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HUA2 is required for the expression of floral repressors in *Arabidopsis thaliana*

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Summary

The *HUA2* gene acts as a repressor of floral transition. Lesions in *hua2* were identified through a study of natural variation and through two mutant screens. An allele of *HUA2* from Landsberg *erecta* (L*er*) contains a premature stop codon and acts as an enhancer of *early flowering 4* (*elf4*) mutants. *hua2* single mutants, in the absence of the elf4 lesion, flower earlier than wild type under short days. *hua2* mutations partially suppress late flowering in *FRIGIDA* (*FRI*)-containing lines, autonomous pathway mutants, and a photoperiod pathway mutant. *hua2* mutations suppress late flowering by reducing the expression of several MADS genes that act as floral repressors including *FLOWERING LOCUS C* (*FLC*) and *FLOWERING LOCUS M* (*FLM*).

Keywords: Arabidopsis, HUA2, FLC, flowering, natural variation.

Introduction

Proper timing of the switch from vegetative to reproductive growth in flowering plants is critical for achieving maximal reproductive fitness. Most plants have an optimal time of year to flower and set seed. In species with a large geographic distribution, the mechanisms that underlie the control of flowering must be adaptable in order to adjust to factors that may be encountered in different habitats.

Two environmental factors used by plants to assess seasonal change are prolonged periods of cold temperatures and relative changes in photoperiod. The promotion of flowering in response to a prolonged period of cold is known as vernalization. Vernalization often requires several weeks of cold (0–6°C) for a maximum response (Michaels and Amasino, 2000). This helps ensure that the winter season has passed and allows for rapid flowering in the spring. Floral induction in response to lengthening days also leads to flowering in the spring. Plants with such a response, including *Arabidopsis thaliana*, are known as long day plants (LDPs). Short day plants (SDPs), on the other hand, flower in the autumn in response to shortening days and lengthening nights (Thomas and Vince-Prue, 1997). The response to daylength in both SDP and LDP is closely associated with the plant circadian clock.

The geographic range of Arabidopsis includes areas with vast differences in daylength and ambient temperature throughout the year. Not surprisingly, populations of Arabidopsis from different regions often display differences in the regulation of flowering. Studies of natural variation in Arabidopsis have revealed loci involved in the regulation of flowering time (Alonso-Blanco *et al.*, 1998; Kowalski *et al.*, 1994). Variation between two rapid-flowering accessions, Landsberg *erecta* (L*er*) and Cape Verdi Islands (Cvi), has been studied using recombinant-inbred lines (RILs) (Alonso-Blanco *et al.*, 1998). One of the flowering quantitative trait loci (QTL) encodes the blue light receptor *CRYPTOCHROME2* (*CRY2*). The Cvi allele of *CRY2* contains a single amino acid change that leads to a more stable protein. This change affects daylength perception as L*er* plants containing an introgressed *CRY2* from Cvi are daylength-insensitive (El-Din El-Assal *et al.*, 2001). This same collection of RILs was used in a separate experiment to isolate QTL that function in the control of circadian period. The circadian clock is closely linked to the control of flowering and many of the circadian QTL found in this study co-localized with known flowering-time genes (Swarup *et al.*, 1999).

The most striking difference in flowering time among natural populations of Arabidopsis is seen between rapid-flowering and winter-annual accessions. Winter-annual accessions are late flowering unless vernalized. Crosses between plants with these different flowering habits revealed that dominant alleles of two loci, *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) make a major contribution to the delay in flowering in winter annuals (Burn *et al.*, 1993; Clarke and Dean, 1994; Koornneef *et al.*, 1994; Lee *et al.*, 1993, 1994b). *FRI* acts to upregulate *FLC*, a MADS-box transcription factor that is sufficient to repress flowering (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). Vernalization leads to a stable decrease in *FLC* levels. Molecular analysis of *FRI* and *FLC* in rapid-flowering accessions has shown that many contain null mutations in *FRI* (Gazzani *et al.*, 2003; Johanson *et al.*, 2000; Le Corre *et al.*, 2002), and some contain mutations in *FLC* that result in lower expression (Gazzani *et al.*, 2003; Michaels *et al.*, 2003).

Plants that contain an active allele of *FRI* have elevated levels of *FLC*. Flower promotion via *FLC* repression occurs through a number of pathways. Vernalization turns off *FLC* through chromatin remodeling (Bastow *et al.*, 2004; Sung and Amasino, 2004). Another group of genes, the autonomous pathway, acts to repress *FLC* independently of daylength and temperature. Mutations in members of the autonomous pathway are late flowering due to elevated *FLC* levels (Michaels and Amasino, 2001). Some members of the autonomous pathway including *FLD* and *FVE* are required for de-acetylation of *FLC* chromatin (Ausin *et al.*, 2004; He *et al.*, 2003). Many of the other members of the autonomous pathway are genes believed to function in RNA binding and processing. *FY* encodes a 3′ end-processing factor (Simpson *et al.*, 2003), and *FPA*, *FCA*, and *FLK* are all predicted to bind mRNA (Lim *et al.*, 2004; MacKnight *et al.*, 1997; Schomburg *et al.*, 2001). The homeodomain of LUMINIDEPENDENS (LD) may act in RNA or DNA binding (Lee *et al.*, 1994a).

The *HUA2* gene plays a role in RNA processing (Chen and Meyerowitz, 1999; Cheng *et al.*, 2003). In this study we identified *HUA2* as a gene involved in the control of flowering by two independent mutant screens and by studies of natural variation. *HUA2* is a repressor of flowering and appears to function by enhancing the expression of several genes that delay flowering including *FLC*.

