

Chapter 2. A role for the *EEP1* miRNA in *Arabidopsis* flower development

2.1 Introduction

In many plant species, the petals of the flower are the principal visual attractant for pollinators, so petal color, number, and arrangement are critical for reproductive success. In order to examine the genetic basis of petal number and position, we have taken advantage of the powerful genetics and molecular biology of *Arabidopsis thaliana*.

The *Arabidopsis* flower has a stereotyped pattern of four concentric whorls consisting of four sepals, four petals, six stamens, and two fused carpels (Figure 2.1, A and B). Sepals form first, at four positions: adaxially (adjacent to the stem), abaxially (opposite the stem), and two in lateral positions. Petals form at alternating positions relative to the sepals (in other words, no petal arises just interior to a sepal). The six stamens emerge at positions analogous to the sepals: two pairs of long medial stamens, and one lateral stamen on each side. Although *Arabidopsis* is self-pollinating and has very unassuming flowers, there is evidence that the petals enjoy special status in terms of the regulation of their number and position. There are several known mutations which affect only petal number, or result in opposing effects on the number of the other organ types.

There are several possible constraints on petal number. Genetic data suggest that a sufficient supply of cells is required for a primordium to form at a given location. Other crucial inputs likely include the positions of the sepals and the location of any petal primordia already formed or forming. These last two factors are probably interdependent in the determination of petal number, since

no mutants have been observed that show defects in just one mechanism (Figure 2.1, C and D).

The requirement for a sufficient pool of cells for primordium initiation is illustrated by a class of mutants that affect the balance of cell proliferation and differentiation in shoot and floral meristems. This set of mutants includes *clavata1* (*clv1*), *clavata3* (*clv3*), and *wuschel* (*wus*). *clv1* and *clv3* plants have taller shoot and floral meristems, resulting in broader stems and extra floral organs in all four whorls, with the most dramatic increase in the fourth whorl (Clark et al., 1993; Clark et al., 1995). Conversely, *wus* meristems are smaller and flatter, due to a reduced stem cell population, and the numbers of flowers and floral organs are reduced, particularly in the inner two whorls (Laux et al., 1996). The three corresponding wild-type proteins are known to act in a negative feedback loop to regulate meristem size. CLV1 and CLV3 constitute a likely receptor-ligand pair (Clark et al., 1997; Fletcher et al., 1999) that inhibit cell proliferation in the meristem. Signaling through CLV1 indirectly represses the expression of *WUS*, which encodes a relative of the homeodomain proteins (Mayer et al., 1998). *WUS* activity in turn is required and sufficient for expression of *CLV3* in the stem cells above the *WUS* expression domain (Brand et al., 2000; Schoof et al., 2000).

Additional mutants with similar phenotypes have also been characterized, although they are believed to act in pathways independent of CLV1/CLV3/WUS. Both *ultrapetala*(*ult*) and *enhanced response to abscisic acid/wiggum*(*era1/wig*) mutants have wider meristems and more floral organs (particularly sepals and petals) than wild-type (Fletcher, 2001; Running et al., 1998). *ERA1/WIG* encodes the beta subunit of farnesyltransferase, implying that post-translational modifications such as farnesylation may be important for

proper signaling within the meristem (Cutler et al., 1996; Ziegelhoffer et al., 2000). *ULT* encodes a novel protein with no recognizable motifs (J. Fletcher, personal communication).

There are several mutants which do not appear to affect cell number in the meristem, but which show whorl-specific alterations in organ number. This group of mutants includes *perianthia* (*pan*), *ettin* (*ett*), *pinoid* (*pid*), *pinformed* (*pin1*), and *pinhead* (*pnh*) (Bennett et al., 1995; Lynn et al., 1999; Okada et al., 1991; Running and Meyerowitz, 1996; Sessions et al., 1997). However, there is no evidence yet to suggest that the gene products disrupted by these mutants act with each other in common pathways.

The flowers of *pan-2* mutants have 4–5 sepals, 4–5 petals, 4–7 stamens, and the normal number of carpels (two). A subset of the flowers on each inflorescence have pentameric symmetry in the first three whorls (Running and Meyerowitz, 1996). Some flowers have four sepals and five petals, or five sepals and four petals, suggesting that the phenotype is not simply a domino effect after sepal primordia are initiated incorrectly. The same is true for *ett* loss-of-function mutants, which occasionally make five sepals and/or five petals in a single flower. Both PAN and ETT are involved in transcriptional regulation: PAN belongs to the basic region / leucine zipper (bZIP) family of transcription factors, while *ETT* encodes ARF3, a member of a family of auxin response factors which act to activate or repress transcription of target genes downstream of auxin signaling (Sessions et al., 1997; Ulmasov et al., 1997). One or both of these proteins could be part of a transcriptional network required for the production or reception of a cell-to-cell signal that allows primordia to form in the correct positions.

Plants mutant for *pid* and *pin* have flowers with extra petals, but fewer sepals, stamens, and carpels. There are slight differences within the second whorl, however: *pin1* petals vary greatly in width, with individual petals often two or three times wider than normal. For both *pin1* and *pid* flowers, the positions of the organs in the first three whorls are disrupted—in other words, the organs are not symmetrically distributed within their whorl. The most dramatic defect in both mutants (and the one for which they are named) occurs not in the flower, but in the shoot: *pin1* and the strongest *pid* mutant (*pid-9*) have defects in organ production on the flanks of the meristem, leading to disordered rosettes and naked, pin-shaped inflorescences. The combination—in two different mutants—of defects in organ formation in the shoot, and defects in floral organ number and position, suggest that the shoot and flower may rely on a common mechanism for setting up proper phyllotaxy.

Plants lacking PIN1 activity form tall, pin-like inflorescences which produce few or no late-arising flowers, often from axial rather than primary shoots (Bennett et al., 1995; Okada et al., 1991). The addition of the plant hormone auxin to any lateral position on the *pin1-6* apex results in organ outgrowth at that position; application of auxin to the top of the *pin1-6* apex causes tissue outgrowth in a ring (Reinhardt et al., 2003). The primary biochemical defect of *pin1* mutants is the absence of polar auxin transport (PAT), the mechanism by which auxin is actively directed to specific tissues. The *pin1* phenotype can be mimicked by treatment of wild-type plants with chemical PAT inhibitors (Okada et al., 1991). The PIN1 protein is a putative auxin efflux carrier: it is homologous to bacterial and eukaryotic transmembrane transporters, is found in the plasma membrane, and shows polar localization in the direction

of auxin flow (Galweiler et al., 1998). PIN1 localization in the inflorescence meristem is dynamic, with high PIN1 activity directing the transport of auxin to the apical ends of primordia (Reinhardt et al., 2003).

The strongest mutant allele of *PID*, *pid-9*, forms an inflorescence much like that of *pin1* (Christensen et al., 2000), whereas other strong and intermediate alleles allow the production of 1–15 flowers before the primary inflorescence terminates in a pin. Even the *pid* mutants which are capable of making several flowers have disrupted inflorescence phyllotaxy, with multiple flowers arising at the same height on the stem. *pid* mutants have no vegetative or root phenotypes, although a portion of *pid* embryos develop three cotyledons instead of two (Bennett et al., 1995). Recent experiments on the *pid-9* shoot have shown that adding auxin to the top of the pin-shaped apex causes production of distinct organs, not the ring-shaped structure induced in the same assay on *pin1*. This response requires that the ectopic auxin be in a form that can be actively transported; application of 2,4-D, a synthetic auxin analog, caused a ring-shaped structure to form (Reinhardt et al., 2003). These data suggest that the *pid-9* apex is still competent to respond to auxin, and also still capable of polar transport. The exact function of *PID* remains elusive, however. *PID* encodes a serine-threonine kinase, which, when overexpressed, causes defects in the two tissues where there is no loss-of-function phenotype—roots and vegetative tissues. A range of experiments investigating the behavior of *35S::PID* in roots (either alone or after treatments with PAT inhibitors) have resulted in two separate interpretations of *PID* function: positive regulation of PAT (Benjamins et al., 2001) or negative regulation of the auxin response (Christensen et al., 2000).

