Chapter 1. Transcription and modulation of transcriptional states in *Arabidopsis*

1.1 Transcription in *Arabidopsis* development

Variation at the level of transcription unarguably contributes enormously to differences between cells—it is, after all, the primary mechanism by which two cells with the exact same genome produce unique sets of proteins and thus acquire completely different morphologies, behaviors, and functions. Coordinated gene expression is particularly important for proper development and acquisition of specific cell fates. Of course, transcriptional networks do not occur in a vacuum, nor are they the absolute determinant of protein accumulation within a cell. The activity of transcription factors can be regulated by intracellular events as well as extracellular signaling, and there are several levels of post-transcriptional regulation that determine whether a protein is translated, stable, and properly modified. We focus here on some basic transcriptional networks in plants, as well as two mechanisms by which transcriptional states are modulated: extracellular signaling, and microRNA activity.

The proteins encoded by the plant homeotic MADS box genes are classic examples of transcription factors that cooperate to activate downstream targets. Organ identity in the flower is instructed by the overlapping expression patterns of floral homeotic genes. These genes encode members of a family of transcription factors called MADS box genes, which share homology with transcription factors in yeast and animals (Hayes et al., 1988; Jarvis et al., 1989). The floral homeotic proteins are classified into three groups (A, B, and C), based

on the organ type they are required to set up: sepals (A), petals (A + B), stamens (B + C), and carpels (C) (recently reviewed in (Eckardt, 2003; Lohmann and Weigel, 2002). Recent work has revealed that these proteins likely operate in complexes of 3 or 4 proteins to bind DNA and regulate expression of downstream targets (Honma and Goto, 2001). As has been the case with Hox proteins in animals, the identities of the downstream genes have long been elusive. More sophisticated experiments have made use of protein fusions with the glucocorticoid receptor (GR) – these allow a transcription factor to be held inactive outside the nucleus until treatment with dexamethasone, so that analysis of the resulting changes in gene expression (recently, through the use of microarrays) can accurately reflect the most immediate, direct targets. Such methods have successfully enabled the identification of direct targets of AGAMOUS (AG), the C class protein. In particular, AG is now known to activate the expression of *NOZZLE* (*NZZ*) in developing stamens (Ito et al. in preparation).

Several developmental processes in plants are set up and maintained by transcription factors which act in opposition to each other. These mechanisms can have the effect of reinforcing boundaries between different populations of cells. One such arrangement allows the stem cells in the shoot apical meristem to maintain their proliferative state, while still enabling organ primordia to form on the lateral edges.

SHOOT MERISTEMLESS (*STM*) is expressed in the shoot apical meristem and encodes a KNOTTED1-class homeodomain protein which functions to prevent differentiation of the stem cells. *stm* loss-of-function mutants arrest at the seedling stage, having no apical meristem between the cotyledons (Long et

al., 1996). STM represses transcription of *AS1* and *AS2*, two myb domain transcription factors which negatively regulate expression of two other genes in the same family as *STM*: *KNAT1* and *KNAT2*. This system of gene regulation resolves into a pattern in which STM, KNAT1, and KNAT2 are active in the center of the shoot apical meristem, while AS1 and AS2 function predominantly in lateral organ primordia (Byrne et al., 2002; Semiarti et al., 2001). Not surprisingly, ectopic expression of *STM* in leaf primordia results in transcription of *KNAT1* and *KNAT2* in leaf tissue, creating densely-staining cells which resemble meristematic cells (Lenhard et al., 2002).

It is thought that a similar strategy enables cells in lateral organs to acquire the appropriate fate, according to their position, particularly for abaxial-adaxial polarity and resulting organ outgrowth (the adaxial side is nearest the stem, and the abaxial side is farthest). The three HD-ZIP proteins PHAVOLUTA (PHV), PHABULOSA (PHB), and REVOLUTA (REV) are found on the adaxial side of lateral organs (McConnell et al., 2001). In contrast, the *KANADI* (*KAN*) genes are expressed in abaxial tissues. Although there is no evidence for direct transcription repression of *KAN1*, *KAN2*, and *KAN3* by the HD-ZIP proteins, or vice versa, there are data suggesting that such a mechanism might be in place. Dominant, gain-of-function mutations in *PHV*, *PHB*, and *REV* cause abaxialization of leaves, a phenotype similar to the triple loss-of-function mutant *kan1*; *kan2*; *kan3* (Emery et al., 2003; McConnell et al., 2001). Loss-of-function mutations in *KAN* result in ectopic expression of *REV* and *PHV* (Eshed et al., 2001), while *35S::KAN1*, *KAN2*, or *KAN3* mimics the triple mutant *phb*; *phv*; *rev* (Emery et al., 2003; Eshed et al., 2001).

1.2 Various inputs from outside the cell can alter the transcriptional program

An important kind of input affecting transcription is derived from information from outside the cell. Extracellular signals can convey information about the cell's external environment, and the identity and/or arrangement of its neighbors. Typical signaling pathways require the function of several proteins (often kinases or phosphatases) between the plasma membrane and the nucleus.

The predominant type of membrane-spanning receptor kinase found in plants contains an extracellular domain, a single-pass transmembrane domain, and an intracellular serine-threonine kinase domain. There are over 400 of these proteins, termed receptor-like kinases (RLKs), and they can be grouped on the basis of motifs in their extracellular domain (Shiu and Bleecker, 2001). A particularly large subset of this receptor family (200+) features proteins with extracellular leucine-rich repeats (LRRs); this motif is believed to be involved in protein-protein interactions. Several LRR-RLKs are known to function in various aspects of plant development, including plant architecture (ERECTA), floral organ abscission (HAESA), cell fate determination in stomatal precursors (TOO MANY MOUTHS), and regulation of cell division and growth in embryos and stamens (EXTRA SPOROGENOUS CELLS) (Canales et al., 2002; Jinn et al., 2000; Nadeau and Sack, 2002; Shpak et al., 2001; Torii et al., 1996; Zhao et al., 2002). In some cases, RLKs function by altering the transcription of downstream genes, rather than simply modifying cell structure or behavior directly.