Results

The Arabidopsis accession Landsberg erecta *contains an enhancer of* elf4-1

The *EARLY FLOWERING 4* (*ELF4*) gene encodes a small protein that is essential for circadian clock function (Doyle *et al.*, 2002). *elf4-1* mutants in the Ws accession are early flowering in short days (SD) (Figure 1a) and display aberrant circadian outputs. As part of a genetic analysis, crosses were made between *elf4* and mutants in light perception and circadian regulation. Certain mutants were not available in Ws, and thus several crosses were made between *elf4-1* and mutants in L*er*. All F_2 populations resulting from crosses to L*er* contained *elf4* mutants that flowered extremely early (Figure 1b), although the extremely early flowering plants did not correspond to the double mutants. The frequency of the extreme phenotype was approximately 1/16, suggesting that the phenotype was the result of a single recessive locus contributed by L*er*. Therefore, we crossed *elf4-1* to wild-type L*er*. The resulting F_2 population also contained extremely early flowering plants indicating that L*er* contained an enhancer of *elf4*, which we referred to as the *eel* locus.

The *elf4 eel* phenotype was first observed in F_2 plants grown in SD under high-pressure sodium lamps. Under these conditions the *elf4 eel* phenotype was extremely early flowering (Figure 1b). When this same F_2 was grown in SD under fluorescent lamps, the *elf4 eel* plants flowered slightly later but were still much earlier than *elf4* single mutants. This shift to later flowering is observed in wild-type plants as well. All flowering and mapping data presented below were collected from plants grown under fluorescent lights.

The eel *locus encodes* HUA2*, a gene involved in the regulation of a floral homeotic gene*

A backcross of an *elf4*/*eel* plant from the original F_2 population to *elf4-1* in Ws produced F_2 populations that segregated 3 *elf4*-like:1 *elf4*/*eel*. Analysis of 600 F_2 plants from such a population enabled us to narrow the *eel* locus to a 40-kb region that spanned two BAC clones, MYJ24 and MKD15. This region contains the *HUA2* gene (Chen and Meyerowitz, 1999). An *HUA2* clone from the Ws accession was able to rescue the *eel* phenotype (Figure 1c). To further confirm the identity of *HUA2* as the *elf4* enhancer, two additional alleles of *HUA2* were crossed into the *elf4-1* background: the original *hua2-1* allele which disrupts a splice junction at the sixth intron (Chen and Meyerowitz, 1999), and *hua2-4*, a T-DNA allele from Columbia (Figure 1d). In the presence of *elf4-1*, both *hua2* alleles result in an *eel* phenotype (Table 1).

The *HUA2* gene was first characterized in a screen for enhancers of *ag-4*, a weak allele of the floral homeotic gene *AGAMOUS* (*AG*). The original *hua2* allele was isolated in combination with a mutation in another gene, *hua1*. The enhancer of *ag-4* phenotype caused by mutations in *hua2* is more apparent in a *hua1 hua2 ag4* mutant background. However, *hua2-1 ag-4* double mutants do display petaloid stamens and slightly enlarged anthers when compared with *hua2-1* and *ag-4* single mutants (Chen and Meyerowitz, 1999). The HUA2 protein sequence contains an RPR domain, a motif found in proteins that function in RNA metabolism, and HUA2 affects *AG* pre-mRNA processing in certain genetic backgrounds (Cheng *et al.*, 2003).

