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HUA1, a Regulator of Stamen and Carpel Identities in Arabidopsis, Codes for a Nuclear RNA Binding Protein

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Stamen and carpel identities are specified by the combinatorial activities of several floral homeotic genes, *APETALA3*, *PISTILLATA*, *AGAMOUS* (*AG*), *SEPALLATA1* (*SEP1*), *SEPALLATA2* (*SEP2*), and *SEPALLATA3* (*SEP3*), all of which code for MADS domain DNA binding proteins. *AG* and the *SEP* genes also control floral determinacy. *HUA1* and *HUA2* were identified previously as regulators of stamen and carpel identities and floral determinacy because the recessive *hua1-1* or *hua2-1* allele affected these processes in plants with a lower dosage of functional *AG* (either homozygous for the weak *ag-4* allele or heterozygous for the strong *ag-1* allele). *HUA2* was cloned previously and shown to code for a novel protein. We isolated the *HUA1* gene using a map-based approach and show that it encodes a protein with six CCCH-type zinc finger motifs that is also found in yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammalian proteins. Several such genes from invertebrates and mammals are known to play key regulatory roles in development. Therefore, *HUA1* are another example of non–MADS domain proteins involved in organ identity specification. We demonstrated that HUA1 binds ribohomopolymers, preferentially poly rU and poly rG, but not double-stranded DNA in vitro. This finding suggests that HUA1, like several mammalian CCCH zinc finger proteins, is an RNA binding protein. Therefore, *HUA1* likely participates in a new regulatory mechanism governing flower development.

INTRODUCTION

The identities of the four floral organ types in Arabidopsis (sepal, petal, stamen, and carpel) are specified by the combinatorial activities of several classes of homeotic genes (Coen and Meyerowitz, 1991; Meyerowitz et al., 1991; Pelaz et al., 2000). For example, stamen identity requires *APETALA3* (*AP3*), *PISTILLATA* (*PI*), *AGAMOUS* (*AG*), and one of three *SEPALLATA* genes, *SEPALLATA1*, *2*, or *3* (*SEP1*, *SEP2*, or *SEP3*). *AG* and the *SEP* genes determine carpel identity and, in addition, confer determinacy to the floral meristem. These floral homeotic genes code for MADS domain proteins (Yanofsky et al., 1990; Ma et al., 1991; Jack et al., 1992; Mandel et al., 1992; Goto and Meyerowitz, 1994; Mandel and Yanofsky, 1998). The combinatorial interaction among these genes may result from physical interaction among the floral homeotic proteins (Honma and Goto, 2001). Although it is likely that these MADS domain proteins act as transcription factors, it is unclear how these potential transcription factors specify organ identity at the molecular level. Little is known about other players in the homeotic pathways.

HUA1 and *HUA2* were identified as regulators of stamen/carpel identities and floral determinacy in a sensitized screen in the *ag-4* background (Chen and Meyerowitz, 1999). Although flowers of severe loss-of-function *ag* alleles (such as *ag-1*) show stamen-to-petal transformation in the third whorl (Bowman et al., 1989), flowers of the weak *ag-4* allele contain stamens in the third whorl (Sieburth et al., 1995). Recessive *hua1-1* and *hua2-1* mutations alter the identity of the third whorl organs in *ag-4* flowers. *ag-4 hua1-1* or *ag-4 hua2-1* flowers contain petaloid stamens in the third whorl. *ag-4 hua1-1 hua2-1* flowers have petals in the third whorl. That *HUA1* and *HUA2* also play a role in carpel identity and floral determinacy is obvious in the *ag-1*/+ background: both carpel identity and floral determinacy are lost in *ag-1*/+ *hua1-1 hua2-1* flowers but not in *ag-1*/+ flowers. *HUA2* codes for a novel protein with a conserved domain found in mammalian growth factors and transcription coactivators (Chen and Meyerowitz, 1999).

To begin to understand the molecular function of *HUA1*, we isolated the *HUA1* gene using a map-based approach. With the knowledge of the *HUA1* sequence, we screened T-DNA insertion libraries and isolated another *hua1* allele, *hua1-2*. We showed that *hua1-2*, like *hua1-1*, also caused stamen-to-petal transformation in *ag-4* flowers, confirming the role of *HUA1* in stamen identity specification. *HUA1* belongs to a family of nine Arabidopsis genes containing multiple, tandem, CCCH-type zinc finger motifs. Proteins with similar zinc fingers are found in diverse eukaryotes from yeast to human. Several such proteins from *Caenorhabditis elegans* and mouse play regulatory roles in development.

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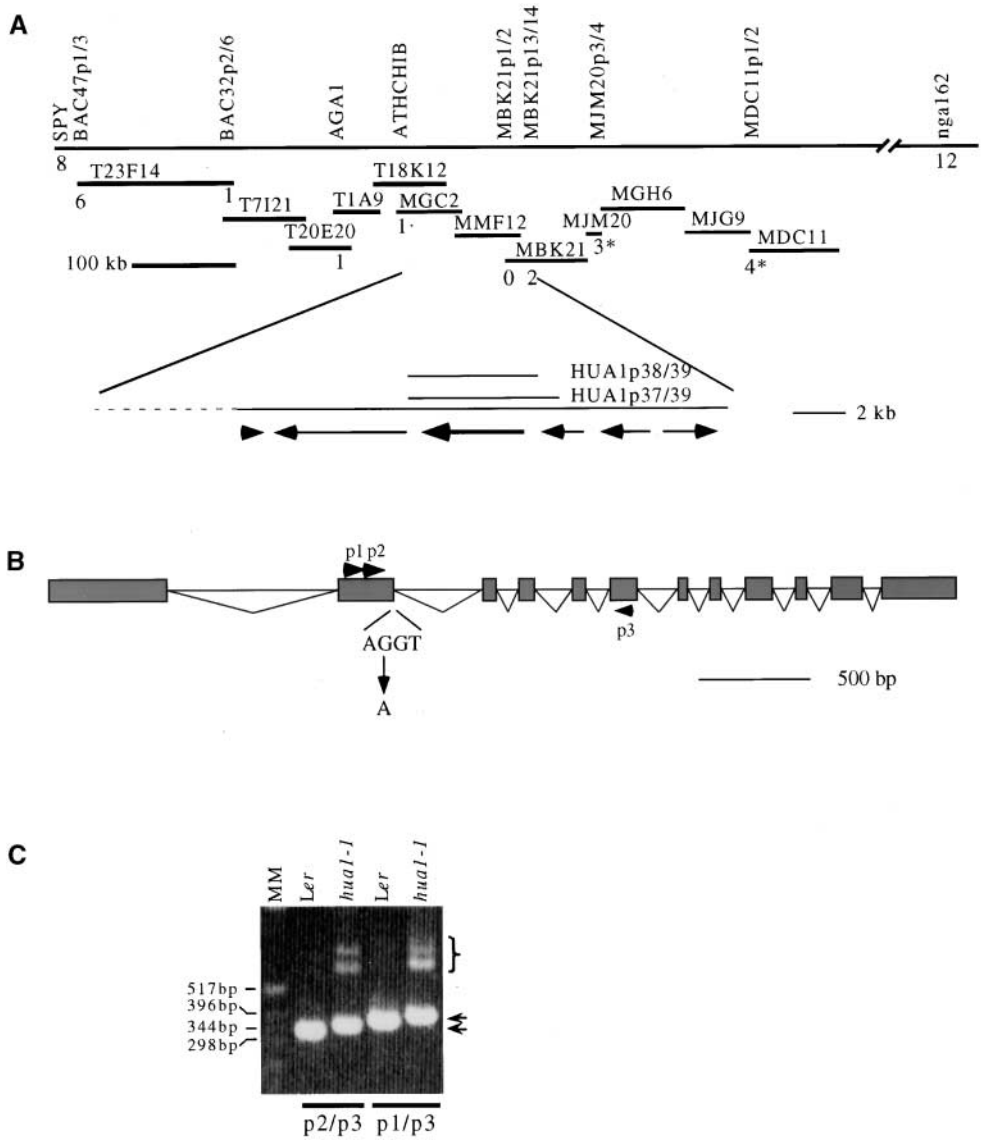