One of the best-characterized LRR-RLKs contributes to the long-range signaling mediated by hormones. Unlike in animals, plant hormones are not produced in a particular tissue, but can be manufactured almost anywhere. The steroid hormone brassinolide (BL) is a well-studied example: it is known to be

required in several processes in plant development, including pollen tube growth, stem elongation, and photomorphogenesis (Li and Chory, 1999). The receptor for BL is termed BRASSINOLIDE-INSENSITIVE1 (BRI1) and encodes an RLK with 25 extracellular leucine-rich repeats (LRRs) (Li and Chory, 1997). Multiple experiments have confirmed that the extracellular domain binds BL, and that it does so with a high degree of specificity (Wang et al., 2001). A particularly elegant experiment showed that BL could induce a hypersensitive response in rice cell lines expressing a fusion protein containing the extracellular domain of BRI1 and the intracellular domain of XA21 (required in rice for resistance to particular strain of *Xanthomonas oryzae*) (He et al., 2000). Another interesting result is that BRI1 kinase activity is not required for BL binding (Wang et al., 2001).

Much of our understanding of RLK function at the biochemical level comes from research done on BRI1 signaling. Based on genetic results, BRI1 seemed unlikely to function as a homodimer, and a yeast two-hybrid screen did in fact reveal that BRI1 was capable of binding another RLK. This protein, named BAK1 for BRI1-associated kinase, interacts with BRI1 in yeast and co-precipitates with BRI1 *in vivo*. Furthermore, BAK1 and BRI1 show kinase activity if co-expressed in yeast, but not if expressed singly. The phenotypes of loss-of-function alleles of *BAK1*, and *BAK1* overexpression, are consistent with its function as part of a receptor complex for BL (Li et al., 2002; Nam and Li, 2002).

The BRI1 signaling pathway extends into the cell via inhibition of BIN1, which in turn negatively regulates two nuclear phosphoproteins, BZR1 and BES1, containing shared domains but no motifs conserved among transcription factors (He et al., 2002). Although the exact biochemical function of these two

proteins remains unclear, their activity shows strong positive correlation with transcript levels of genes upregulated by BL induction (Wang et al., 2002; Yin et al., 2002). This suggests that BZR1 and BES1 may act in the nucleus to enable BL-induced changes in gene expression, from a signal that is communicated across the plasma membrane by BRI1 and BAK1.

In addition to signals which act over long ranges, like hormones, there are extracellular signals which are more local, because they involve ligands which are either membrane-bound or secreted only a short distance. A well-studied example of such a mechanism is the CLAVATA1 (CLV1) / CLAVATA3 (CLV3) pathway. These genes were discovered by virtue of their similar, noticeable loss-of-function phenotypes: enlarged shoot apical meristems, larger floral meristems, and extra floral organs (most notably carpels) (Clark et al., 1993; Clark et al., 1995). This phenotype suggested that CLV1 and CLV3 were required for proper restriction of cell division in shoot and floral meristems. Molecular cloning of these genes revealed that *CLV1* encoded a LRR-RLK, whereas *CLV3* encoded a small secreted protein with no known motifs. *CLV1* and *CLV3* are expressed in overlapping domains within the meristem: the former is found in a central cone-shaped domain containing the stem cells, while the expression pattern of the latter is wider and below the top layers of the central zone (Clark et al., 1997; Fletcher et al., 1999).

The CLV3 protein has a cleavable signal sequence and has been shown to be localized to the extracellular space in transfected onion cells (Rojo et al., 2002; Sharma et al., 2003). Biochemical studies demonstrating that CLV3 and CLV1 belonged to the same protein complex have been retracted (Trotochaud et al., 2000); at this writing, the primary evidence that CLV3 is a CLV1 ligand is genetic.

Another possible candidate for a protein functioning in this proposed signaling complex is CLV2. Loss-of-function *clv2* alleles resemble weak alleles of *clv1* and *clv3*, suggesting that the requirement for CLV2 is not as stringent. However, recent work has indicated that alleles of *CLV1* more likely to be null alleles show a weaker phenotype than the alleles with seemingly subtle missense mutations (Dievart et al., 2003). These results indicate that the stronger *clv1* alleles may be interfering with the function of one or more additional receptors, perhaps by binding them in non-functional heterodimers, or by outcompeting them for downstream effectors.

When *CLV3* is overexpressed, the resulting plants have a terminated meristem phenotype which requires wild-type CLV1 function (i.e., *clv1* is epistatic to *35S::CLV3*). The *wuschel* (*wus*) loss-of-function mutant has a nearly identical phenotype (Laux et al., 1996). *WUS* was found to encode a novel homeodomain protein, and the gene is expressed in a sub-region of the *CLV1* expression domain (Mayer et al., 1998). Consistent with the phenotypic data, *WUS* is ectopically expressed in *clv1* and *clv3* mutants, but downregulated in *35S::CLV3*-containing plants (Brand et al., 2000). WUS therefore seems to be negatively regulated at the transcriptional level by CLV3/CLV1 signaling, although the transcription factor or factors which link CLV3 and CLV1 to the regulation of *WUS* expression have not yet been identified. WUS in turn activates the expression of *AGAMOUS* (*AG*) in floral meristems (Lenhard et al., 2001; Lohmann et al., 2001). In addition, *WUS* misexpression from the *CLV1* or *AINTEGUMENTA* (*ANT*) promoter is sufficient to turn on ectopic *CLV3* expression in the cells in nearby layers, suggesting that *WUS*, *CLV1*, and *CLV3* act in a negative feedback loop (Brand et al., 2000; Schoof et al., 2000). This type

of self-regulating pathway is necessary for maintaining the appropriate balance of cell proliferation and differentiation in a fluid, dynamic structure such as the meristem. Through the action of CLV3 and CLV1, cells are informed of the choices their neighbors are making, and can change transcriptional states to increase or decrease the size of the stem cell population.

1.3 MicroRNAs can modulate gene expression post-transcriptionally

An important mechanism for immediately modifying transcriptional output relies on developmentally regulated endogenous microRNAs (miRNAs), which act on specific target genes to destabilize their mRNA or prevent the mRNA from being translated. Such miRNAs were first discovered in *C. elegans* (Lau et al., 2001; Lee and Ambros, 2001; Olsen and Ambros, 1999; Pasquinelli et al., 2000; Reinhart et al., 2000), although a related type of small RNA, small interfering RNA (siRNA), was described earlier in plant post-transcriptional gene silencing (PTGS) (Hamilton and Baulcombe, 1999; Metzlaff et al., 1997; Smith et al., 1994; Vaucheret et al., 2001). The two kinds of small RNAs are both about 19-24 nucleotides long, and are processed from longer precursors by an enzyme called Dicer (Hutvagner et al., 2001; Ketting et al., 2001; Zamore et al., 2000). siRNAs are capable of guiding cleavage of the protein-coding target mRNA (Martinez et al., 2002; Yang et al., 2000). The two types differ in source and structure, however. siRNAs are double-stranded and generated in the presence of high copy number of a transgene (as in PTGS), or from an exogenous double-stranded precursor (injected dsRNA or a sense-linker-antisense transgene), whereas miRNAs are single-stranded and processed from an endogenous non-coding RNA folded into a stem-loop structure. Most animal

miRNAs actually act not by promoting cleavage of the target mRNA, but by blocking its translation (recently reviewed in (Pasquinelli and Ruvkun, 2002).