In this chapter, we discuss a novel loss-of-function mutant called *early extra petals*, or *eep1*, whose early flowers have significantly more petals than wild-type. In double mutants, *eep1* causes interesting and divergent effects on *pid* and *pin1*. The *EEP1* gene was mapped, cloned, and found to consist of a microRNA very similar to *MIR164a* and *b* (Reinhart et al., 2002). *EEP1*, like *MIR164*, is predicted by sequence analysis to target the transcripts of six genes in the NAC family of transcription factors (Rhoades et al., 2002). This group of 6 genes includes *CUC1* and *CUC2*, two functionally redundant genes known to be required for proper organ separation in the embryo and the flower (Aida et al., 1997; Takada et al., 2001). When *EEP1* is constitutively overexpressed, the resulting phenotype bears a strong resemblance to the *cuc1; cuc2* double mutant in all but the fused-cotyledon defect in the embryo (Aida et al., 1997). Indeed, both *35S::EEP1* and *cuc1; cuc2* have a reduced number of petals, suggesting that *CUC1* and *CUC2* may be the endogenous targets of *EEP1* within the second whorl. *CUC1* and *CUC2* transcripts are less abundant in the single strong *EEP1* overexpression line tested in comparison to wild type, implying that *EEP1* can act to promote cleavage of the *CUC1* and *CUC2* mRNAs. In summary, this research reveals a role for miRNA-mediated regulation of floral organ number and position.

2.2 Results

The *eep1* mutant has extra petals in early flowers

early extra petals (eep1) was identified as a spontaneous recessive mutation in the Landsberg-*erecta* (*L-er*) background. *eep1* plants have more petals per flower

than wild-type, particularly in the first 10 flowers (5.76 ± 0.12 vs. 4.0 ± 0 ; Figure 2.1 E and F). The extra petals in *eep1* are found in the correct whorl, but are disorganized (as opposed to symmetric) in distribution. Occasional petals are up to 50% wider than normal. To test whether the *eep1* phenotype was whorl-specific or organ-specific, we crossed it to *apetala3-3* (*ap3-3*), in which the petals are transformed into sepals in the second whorl. The *eep1; ap3-3* double mutant showed only a very mild increase in second-whorl sepals (data not shown), suggesting that the *eep1* phenotype actually requires wild-type *AP3* function, and therefore proper petal identity as well.

Since the neighboring whorls in *eep1* flowers are completely unaffected, two possible explanations for the petal phenotype can be ruled out. For example, if the extra petals resulted from a homeotic mutation, another organ type (stamens or sepals) should be completely missing. If the phenotype was caused by a shift in a whorl boundary (to expand the second whorl at the expense of the first or third), organ number in the compacted whorl would be reduced.

In addition to the petal number phenotype, *eep1* also causes a slight defect in septum formation. This defect is most apparent after the dried silique (seed pod) shatters (Figure 2.1, G and H). Otherwise, vegetative development is indistinguishable from wild-type, and fertility is normal as well. The roots of *eep1* are indistinguishable from wild-type when grown vertically on MS plates, on the basis of primary root length and the number of lateral roots (data not shown).

To learn more about any possible signal transduction pathway through which *EEP1* might act, we constructed double mutants with *eep1* and several of the extra-organ or extra-petal mutants mentioned above.

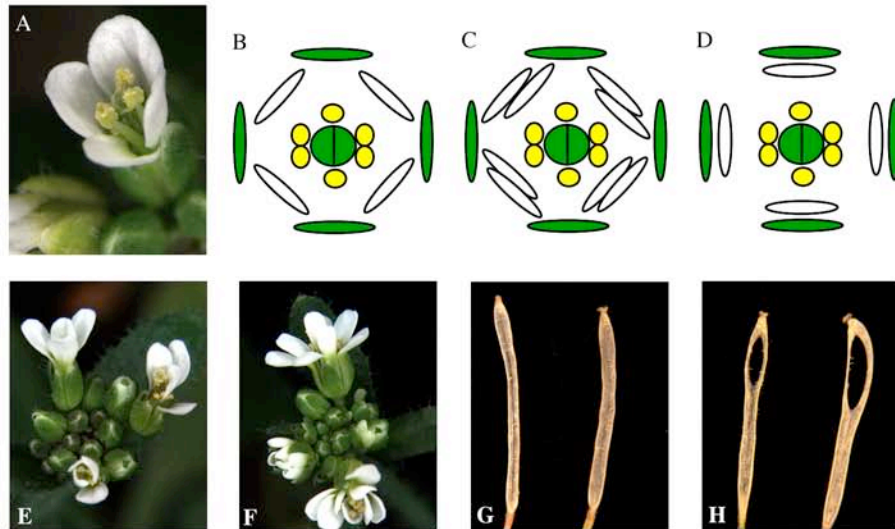


Figure 2.1 *eep1* has extra petals in early flowers and slightly defective septa.

A) *L-er* flower.

B) Diagram of wild-type flower. From outside: sepals, petals, stamens, and two fused carpels.

C, D) Diagrams of two phenotypes predicted for petals if position within the whorl and position relative to the preceding whorl were independent (see text).

E) *L-er* inflorescence.

F) *eep1* inflorescence, showing extra petals in several flowers.

G, H) Septa of dried, mature siliques. G) *L-er* H) *eep1*. Note the defect in apical septum formation in *eep1*.

Nearly all combinations of *eep1* with other extra-petal mutants affect at most the second and fourth whorls.

Almost all of the phenotypes of *eep1* double mutants are not new or unexpected; the changes wrought by *eep1* on each single mutant are (with two exceptions, discussed later), restricted to the second and fourth whorls. For example, the *clv1-1* single mutant has extra organs in all 4 whorls, and the presence of *eep1* in the double mutant merely gives rise to additional extra petals, plus partially unfused carpels. Most of these genetic interactions therefore qualify as “additive,” in the sense that the phenotypic difference between the double mutant and wild type is approximately the “sum” of the differences in each single mutant. This term is most often used to analyze combinations of non-equivalent traits such as organ number and organ identity. It is perhaps somewhat dangerous to use this term with a quantifiable trait like petal number. Thus I will avoid using the term “additive” to categorize the second-whorl phenotypes, but I will still attempt to group the double mutant classes on a quantitative as well as descriptive basis.

The *eep1* double mutant phenotypes (Figure 2.2) can be scored according to two sets of criteria: 1) average petal number (for the first 10 flowers) and 2) severity of phenotype as a function of the order in which flowers arise. These traits do not show much correlation. For example, *eep1* in combination with *pan-2*, *ett-3*, or *clv1-1* does not result in a dramatic increase in petal number, while petal number in *eep1* double mutants with *pnh-1* and *ult-2* is much higher than in any of the single mutants (Table 2.1). Yet the decreasing severity of the phenotype in late-arising *eep1* flowers is seen in *ett-3*, *pnh-1*, and *sno* double mutants, but not those of *clv1-1* or *ult-2*. It is difficult to interpret any of these

results as indications that *EEP1* acts in a pathway together with one or more of the genes represented by these mutants—in fact, without evidence of physical interaction, or transcriptional regulation, the most conservative assumption is that *EEP1* functions independently of all of them. The two classes of double mutants (as judged by petal number alone) may reveal something about common strategies for determining the number of petals. As mentioned previously, there are at least three likely inputs which affect petal number and position: 1) cell number and availability within the meristem, 2) positions of the sepals, and 3) positions of the other petal primordia. It is possible that more than one pathway transduces information for each type of input. We predict that the double mutants with mild effects on the second whorl are the result of mutations in two pathways with the same type of input, whereas double mutants with severe effects on petal number and position likely represent mutations in pathways with distinct types of input.

		n			n
<i>clv1-1</i>	4.34±0.08	41	<i>clv1-1; eep1</i>	6.27±0.19*	30
<i>ett-3</i>	4.27±0.09	41	<i>ett-3; eep1</i>	6.13±0.21*	30
<i>pan-2</i>	4.69±0.07	45	<i>pan-2; eep1; er</i>	4.77±0.14*	30
<i>pid-2</i>	7.68±0.27	25	<i>pid-2; eep1</i>	7.83±0.65#	23
<i>pnh-1</i>	4.06±0.04	49	<i>pnh-1; eep1</i>	7.88±0.25*	40
<i>ult-2</i>	4.57±0.13*	30	<i>ult-2; eep1</i>	8.10±0.23*	40

Table 2.1 Average petal number (\pm standard error) for each single mutant alone and in combination with *eep1*. An asterisk (*) denotes values for which the data set included only the first 10 flowers per plant; a pound sign (#) indicates that only the first 1-3 flowers were considered, by necessity.



Figure 2.2 Double mutants with *eep1* and other extra-petal mutants.
 A) *pan-2* B) *pan-2; eep1* C) *ett-3* D) *ett-3; eep1* E) *clv1-1* F) *clv1-1; eep1* G) *ult-2* H) *ult-2; eep1*
 I) *pnh-1* J) *pnh-1; eep1*.