Figure 1. Map-Based Cloning of *HUA1*.

(A) The chromosomal region containing *HUA1* is represented by the top line with the cleaved-amplified polymorphic sequence/(CAPS) and simple sequence length polymorphism (SSLP) markers written above. The TAMU BAC (those starting with the letter T) and P1 (those starting with the letter M) clones are represented by the shorter, overlapping lines. The numbers below the corresponding markers indicate the numbers of recombination break points between these markers and *HUA1* in 1448 chromosomes or 1038 chromosomes (numbers with asterisks). *HUA1* was eventually mapped between ATHCHIB and MBK21p3/4. The potential genes in this region are indicated by arrows. A mutation is found in one of the genes (the thicker arrow) in *hua1-1*. Two genomic clones, HUA1p38/39 and HUA1p37/39, are used for plant transformation.

(B) The genomic organization of this gene, with exons represented by boxes and introns represented by triangles. The G-to-A mutation is at the end of the second exon at the exon-intron boundary. p1, p2, and p3 are three primers used in RT-PCR shown in **(C)**.

(C) RT-PCR with p2 and p3 or p1 and p3 from L*er* and *hua1-1* samples. Although only one band is found in L*er* samples, at least three bands are present in *hua1-1* samples, one of which (indicated by arrows) is slightly larger than the corresponding L*er* band. The larger products are indicated by a bracket. MM, molecular mass.

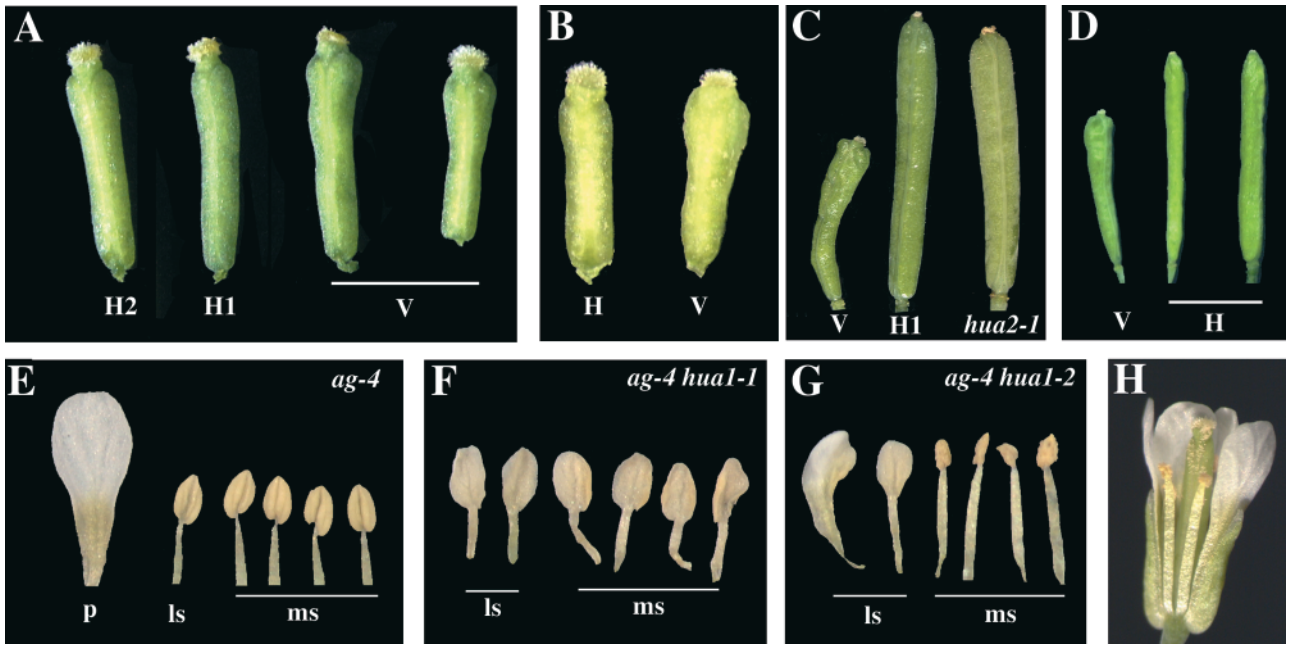

Figure 2. Plant Phenotypes.

(A) to **(D)** Phenotypic rescue by *HUA1* genomic or *GFP-HUA1* clones in *hua1-1 hua2-1*.
(A) Stage 12 gynoecia of *hua1-1 hua2-1* transgenic plants containing *HUA1* genomic clones *HUA1p38/39* (H2) or *HUA1p37/39* (H1) (see Figure 1A) or the vector alone (V).
(B) Stage 12 gynoecia of *hua1-1 hua2-1* transgenic plants containing *35S*::*GFP-HUA1* (H) or the vector alone (V).
(C) Mature siliques of *hua1-1 hua2-1* transgenic plants containing vector alone (V) or *HUA1p37/39* (H1) and a *hua2-1* silique for comparison.
(D) Mature siliques of *hua1-1 hua2-1* transgenic plants containing vector alone (V) or *35S*::*GFP-HUA1* (H).
(E) to **(G)** Dissected second and third whorl organs from *ag-4*, *ag-4 hua1-1*, and *ag-4 hua1-2* flowers, respectively. p, petal; ls, lateral stamen; ms, medial stamen.
(H) A *hua1-2* flower.

We demonstrated that HUA1 binds RNA and single-stranded DNA but not double-stranded DNA in vitro, suggesting that HUA1, like several mammalian CCCH zinc finger proteins, is an RNA binding protein.