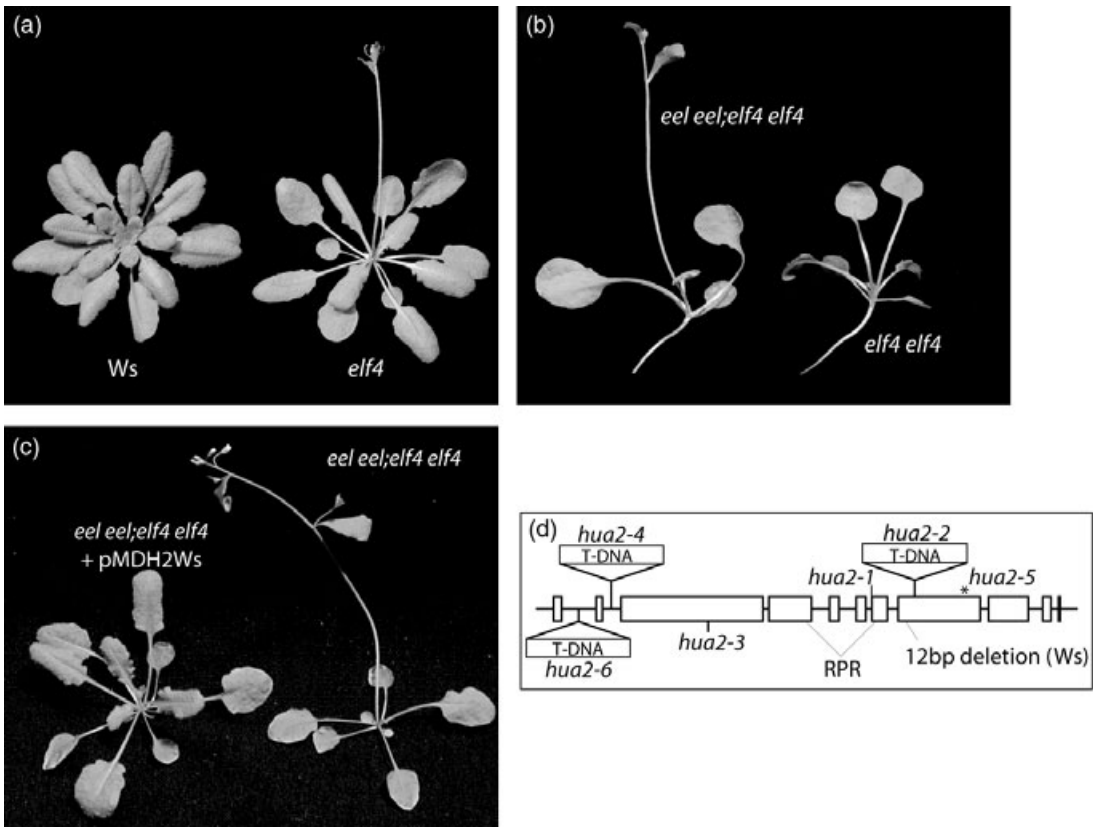

Figure 1. *hua2-5* enhances the *elf4-1* phenotype.
(a) Short day-grown Ws and *elf4-1*.
(b) A plant with the *eel* phenotype and a non-*eel elf4-1* plant from the same segregating F_2 population.
(c) Two plants with the *eel1* genotype: *elf4*/*elf4*; *hua2-5*/*hua2-5*. An extra copy of *HUA2* from Ws (pMDH2Ws) has been added to the plant on the left.
(d) Schematic showing the exon/intron structure of *HUA2* and the position of various mutations within the gene.

The eel *lesion in* HUA2 *is not present in all strains of* L*er*

HUA2 was sequenced from both the Ws and L*er* ecotypes. The sequence data revealed two polymorphisms that resulted in differences between the Ws and L*er* amino acid sequences. The Ws sequence contained a 12-bp deletion relative to L*er* and Col resulting in the removal of amino acids 967–970. The L*er* allele of *HUA2* contained a premature stop codon. This lesion is located in exon 8 and results in the removal of 280 amino acids from the C-terminal end (Figure 1d). Given the recessive nature of the L*er* allele, we concluded that the premature stop codon was the cause of the *eel* phenotype.

To clarify further discussion, our *HUA2* allele containing the premature stop codon will be referred to as *hua2-5*. The truncated protein in *hua2-5*, if it is produced, contains several motifs including a PWWP domain, the RPR domain, and four putative nuclear localization sequences. The 280 C-terminal amino acids missing in *hua2-5* do not contain any recognizable protein motifs.

The *hua2-1* lesion disrupts a splice junction at the sixth intron (Chen and Meyerowitz, 1999). Interestingly the screen that produced the *hua2-1* mutant was carried out in a L*er* genetic background. Given that we detected a stop codon in the *HUA2* gene in L*er*, it was surprising that *hua2-1* was

Table 1 Flowering phenotype of *eel* plants in short day (SD) conditions

	Total leaf number
Ws	38.0 ± 1.8
L*er*	47.3 ± 3.0
elf4-1 (Ws/L*er*)	16.6 ± 3.1
elf4-1 hua2-5 (*eel*) (Ws/L*er*)	8.7 ± 1.9
elf4-1 hua2-1 (Ws/L*er*)	6.4 ± 0.5
Col	64.3 ± 3.1
elf4-1 (Ws/Col)	17.2 ± 2.4
elf4-1 hua2-4 (Ws/Col)	9.0 ± 1.3

Values represent total leaf number ± standard deviation. All plants were grown under SD conditions (8 h light:16 h dark). Total leaf number is equal to the number of rosette leaves plus the number of cauline leaves. At least eight plants were analyzed for each entry. The *elf4-1* (Ws/Col) and *elf4-1 hua2-4* (Ws/Col) represent the two clear early-flowering classes that appeared in this F_2. The frequency of early flowering plants, however, was less than expected suggesting that Col may contain a suppressor of *elf4*.

Table 2 Genotype of various L*er* lines at *HUA2*

L*er* lines containing *hua2-5* (premature stop codon)
*gi-3 (fb)**, *fd**, *ft**, *co-3**, *fy**, *fwa**, *fpa**, CVL135†, CVL40†
L*er* lines containing wild-type *HUA2* (no *hua2-5* lesion)
*fca**, *fve*$^{-}$*, *clf-2* (Goodrich *et al.*, 1997) *ag-4* (Chen and Meyerowitz, 1999)
Accessions containing wild-type *HUA2* (no *hua2-5* lesion)
Cvi, Col, Ws, Ema-1, Seattle-0, Limeport, H55, Petergof, Litva, Oy-1, Bla-2, Nd-1, Ber, Condara, Cnt-1, Di-G, ENF, Li-5, Je54, M3385S, Est, Berkeley, Da(1)-12, Abd-0, Gr3, Co, Shahdara, Wei-0, LIN,

*Lines believed to be those described in Koornneef *et al.* (1991) *Mol. Gen. Genet.* **229**, 57–66.
†Individual recombinant-inbred lines from the L*er* × Cvi population that are homozygous L*er* at *HUA2* (Alonso-Blanco *et al.*, 1998).
Genotype of various L*er* lines at the *HUA2* locus.

isolated in L*er*. We sequenced exon 8 from *hua2-1* and did not find the stop codon. This indicates that the parental L*er* line of *hua2-1* contains a polymorphism with our L*er* strain containing *hua2-5*. The *hua2-5* base change was not present in any other Arabidopsis accessions that were examined (Table 2). However, we did find examples of L*er* lines that contained the *hua2-5* lesion including the L*er* used in the L*er* × Cvi RIL population (Table 2) (Alonso-Blanco *et al.*, 1998).