***eep1* enhances the *pid-2* phenotype in the shoot and in the first floral whorl**

The two mutants not yet mentioned (*pid* and *pin1*) have a different type of interaction with *eep1*: they are essentially epistatic, at least in terms of petal number (other aspects of the phenotype are discussed later). Plants homozygous for *pid-2*, an intermediate allele, make abundant petals, and the number does not change significantly with the addition of *eep1* (Table 2.1 and Figure 2.3). For *pin1*, the number of petals is not the only factor to consider, as petal size varies considerably (occasionally, the entire second whorl consists of a single, 360° petal). Visually, petal number and shape in *pin1-4* and *eep1; pin1-4* are indistinguishable (Figure 2.4, B and C).

The phenotypic effects of *eep1* on *pid-2* are not limited to the second whorl, nor are they restricted to the flower. *pid-2* flowers have a reduced number of stamens, sepals, and carpels, and these defects are enhanced in the *eep1; pid-2* double mutant, with the change in sepal number being significant ($p < 0.01$; Figure 2.3, B and C). Even more dramatic, however, is the enhancement by *eep1* of defects in the *pid-2* inflorescence. *pid-2* plants make an average of 11.2 ± 0.5 flowers before the inflorescence terminates prematurely, but *eep1; pid-2* double mutants produce 1.6 ± 0.2 flowers on average ($p < 0.001$). These rare flowers in double mutants also arise later than those of *pid-2* (Figure 2.3, E). These data suggest that *EEP1* can function in the shoot and in the first whorl, at least in the *pid-2* mutant background.

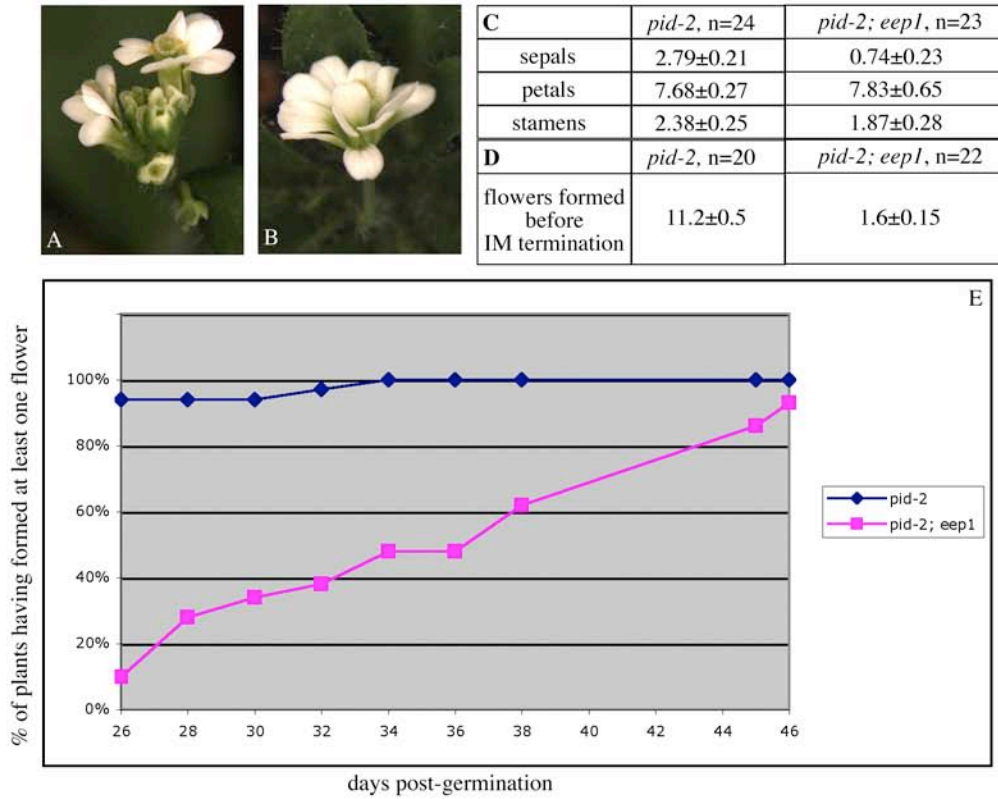


Figure 2.3 *eep1* severely enhances the *pinoid* (*pid*) phenotype in both the shoot and flower.
 A) *pid-2* homozygotes produce several flowers before terminating abruptly. These flowers usually have extra petals but a reduced number of sepals, stamens, and carpels.
 B) *pid-2; eep1* double mutants terminate after making 1–3 flowers. In addition, organ number is even more sharply affected than in the *pid-2* single mutant.
 C) Average number of organs in each whorl. n=number of flowers.
 D) Average number of flowers produced before termination of the inflorescence meristem. n=number of plants.
 E) The *pid-2; eep1* double mutant flowers later than *pid-2* alone. n=34 for *pid-2*, and n=29 for *pid-2; eep1*.

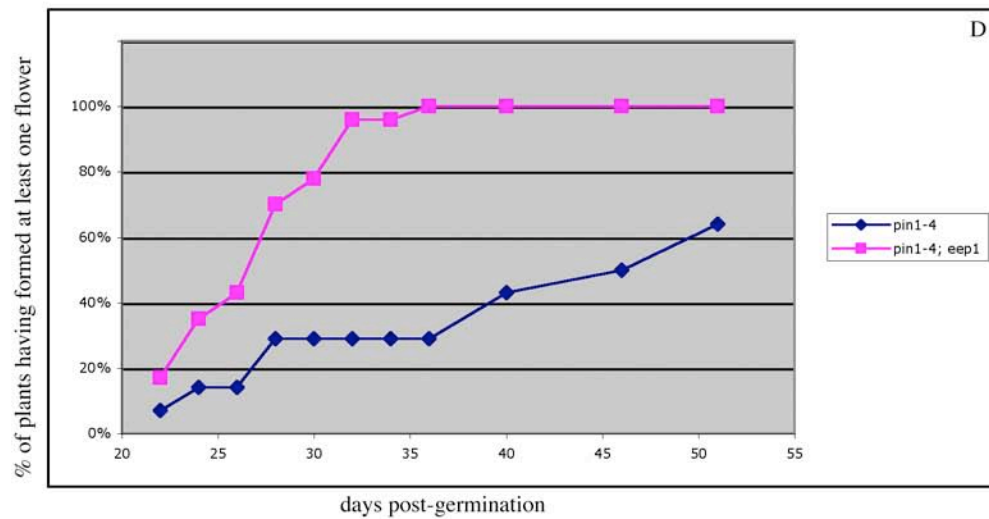


Figure 2.4 *eep1* partially suppresses the delayed flowering of *pinformed* (*pin1*) and further reduces sepal number. A) *pin1* inflorescences are replaced by pin-like structures, which can produce very few flowers apiece, and then only after a long delay. B) *pin1-4* flowers have more petals than wild-type, but fewer sepals, stamens, and carpels. C) Except for a decrease in sepal number, flowers of *pin1-4; eep1* plants are indistinguishable from those of *pin1-4* alone. D) *pin1-4; eep1* plants flower earlier than *pin1-4*. n=14 for *pin1-4*; n=24 for *pin1-4; eep1*.

The *eep1*; *pin1-4* double mutant produces flowers earlier than *pin1-4*

Plants homozygous for the *pin1-4* mutation produce a pin-shaped inflorescence, from which 1–3 late flowers may emerge. A small number of flowers may also arise from pin-shaped axial inflorescences. When *pin1-4* is combined with *eep1*, petal number and size do not perceptibly change, but flowers are produced earlier than usual (Figure 2.4, D). This effect indicates, again, that *EEP1* is capable of acting in the shoot in particular mutant backgrounds. Preliminary data indicate that the *pin1-4* phenotype, like that of *pid-2*, may be enhanced by *eep1* in the first whorl (exact numbers of sepals in *pin1-4* vs. *pin1-4*; *eep1* will require a larger sample size).

The mapping and genomic rescue of the *eep1* phenotype

In order to identify the *EEP1* gene product, we mapped the locus via positional cloning. A mapping population was created by outcrossing *eep1* (originally in the *L-er* ecotype) to Columbia, to take advantage of sequence polymorphisms between the two different ecotypes. F2 plants with the *eep1* extra-petal phenotype were used for checking linkage to markers on all five chromosomes. Tight linkage was observed between *eep1* and a cleaved amplified polymorphic sequence (CAPS) marker at the 3' end of BAC T1G16 (1 recombinant chromosome out of 342); this chromosome was not recombinant at the next 5' marker on the same BAC. Conversely, another single chromosome was recombinant at a marker in the middle of BAC T1G16, but not the next 3' marker. These recombination events demarcated a 50 kb region between 42 and 92 kb on T1G16 (Figure 2.5, A). Using genomic fragments subcloned from this BAC, the

eep1 phenotype was rescued by a 12 kb clone consisting of 70–82 kb of T1G16 (Figure 2.5, B).