RESULTS

Positional Cloning of *HUA1*

HUA1 was mapped in an 86-kb region between polymorphism markers ATHCHIB and MBK21p3/4 on chromosome III (see Methods; Figure 1A). Three candidate genes from this region were sequenced from Landsberg *erecta* (L*er*) and *hua1-1*. A G-to-A mutation was found in one of the genes. The mutated nucleotide resides immediately next to a potential intron splice donor site (Figure 1B), changing the sequence next to the splice donor site from a preferred (AG) to a rare (AA) context in Arabidopsis genes (Brown et al., 1996). To determine whether this potential intron was in fact an intron and whether this mutation affected the splicing of this intron, we isolated cDNAs corresponding to this gene. The identity of the intron was confirmed by comparison of the cDNA and genomic sequences (Figure 1B). Reverse transcriptase–mediated polymerase chain reaction (RT-PCR) was performed using RNA from L*er* and *hua1-1* inflorescences with two sets of primers that flanked the intron (Figure 1B). Whereas a single PCR fragment of the expected size was obtained from L*er* with each set of primers, several fragments of larger sizes were found in *hua1-1* samples (Figure 1C). No products of the correct size were obtained from *hua1-1*. The p2/p3 fragment that was slightly larger than the corresponding L*er* fragment was cloned and sequenced. A cryptic splice donor site 22 nucleotides downstream of the wild-type donor site apparently was used (data not shown). One of the much larger fragments also was cloned and sequenced and found to correspond to the

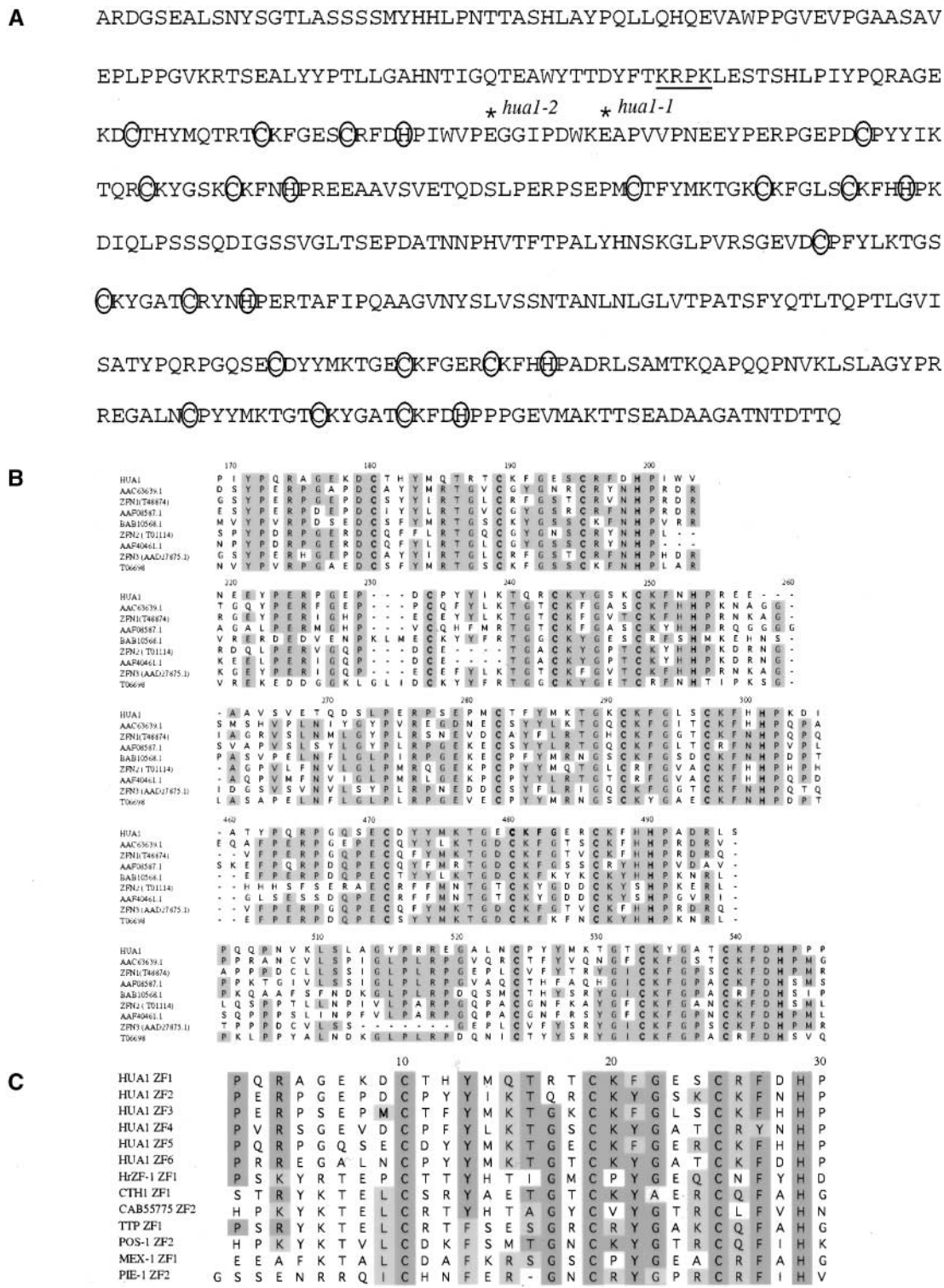

Figure 3. The HUA1 Protein.

(A) The HUA1 amino acid sequence with the critical CCCH zinc finger residues circled. The putative nuclear localization signal is underlined. The positions of the *hua1-1* and *hua1-2* mutations are indicated.

inclusion of the entire intron (data not shown). These *hua1-1* RNA species would result in premature translation termination in the polypeptide.

To confirm that this is the *HUA1* gene, we transformed *hua1-1 hua2-1* plants with two genomic clones that contained only this gene (Figure 1A). The two clones differed in the amount of the potential promoter region of the gene (1.3 kb versus 540 bp). In each case, ~50% of the transgenic lines (more than 40 for each) showed phenotypes that indicated complete rescue, and the remaining 50% showed partial rescue or no rescue. None of the 30 transgenic plants containing the cloning vector alone showed any sign of rescue (Figures 2A and 2C). *hua1-1 hua2-1* gynoecia are consistently (100%) enlarged in the top portion of the ovary (Chen and Meyerowitz, 1999) starting from stage 12 (stages according to Smyth et al. [1990]). Plants showing full rescue exhibited completely normal stage 12 gynoecia (Figure 2A), as found in wild-type plants. The phenotypic rescue was most evident in mature flowers. The rescued siliques were much longer than *hua1-1 hua2-1* siliques at maturity and comparable in length to *hua2-1* siliques (Figure 2C). Phenotypic rescue also was observed, although at a lower frequency, in *hua1-1 hua2-1* plants transformed with *35S*::*HUA1cDNA* (data not shown). Therefore, we most likely cloned the *HUA1* gene. However, it remains to be determined whether this gene rescues the floral homeotic phenotype of *ag-4 hua1-1*.

hua1-2

Previous genetic analyses of *HUA1* were performed on the single *hua1-1* allele. To test the conclusions from these analyses, we isolated another *hua1* allele, *hua1-2*, from the Tom Jack T-DNA collection (Arabidopsis Stock Center, Ohio State University, Columbus) using a PCR-based approach. An ~5-kb T-DNA was inserted in the second exon 24 bp upstream of the point mutation found in *hua1-1* (data not shown).