hua2 *mutants flower early in the absence of* elf4-1 *mutations*

hua2-5 was identified due to its ability to enhance the *elf4-1* phenotype. Thus, it was of interest to see whether this effect on flowering time was *elf4*-specific or whether mutations in *hua2* affected flowering in the absence of *elf4*. A Col insertion allele, *hua2-4*, flowered early under both LD and SD relative to wild type. Both *hua2-1* and *hua2-5* in L*er* also flowered early in SD but flowered similar to wild type in LD (Figure 2a). *hua2-1* flowered earlier than *hua2-5* suggesting that *hua2-5* is a weaker allele. *hua2-1* plants were crossed to both *hua2-5* and L*er*. F_1 plants from *hua2-1* × *hua2-5* flowered with an intermediate number of leaves. F_1 plants from the *hua2-1* × L*er* flowered similar to L*er* (Figure 2a). Thus, *hua2-1* is recessive when paired with a wild-type allele in L*er*. Other strong *hua2* alleles in Ws and Col exhibit a heterozygous phenotype (see below). The lack of a heterozygous phenotype with *hua2-1* in L*er* may be due to the L*er* genetic background or a unique feature of the *hua2-1* allele.

In addition to isolating *hua2* as an *elf4* enhancer, a *HUA2* mutant, *hua2-6*, was recovered in a screen for early-flowering mutants in the Ws background. Plants homozygous for the *hua2-6* mutation flowered earlier than wild type under both LD and SD, but the difference was more pronounced under SD. Plants heterozygous for *hua2-6* flower with an intermediate number of leaves when compared with *hua2-6* homozygous and wild-type plants (Figure 2b).

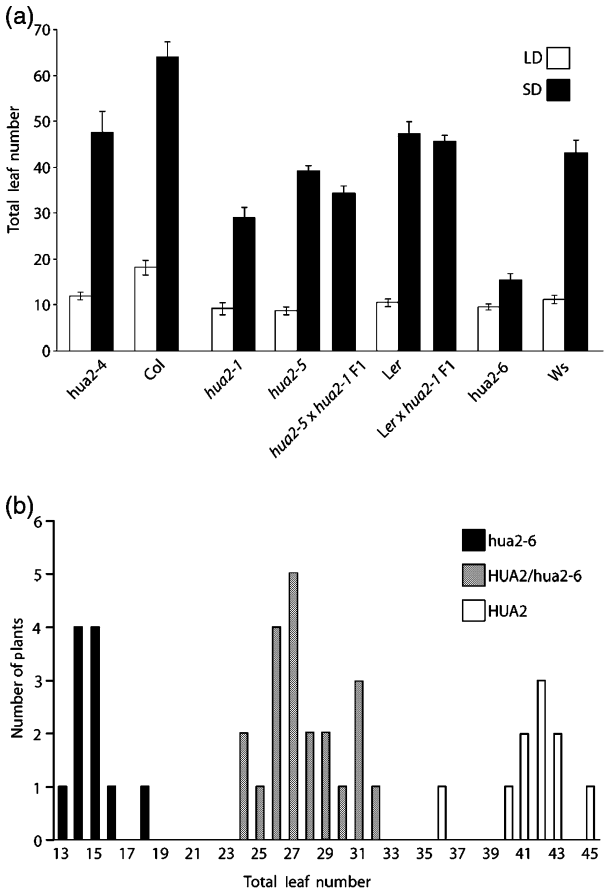

Figure 2. Effect of *hua2* mutants on flowering in long day (LD) and short day (SD) conditions.
(a) All *hua2* alleles tested flower earlier than the respected wild type: *hua2-4* (Col), *hua2-1* and *hua2-5* (L*er*), *hua2-6* (Ws). White bars represent the total leaf number (rosette + cauline) in plants grown in LD. Black bars represent the total leaf number in plants grown in SD. Error bars represent ±standard deviation.
(b) Distribution of a population that segregates for hua2-6 grown in SD. Black, gray, and white bars represent hua2-6 homozygotes, heterozygotes, and wild type, respectively.

HUA2 *plays a role in the regulation of the floral repressor* FLC

Columbia plants containing an active *FRI* gene from the San Feliu-2 ecotype (*FRI*-Col) show a delay in flowering unless vernalized (Lee *et al.*, 1994a). In addition to our identification of one *hua2* allele (*hua2-5*) as an enhancer of *elf4* and another (*hua2-6*) as a mutant that flowers early in SD, two additional *hua2* alleles were uncovered as suppressors of *FRI*-mediated late flowering. *hua2-2* contains a T-DNA in exon 8 (Figure 1d). *hua2-3* was recovered from a fast neutron population and contains an insertion in exon 3. A wild-type copy of the *HUA2* gene restored *FRI*-like late flowering when introduced into the *hua2-2* and *hua2-3* mutant backgrounds (data not shown).