Subsequent and more precise rescue experiments were performed using overlapping 3 kb fragments. Transformants were scored as positive for phenotypic rescue of *eep1* if a) the general appearance was wild-type and b) the first two flowers had no more than a total of one extra petal. As judged by this stringent definition of rescue, over 50% of plants carrying either of the first two fragments and 25% of plants carrying the third showed the rescued phenotype (Figure 2.5, C, bars with vertical and diagonal cross-hatching, respectively).

In the process of amplifying sequences in this region from *eep1* for sequencing, we observed that certain PCR reactions were consistently failing in *eep1* but working in *L-er*. All primer pairs in this set of reactions spanned a particular region, suggesting that there might be a substantial disruption in the sequence. To identify the disruption, we employed TAIL-PCR to amplify across the 500 bp gap in *eep1* and subcloning the resulting product. Gel-purified TAIL-PCR product was cloned and sequenced, revealing the presence of sequence corresponding to the *Arabidopsis* transposon Tag1 (accession # U12220). Subsequent long-range PCR with closely flanking primers confirmed the presence of a 3.2 kb insertion in this region in *eep1*, but not *L-er* (location noted in Figure 2.5, C).

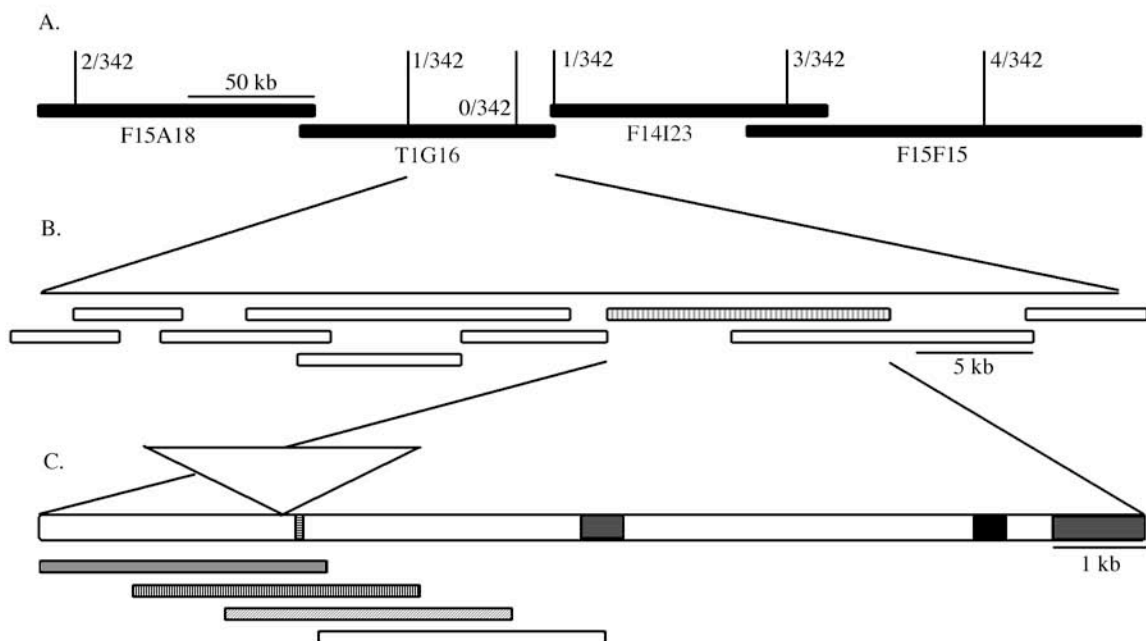


Figure 2.5 The mapping and identification of the *eep1* mutation.

A) Details of the chromosome walk, with black bars representing BAC clones in the TAIR database. Recombination data are provided in terms of the number of chromosomes recombinant at a given marker, over the total number of chromosomes tested.

B) Diagram of genomic clones used to test for rescue of the *eep1* phenotype. The one clone which did rescue is highlighted with vertical bars.

C) Details of the 12 kb rescuing region. Black sections represent putative protein-coding loci; the section with horizontal bars is a predicted microRNA. The location of a 3.2 kb *Arabidopsis* transposon, present in *eep1* but not *L-er*, is indicated by the large triangle. The four 3 kb genomic fragments tested for rescue are shown underneath. The two regions marked with vertical lines rescue twice as well as the one with diagonal lines; the region marked with a hollow bar did not rescue at all.

***Eep1* encodes a putative microRNA, homologous to *MIR164a* and *b*.**

There are no annotated open reading frames in this region, suggesting that the transposon insertion found in *eep1* does not affect a protein-coding gene. However, the transposon insertion site is located 160 bp from a 21 nt sequence that is identical along 20 contiguous nt with two published microRNAs, *MIR164a* and *b* (Figure 2.6, A; (Reinhart et al., 2002)). To investigate whether this might represent a true miRNA, we ran the sequence in the region through the RNA folding prediction program MFOLD (<bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html>). The program identified a low-energy stem-loop fold which included the 20 nt sequence of interest in a position similar to that of *MIR164a* and *b* in their respective stem-loop structures (Figure 2.6, B; (Reinhart et al., 2002)). Since we have not attempted to clone this miRNA, we do not know the exact length of the mature product. Judging from published plant miRNAs, it is likely to be 20-23 nt. We have assumed here that the mature form starts at the same nucleotide as *MIR164a/b*, since the majority of both plant and animal miRNAs found to date begin with U (Lagos-Quintana et al., 2002; Lau et al., 2001; Reinhart et al., 2002).

The locations and relative potency (50% vs. 25%) of the rescuing clones suggest that there may be cis-regulatory sequences 5' of the microRNA (between 700 and 1700 bp upstream) which aid in proper expression but are not absolutely required for transcription. We predict that the transposon insertion in *eep1* predominantly affects the transcript or the basal promoter; in addition, the insertion of 3 kb of transposon sequence between the gene and the positive cis-regulatory elements mentioned above may have an adverse effect on transcription. Several lines of evidence point to the likelihood that *eep1* is a loss-

of-function rather than gain-of-function allele: 1) *eep1* is recessive; 2) the *eep1* phenotype can be rescued by an *EEP1*-containing genomic fragment; and 3) the overexpression phenotype of *EEP1*, as will be discussed later, bears no resemblance to *eep1*. Efforts to gain molecular confirmation of the effect of the transposon insertion on the *EEP1* transcript were unsuccessful, as the transcript could not be amplified by RT-PCR from either wild-type or *eep1* RNA.

Since the *EEP1* predicted 21mer is nearly identical to *MIR164a* and *b*, *in situ* hybridization and Northern blots are not useful in determining the specific expression pattern of *EEP1* itself. Therefore, we have investigated the *EEP1* expression pattern through the use of promoter-GUS reporter lines. For the first, we used 1.8 kb of 5' sequence, up to but not including the predicted mature 20-23mer. We also made a construct with GUS downstream of the second 3 kb rescuing fragment shown in Figure 2.5 (C), in case some cis-regulatory sequences 3' of the gene were required. It is important to keep in mind, however, that these reporter lines may not be a true read-out of the location where the mature *EEP1* is active, since some unprocessed miRNAs are found in a broader region than are their corresponding mature forms (Park et al., 2002).

In *pEEP::GUS* inflorescences, GUS activity is detected throughout young floral buds, with decreasing signal in sepals as they grow out. GUS activity lingers in the developing gynoecium and is seen in later flowers only in the small region between fully-formed sepals (Figure 2.7).