We crossed *hua1-2* to *ag-4* to determine if *hua1-2* affects organ identity in the third whorl of *ag-4* flowers, as *hua1-1* does. Indeed, *ag-4 hua1-2* third whorl organs differed from those in *ag-4* in that the lateral stamens were transformed to petals or petaloid stamens (cf. Figures 2G and 2E). The medial stamens appeared normal. This phenotype was consistent in all flowers of *ag-4 hua1-2* plants. To rule out the possibility that this phenotype is attributable to differences in the genetic background of the two parental strains (*hua1-2* in Columbia [Col] and *ag-4* in L*er*), we analyzed 55 *ag-4* homozygous plants in the F2 population. Six were homozygous for *hua1-2*, whereas the others were *hua1-2*/+ or +/+. Only *ag-4 hua1-2* plants exhibited the third whorl phenotype, suggesting that the homeotic transformation is caused by *hua1-2* and that *hua1-2* is recessive. *hua1-2* seemed weaker than *hua1-1*, because all six stamens in *ag-4 hua1-1* appeared petaloid (Figure 2F) (Chen and Meyerowitz, 1999). Nonetheless, this confirmed the role of *HUA1* in stamen identity specification. *hua1-2 hua2-1* double mutant flowers exhibited the same defects in the gynoecia as *hua1-1 hua2-1* flowers (data not shown). Like *hua1-1* flowers, single mutant *hua1-2* flowers have no obvious phenotypes (Figure 2H).

The HUA1 Protein

The most prominent feature of the 524–amino acid HUA1 protein is the presence of six tandem CCCH-type zinc finger motifs that span the C-terminal two-thirds of the protein (Figure 3A). A potential nuclear localization signal (KRPK) is found in the N-terminal one-third of the protein (Figure 3A). The *hua1-1* mutation that results in aberrant splicing of the transcript presumably would cause translation termination after the first zinc finger motif.

HUA1 belongs to a family of nine Arabidopsis genes with highly similar, tandem, CCCH-type zinc finger motifs. The other members of the family include three previously cloned genes of unknown function, *ZFN1*, *ZFN2*, and *ZFN3*, and five genes predicted from the genome sequence (Figure 3B). These proteins all contain five tandem zinc fingers, whereas HUA1 has six. When these protein sequences are aligned, similarity is found only in the zinc finger regions, with HUA1 zinc fingers 1, 2, 3, 5, and 6 aligning with zinc fingers 1, 2, 3, 4, and 5 of the other proteins, respectively. The similarity is not confined to the cysteine and histidine residues of the zinc fingers but instead persists at least over a block of 30 amino acids. Interestingly, the six HUA1 zinc finger regions are less similar among themselves than they are

Figure 3. (continued).

(B) A Clustal W alignment of the HUA1 family members at the corresponding zinc finger regions. GenBank accession numbers for the HUA1 paralogs are indicated. The cysteine and histidine residues of the zinc fingers are in boldface letters. Shaded letters represent identical residues.
(C) A Clustal W alignment of the six HUA1 zinc fingers with those from animal proteins. Identical and similar residues are shaded with darker and lighter gray, respectively. HrZF-1 ZF1, zinc finger 1 of HrZF1 (BAA81905) from *Halocynthia roretzi*; CTH1 ZF1, zinc finger 1 of CTH1 (CAA71245) from *Cyprinus carpio*; CAB55775 ZF2, zinc finger 2 of a zebrafish protein; TTP ZF1, POS-1 ZF2, MEX-1 ZF1, and PIE-1 ZF2 are the indicated zinc fingers from the mouse TTP (S04743) and the *C. elegans* POS-1 (T37246), MEX-1 (U81043), and PIE-1 (AAB17868) proteins, respectively.

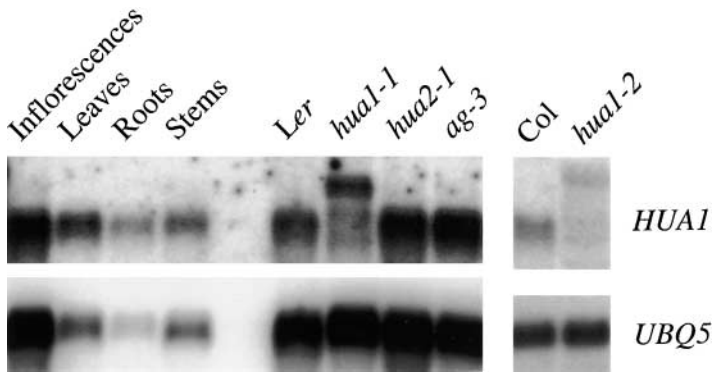

Figure 4. *HUA1* RNA Accumulation in Various Organs and in Inflorescences of Various Genotypes as Indicated.

UBIQUITIN5 (*UBQ5*) RNA was used to monitor the total amount of RNA in the samples. L*er* is the wild-type control for *hua1-1*, *hua2-1*, and *ag-3*. Col is the wild-type control for *hua1-2*.

to their counterparts in the paralogous proteins (cf. Figures 3B and 3C). Genes with zinc fingers of the same type and organization are found in other plant species such as rice and garden pea. There exist other Arabidopsis CCCH-type zinc finger proteins that do not belong to this family. One example is ZFWD1 (Terol et al., 2000), which contains a single CCCH-type zinc finger distantly related to the HUA1 zinc fingers (data not shown).

Besides plant proteins, BLAST searches revealed that many animal proteins contain similar zinc fingers, although the similarity between the HUA1 zinc fingers and the zinc fingers from these animal proteins is not as extensive as that among the HUA1 family members (Figure 3C). These proteins are found in diverse organisms such as *Halocynthia roretzi*, *C. elegans*, zebrafish, mouse, and human.

HUA1 RNA Is Found in All Major Plant Organs

Among the known floral homeotic genes, those encoding MADS domain proteins are expressed primarily in flowers (Yanofsky et al., 1990; Ma et al., 1991; Jack et al., 1992; Mandel et al., 1992; Goto and Meyerowitz, 1994; Mandel and Yanofsky, 1998), whereas *AP2* and *HUA2* are expressed in nonfloral as well as floral tissue (Jofuku et al., 1994; Chen and Meyerowitz, 1999). We examined the expression of

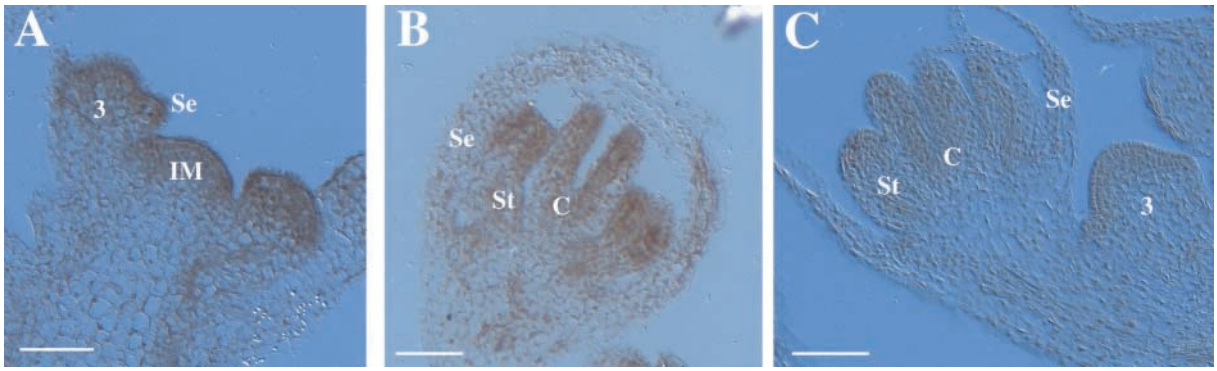

Figure 5. *HUA1* RNA Localization in Wild-Type Inflorescences as Determined by in Situ Hybridization.