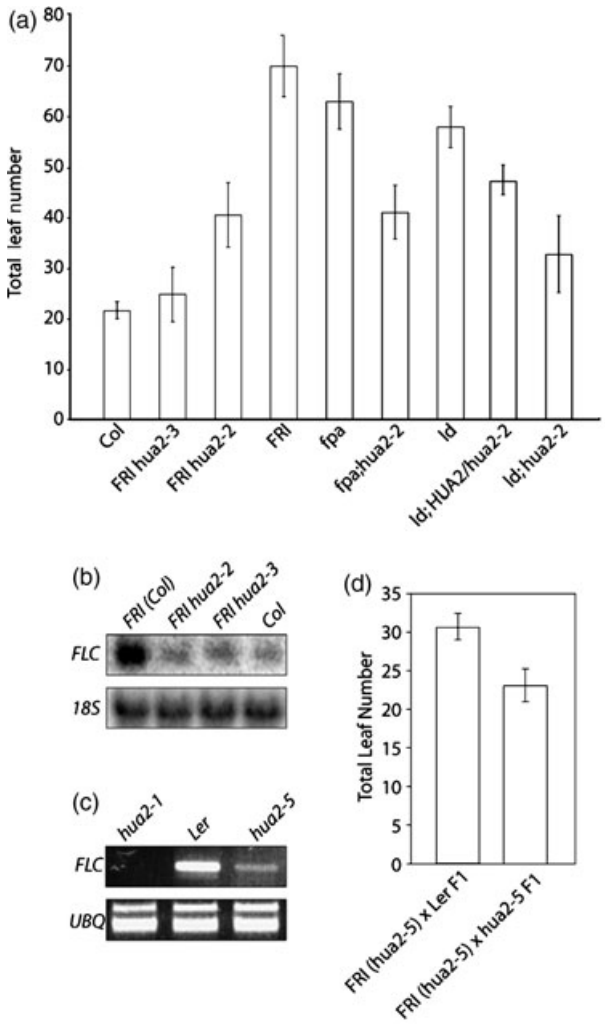

Figure 3. Suppression of *FLC*-mediated late flowering by mutations in *HUA2*.
(a) *hua2* mutants suppress late flowering in *FRI*-containing plants as well as autonomous pathway mutants.
(b) *hua2* mutations reduce the steady-state level of *FLC* mRNA in an *FRI* background. The blot was probed first for *FLC* and then with 18S rRNA as a loading control.
(c) *hua2* mutations in L*er* reduce steady-state levels of *FLC* mRNA. *Ubiquitin* (*UBQ*) was used as a control.
(d) The *hua2-5* mutation suppresses the effect of *FRI* in a L*er* background. Error bars represent ±standard deviation. Plants in (a) and (d) are in the Col and L*er* backgrounds, respectively. All plants were grown under long days.

hua2-2 and *hua2-3* vary in their ability to suppress *FRI*-mediated late flowering. *hua2-3* suppressed *FRI* to a greater extent than *hua2-2* (Figure 3a). The *hua2-3* lesion occurs near the N-terminal end of the protein whereas *hua2-2* contains a mutation in exon 8 and may produce a truncated but partially functional protein product. The relative strength of the *hua2-3* and *hua2-2* lesions with respect to *FRI* suppression is reminiscent of the relative strength of *hua2-1* and *hua2-5* in L*er*, which also truncate the protein at different places.

Plants containing *FRI* are late flowering due to elevated levels of *FLC* (Michaels and Amasino, 2001). The fact that *hua2* mutations partially suppress *FRI*-mediated late flowering suggests that *hua2* mutations may alter *FLC* levels. RNA blot analysis revealed that *FLC* levels are in fact reduced in *FRI* plants containing *hua2* mutations (Figure 3b). There was also reduced expression of *FLC* in L*er* lines containing *hua2* lesions. In L*er*, *FLC* levels are low due both to the lack of *FRI* activity (Johanson *et al.*, 2000) and to the insertion of a transposable element in the first intron of *FLC* (Michaels *et al.*, 2003). Thus, RT-PCR was used to determine the effect of *hua2* mutations on *FLC* levels in L*er*. *FLC* was reduced in *hua2-1* compared with wild type. Consistent with the flowering phenotype, *hua2-5* mutants showed an intermediate level of *FLC* (Figure 3c).

Genes in the autonomous pathway act to repress *FLC* expression. Mutations in any member of this pathway result in late flowering due to elevated levels of *FLC* (Michaels and Amasino, 2001). Mutants in two members of the autonomous pathway, *fpa* and *ld*, were crossed to the *hua2-2* mutants to evaluate whether *hua2* could suppress the late flowering effect of autonomous-pathway mutants. In both cases, late flowering was partially suppressed (Figure 3a).

The effect of *hua2-5* on *FRI*-mediated late flowering in L*er* was tested genetically. A previously reported L*er* line containing an introgressed copy of *FRI* is also homozygous for the *hua2-5* lesion (Lee *et al.*, 1994a). This line was crossed to the strain of L*er* with a lesion-free *HUA2* gene and to *hua2-5*. Due to the dominant nature of *FRI*, F_1 plants could be evaluated for flowering time. Plants containing a wild-type allele of *HUA2* (*FRI*/*fri*; *HUA2*/*hua2-5*) flowered later than plants homozygous for *hua2-5* (*FRI fri*; *hua2-5 hua2-5*) (Figure 3d).

HUA2 *affects flowering independently of* FLC

Mutations in *hua2* lead to a decrease in *FLC*, which likely contributes to the early flowering phenotype of *hua2* mutants. Although *flc* null mutants flower slightly earlier than wild type in SD (Michaels and Amasino, 2001), *hua2* mutants flower even earlier than *flc* null mutants (Figure 4a). In addition, *hua2* mutations suppress the late flowering of *co* mutants in a dose-dependent manner (Figure 4b). Mutations in *flc* have no effect on the *co* mutant phenotype (Michaels and Amasino, 2001). Thus, the overall effect of *HUA2* on flowering time cannot be entirely explained through its interaction with *FLC*. *HUA2* must also interact with other components of flowering time regulation.