Mutations in genes known to act in miRNA metabolism cause a variety of developmental defects. For example, *carpel factory* (*caf*) mutants have extra carpels, defective ovules, abnormal leaf and floral organ shape, and indeterminate growth in the floral meristem (Jacobsen et al., 1999). Stronger alleles of the same gene (*sin1*, *sus1*) cause severe embryo defects (Golden et al., 2002; Ray et al., 1996; Schwartz et al., 1994). The CAF/SIN/SUS1 protein is an RNA helicase/ RNAse III which is homologous to Dicer. In fact, mature miRNAs do not accumulate to normal levels in *caf* mutants, although the abundance of precursors remain the same (Park et al., 2002). This suggests that CAF (recently renamed DICER-LIKE1, or DCL1) is required for processing plant miRNAs into their mature form. The *hen1-1* mutation causes a pleiotropic phenotype reminiscent of *caf*, and accumulation of mature miRNAs in *hen1-1* is similarly lacking. *HEN1* was found to encode a novel protein with homologs in fungi, bacteria, and metazoans (Park et al., 2002). Subsequent work has shown that HEN1 functions in both miRNA accumulation and some forms of PTGS (Boutet et al., 2003).

Work from several labs has resulted in the identification of 24 distinct miRNA sequences in *Arabidopsis*. Most sequences are found in multiple copies in the genome, sometimes quite near each other (for example, *MIR166c* and *d*) (Llave et al., 2002a; Park et al., 2002; Reinhart et al., 2002). Confirming the relevance of miRNAs to proper plant development, several miRNAs are spatially and/or temporally regulated, as detected on Northern blots (Park et al., 2002; Reinhart et al., 2002).

Animal miRNAs predominantly target heterochronic genes – genes which control the timing of developmental events (Abrahante et al., 2003; Grishok et al., 2001; Lee et al., 1993; Lin et al., 2003; Olsen and Ambros, 1999; Pasquinelli et al., 2000; Reinhart et al., 2000) – or genes required for proper morphogenesis (Brennecke et al., 2003; Xu et al., 2003). Similarly, the targets of plant miRNAs are likely to be transcription factors with crucial roles in developmental processes. Recent work to determine the candidate targets for miRNAs has shown that the list includes genes encoding 3 SCARECROW-like transcription factors, 5 NAC transcription factors, 5 MYB proteins, 5 HD-Zip proteins, AP2, and at least one AP2-like protein (Park et al., 2002; Reinhart et al., 2002; Rhoades et al., 2002). The majority of the miRNA-binding sites in the predicted target mRNAs are in the coding region, with the remainder located in the 3' untranslated region (Rhoades et al., 2002).

The first efforts to investigate plant miRNA function led to the surprising conclusion that, unlike the majority of animal miRNAs, plant miRNAs could behave like siRNAs by inducing cleavage of the target mRNA. Llave et al. focused on *MIR39*, which is predicted to target three *SCARECROW* (*SCR*) –like (*SCL*) genes. *MIR39* is expressed most strongly in inflorescences, and it is in this tissue that truncated transcripts (as well as the expected full-length transcripts) of two *SCL* genes can be detected. Further evidence to demonstrate a direct relationship was provided by co-transformation of *N. benthamiana* leaf tissue with *35S::SCL6* and *35S::MIR39* and detection of cleaved versus full-length *SCL6* transcripts. An additional insight was provided by careful analysis of the truncated transcripts: the cleavage event, while showing some preference for

particular base pairs, does not always occur at the same position (Llave et al., 2002b).

A study using wheat germ extract produced some additional exciting results. First, the extract was shown to include an RNA-induced silencing complex (RISC) pre-loaded with endogenous wheat miRNA. Using this extract in an *in vivo* assay, the authors observed cleaved wild-type *PHAVOLUTA* (*PHV*) mRNA, but not *PHV* mRNA containing a dominant mutation in the miRNA recognition sequence. This result confirmed the hypothesis, initially put forth by Rhoades et al. (2002), that the dominant alleles in *PHV* and *PHABULOSA* (*PHB*) are resistant to down-regulation by the activity of MIR165 and 166, the miRNAs predicted to bind *PHV* and *PHB* mRNAs (Tang et al., 2003). Since *PHV* and *PHB* are known to be required for proper polarity of lateral organs, these experiments uncovered a functionally relevant miRNA. Finally, this work showed that perfect miRNA:mRNA complementarity is not required for the cleavage-based mechanism of miRNA action.

MIR172 is the first published example of a plant miRNA acting through translational repression (rather than cleaving the target mRNA). *MIR172* is found in several copies in the genome and is predicted to target *AP2* and 3 genes in the same family. When this miRNA is overexpressed, the resulting phenotype resembles that of *ap2* loss-of-function alleles. *AP2* mRNA levels are unaffected, while AP2 protein is missing. In the *hen1* and *dcl1* mutant backgrounds, where miRNAs do not accumulate to wild-type levels, AP2 protein levels are higher than normal, but the *AP2* message remains at wild-type abundance. The same is true when a *35S::AP2* construct with mutations in the miRNA recognition site is transformed into plants (*35S::AP2ml*) (Chen, 2003). Overexpression of *MIR172*

results in a phenotype resembling an *ap2* loss-of-function mutant (with no change in *AP2* mRNA levels – only protein), and these plants also flower earlier than wild-type. This aspect of the phenotype suggests that *MIR172* does, in fact, target another of the *AP2*-like genes (in particular, At2g28550, which is known to block the transition to flowering) (Aukerman and Sakai, 2003; Chen, 2003).

Additional miRNAs have been shown to function by negatively regulating their predicted target mRNAs; for example, *miRJAW* is complementary to sequences in the transcripts of several TCP transcription factors, and has been shown to promote cleavage of *TCP4* (Palatnik et al., 2003).

A slight gap in our understanding of plant miRNA function stems from the fact that no loss-of-function alleles in miRNAs have been identified. The best genetic evidence for miRNA function, outside of the results from overexpressing miRNA-resistant target mRNAs, has been the *caf/dcl1* and *hen1* mutants mentioned above. Although these two mutants have a general defect in miRNA metabolism, the phenotypes do not always parallel that of the dominant gain-of-function alleles or overexpressed, mutated transgenes. For example, overexpression of miRNA-resistant *AP2* causes homeotic transformations in the third and fourth whorls, but for *hen1*, such phenotypes are only visible in a sensitized background (*hua1-1*; *hua2-1*) (Chen, 2003).