(A) and **(B)** Longitudinal sections of an inflorescence and a stage 8 to 9 flower, respectively, reacted with a *HUA1* antisense probe.
(C) A longitudinal section of a stage 3 and a stage 7 flower reacted with a *HUA1* sense probe.
Se, sepal; St, stamen; C, carpel; IM, inflorescence meristem; 3, a stage 3 flower. Bars = 50 μm.

HUA1 with RNA filter hybridization. *HUA1* RNA was found throughout the plant, in inflorescences, stems, leaves, and roots (Figure 4).

To determine if *HUA1* expression is regulated by *AG* or *HUA2*, two other genes with similar floral homeotic functions, we examined *HUA1* RNA abundance in wild-type, *ag-3*, and *hua2-1* inflorescences. Comparable levels of *HUA1* RNA were found in these genotypes (Figure 4), suggesting that *HUA1* is not regulated at the transcript level by either *AG* or *HUA2*.

In *hua1-1*, the major RNA species was ~400 nucleotides longer than the wild-type *HUA1* RNA (Figure 4), suggesting that the second intron was retained in most *hua1-1* RNA. This major *hua1-1* transcript was detected at 12% of the abundance of wild-type *HUA1* RNA. Other *hua1-1* RNA species of smaller sizes and lower abundance also were detected (Figure 4). The prevalence of the RT-PCR products 22 bp longer than the wild-type products in Figure 1C likely was caused by preferential amplification by PCR. In *hua1-2*, a major transcript larger than the wild-type transcript was detected at 22% of the wild-type abundance (Figure 4).

We also examined the localization of *HUA1* transcript in wild-type inflorescences by in situ hybridization. Like *HUA2* RNA, *HUA1* RNA was detected with radioactive probes in all cells in the inflorescence meristem, the inflorescence stem, and flowers of all stages (data not shown). With nonradioactive probes that are less sensitive, *HUA1* RNA was detected throughout the inflorescence meristem and young floral primordia (Figures 5A and 5C). In stage 7 and older flowers, *HUA1* RNA was more concentrated in petals (data not shown), stamens, and carpels (Figures 5B and 5C).

HUA1 Is a Nuclear Protein

Because HUA1 has a poor potential nuclear localization signal and several *C. elegans* proteins with similar zinc fingers are found in the cytoplasm, we examined the subcellular localization of HUA1 by fusing the full-length cDNA to the green fluorescent protein (GFP) gene. The fusion protein was functional because it was able to rescue the *hua1-1 hua2-1* mutant phenotype (Figures 2B and 2D). In onion epidermal cell transient expression assays, GFP-HUA1 was found to be in the nucleus, whereas GFP alone was present throughout the cell (Figure 6). The N-terminal one-third of the protein without the zinc fingers (HUA1NT) and the C-terminal two-thirds containing the zinc fingers (HUA1CT) also were fused separately to GFP. The GFP-HUA1NT fusion protein was found exclusively in the nucleus, and the GFP-HUA1CT fusion protein was present throughout the cell (data not shown), suggesting that the N-terminal one-third of the protein contains signals for nuclear import or protein degradation in the cytoplasm. GFP-HUA1 localization also was examined in the roots of transgenic Arabidopsis plants, where GFP fluorescence was detected only in the nuclei (data not shown).

Nucleic Acid Binding Properties of HUA1

Many CCCH-type zinc finger proteins from animals have been shown to bind RNA and/or to be involved in post-transcriptional regulation (see Discussion). To begin to understand the molecular functions of HUA1, we examined the nucleic acid binding properties of HUA1 using recombinant HUA1 proteins purified from *Escherichia coli*. Under moderate salt concentrations (0.1 and 0.25 M) that resemble the in vivo situation, HUA1 was found to bind ribohomopolymers but not double-stranded DNA (Figure 7). Specifically, at 0.1 M NaCl, the full-length protein bound poly rG, poly rU, poly rA, and single-stranded DNA but not double-stranded DNA. At 0.25 M NaCl, the full-length protein bound only poly rU and poly rG. The binding to poly rU can be detected at salt concentrations as high as 0.5 M (data not shown). An N-terminally truncated HUA1 protein with only the zinc fingers (HUA1CT) bound only poly rG under the two salt concentrations. An unrelated protein (APA1; X. Chen and E.M. Meyerowitz, unpublished results) did not bind any nucleic acid under the same conditions. This finding suggests that HUA1 is an RNA binding protein and that the N-terminal portion of the protein affects the HUA1 RNA binding properties.

Expression of Floral Homeotic Genes in *hua1-1* Flowers

The RNA localization patterns of *AP1* and *AG* in *hua1-1 hua2-1* flowers were reported previously (Chen and Meyerowitz, 1999). Those of the two B function genes, *AP3* and *PI*, are similar in *hua1-1 hua2-1* and wild-type flowers (X. Chen and E.M. Meyerowitz, unpublished results). We examined the RNA accumulation patterns of the *SEP1*, *SEP2*, and *SEP3* genes, which were recognized recently as regulators of floral organ identities and potential partners of *AG* (Pelaz et al., 2000; Honma and Goto, 2001). The expression patterns of all three *SEP* genes in *hua1-1 hua2-1* were similar to those in wild-type controls and to those reported previously (data not shown; Ma et al., 1991; Mandel and Yanofsky, 1998), suggesting that the *HUA* genes do not regulate the expression domains of the *SEP* genes.

Because HUA1 is a nuclear RNA binding protein, it is conceivable that *HUA1* regulates *AG* at the level of RNA splicing. We performed RT-PCR to determine if partially spliced *AG* RNAs could be detected in *hua1-1* but not wild-type inflorescences. No difference was found between the *hua1-1* and wild-type samples (data not shown).