An additional candidate for *HUA2* interaction is *FLOWERING LOCUS M* (*FLM*/*MAF1*). *FLM* is a MADs box gene that is closely related to *FLC* both at the amino acid level and in exon/intron gene structure (Ratcliffe *et al.*, 2001; Scortecci *et al.*, 2001). In addition, *FLM*, like *FLC*, acts as a negative regulator of the floral transition. Like *hua2* mutants, *flm*

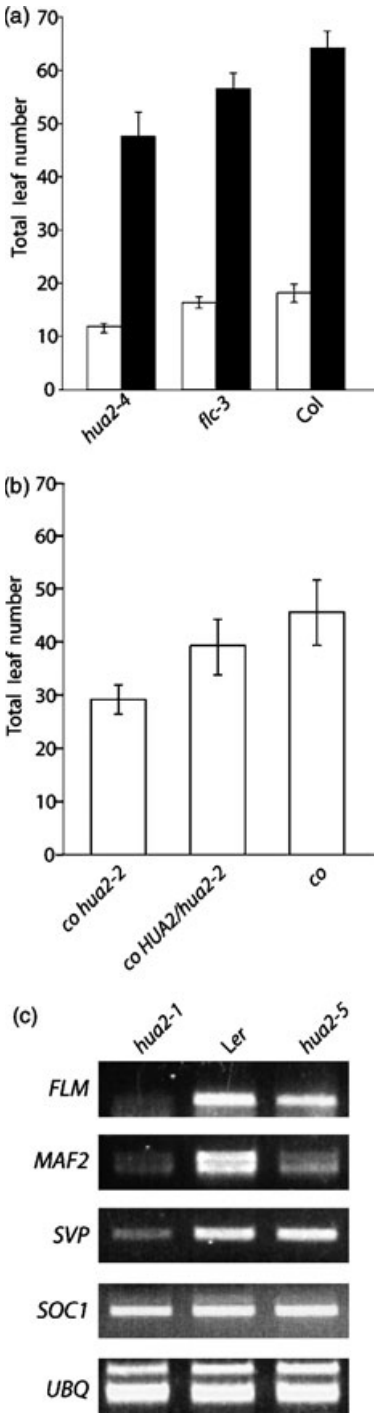

Figure 4. Interaction between *HUA2* and floral regulators other than *FLC*.
(a) *hua2* mutants flower earlier than an *flc* null mutant in both long day and short day plants.
(b) *hua2* mutations suppress the effect of *co* mutations.
(c) *hua2* mutations in L*er* reduce the mRNA levels of *FLM*, *SVP*, and *MAF2*. The two bands seen with *MAF2* represent two splice variants. *UBQ* was used as a control. Bands for *SOC1* represent basal levels of gene expression. The tissue was harvested prior to the increase in *SOC1* seen at the time of floral transition.

mutants have a pronounced early-flowering phenotype in SD compared with wild type, and *flm* mutations, like *hua2* mutations, partially suppress *co* (Scortecci *et al.*, 2003). *FLM* RNA levels were examined in L*er*, *hua2-1*, and *hua2-5* seedlings grown under SD. Like *FLC*, *FLM* was reduced in the *hua2-1* mutant compared with wild type. *hua2-5* mutants displayed an intermediate level of *FLM* expression (Figure 4c). Thus, early flowering in *hua2* mutants is due, at least in part, to the combined reduction in *FLC* and *FLM* expression. Expression of a third member of the *FLC* clade, *MAF2*, is also reduced by *hua2* mutations in a manner similar to *FLC* and *FLM* (Figure 4c).

In addition to *FLC*, *FLM*, and *MAF2*, the expression of two additional MADS genes involved in flowering time regulation were studied. *SHORT VEGETATIVE PHASE* (*SVP*) is a repressor of flowering (Hartmann *et al.*, 2000). Like mutants in *flm*, *svp* mutants are early flowering, and the early-flowering phenotype is most apparent in SD. *hua2-5* mutations did not appear to have an effect on *SVP* levels. *SVP* levels were, however, reduced in a *hua2-1* background although the degree of reduction was less than that seen for genes in the *FLC* clade (Figure 4c). *SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) is a promoter of flowering (Borner *et al.*, 2000; Lee *et al.*, 2000; Onouchi *et al.*, 2000). Mutations in *hua2* did not alter the expression of *SOC1* (Figure 4c).

Discussion

The switch from vegetative growth to flowering results from the accurate perception of specific environmental signals such as photoperiod and prolonged periods of cold temperature. The environmental parameters that define the optimal time to flower can vary substantially throughout the geographic range of a species, especially one as vast as that of Arabidopsis. Arabidopsis accessions collected from a variety of habitats provide a wealth of variation that can be used for gene discovery.

Using natural variation as a tool for gene discovery has revealed several genes involved in regulating flowering time. For example, crosses between winter-annual and rapid-flowering accessions led to the identification of *FRI* and *FLC*, two loci required for the winter-annual habit (Michaels and Amasino, 2000). Although the loss of *FRI* results in a major switch in reproductive strategy (Johanson *et al.*, 2000), most polymorphisms in flowering-time genes among natural populations are likely to result in a far subtler effect.