1.4 New insight into miRNA function in plant development

In this work, we describe a new miRNA, called *EARLY EXTRA PETALS* (*EEP1*), which plays a role in organ formation and patterning in the shoot and flower. Plants homozygous for the recessive *eep1* mutation have extra petals in the first ten flowers, and *eep1* enhances the floral phenotype of *pinformed* (*pin1*)

and *pinoid* (*pid*), as well as the *pid* phenotype in the shoot. The miRNA encoded by *EEP1* is closely related to published miRNAs *MIR164a* and *b* (Reinhart et al., 2002); all three are predicted to bind the mRNAs of six target genes in the NAC family of transcription factors (Rhoades et al., 2002). Included in this group of six genes are *CUC1* and *CUC2*, which mark boundaries between organs in the flower and between cotyledons in the developing embryo (Aida et al., 1997; Takada et al., 2001). Ubiquitous overexpression of *EEP1* from the cauliflower mosaic virus *35S* promoter leads to phenotypes resembling the *cuc1*; *cuc2* double mutant (Aida et al., 1997); furthermore, *CUC2* mRNA is significantly less abundant in *35S::EEP1* compared to wild type. These data suggest that *EEP1*can negatively regulate *CUC1* and *CUC2* post-transcriptionally, and that it acts via an mRNA cleavage-based mechanism, at least for *CUC2*. We discuss the possible implications of this negative regulation, as it relates to *pin1*, *pid*, and the interactions of each mutant with *eep1*.

1.5 Advances in functional characterization of proteins from the CLE family

In second part of this work, we describe a set of reverse genetic experiments designed to test whether several CLV3-like proteins might function as ligands for one or more RLKs. The genes encoding these proteins belong to a family of 25 *Arabidopsis CLV3/ERS* (*CLE*) genes, all of which code for predicted secreted or membrane-bound proteins of approximately 100 amino acids (Cock and McCormick, 2001). They share a small conserved domain in the C terminus – otherwise, there is little or no sequence conservation among them. We sought to investigate the function of five of these proteins, using overexpression, double-stranded RNA interference (dsRNAi), and reporter lines. We found that CLE11

and CLE12 were capable of mimicking CLV3 when ubiquitously overexpressed, in that they caused meristem termination and resulted in rare, defective flowers. CLE16 and CLE17, when overexpressed, cause defects in leaf shape but do not affect the meristem. We hoped to shed light on the endogenous functions of these proteins via the dsRNAi experiments, but they yielded no mutant phenotypes. Expression analysis indicates that the *CLE* genes are transcribed in restricted but overlapping patterns, predominantly in reproductive tissues. We discuss several explanations for the results, both positive and negative, that we have generated with these experiments.

1.6 References

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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*(*era1I/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.

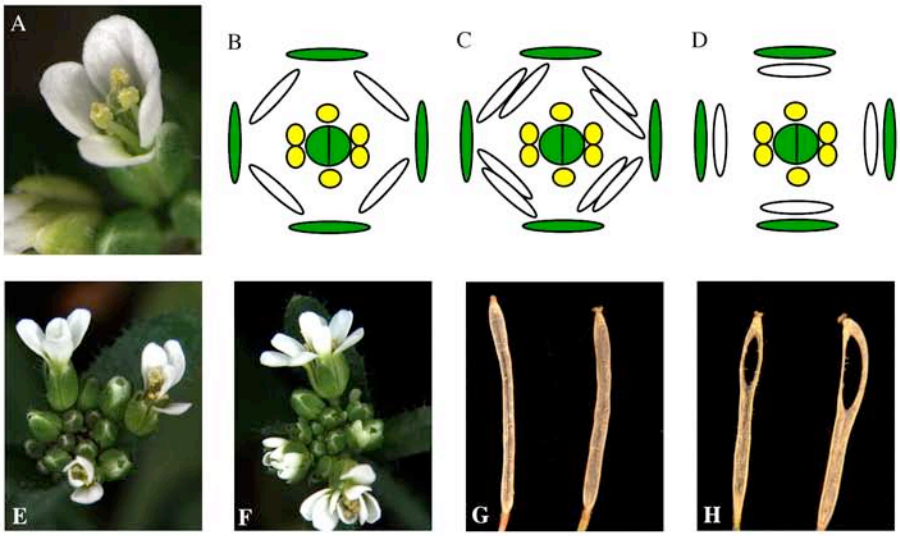

Figure 2.1 *eep1* has extra petals in early flowers and slightly defective septa.
A) L-*er* flower.
B) Diagram of wild-type flower. From outside: sepals, petals, stamens, and two fused carpels.
C, D) Diagrams of two phenotypes predicted for petals if position within the whorl and position relative to 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
clv1-1	4.34±0.08	41	*clv1-1; eep1*	6.27±0.19*	30
ett-3	4.27±0.09	41	*ett-3; eep1*	6.13±0.21*	30
pan-2	4.69±0.07	45	*pan-2; eep1; er*	4.77±0.14*	30
pid-2	7.68±0.27	25	*pid-2; eep1*	7.83±0.65#	23
pnh-1	4.06±0.04	49	*pnh-1; eep1*	7.88±0.25*	40
ult-2	4.57±0.13*	30	*ult-2; eep1*	8.10±0.23*	40

Table 2.1 Average petal number (± 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.