DISCUSSION

Biological Role of *HUA1*

HUA1 was identified initially as a gene involved in conferring reproductive organ identities as well as floral determinacy in

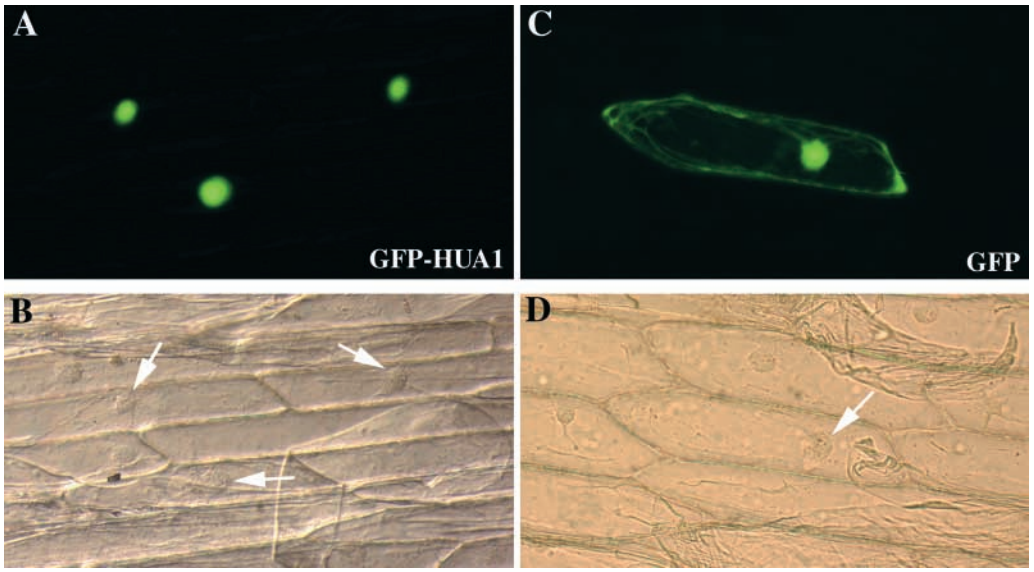

Figure 6. Onion Epidermal Cell Transient Expression Assay for HUA1 Subcellular Localization.

(A) and **(B)** Fluorescent and light field images, respectively, of cells expressing GFP-HUA1. Arrows indicate nuclei.
(C) and **(D)** Fluorescent and light field images, respectively, of cells expressing GFP. Arrows indicate nuclei.

Arabidopsis. Although these proposed roles were based on analyses of the single *hua1-1* allele, the fact that *hua1-2* and *hua1-1* show similar effects in the *ag-4* and *hua2-1* backgrounds suggests that the previously analyzed *hua1-1* defects likely reflect the true biological functions of *HUA1*.

In the flower, available genetic evidence suggests that *HUA1* acts only in the third and fourth whorls. However, *HUA1* RNA is clearly detected in all four floral whorls in early flowers and is concentrated in the inner three whorls in later flowers. Translational or post-translational processes theoretically can account for the whorl-specific activities of *HUA1*. Alternatively, *HUA1* also plays a role in the perianth organs, a role not recognized because of the presence of one or more genes with overlapping functions. It is worth noting that the *HUA1* expression patterns in the flower resemble those of *SEP1*, *2*, or *3*, although the functional significance of this, if any, remains to be determined.

HUA1 also regulates plant size (Chen and Meyerowitz, 1999). Although *hua1-1* or *hua2-1* single mutants show no obvious phenotypes, *hua1-1 hua2-1* plants are shorter and smaller than are wild-type plants. Further backcrosses are necessary to determine if *hua1-2 hua2-1* plants exhibit similar phenotypes, because the two alleles are from two ecotypes that differ in size (L*er* and Col). The fact that *HUA1* is expressed in stems and leaves is consistent with its proposed role in vegetative development. Both *HUA1* and *HUA2* (Chen and Meyerowitz, 1999) are expressed in the root. No obvious root defects have been observed with either the *hua1-1* or *hua2-1* single mutant or the double mutant on sucrose-containing plates (T. Western and X. Chen, unpublished results).

HUA1 Belongs to a Gene Family

HUA1 belongs to a family of nine genes in Arabidopsis that contain tandem CCCH-type zinc fingers. Genes of this family also are found in other higher plants. Not all Arabidopsis genes containing CCCH-type zinc fingers belong to this family. This family of genes is unique in that the CCCH-type zinc fingers are found in tandem and the corresponding zinc fingers among genes are more similar than the zinc fingers in any one gene. *HUA1* is the only gene of this family with a known biological function. Proteins with similar zinc fingers are found in invertebrates and vertebrates. However, the degree of similarity is much less than that among *HUA1* family members, and most of these animal proteins contain only two tandem zinc fingers.

Despite the established function of *HUA1* in conferring reproductive organ identity and floral determinacy in flower development, neither *hua1-1* nor *hua1-2* single mutant flowers exhibit any floral defects. It is likely that neither *hua1* nor *hua2* is a null allele (transcript found in both) and that the lack of floral phenotypes is attributable to the residual activities of the mutant proteins. Alternatively, the lack of floral phenotypes in the *hua1* single mutants can be attributable mainly to the presence of other genes with overlapping

functions. Given that examples of structurally related genes with redundant or similar functions exist (e.g., *SEP1*, *SEP2*, and *SEP3* [Pelaz et al., 2000]; *SCARECROW* and *SHORT ROOT* [Di Laurenzio et al., 1996; Helariutta et al., 2000]), it is tempting to postulate that some of the *HUA1* family members share overlapping roles with *HUA1* in flower development. This hypothesis can be tested by the loss-of-function phenotypes of these genes.

Potential Molecular Functions of *HUA1*

The presence of the CCCH-type zinc fingers in HUA1 provides some hints to the potential molecular functions of *HUA1*. Although no conclusive evidence exists for CCCH-type zinc fingers being involved in DNA binding, many proteins with this type of zinc finger have been shown to bind RNA or to be associated with RNA metabolism. The murine TTP protein (Figure 3C) regulates the expression of tumor necrosis factor α (*TNF*-α) at the post-transcriptional level. TTP binds to an AU-rich sequence in the 3′ untranslated region of the *TNF*-α mRNA and destabilizes it (Carballo et al., 1998). Both zinc fingers in TTP are required for this RNA–protein interaction (Lai et al., 1999). Three other mammalian proteins and one *Xenopus laevis* protein with double zinc fingers also were shown to bind to *TNF*-α RNA (Lai et al., 2000). In *C. elegans*, three genes encoding proteins with double zinc fingers of the CCCH type (Figure 3C), *Pie-1*, *Mex-1*, and *Pos-1*, specify the identity of germline blastomeres in early embryonic development with distinct mechanisms. *Pie-1* is a bifunctional protein that acts in the nucleus to repress germline transcription and in the cytoplasm to regulate the expression of *nanos-2* RNA at the post-transcriptional level (Tenenhaus et al., 2001). The two zinc fingers in the protein are not required for transcription repression, but the second zinc finger is required for the cytoplasmic function of PIE-1 (Tenenhaus et al., 2001). *Mex-1* acts to restrict the localization of PIE-1 (Guedes and Priess, 1997). *Pos-1* appears to regulate the translation of maternal RNAs in the germline blastomeres (Tabara et al., 1999). All three proteins are known to be associated with large, germline-specific RNA–protein complexes known as the P granules in the cytoplasm (Mello et al., 1996; Guedes and Priess, 1997; Tabara et al., 1999). In addition, several yeast, *Drosophila melanogaster*, and mammalian proteins with CCCH fingers have been demonstrated to function in splicing,