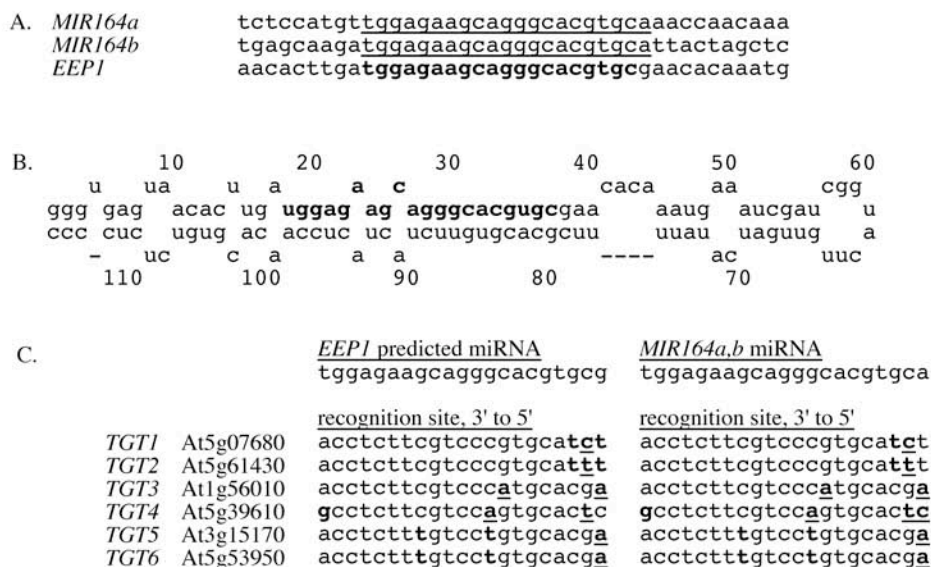


Figure 2.6 *EEP1* is a putative microRNA closely related to *MIR164a* and *b*.

A) Alignments of two published microRNAs with sequence 160 bp downstream of the transposon insertion site. The mature forms of the known miRNAs are underlined; the homologous sequence in *EEP1* is marked in bold type.
 B) The MFOLD RNA structure prediction for the region near the insertion site. Bold type marks the 20 bp of homology with *MIR164*.
 C) Alignments of *MIR164* and *EEP1* with the complementary sequences in putative target genes. *TGT3*, *TGT5*, and *TGT6* are *NAC1*, *CUC1*, and *CUC2*, respectively. The sequence of the predicted 21 nt mature product is shown for both *EEP1* and *MIR164*. The target sequences are listed as the reverse of the sense strand, to highlight complementarity with the miRNA above. Bold, underlined text marks the bases at which the miRNA cannot bind the proposed target; bold text alone marks the bases which can form non-canonical G-U pairs.

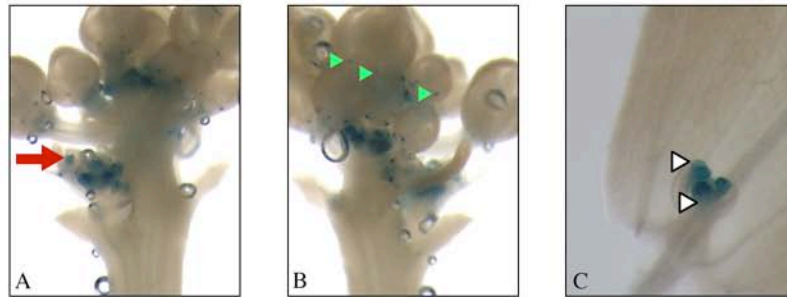


Figure 2.7 The *EEPI* promoter drives *GUS* expression in young floral buds of wild-type plants. A, B) Opposite views of one *pEEPI::GUS* inflorescence. *GUS* activity is visible in early flowers, with lingering expression in developing carpels (arrowhead). Expression in later flowers is restricted to discrete domains between mature sepals (arrows). C) Closeup of secondary inflorescence in a leaf axil. Three young floral buds are visible, with stage 4 flowers showing reduced staining in the sepals (arrowheads).

The *EEP1* microRNA has six possible mRNA targets for degradation or translational repression.

Based on genome-wide sequence analysis, Rhoades and colleagues (2002) identified five protein-coding genes that could be potential targets for negative regulation by *MIR164a* and *b*. These same five genes (plus a sixth) constitute likely targets for *EEP1*, as well (Figure 2.6, C). Provided that *EEP1* is a 21mer in its mature form, the predicted alignments of *EEP1* with each target sequence are shown. In each case, mismatches can include or exclude the non-canonical G-U base-pairing observed in RNA secondary structure (see figure legend). The alignments for *MIR164a* and *b* are also shown, for comparison.

All six genes are members of the NAC family of transcription factors, a group which includes CUP-SHAPED COTYLEDONS1 and 2 (*CUC1*, *CUC2*), *NAC1*, *NAP*, and founding member NO APICAL MERISTEM (*NAM*) from *Petunia* (Aida et al., 1997; Sablowski and Meyerowitz, 1998; Souer et al., 1996; Takada et al., 2001; Xie et al., 2000). Three of the six putative target genes are among those mentioned above: *NAC1* (TGT3), *CUC1* (TGT5), and *CUC2* (TGT6). *NAC1* is known to act in the root to promoter lateral outgrowth in response to auxin (Xie et al., 2000). *CUC1* and *CUC2* function redundantly in the embryonic shoot meristem and in flowers. In the absence of both proteins, fusion of cotyledons, sepals, and stamens results. *CUC1* is expressed in a domain separating the cotyledons, in the zone between the inflorescence meristem and each emerging floral primordia, and in boundary regions of flowers (between whorls and between individual organ primordia) (Aida et al., 1997; Takada et al., 2001). The *CUC2* expression pattern is quite similar, as described in Ishida et al., 2000.

Overexpression of *EEP1* results in a distinct floral phenotype similar to that of the *cuc1; cuc2* double mutant

Plants expressing *EEP1* from the constitutive 35S promoter produce flowers with partially fused sepals, reduced stamen number, and reduced female fertility (Figure 2.8, A). One line was even stronger, with completely fused sepals, fused stamens, and slightly reduced petal number (Figure 2.8, B-E). The phenotype of this strong line shows a striking resemblance to that of the *cuc1; cuc2* double mutant (Aida et al., 1997). The embryonic phenotype of *cuc1; cuc2* has not yet been observed in *35S::EEP1*, but the transformants have only been grown in soil at present. Seedlings which arrest with fused cotyledons are far easier to identify on tissue culture plates, so T2 seeds will be sown on selective plates for that purpose.

A critical question for understanding *EEP1* function is whether the miRNA operates by promoting cleavage of target mRNAs, or by repressing their translation. Several published plant miRNAs act via the first mechanism (Emery et al., 2003; Llave et al., 2002; Palatnik et al., 2003; Tang et al., 2003), while at least one functions via translational repression (Aukerman and Sakai, 2003; Chen, 2003). To test whether the *EEP1* miRNA can act to regulate the RNA stability of *CUC1* and/or *CUC2*, or any of the other four predicted targets, we performed RT-PCR on each transcript from *L-er*, *eep1*, and *35S::EEP1* inflorescence RNA. RT-PCR products from the first four predicted targets were found at uniformly low levels from all three sources (data not shown; more careful scrutiny of the expression of these genes is needed—both stricter DNase treatment of RNA samples and more PCR amplification cycles should allow meaningful analysis). *CUC1* and *CUC2* transcripts were relatively more abundant in inflorescence

tissue. There was no detectable difference in expression between *L-er* and *eep1*, but expression of *CUC1* and *CUC2* was significantly reduced in *35S::EEP1* (Figure 2.8, F and G). This RT-PCR result suggests that the *EEP1* miRNA can regulate *CUC1* and *CUC2* by promoting cleavage of the mRNA, rather than by blocking translation. The absence of a significant increase in *CUC1* or *CUC2* mRNA in *eep1* compared to wild type may indicate that they are not endogenous targets of *EEP1* in wild type, or it may indicate that any expansion in *CUC1* and *CUC2* expression in the mutant is too subtle (i.e., spatially restricted) to be distinguishable over normal transcript levels. Performing *in situ* hybridization experiments on *eep1* and *L-er* with *CUC1* and *CUC2* probes will help resolve this issue.

Published work describing lines overexpressing *CUC1* has detailed phenotypic effects in the cotyledons (including the formation of ectopic meristems) but no defects in inflorescence development (Takada et al., 2001). Additional work performed on callus tissue has shown that high levels of *CUC1* or *CUC2* can promote the appearance of adventitious shoots in this context as well (Daimon et al., 2003), but no floral phenotype was reported. This set of published data does not necessarily rule out *CUC1* or *CUC2* as true targets of *EEP1*, though, if recent work with *APETALA2* (*AP2*) and *PHABULOSA* (*PHB*) is any indication. Overexpression of *AP2* does not result in a phenotype in the third and fourth whorls, probably because the *MIR172* miRNA is present in those whorls to repress its translation. Only when the *AP2* transcript is mutated to prevent *MIR172* from binding does *35S::AP2* show an effect in all four whorls (Chen, 2003). In the case of *PHB*, overexpression from the *35S* promoter is

sufficient for ectopic adaxial fates only if the cDNA is mutated in the miRNA-binding site (McConnell et al., 2001; Tang et al., 2003).