C	*pid-2*, n=24	*pid-2; eep1*, n=23
sepals	2.79±0.21	0.74±0.23
petals	7.68±0.27	7.83±0.65
stamens	2.38±0.25	1.87±0.28
D	*pid-2*, n=20	*pid-2; eep1*, n=22
flowers formed before IM termination	11.2±0.5	1.6±0.15

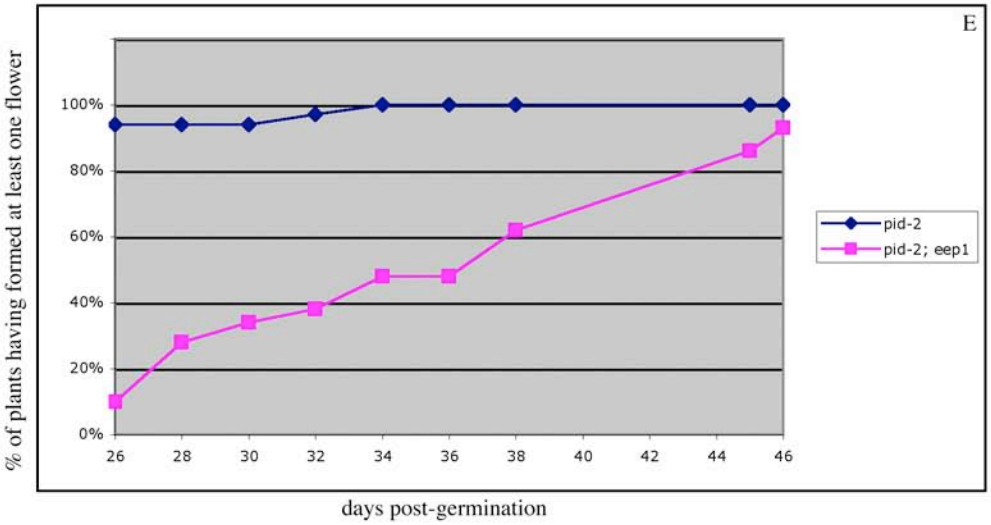

Figure 2.3 *eep1* severely enhances the *pinoid* (*pid*) phenotype in both the shoot and flower.
A) *pid-2* homozygotes produce several flowers before terminating abruptly. These flowers usually have extra petals but a reduced number of sepals, stamens, and carpels.
B) *pid-2*; *eep1* double mutants 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*.

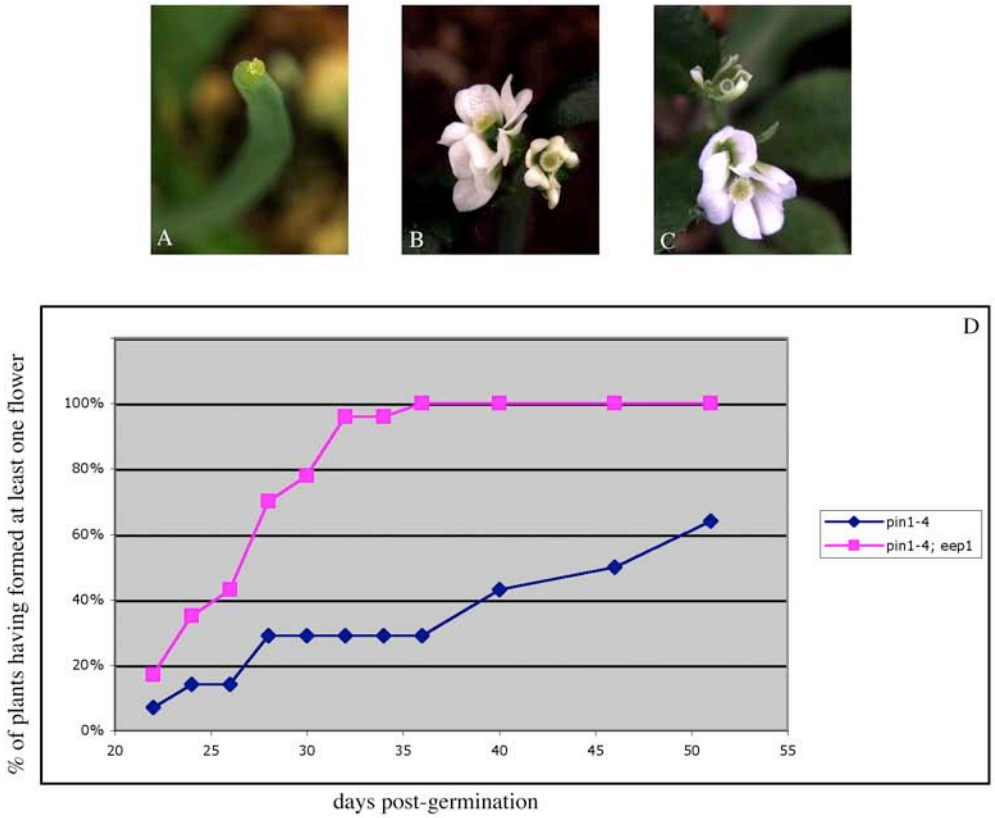

Figure 2.4 *eep1* partially suppresses the delayed flowering of *pinformed* (*pin1*) and further reduces sepal number.
A) *pin1* inflorescences are replaced by pin-like structures, which can produce very few flowers apiece, and then only after a long delay.
B) *pin1-4* flowers have more petals than wild-type, 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 #L12220). 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).

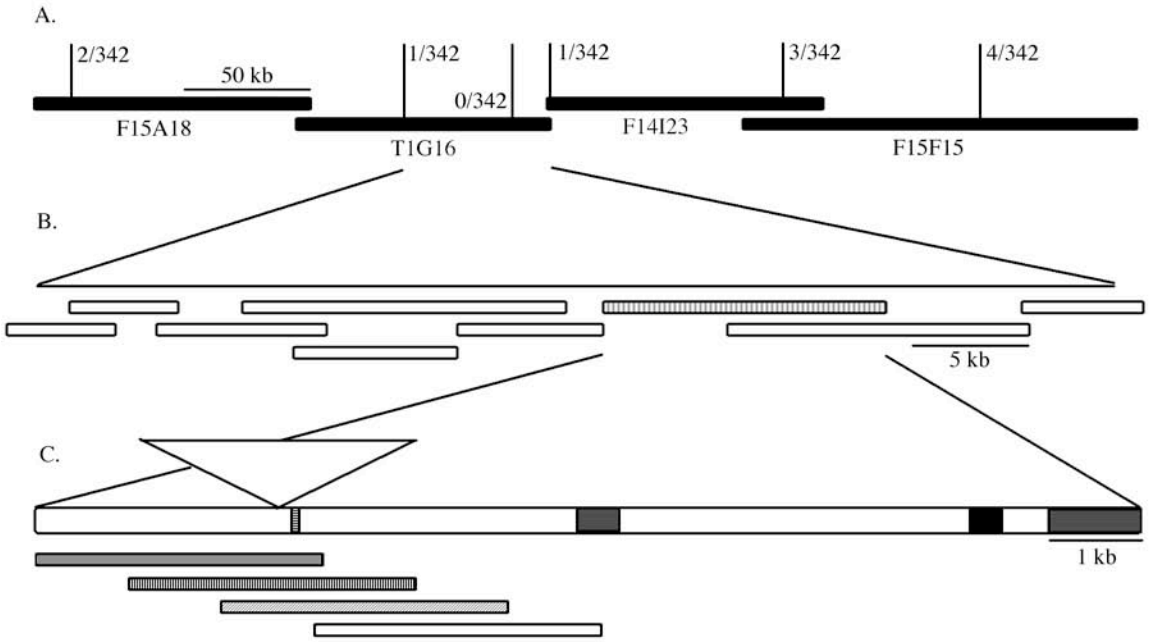

Figure 2.5 The mapping and identification of the *eep1* mutation.
A) Details of the chromosome walk, with black bars representing BAC clones in the TAIR database. Recombination data are provided in terms of the number of chromosomes recombinant at a given marker, over the total number of chromosomes 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).