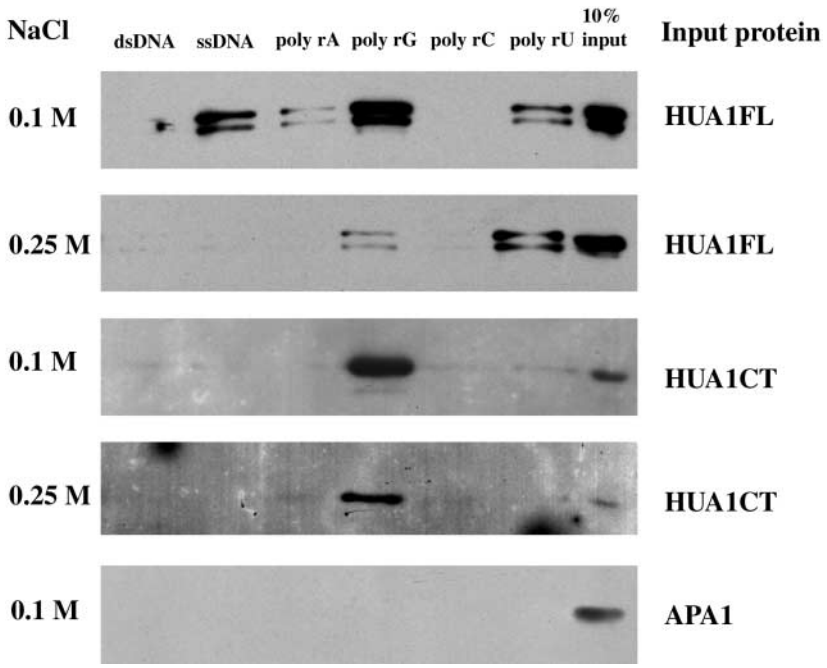

Figure 7. In Vitro Nucleic Acid Binding by HUA1.

The full-length HUA1 protein (HUA1FL), an N-terminally truncated version that contains all six zinc fingers (HUA1CT), and an unrelated protein (APA1) were expressed as His-tagged proteins, purified, and incubated with various nucleic acids attached to agarose or cellulose beads. HUA1FL binds single-stranded DNA (ssDNA), poly rA, poly rG, and poly rU at 0.1 M NaCl and poly rG and poly rU at 0.25 M NaCl. HUA1CT binds poly rG under both conditions. APA1 does not bind any nucleic acid under these conditions. Ten percent of the input proteins is shown in the right lane. dsDNA, double-stranded DNA.

polyadenylation, or RNA cleavage (Zhang et al., 1992; Bai and Tolias, 1996; Barabino et al., 1997).

The fact that HUA1 binds poly rG, poly rU, and single-stranded DNA in vitro suggests that HUA1 is an RNA binding protein. This and the nuclear localization of HUA1 point to a potential role in nuclear RNA metabolism, such as RNA processing, RNA stability, or RNA export.

Molecular Function of *HUA1* in the *AG* Pathway

The previously isolated floral homeotic genes all seem to code for potential transcription factors. AP1, AP3, PI, AG, and the SEP proteins contain the MADS domain known to bind to DNA (Huang et al., 1993; Shiraishi et al., 1993). AP2 contains another DNA binding domain, the AP2 domain (Weigel, 1995). HUA2 does not have any known DNA binding domain, but it has been shown to possess transcription activation activity in yeast (X. Chen, unpublished results). Two other genes that play a role in carpel development, *CRABS CLAW* (*CRC*) and *SPATULA* (*SPT*), both encode proteins with canonical DNA binding motifs (Bowman and Smyth, 1999; Heisler et al., 2001). Although we cannot exclude the possibility that HUA1 also functions in transcriptional regulation, HUA1 likely functions in post-transcriptional processes at the RNA level.

How would HUA1, a nuclear, potential RNA binding protein, act in the floral homeotic *AG* pathway? One hypothesis is that HUA1 promotes the expression of *AG* at the post-transcriptional level, such as through RNA processing (capping, splicing, and polyadenylation), RNA stability, or RNA export from the nucleus. The *hua1-1* mutation does not affect the spatial arrangement of *AG* RNA in flowers (Chen and Meyerowitz, 1999). Protein gel blot experiments with dissected inflorescences containing stage 6 and younger flowers showed that *hua1-1* does not affect AG protein levels in these young flowers (Chen and Meyerowitz, 1999). However, it is possible that HUA1 acts to maintain *AG* expression in later floral stages. Although no aberrantly spliced forms of *AG* RNA can be detected in *hua1-1* flowers, it is possible that these forms are unstable and rapidly degraded. Therefore, it is still possible that HUA1 regulates *AG* expression at the level of RNA splicing. A second hypothesis is that *HUA1* acts together with *AG* in regulating the expression of downstream targets. It is well documented that transcription and RNA processing are closely coordinated (Ladomery, 1997; Proudfoot, 2000). It is possible that HUA1 acts as an RNA processing factor that works on *AG* target gene transcripts.

In addition, many genes known to play a role in the development of the gynoecium may be regulated by *HUA1* or serve as regulators of *HUA1*. Examples are *CRC* (Alvarez and Smyth, 1999; Bowman and Smyth, 1999), *SPT* (Alvarez and Smyth, 1999; Heisler et al., 2001), *ETTIN* (Sessions and Zambryski, 1995), *SHATTERPROOF1* and *-2* (Liljegren et al., 2000), *FRUITFUL* (Férrandiz et al., 2000), *LEUNIG* (Liu et al., 2000), and *AINTEGUMENTA* (Liu et al., 2000). The relationships between *HUA1* and these genes are unknown at present.

METHODS

Strains and Transgenic Lines

The mutant strains used in this study, *ag-3* (Bowman et al., 1991), *ag-4* (Sieburth et al., 1995), *hua1-1* (Chen and Meyerowitz, 1999), *hua2-1* (Chen and Meyerowitz, 1999), and *hua1-1 hua2-1*, are in the Landsberg *erecta* (L*er*) background of *Arabidopsis thaliana*. *hua1-2*, isolated in this study, is in the Columbia (Col) background. Plants were grown at 23°C in Pro-mix BX (a peat-based growing medium from Premier Horticulture Inc., Red Hill, PA) under 16-hr-light/8-hr-dark cycles.

hua1-2 was isolated from the Tom Jack T-DNA lines using a polymerase chain reaction (PCR) screening approach. A *HUA1*-specific primer (HUA1p24; 5′-aaggcatcaaacttttttgagtcctccttt-3′) was used in combination with a T-DNA border primer (JL-202; 5′-cattttataataacgctgcggacatctac-3′) to amplify from pooled DNA obtained from the Arabidopsis Stock Center (Ohio State University). The PCR product was confirmed by DNA filter hybridization and sequencing. An individual homozygous line was identified from one of the subpools.

hua1-1 hua2-1 plants were transformed with *HUA1* genomic DNA, *35S*::*HUA1cDNA*, and *35S*::*GFP-HUA1* fusion constructs by 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 with a Fuji (Tokyo, Japan) HC300-Z digital camera.

Positional Cloning of *HUA1*

The mapping population was derived from the cross *ag-4*/+ *hua1-1 hua2-1* × Col. In the F2 population of this cross, *ag-4*/+ *hua1-1 hua2-1* and *ag-4 hua1-1 hua2-1* plants were chosen based on their floral phenotypes. The *HUA1* mapping population consisted of 724 such plants, among which 529 were used previously for the positional cloning of *HUA2*.