Analysis of selected overexpression and T-DNA insertion lines

In order to determine whether any of the other *NAC* genes are *in vivo* targets of *EEP1* in the second whorl, we first searched for loss-of-function lines. T-DNA insertion lines for *TGT2* and *TGT3* were retrieved by BLAST searches at <www.arabidopsis.org/BLAST>. Plants homozygous for each insertion were identified by PCR genotyping, and no floral or shoot phenotypes were observed (data not shown). For *NAC1*, this is consistent with its expression pattern (restricted to the root) and the experiments done using RNA interference (Xie et al., 2000). In the case of *TGT2*, this result suggests either that it does not act in the shoot or flower, or that it has a close homolog which can functionally compensate for its absence. The most similar protein to *TGT2* is *TGT1*, which is 80% identical. Unfortunately, no T-DNA insertions in or near *TGT1* are available at this writing.

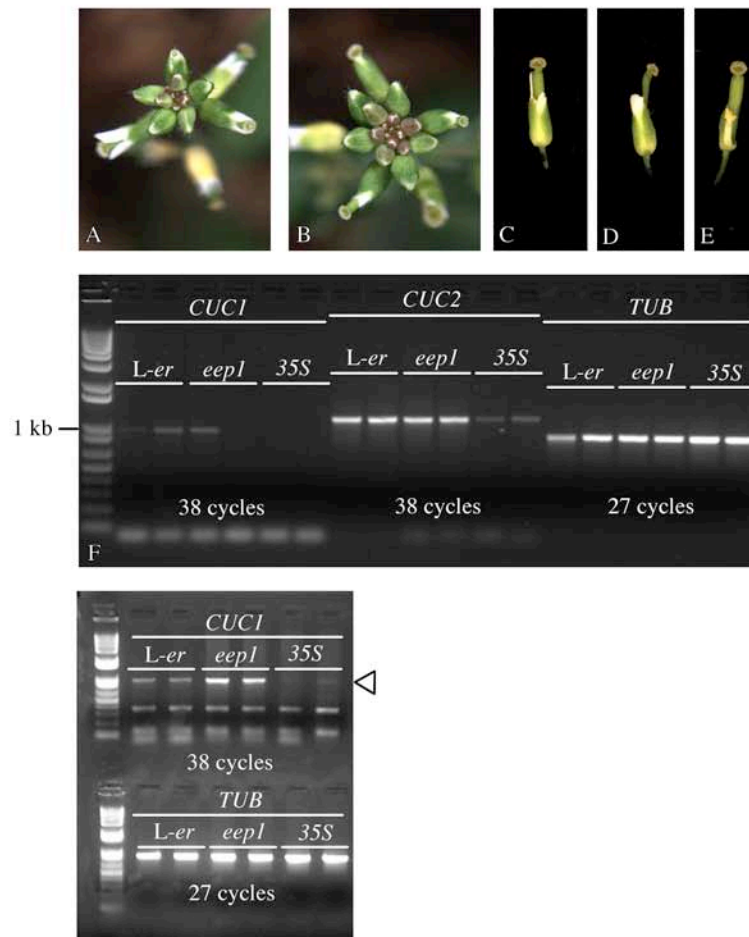


Figure 2.8 A strong line of *35S::EEP1* is very similar to *cuc1; cuc2* double mutants, and *CUC1* and *CUC2* mRNA levels are decreased in this line relative to wild type.
 A) A *35S::EEP1* line with an intermediate phenotype. Sepals are partially fused, and stamen number is reduced. B, C, D, E) A single *35S::EEP1* line shows a strong phenotype resembling *cuc1; cuc2*. The sepals are almost completely fused, petal number is reduced, and stamens are severely fused or missing (fused stamens are visible in E).
 F) RT-PCR amplification of *CUC1*, *CUC2*, and tubulin transcripts from *L-er*, *eep1*, and *35S::EEP1* inflorescence RNA. *CUC2* transcripts are clearly decreased in *35S::EEP1*, compared to *L-er* (no such difference is apparent in the tubulin controls). Abbreviation: *35S*=*35S::EEP1*.
 G) RT-PCR of *CUC1* (repeated with different forward primer, TGT5-F3) and tubulin. Expected product is 890 bp (arrowhead).

In similar experiments to those performed elsewhere on *CUC1* and *CUC2*, we overexpressed the other four predicted targets (*TGT1-4*) to test whether misexpression of one or more of these genes might be responsible for the *eep1* extra-petal phenotype. None of these four predicted targets produced a phenotype when overexpressed. As with *CUC1* and *CUC2*, this does not rule out the possibility that one or more of these genes are the endogenous targets of *EEP1*, since the *EEP1* miRNA is likely to downregulate even high levels of target mRNAs.

2.3 Discussion

We have shown that plants homozygous for the recessive allele *eep1* have extra petals in early flowers, and that double mutants of *eep1* with *pin1-4* and *pid-2* uncover functions for *EEP1* outside of the second whorl, in such tissues as the inflorescence meristem, and the first and third whorls of the flowers. These genetic interactions (enhancement of the shoot and floral phenotype *pid-2*, enhancement of the *pin1-4* floral phenotype, and partial suppression of the *pin1-4* delay in flowering) with two mutants known to be defective in auxin transport and/or signaling suggest that *EEP1* may function to regulate organ formation and phyllotaxy in the shoot and flower by auxin-related mechanisms. Alternatively, *EEP1* may represent an auxin-independent type of control of the same genes regulated by *PIN1* and *PID*.

The *eep1* locus was mapped by positional cloning, with rescue of the *eep1* phenotype by several nested genomic fragments. The lesion was identified as a Tag1 transposon insertion in *eep1* but not *L-er*; this 3 kb insertion does not disrupt

any open reading frames, but it lies just 5' of a putative miRNA. The *EEP1* miRNA is identical along the first 20 nt to published miRNAs *MIR164a* and *b*, and all three are predicted to regulate one or more of six genes in the NAC family of transcription factors (Rhoades et al., 2002). Several plant miRNAs have been functionally characterized, including *MIR165* and *166*, *MIR39*, *MIRJAW*, and *MIR172*, which target (respectively) *PHABULOSA*, *PHAVOLUTA*, and *REVOLUTA*; *SCARECROW*-like genes; *TCP4*; and *AP2* and related genes. With the exception of *MIR172*, which acts to repress the translation of its targets, these miRNAs function by promoting cleavage of the target mRNA at sequences within the miRNA binding site (Chen, 2003; Emery et al., 2003; Llave et al., 2002; Palatnik et al., 2003; Tang et al., 2003). The *eep1* allele represents the first known loss-of-function mutant in a plant miRNA.

There are six predicted mRNA targets for *EEP1* and *MIR164a* and *b*. All six are in the NAC family of transcription factors, whose founding member is the *NO APICAL MERISTEM* gene in *Petunia* (Souer et al., 1996). Among these six genes are three which have been functionally characterized: *CUC1*, *CUC2*, and *NAC1* (Aida et al., 1997; Takada et al., 2001; Xie et al., 2000). *CUC1* and *CUC2* are redundantly required for several developmental processes (Aida et al., 1997), and a strong *EEP1* overexpression line bears a striking resemblance to the *cuc1; cuc2* double mutant, suggesting that *EEP1* is capable of negatively regulating these two genes post-transcriptionally. Indeed, levels of *CUC1* and *CUC2* transcripts are decreased in *35S::EEP1* relative to wild type, implying that *EEP1* can direct cleavage of these two mRNAs.

Experiments for a) confirming that *CUC1* and *CUC2* are endogenous targets of *EEP1*, and b) testing whether *EEP1* also regulates any of the other

predicted target genes, include improved loss-of-function and gain-of-function analysis. For example, if the *cuc1; cuc2; eep1* triple mutant shows that the *cuc* mutations are completely epistatic to *eep1* in the second whorl, then these two genes are probably the only targets of *EEP1* in that whorl. If the combination of *cuc1; cuc2* and *eep1* is additive in the second whorl, and double-stranded RNA interference (dsRNAi) of one or more of targets 1 through 4 suppress the *eep1* phenotype, then those genes are in fact the true targets of *EEP1* in the second whorl.