A.
MIR164a tctccatgttggagaagcagggcacgtgcaaaccaacaaa
MIR164b tgagcaagatggagaagcagggcacgtgcattactagctc
EEP1 aacacttga**tggagaagcagggcacgtgc**gaacacaaatg

B.
```
         10        20        30        40        50        60
     u   ua    u  a     a  c              caca     aa       cgg
  ggg gag  acac ug uggag ag agggcacgugcgaa     aaug  aucgau    u
  ccc cuc  ugug ac accuc uc ucuugugcacgcuu     uuau  uaguug    a
     -   uc    c  a     a  a                ----     ac       uuc
    110       100        90        80             70
```

C.

		EEP1 predicted miRNA	*MIR164a,b* miRNA
		tggagaagcagggcacgtgcg	tggagaagcagggcacgtgca
		recognition site, 3' to 5'	recognition site, 3' to 5'
TGT1	At5g07680	acctcttcgtcccgtgca**tct**	acctcttcgtcccgtgca**tc**t
TGT2	At5g61430	acctcttcgtcccgtgca**ttt**	acctcttcgtcccgtgca**tt**t
TGT3	At1g56010	acctcttcgtccc**a**tgcacg**a**	acctcttcgtccc**a**tgcacg**a**
TGT4	At5g39610	**g**cctcttcgtcc**a**gtgcac**t**c	**g**cctcttcgtcc**a**gtgcac**tc**
TGT5	At3g15170	acctctt**t**gtcc**t**gtgcacg**a**	acctctt**t**gtcc**t**gtgcacg**a**
TGT6	At5g53950	acctctt**t**gtcc**t**gtgcacg**a**	acctctt**t**gtcc**t**gtgcacg**a**

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-canoncial G-U pairs.

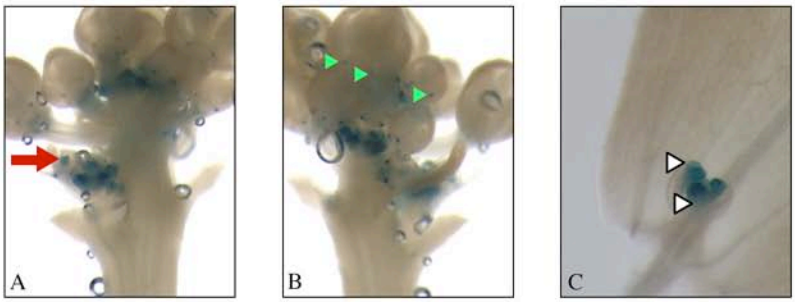

Figure 2.7 The *EEP1* promoter drives *GUS* expression in young floral buds of wild-type plants.
A, B) Opposite views of one *pEEP1::GUS* inflorescence. GUS activity is visible in early flowers, with lingering expression in developing carpels (arrowhead). Expression in later flowers is restricted to 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., 2002b; 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.

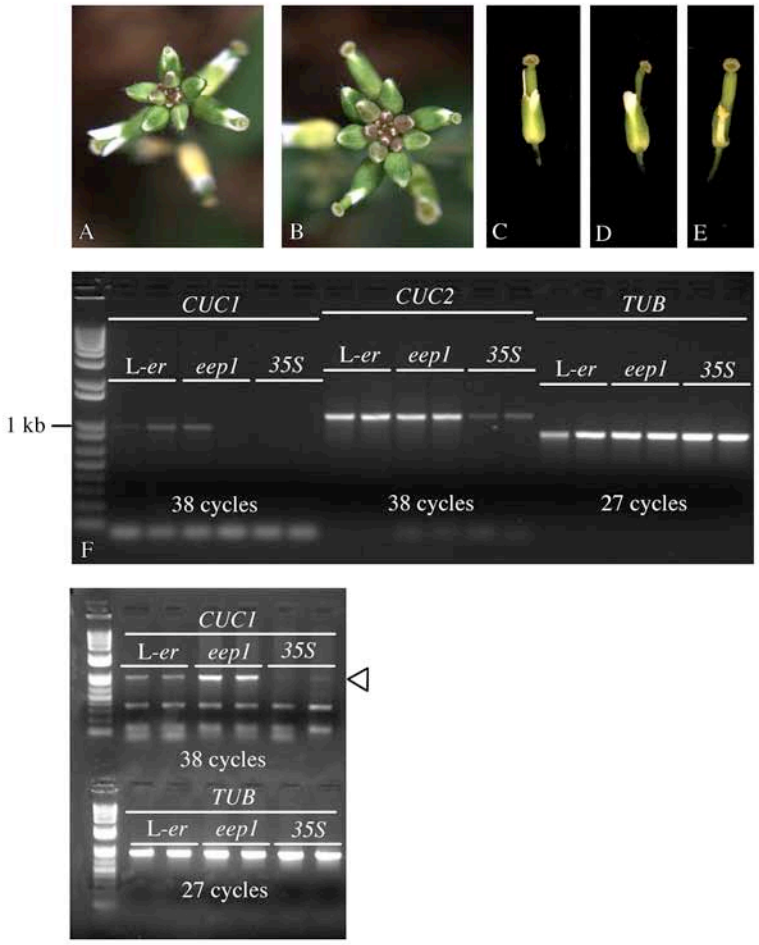

Figure 2.8 A strong line of *35S::EEP1* is very similar to *cuc1*; *cuc2* double mutants, and *CUC1* and *CUC2* mRNA levels are decreased in this line relative to wild type.
A) A *35S::EEP1* line with an intermediate phenotype. Sepals are partially fused, and stamen number is reduced. B, C, D, E) A single *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 amplication 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 regulated 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., 2002b; 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 μl of template DNA was used for each 25 μl 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 μl of the 10 μl 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′-atggatgttgatgtgtttaacgg-3′, TGT5-F3, 5′-gatgaatcccttatgccacc-3′, TGT5-R, 5′-tgtggccgtttactctctga-3′, CUC2-F, 5′-atggacattccgtattaccac-3′, and CUC2-R, 5′-tcagtagttccaaatacagtcaag-3′. Tubulin transcripts were amplified as a control, with the following primer pair: TUB2-P1, 5′-atccgtgaagagtacccagat-3′ and TUB2-P2, 5′-tcaccttcttcatccgcagtt-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′-ttaagtcatccgcaatgcctac -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'–tcttctatttggatgatacattta–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 Na_2HPO_4, 15.8 mM NaH_2PO_4, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4FE(CN)_6$, 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.