DNA was isolated from each F2 plant either by the hexadecyl-trimethyl-ammonium bromide method (Reiter et al., 1992) or by a simpler preparation method (Edwards et al., 1991). *HUA1* was first mapped to the top arm of chromosome III close to simple sequence length polymorphism (SSLP) markers nga162 and ATHCHIB and to a cleaved-amplified polymorphic sequence (CAPS) marker in a gene named *AGA1* (X. Chen and E.M. Meyerowitz, unpublished results). *AGA1* was used as a probe to screen Texas A&M University (TAMU) bacterial artificial chromosome (BAC) filters. One of the clones that hybridized to *AGA1*, T20E20, was sequenced from the ends. The end sequence was used to screen for overlapping BACs until eventually a contig of five TAMU BACs that overlapped with the Kazusa P1 contig in this region (see Figure 1) was established. CAPS markers were generated by sequencing the BAC or P1 ends in L*er* and Col (in cases in which the Col sequence was not yet available from the Arabidopsis Genome Initiative) backgrounds. Eventually, *HUA1* was mapped between markers ATHCHIB and MBK21p3/4. Information on the CAPS markers shown in Figure 1 as well as additional markers developed in this study can be viewed at the Chen laboratory World Wide Web site (http://waksman.rutgers.edu/~xuemei).

HUA1 genomic DNA for mutant rescue was amplified with HUA1p39 (5′ BglII-aaggcatcaaacttttttgagtcctccttt 3′) in combination with either HUA1p37 (5′ BglII-cacaaaagcttctctgagt 3′) or HUA1p38 (5′ BglII-ttgctgactccatttttggtc 3′). The resulting 5.7- and 5-kb fragments, respectively, were cloned separately in the BamHI site of the plant transformation vector pPZP211 (Hajdukiewicz et al., 1994). The full-length *HUA1* cDNA (see below) was amplified with HUA1p34 (5′ XbaI-gtcaaatcaagttttatggatc 3′) and HUA1p6 (5′ XbaI-tcattgagtagtgtcggtgttg 3′), cloned into pCGN1547 (McBride and Summerfelt, 1990) containing the 35S promoter of the *Cauliflower mosaic virus* and the nopaline synthase 3′ end, and sequenced.

HUA1 cDNA

The Weigel cDNA library was screened with a *HUA1* genomic DNA probe. Six cDNA clones were obtained from 500,000 plaques screened. One cDNA clone, HUA1cDNA6, was full-length but contained a one-nucleotide deletion in the coding region. 5′ rapid amplification of cDNA ends (Gibco BRL) was performed according to the manufacturer's instructions to obtain a 650-bp fragment corresponding to the 5′ one-third of the *HUA1* RNA. This fragment was cloned into pCR-Blunt (Invitrogen, Carlsbad, CA), and the resulting plasmid, pCR-MBKp46/50, was sequenced. A 350-bp AgeI-AccI fragment from pCR-MBKp46/50 was used to replace the corresponding fragment in HUA1cDNA6 to repair the one-nucleotide-deletion defect.

RNA Filter and in Situ Hybridization

Total RNA from various organs and genotypes was isolated using the Tri-reagent (Molecular Research Center, Cincinnati, OH). Poly(A^+) RNA was isolated using a Rapid mRNA Purification Kit (Amresco, Solon, OH). Approximately 2 μg of poly(A^+) RNA was separated on a 1.2% formaldehyde/agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled PCR fragments corresponding to either the full-length or the 3′ half of the *HUA1* cDNA. Hybridization signals were quantified with a phosphorimager. The *UBIQUITIN5* gene was used as a control for the amount of RNA used.

In situ hybridization with radioactive probes was performed as described (Chen and Meyerowitz, 1999). In situ hybridization with digoxigenin-labeled probes was performed according to www.wisc.edu/genetics/CATG/barton/protocols.html, except that UTP and digoxigenin-UTP were used at a 3:7 ratio in probe synthesis. A 1.55-kb fragment was amplified from *HUA1* cDNA using primers HUA1p19 (5′ BglII-atggcacatcgtcaattgt 3′) and HUA1p6 and cloned into pCR-Blunt (Invitrogen). Two plasmids, pHUA1p19/6A and pHUA1p19/6S, with different orientations of the insert, were obtained. These two plasmids were digested with SpeI and transcribed with T7 RNA polymerase to generate the antisense and the sense probe for *HUA1*, respectively.

HUA1 Protein Localization

The full-length *HUA1* coding region was amplified with Pwo polymerase (Roche Molecular Biochemicals, Indianapolis, IN) using primers HUA1p19 and HUA1p6 and cloned into pAVA321 (von Arnim et al., 1998), resulting in a fusion of green fluorescent protein (GFP) to the N terminus of HUA1 (pAVA321-HUA1). The *35S*::*GFP-HUA1* cassette was excised with SacI and KpnI and cloned into pPZP211 for plant transformation.

Transient expression assays were performed as described (von Arnim and Deng, 1994). Either pAVA321 or pAVA321-HUA1 DNA was delivered into onion epidermal cells by bombardment with the Bio-Rad Biolistic PDS-1000/He system. After bombardment, the epidermal peels were cultured at 22°C in darkness for 16 hr and observed under a Zeiss (Jena, Germany) Axioplan Universal transmitted light microscope. Images were captured with a digital 1X HRD100-N/K camera from Diagnostic Instruments (Sterling Heights, MI).

In Vitro Nucleic Acid Binding Assay

The full-length version and an N-terminally truncated version of the *HUA1* gene were cloned into the pRSETA protein expression vector (Invitrogen). An unrelated gene, *APA1*, also was cloned into the same vector (X. Chen and E.M. Meyerowitz, unpublished results). The 6His-tagged proteins were purified from *Escherichia coli* using QIAexpressionist (Qiagen, Valencia, CA).

In vitro nucleic acid binding assays were performed as described (Yang et al., 1998). Five hundred nanograms of purified 6His-tagged HUA1 full-length protein, an N-terminally truncated HUA1 protein, and an unrelated protein (APA1) were incubated with 20 μL of poly rA, poly rG, poly rC, and poly rU attached to agarose beads and double-stranded and single-stranded calf thymus DNA attached to cellulose beads (Sigma) in 500 μL of RHPA binding buffer (10 mM Tris, pH 7.4, 2.5 mM $MgCl_2$, 0.5% Triton X-100, and NaCl at various concentrations) with 1 mg/mL heparin. The concentrations of the nucleic acids were 0.35 to 0.7 mg/mL (poly rA), 0.25 to 1 mg/mL (poly rC), 1.5 to 4.5 mg/mL (poly rG), 0.1 to 1 mg/mL (poly rU), 0.75 to 1.25 mg/mL (single-stranded DNA), and 0.75 to 2 mg/mL (double-stranded DNA). After incubation at 4°C for 10 min, the beads were washed five times in RHPA buffer and then boiled in SDS loading buffer. The proteins were separated by SDS-PAGE and detected with a monoclonal anti-His antibody (Pharmacia).

GenBank Accession Number

The *HUA1* cDNA sequence is in GenBank under accession number AY024357.

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HUA1, a Regulator of Stamen and Carpel Identities in Arabidopsis, Codes for a Nuclear RNA Binding Protein

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