Informative gain-of-function analysis requires overexpression of a mutated version of each target. Specifically, the mutations would not change the protein sequence but would alter the miRNA-binding site in the mRNA, so that *EEP1* could not bind as effectively. Such experiments have been done with great success on the targets of *MIR172* and *MIRJAW* (Aukerman and Sakai, 2003; Chen, 2003; Palatnik et al., 2003), and dominant gain-of-function alleles in *PHAVOLUTA* (*PHV*), *PHABULOSA* (*PHB*), and *REVOLUTA* (*REV*) are now known to alter the miRNA binding sites in the mRNAs as well (Emery et al., 2003; McConnell et al., 2001; Tang et al., 2003). As noted earlier, overexpression of the wild-type sequence is often not sufficient to overcome the miRNAs in the tissues where the latter are expressed. If *CUC1* and *CUC2* are the only second-whorl targets of *EEP1*, then simultaneous overexpression of mutated transcripts from both genes (*CUC1-m* and *CUC2-m*) should mimic the *eep1* extra-petal phenotype. Otherwise, overexpression of another predicted target or targets may re-create the *eep1* second-whorl phenotype.

These *35S::TGT-m* lines will also be useful in investigating the possibility that the *EEP1*-like miRNAs *MIR164a* and *b* act on different mRNAs and/or in

different locations from *EEP1*. Overexpression analysis of *MIR164a* and *b* in *L-er* and *eep1* is underway; we will also examine the expression pattern of these two miRNAs, using promoter-GUS reporter lines.

Analysis of the *eep1* genetic interactions and their possible significance

The data presented here hint that *EEP1* may function in the second whorl to regulate *CUC1* and *CUC2* expression, and that *EEP1* may also be capable of functioning in the shoot and in whorls 1 and 3. Possible explanations for the absence of an *eep1* single mutant phenotype in the latter set of tissues include 1) genetic redundancy (either at the level of regulating specific target genes, or at a broader level of controlling the activity of proteins required for proper phyllotaxy) and 2) absence of *EEP1* transcription in these tissues in wild type. The most significant challenge in understanding *EEP1* function will be the resolution of its regulation of *CUC1* and *CUC2* with the genetic interactions of *eep1* with *pid* and *pin1* in the shoot and flower. Here we discuss some possible models for the action of *EEP1*, *PIN1*, and *PID* in regulating and/or responding to the *CUC* genes, given the data currently available.

As mentioned earlier, published analysis of *CUC1* expression indicates that it is expressed in a complex pattern in the inflorescence. *CUC1* transcripts are found between the inflorescence meristem (IM) and floral primordia, in the boundaries between whorls, and in the boundary regions between primordia within the same whorl (Takada et al., 2001). *CUC2* has a very similar expression pattern (Ishida et al., 2000). In *pin1-6* and *pid-9*, *CUC2* is ectopically expressed in the IM, in a ring-shaped domain; *CUC1* expression has not been characterized in these mutants (Reinhardt et al., 2003; Vernoux et al., 2000). This is an intriguing

result, particularly given the interaction between *eep1* and *pid-2* in the IM, but there are additional valuable pieces of information that are lacking. For examples, it will be informative to identify the default expression pattern of *CUC1* and *CUC2* in the absence of miRNA control; this will be achieved by using *pCUC1::GUS* and *pCUC2::GUS* (a published GUS reporter line for *CUC1* was constructed using a *CUC1*-GUS fusion protein under the control of the endogenous promoter—thus, the reporter gene mRNA was presumably still sensitive to miRNAs (Takada et al., 2001)).

In addition, the functions of *CUC1* and *CUC2* in the wild-type IM are not yet known, since the *cuc1; cuc2* double mutant produces inflorescences with normal structure after the induction of flowering on callus tissue (Aida et al., 1997)). Given the high level of redundancy in the NAC family of proteins, a third protein may be able to compensate for the absence of *CUC1* and *CUC2* in the IM (but not in the flower).

The consequences of the misexpression of *CUC1* and *CUC2* in the IM are also unknown. *In situ* hybridization experiments to examine the expression of these genes in *eep1* meristems is crucial. A published report of *35S::CUC1* indicates that inflorescence development is wild-type; this data may be misleading, however, as any miRNAs in the IM may still be able to regulate *CUC1* transcript levels (Takada et al., 2001). If one or both genes are found to be misexpressed in the *eep1* IM, then there is clearly no phenotypic consequence of misexpression of these genes, at least in that domain. On the other hand, if *CUC1* and *CUC2* expression is normal in *eep1* inflorescence meristems, it suggests that another pathway may be functionally redundant with that in which *EEP1* acts—possibly mediated by *MIR164a* or *b*. Similarly, it is not yet clear whether

the misexpression of *CUC2* makes a substantial contribution to the *pid-9* and *pin1-6* shoot phenotypes. In fact, there are several genes which are misexpressed in a ring in the periphery of the IM in one or both mutants, such as *AINTEGUMENTA* (*ANT*), *LEAFY* (*LFY*) (both transcription factors), and *PIN1* itself (Reinhardt et al., 2003; Vernoux et al., 2000).

Additional, potentially helpful information that is not yet available includes the precise expression pattern of *EEP1* in the IM (assuming that *EEP1* is transcribed there). It will be important to determine whether the region of *CUC2* misexpression in *pin1-6* and *pid-9* overlaps with the domain of *EEP1* expression; if so, then it is possible that misexpression of *CUC2* in *pin1-6* and *pid-9* is due at least in part to compromised *EEP1* activity or expression. We would continue this line of investigation by checking *EEP1* transcription in *pid-2*, *pid-9*, and *pin1-4* by transforming them with the *pEEP1::GUS* reporter construct. Finally, we need more information on *pid* alone, and the interaction of *pid* with *eep1*. In particular, we have made the *pid-2; eep1* double but not *pid-9; eep1*; conversely, we know the expression pattern of *CUC2* in *pid-9* IMs (Reinhardt et al., 2003) but not in *pid-2*.

The overexpression of mutated putative target mRNAs in *pid-2* may help confirm any conclusions drawn on the basis of the *CUC1*, *CUC2*, and *EEP1* expression patterns in *pid-2* and *pid-9* IMs. For example, if co-expression of *35S::CUC1-m* and *35S::CUC2-m* in *pid-2* result in a *pid-2; eep1*-like phenotype, then it suggests that 1) *CUC1* and *CUC2* are targets of *EEP1* in the shoot and 2) their misexpression may contribute to the strong *pid-9* phenotype. In the case of *pin1-4*, we suspect it is more probable that *EEP1* is regulating a different target mRNA—one where the activity of the target in question is downregulated in *pin1-4*, and where removal of *EEP1* activity gives partial relief (enough to allow

earlier flowering in *pin1-4; eep1* than in *pin1-4* alone). In the interest of investigating all possible predicted targets, we will perform all of the above experiments for each individual remaining target (*TGT1-4*), in addition to *CUC1* and *CUC2*.

Understanding the genetic interactions of *eep1* with *pid-2* and *pin1* in the flower poses a different set of challenges. Most importantly, we do not yet know whether there are any changes in the expression or activity of *CUC1* and/or *CUC2* in *pid-2* or *pin1* flowers. We do know, however, that both proteins are required for maintaining boundaries between sepal primordia and between stamen primordia, as is obvious from the severe organ fusion defects in the *cuc1; cuc2* double mutant (Aida et al., 1997). The floral phenotypes of *pin1-4* and *pid-2* are for the most part consistent with possible expansion of expression of any genes (such as *CUC1* and *CUC2*) marking boundary regions—both *pin1-4* and *pid-2* have reduced stamen numbers, and *pid-2* has fewer sepals than wild type. In the first whorl of *pin1-4* flowers, the phenotype is less straightforward: the mutant has variable numbers of sepals, some of which are fused. Since combining *eep1* with either *pin1-4* or *pid-2* causes a reduction in sepal number, and *eep1; pid-2* also makes fewer stamens than *pid-2* alone, it is likely that if there is misregulation of boundary genes in *pin1-4* and *pid-2*, it is largely independent of *EEP1*. It will be particularly useful to examine *CUC1* and *CUC2* expression in *pin1-4*, *pid-2*, *pin1-4; eep1*, and *pid-2; eep1* flowers, and then compare that to the wild-type pattern of *EEP1* expression. Also, inducing flowers from *cuc1; cuc2; pid-2* and *cuc1; cuc2; pin1-4* callus tissue will allow us to determine whether *CUC1* and *CUC2* are required for all or part of the *pid-2* and *pin1-4* floral phenotypes.