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Chapter 3. Functional analysis of five CLE proteins by reverse genetics

3.1 Introduction

A major class of transmembrane receptors in plants is made up of receptor-like kinases, or RLKs. The *Arabidopsis thaliana* genome contains over 400 genes encoding these receptors, which are subdivided into smaller groups based on motifs in the extracellular domain. Slightly over half of the RLK family is made up of receptors with leucine-rich repeats (LRRs) in their extracellular portion (Shiu and Bleecker, 2001). LRR-RLKs have been shown to be instrumental in a number of developmental processes, including hormone response, shoot architecture, and floral organ abscission (Jinn et al., 2000; Li and Chory, 1997; Torii et al., 1996).

One of the first LRR-RLKs to be functionally characterized was *CLAVATA1* (*CLV1*), a receptor required to control cell proliferation in shoot and floral meristems (Clark et al., 1993; Clark et al., 1997; Jinn et al., 2000). Loss-of-function *clv1* alleles give rise to plants with enlarged, fasciated shoot meristems and flowers with increased organ number (Clark et al., 1993). Similar phenotypes are observable in *clv3* loss-of-function mutants (Figure 3.1, B). Since *CLV3* encodes small secreted protein of 96 amino acids (Fletcher et al., 1999; Rojo et al., 2002), it is considered a likely candidate for the CLV1 ligand. The predicted ligand-receptor relationship is supported by several pieces of genetic data. First, plants trans-heterozygous for mutant alleles of *clv1* and *clv3* show a *clv* phenotype, a result which in some situations points to physical interaction (direct or indirect) between the two proteins (Clark et al., 1995). Second,

constitutive overexpression of *CLV3* causes meristem termination in wild-type plants (Figure 3.1, C), but not in the absence of CLV1 activity, suggesting that CLV1 functions downstream of CLV3 (Fletcher et al., 1999). Third, *CLV3* expression in the stem cells partially overlaps with *CLV1* expression. Fourth, CLV3 can act non-cell-autonomously in the floral meristem (Fletcher et al., 1999; Lenhard and Laux, 2003; Rojo et al., 2002).

In addition to CLV1 and CLV3, a third protein may function as part of this signaling complex. Loss-of-function mutations in *CLV2* give rise to a weak *clavata*-like phenotype (Kayes and Clark, 1998). *CLV2* encodes an LRR-containing receptor-like protein, which lacks the kinase domain present in LRR-RLKs (Jeong et al., 1999). Although there is no biochemical evidence for the presence of CLV2 in this complex, work on the brassinolide (BL) receptor BRI1 has indicated that LRR-RLKs can indeed function as heterodimers. BRI1 is known to bind BAK1; both are RLKs and contain 25 and 5 LRRs, respectively (Li et al., 2002; Li and Chory, 1997).

There are very few known ligands that act on LRR-RLKs (all but five of the LRR-RLKs which have been functionally characterized are "orphan" receptors). They include hormones (BL, systemin [in tomato], and phytosulfokine) and exogenous proteins found in pathogens (flagellin, and an epitope from the rice pathogen *Xanthomonas oryzae*) (Gomez-Gomez and Boller, 2000; He et al., 2000; Matsubayashi et al., 2002; Montoya et al., 2002; Song et al., 1995). Phytosulfokine and systemin are small peptides (5 and 18 amino acids, respectively). CLV3 is therefore the largest endogenous protein believed to signal through an LRR-RLK.

Work in the McCormick lab has resulted in the characterization of a family of genes similar to *CLV3*, termed the *CLV3/ESR* (*CLE*) genes. There are 26 such genes in *Arabidopsis* (including *CLV3*), and additional genes have been identified in other plant species (maize, rice, soybean, tomato) but not in animals. Each of the *CLE* genes encodes a small protein predicted to be secreted or membrane-bound. All of these proteins also share a small C-terminal conserved domain of 15 amino acids, including an invariant histidine and arginine and a highly conserved glycine (Cock and McCormick, 2001). Subsequent work by Sharma and Fletcher has indicated that all but one of the 26 CLE genes in *Arabidopsis* are expressed at sufficient levels for detection by RT-PCR. In addition, their expression is tissue-specific. When fused with GFP and transiently expressed in leek epidermal cells, all three CLE genes tested were localized to the membrane or extracellular space, as predicted by their protein sequences (Sharma et al., 2003).

Recent studies of *CLE19* and *CLE40* have indicated that multiple CLE proteins may be capable of activating a restricted number of pathways, notably the CLV1 pathway in the shoot, and a potentially related (but not identical) pathway in the root. For example, root-specific overexpression of *CLE19* leads to a gradual reduction in cell number in the root meristem, and an extragenic suppressor of this phenotype has a *clv*-like effect on carpel number (Casamitjana-Martinez et al., 2003). No loss-of-function phenotype has been reported for *CLE19*, however. Also, mention of the widespread *CLE19* expression pattern and unpublished data alluding to additional, non-root phenotypes in *35S::BnLLP1* (the tomato ortholog of *CLE19*) would imply that CLE19 is just as likely to function in other tissues besides the root (Casamitjana-Martinez et al., 2003).

The data for CLE40 suggest that this protein is able to act both in the shoot and the root to limit cell division, and that its signaling capabilities in the shoot are CLV1-dependent. It was also noted in this study that *CLV3*, while not normally expressed in the root, can cause the same root meristem defect (when overexpressed) as *CLE40*. The only phenotype in the single loss-of-function allele of *CLE40* is a increased waving of the primary root, with no observable defect in the structure of the root meristem itself (Hobe et al., 2003).

These experimental data for CLE19 and CLE40 point to the possibility that multiple CLE proteins may be able to bind CLV1 and/or a CLV1-like receptor in the root meristem. A lack of meristematic phenotypes (in the shoot or the root) in *cle40* loss-of-function mutants indicates that a) the overexpression phenotype does not represent the endogenous function of CLE40 or that b) loss of CLE40 activity in the shoot and/or root may be more than adequately compensated for by CLV3. This second possibility has been tested: the *cle40*; *clv3-2* double mutant is indistinguishable from *clv3-2* (Hobe et al., 2003).