Here we identified an *elf4-1* enhancer locus as *HUA2* in a cross between the *elf4* mutant in Ws and one strain of L*er*. The ability to isolate the *hua2-5* lesion was facilitated by evaluating natural variation in the *elf4* mutant background because *hua2-5* caused extremely early flowering when combined with *elf4-1*. However, *hua2-5* mutants alone flower earlier than wild type, and thus, the effect of the

hua2-5 lesion on flowering in the absence of *elf4* could be detectable by standard QTL analyses. In fact, the L*er* strain used to create the L*er* × Cvi RILs contains *hua2-5*, and a flowering QTL identified from these lines, *FLG*, co-localizes with *HUA2* (Alonso-Blanco *et al.*, 1998).

In an *elf4* background, *hua2-5* homozygous mutants may be more apparent because, by attenuating the photoperiod response, the *elf4* mutation decreases the overall number of genes contributing to floral regulation. None of the genes affected by *HUA2* are major factors in the regulation of circadian rhythms and thus the *eel* phenotype results from the attenuation of *ELF4*-independent pathways. A *hua2* mutant may be more pronounced in an *elf4-1* background because an *ELF4*-dependent pathway can no longer compensate for the lack of *HUA2*. Therefore, studies of natural variation that begin with a mutant background could serve to enhance the effect of QTL or reveal additional loci that might not be apparent in wild-type backgrounds. On the other hand, such an approach might result in the loss of loci detectable in a cross between wild types. If, for example, genetic differences between Ws and L*er* affect an *ELF4*-dependent process, one might expect the effect of this polymorphism to be masked in an *elf4* background.

Whether *hua2-5* resulted from natural variation or an induced mutation is not known. The *erecta* mutation is the result of a gamma ray-induced mutagenesis and thus the *hua2-5* lesion could have been induced. Alternatively the original Landsberg strain was not isogenic (Rédei, 1992) and could have contained both *HUA2* alleles. If the original *erecta* strain was heterozygous for the *hua2-5* mutation, the two alleles of *HUA2* could have been fixed in different single seed lineages. It is also possible that the *hua2-5* lesion has arisen more than once and was inadvertently selected due to its subtle affect on the regulation of flowering time. To date, we have not found other accessions of Arabidopsis that contain the *hua2-5* lesion, although we only analyzed a subset of available accessions. However, natural variation at a different site within the *HUA2* gene has been uncovered in another study (V. Grbic, University of Western Ontario, Ontario, Canada, personal communication) suggesting that a *HUA2* variant may have been a target for natural selection at least one other time.

The *HUA2* gene was originally isolated in a screen for enhancers of a weak allele of *AG*, *ag-4* (Chen and Meyerowitz, 1999). The *HUA1* gene, which encodes a putative RNA-binding protein, was also isolated in this screen. *hua1-1 hua2-1 ag-4* triple mutants resemble strong *ag* alleles, but *hua2-1* alone in an *ag-4* background has only a slight enhancing effect. In the absence of *ag-4*, *hua2-1* mutants do not alter floral morphology. Thus, the only phenotype of a *hua2* single mutant reported to date is the alteration of flowering time.

The *AG* gene contains a 3-kb intron. In wild type improperly spliced *AG* transcripts containing regions of this large intron can be seen at low levels (Cheng *et al.*, 2003). In *hua1-1 hua2-1* double mutants these aberrant transcripts accumulate to higher levels than in wild type. Thus, *HUA1* and *HUA2* appear to have a role in processing *AG* pre-mRNA. The gene structure of *AG* has several similarities with the floral repressor *FLC* and other members of the *FLC* clade. These genes encode type II MADS-box transcription factors and contain one large intron. However, unlike the case with *AG*, *FLC* transcripts containing regions of the large intron are not found in wild type or in the *hua1 hua2* double mutant background (Cheng *et al.*, 2003). Thus, there is no evidence that *HUA1* and *HUA2* affect *FLC* splicing, although it possible that aberrantly spliced *FLC* transcripts are turned over too rapidly to be detectable.

In the *hua1 hua2* double mutant background, both *FLC* and *AG* levels are reduced, but *FLC* is reduced to a much greater extent (Cheng *et al.*, 2003). We show that *hua2* single mutants have a large effect on *FLC* mRNA levels and thus the decrease in *FLC* levels previously reported in the *hua1 hua2* double mutant is due primarily to *hua2*. In contrast, the *hua2* single mutant does not appear to affect *AG* mRNA levels (Cheng *et al.*, 2003). In addition, *hua1* mutants in combination with *elf4* do not result in an *eel* phenotype indicating that *HUA1* does not have a role similar to that of *HUA2* in the control of flowering time (M.R. Doyle and R.M. Amasino, unpublished data). Thus, *HUA2* may affect *AG* and *FLC* expression by entirely different mechanisms.

Downregulation of *FLC* only partially explains the early-flowering phenotype in *hua2* mutants. *hua2-4*, a T-DNA mutant in Columbia, flowers earlier than an *flc* null mutant indicating that mutations in *hua2* do more than simply reduce *FLC* levels. *FLM* is a repressor of flowering that is closely related to *FLC* in amino acid sequence and has a similar gene structure containing a large first intron (Ratcliffe *et al.*, 2001; Scortecci *et al.*, 2001). As with *FLC*, *hua2* mutations also result in decreased *FLM* expression. This is also the case with *MAF2*, another gene in the *FLC* clade, although the differences in *MAF2* expression between different *hua2* alleles appear subtler than those seen for *FLC* and *FLM*. Another MADs box gene that represses flowering, *SVP*, is not affected in a *hua2-5* background, but does appear to be downregulated in the *hua2-1* background. Whether the early flowering of *hua2* mutants might result from effects on genes in addition to those identified din this study remains to be determined.