Ironically, it is the function of *EEP1* in the second whorl (the location of the only single-mutant *eep1* defect) which is the least logical of all, particularly if one assumes that *EEP1* is negatively regulating *CUC1* and *CUC2* there. Both *35S::EEP1* and *cuc1; cuc2* have a slight reduction in petal number—a phenotype which is clearly opposite that of *eep1*. This phenotype (especially for the double mutant) is counter-intuitive, considering that *CUC1* and *CUC2* are expressed in cells between or surrounding organ primordia in the first three whorls, and *cuc1; cuc2* has fused sepals and fused stamens, consistent with loss of boundary identity between organs (Aida et al., 1997). In other words, the second-whorl phenotype seems more like a gain of boundary tissue, or a decrease in the ability to make primordia. It is important to note, however, that while *cuc1; cuc2* stamens are often fused, the total number is reduced, hinting at a partial parallel with the effects on the second whorl (Aida et al., 1997). Similarly, intermediate lines of *35S::EEP1* routinely produce flowers with only 4 or 5 stamens. These data from *cuc1; cuc2*, *eep1*, and *35S::EEP1* suggest that *CUC1* and *CUC2* may function in some cases to promote primordia formation and/or outgrowth.

Because the published analyses of *CUC1* and *CUC2* expression do not report any expression in any primordia themselves, some explanation is needed. One possibility is that *CUC1* and/or *CUC2* are expressed in petal primordia very transiently, or at such low levels that they are undetectable by *in situ* hybridization. If this is the case, *CUC1* and *CUC2* may have dramatically different functions within the same whorl (whorl 2 or 3), depending on timing and location of their expression. An alternate possible explanation for the lack of *CUC1* and *CUC2* expression in the primordia is that one or both proteins can function non-cell-autonomously. In fact, there is evidence suggesting that *CUC2*

has a non-cell-autonomous function in gynoecium development (Ishida et al., 2000). A clearer understanding of the function of *CUC1* and *CUC2* in the second whorl will require experiments mentioned above: *in situ* hybridization on *eep1* and *L-er*, and promoter-GUS analysis of the *CUC* genes (*pCUC1::GUS* and *pCUC2::GUS*).

2.4 Materials and Methods

Plant growth conditions

Seeds were sown on a 4:3:2 mixture of potting soil, vermiculite, and perlite. Each flat was watered generously and kept at 4°C for 4 days for seed stratification. Plants were grown under 600 ft-candles of continuous cool white fluorescent light at a temperature varying between 17 and 21°C. Pests such as fungus gnats and aphids were kept under control by treatment with Gnatrol (20 ml in water) and granular Marathon (~ 4 g, post-germination).

Crosses and genetics

For proper identification of double mutants, we checked the F₂s for 1/16 segregation of a novel phenotype, but for further confirmation, we harvested seeds from F₂ plants resembling the *eep1* single mutant and looked for segregation of the double mutant at 1/4 frequency in the progeny. When feasible, we used *L-er* alleles of mutants crossed to *eep1*. The one exception was *pan-2*, which originated in the *Ws* ecotype (*ER/ER*).

PCR, RT-PCR, and TAIL-PCR

DNA was extracted from leaf or inflorescence tissue according to Edwards et al., 1991. Approximately 1–2 μ l of template DNA was used for each 25 μ l PCR reaction. PCR consisted of 35 cycles (94°C, 30 sec; 52–57°C [depending on primers], 30 sec; 72°C, 2 min) in an MJ Research DNA Engine Thermal Cycler.

For RT-PCR, RNA was extracted from inflorescence tissue with Tri Reagent (Molecular Research Center) or the RNA-Easy kit (Qiagen), using 1 inflorescence per sample. The RNA samples prepared with Tri Reagent underwent additional rounds of purification, including treatment for 30 minutes with 5 units RQ1 RNase-free DNase and a subsequent phenol-chloroform extraction. The cDNA first-strand synthesis was performed on ~ 100–150 ng of total RNA, using MMLV reverse transcriptase and either oligo dT or a gene-specific reverse primer. The entire RT reaction (except the enzyme) was heated to 65°C for 5 minutes and then cooled gradually to room temperature; cDNA synthesis was allowed to proceed at 37°C for 30–40 minutes. One μ l of the 10 μ l reaction was used as a template for PCR. Cycle number and annealing temperature were template- and primer-specific, respectively. The following primers were used to amplify *TGT5/CUC1* and *TGT6/CUC2*: TGT5-F, 5'-atggatggtgatgtgttaacgg-3', TGT5-F3, 5'-gatgaatcccttatgccacc-3', TGT5-R, 5'-tgtggccgtttactctctga-3', CUC2-F, 5'-atggacattccgtattaccac-3', and CUC2-R, 5'-tcagtagttccaaatacagtaag-3'. Tubulin transcripts were amplified as a control, with the following primer pair: TUB2-P1, 5'-atccgtgaagagtaccagat-3' and TUB2-P2, 5'-tcaccttctcatccgcagtt-3'.

Thermal-assisted interlaced PCR (TAIL-PCR) was performed according to the published protocol (Liu et al., 1995), except that the nested primers used in this case matched plant genomic DNA: primer1 – 5'-ttaagtcacccgaatgcctac -3', primer2 – 5'-caatgcttactactgtgtatgtct-3', primer3 – 5'-gtgtgtagagaggaagaagag-3'.

Mapping

Since the *eep1* mutation arose in the *L-er* background, we crossed *eep1* to Col and allowed the F1 plants to self-fertilize, thereby creating a mapping population. DNA was extracted from *eep*-like F2 plants for subsequent analysis. To assay the recombination frequency between *eep1* and various chromosomal markers, both cleaved amplified polymorphisms (CAPs) and simple sequence length polymorphisms (SSLPs) were used to take advantage of differences between the *L-er* and Col sequences. Markers were either already published (Konieczny and Ausubel, 1993), described on the TAIR database (<www.arabidopsis.org/servlets/Search?action=new_search&type=marker>), or were designed using information in the Cereon/Monsanto *L-er*/Col polymorphism database (<www.arabidopsis.org/Cereon/index.html>).

Genomic rescue and gene overexpression

We ordered the DNA for BAC T1G16 from the AIMS collection, and subcloned large overlapping fragments covering the 3' half of the region covered by the BAC. The second round of the rescue experiment was simply done by PCR amplification of overlapping 3 kb chunks. In both cases, the fragments were cloned into a modified pZP200 vector (spect) containing the BASTA-resistance (Ba^R) gene. The resulting plasmids were then transformed into the ASE strain of

Agrobacterium tumefaciens (kan chlor), and *eep1* plants were transformed by the floral dip method (Clough and Bent, 1998). Plants were sprayed with BASTA several times, beginning around 6 days post-germination. Ba^R plants were scored for petal number as a measure of rescue. For any plant which was visually wild-type and had a total of 9 or fewer petals in the first two flowers, the *eep1* phenotype was considered rescued.

Overexpression lines were constructed by cloning PCR products into TOPO pCR2.1, then inserting the resulting fragments into the EcoRI site of pBJ36/35S (except for At1g56010/NAC1, which was cut out of pCR2.1 with EcoRV/KpnI and ligated into the SmaI/KpnI sites of pBJ36/35S). The pBJ36/35S vector contains the cauliflower mosaic virus 35S promoter from pHANNIBAL, a NOS terminator, and the amp^R gene. The cDNA of At5g39610 was used, whereas the genomic sequence (coding plus introns) were used for At5g61430 (1.3 kb), At5g07680 (1.3 kb), and At1g56010 (2.3 kb; based on At5g56010.2 cds). For *35S::EEP1*, the region corresponding to the second 3 kb fragment in Figure 5 was used (forward primer: 5'-catttctcttcaccattcttcttc-3'; reverse primer: 5'-tcttctattggatgatacattta-3'). The NotI fragment from each resulting pBJ36/35S construct was ligated into pMLBART, a binary vector containing the Ba^R gene.

GUS staining

Tissues were fixed in ice-cold 90% acetone for 30 minutes, then washed with rinse solution (34 mM Na₂HPO₄, 15.8 mM NaH₂PO₄, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 0.5% Triton X-100). Rinse solution was replaced with a small amount of fresh solution (just enough to cover tissues) to which X-Gluc was added to a concentration of 2 mM (X-Gluc can be purchased as a powder and dissolved in

DMF—100 mg in 1.92 ml to make a 100 mM stock solution). Tissues were vacuum infiltrated to draw solution into cells, then incubated in the dark at 37°C overnight, or until strong staining appeared. Tissues were then washed in 50% ethanol and cleared in 75% ethanol. Individual inflorescences and seedlings were mounted in single-depression slides in 50% glycerol, then photographed.

2.5 Acknowledgments

I am grateful to B. Williams for spotting *eep1* in the first place; F. Wellmer for dusting off the *eep1* project after 3 years, then letting me take it over; J. Long, for announcing a year in advance of the cloning that *EEP1* had to be a miRNA; P. Sieber, for very useful miRNA-related discussions, and O. Redenbacher, for late-night caloric input.

2.6 References

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