The MADS genes *FLC*, *FLM/MAF1*, *SVP*, and *MAF2* all act as repressors of the floral transition; however, these genes act in different pathways of floral regulation. *FLC* is involved primarily in the vernalization response (Michaels and Amasino, 1999; Sheldon *et al.*, 1999). *flc* mutants are early flowering in non-inductive SD, but the effect is not as great as that seen in *flm* and *svp* mutants (Hartmann *et al.*, 2000; Michaels and Amasino, 2001; Scortecci *et al.*, 2001). *MAF2* represses flowering in response to short periods of cold

temperatures (Ratcliffe *et al.*, 2003). Because *hua2* mutations affect the expression of all these genes, *HUA2* either interacts with these genes directly or interacts with an unidentified factor or factors that regulate this group of transcription factors.

The genes that appear to be affected the most by *HUA2* (*FLC*, *FLM*, and *MAF2*) are closely related to one another. Thus, although the specific function of these genes in floral regulation has diverged over time, they still share some of the same regulatory components such as *HUA2* and members of a *PAF*-complex that mediate gene activation (He *et al.*, 2004). The related primary amino acid sequence and gene structure of *FLC*, *FLM*, and *MAF2* indicate that these genes arose from a common ancestral gene that most likely required *HUA2* for expression. Thus, these genes have maintained some regulatory pathways in common despite their functional divergence into different flowering pathways, and mutations in common regulators of these genes such as *hua2* will have a broad-flowering phenotype.

Several other genes have been described that affect flowering via both *FLC*-dependent and *FLC*-independent pathways. These genes include a putative RNA-binding protein, *EARLY FLOWERING 5* (*ELF5*) (Noh *et al.*, 2004); the putative chromatin remodeling proteins *PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1* (*PIE1*) and *EARLY FLOWERING IN SHORT DAYS* (*EFS*) (Noh and Amasino, 2003; Soppe *et al.*, 1999; S.D. Michaels and R.M. Amasino, unpublished data); and a SUMO protease, *EARLY IN SHORT DAYS 4* (*ESD4*) (Murtas *et al.*, 2003; Reeves *et al.*, 2002). It is of interest to determine whether the *FLC*-independent role of these genes in flowering-time regulation is similar to that of *HUA2*. Like *HUA2*, these genes may affect the expression of several floral suppressors including *FLM*, *MAF2*, and *SVP*.

Experimental procedures

Plant materials and growth conditions

The *elf4-1* mutant is in the Ws background and has been previously described (Doyle *et al.*, 2002). The *hua2-1* strain and the strain referred to as L*er* have been described previously (Chen and Meyerowitz, 1999). Other accessions were obtained from the Arabidopsis stock center (http://www.arabidopsis.org). *hua2-4* was isolated from the Salk Institute Genome Analysis Laboratory (SIGnAL) T-DNA collection (reference number:SALK_032281; http://signal.salk.edu/cgi-bin/tdnaexpress). The *hua2-3* fast neutron allele and the *hua2-2* T-DNA allele were isolated from mutagenized populations in a Columbia background containing *FRI*. *hua2* mapping and flowering time analyses were conducted on plants grown in either LD or SD, 16 h light:8 h dark and 8 h light:16 h dark, respectively, under cool-white fluorescent light unless mentioned otherwise. Flowering time data are presented as total leaf number (rosette + cauline) as no difference in the ratio of rosette to cauline leaves was observed in any line.

Cloning and genotyping of HUA2

HUA2 was cloned from the Ws ecotype using PCR. The clone consisted of 2 kb of upstream sequence, the *HUA2* genomic sequence, and 0.8 kb of downstream sequence. This sequence was cloned in two fragments using an internal *Bam*HI site. Primers used to amplify these two fragments were as follows: HUA2L1: 5′-AAAAGCTTCGCTATATGCCACTGCTTTG-3′, HUA2R1: 5′-CTAATTTGGGGAAGCAAGGA-3′, HUA2L2: 5′-CTTTGGGCGATGAGGATTC-3′, HUA2R2: 5′-AAACTCGAGGCAGCGAGACATAACTT-3′. Restriction sites existing at the 5′ends of HUA2L1 and HUA2R1 were used in conjunction with the native *Bam*HI site to clone both fragments into the pPZP221B binary vector (Kang *et al.*, 2001).

The premature stop codon in *hua-5* was detected using the following oligo sequences: HUALerL: 5′-CTTCACAATCATTAACAACTCAG-3′ and HUA LerR: 5′-TGCTGCATAGATCCTGGGTA-3′. These primers produced a 110-bp fragment spanning the region containing the polymorphism for which the sequence was determined.

Expression analysis

Total RNA was isolated from 7-day-old seedlings using TRI Reagent (Sigma, St Louis, MO, USA). Complementary DNA was synthesized using 2 μg of total RNA, an oligo dT primer and Superscript II reverse transcriptase (Gibco, Carlsbad, CA, USA). *FLC*, *FLM*, *MAF2*, *SVP*, *SOC1*, and *UBQ10* were detected by RT-PCR as previously described (Doyle *et al.*, 2002; Michaels *et al.*, 2003; Ratcliffe *et al.*, 2003; Scortecci *et al.*, 2001). RNA blots contained 15 μg of total RNA per lane on a denaturing formaldehyde gel (1% agarose). RNA was transferred to a nylon membrane and probed with *FLC*.

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