In this study, we have used reverse genetics to investigate the functions of five additional *CLE* genes: *CLE11*, *12*, *13*, *16*, and *17*. *CLE11*, *12*, and *13* are of particular interest because their sequences align closely with that of *CLV3* (see Figure 1D and Sharma et al., 2003). We show that CLE11 and CLE12 are capable of signaling through CLV1/CLV2 when overexpressed. Expression analysis and double-stranded RNA interference (dsRNAi) experiments indicate, however, that this phenomenon is likely not representative of the endogenous function of CLE11 and CLE12.

D.

```
CLV3   MDSKSFVLLLLLFCFLFLHDASD
CLE11  MTKQPKPCSFLFHISLLSALFVFLLISFAFTTSYKLKSGIN
CLE12  MLRISSSSSMALKFSQILFIVLWLSLFFLLLHHLYSLNFRRLYSLNAVEPSLLKQHYRSY
CLE13  MATTRVSHVLGFLLWISLLIFVSIGLFGNFSSKPINPFPSPVITLPALYY
CLE16  MEACSRKRRRRRAYTTSTTGYAAVFFCGIFVFAQFGISSSALFAP
CLE17  MTMCFFLFFFVFYVS                    FQIVLSSSAS
CLE19  MLHLFILYAPYSLYINISILILFALLSNVAIYNNPAFAFLHIISPSNKQKQYLTKNRQMKIKGLMILASSLLIL
CLE40  MAAMKYKGSVFIILVI

CLV3   LTQAHAHVQGLSNRKMMMMKMESEWVGANGEAEKAKTKGLGLHEELRTVPSGPDPLHHHVNPPRQPRNNFQLP
CLE11  SLGHKRILASNFDFTPFLKNKDRTQRQRQSPSLTVKENGFWYNDEERVVPSGPNPLHH
CLE12  RLVSRKVLSDRFDFTPFHSRDNSRHNHRSGEQYDGDEIDPRYGVEKRRVPSGPNPLHH
CLE13  RPGRRALAVKTFDFTPFL KDLRRSNHRKALPAGGSEIDPRYGVEKRLVPSGPNPLHH
CLE16  DHYPSLPRKAGHFHEMASFQAPKATVSFTGQRREEENRDEVYKDDKRLVHTGPNPLHN
CLE17      VGYSRLHLVASPPPPPPRKALRYSTAPFRGPLSRDDIYGDDKRVVHTGPNPLHN
CLE19  AFIHQSESASMRSLLMNNGSYEEEEQVLKYDSMGTIANSSALDSKRVIPTGPNPLHNR
CLE40  LLLSSSLLAHSSSTKSFFWLGETQDTKAMKKEKKIDGGTANEVEERQVPTGSDPLHHKHIPFTP
```

Figure 3.1 CLAVATA3, a protein required for proper meristem regulation, is the founding member of the *CLV3/ESR* (*CLE*) gene family.
A) L-*er* (wild type) B) *clv3-2*. Loss-of-function mutations in *clv1* and *clv3* result in meristem fasciation and the production of extra organs in the flowers, particularly in the central whorls.
C) *35S::CLV3* plants have defective apical and axial meristems, each of which terminates after producing 1–3 leaves.
D) Sequence alignment of selected CLE proteins. The conserved C-terminal region is underlined. Functional analyses of CLV3, CLE19, and CLE40 have been published elsewhere (see text); reverse genetics experiments for CLE11-13, 16, and 17 are presented in this work.

3.2 Results

The overexpression of *CLE* genes results in specific developmental defects

To begin to understand the possible functions for the *CLE* genes, we overexpressed *CLE11-13*, *16*, *17*, and *CLV3*, under the control of the constitutive CaMV 35S promoter. As has already been published, plants carrying *35S::CLV3* have early-terminating meristems, both apically and axially. Since each meristem only gives rise to 1–3 leaves, this lends the plant a bushy appearance. The rare flowers produced by *35S::CLV3* have a reduced number of stamens and no central gynoecium (Fletcher et al., 1999). The phenotype of *35S::CLE12* (Figure 3.2, B and F) is remarkably similar, except that a greater proportion of the T1 plants produce flowers. Transgenic lines of intermediate strength are able to recover sufficiently from meristem termination to set seed, albeit at reduced yields compared to wild type. These plants are shorter in stature than wild type (data not shown). All of the misexpression experiments which include *CLE12* were also performed with *CLE11*, with identical results. For the sake of brevity, only the results from *CLE12* are shown.

CLE13, though grouped tightly with *CLV3*, *CLE11*, and *CLE12* (Sharma et al., 2003), does not cause a discernible phenotype when overexpressed (Figure 3.2, C and G). We confirmed that *CLE13* is transcribed at high levels in these lines, but we cannot determine the level of CLE13 protein in the absence of an antibody or tagged CLE13. The *CLE13* transcript may be under strict translational control, such that overexpressing the gene does not actually lead to higher abundance of protein. Alternatively, the CLE13 protein may require post-translational modification. It is also possible that CLE13 is unable to bind and/or

activate the CLV1 receptor. If this last option were true, it would suggest that either the binding specificity of the CLE proteins does not reside in the conserved C-terminal domain, or that unique sequences in the non-conserved portion of the protein actually inhibit or repress the binding of CLE13 to CLV1.

CLE17 (data not shown) and *CLE16* (Figure 3.2, D and H) do not affect either shoot or floral meristems when overexpressed. The only defects are in leaf shape (slightly smaller and narrower than wild type) and stature (as in *35S::CLE11* and *35S::CLE12*). As of this writing, there is no receptor-like kinase which is known to be involved in leaf size, with the exception of BRI1 (Li and Chory, 1997). Neither loss-of-function mutants in *BRI1* nor overexpression lines cause a phenotype that resembles *35S::CLE16* and *35S::CLE17*. Instead, decreases and increases in BRI1 activity cause severe dwarfing and greatly increased cell elongation, respectively (Li and Chory, 1997; Wang et al., 2001).

CLE12 overexpression phenotype requires _CLV1_ and _CLV2_, but not _CLV3_

Since *CLE12* has a similar overexpression phenotype to *CLV3*, we investigated whether CLE12, like CLV3, acts through the CLV1/CLV2 receptor complex. Plants homozygous for loss-of-function mutations in *clv1*, *clv2*, and *clv3* and transgenic for *35S::CLE12* demonstrate that this is in fact the case. *clv1* and *clv2* mutations are epistatic to *35S::CLE12* (as they are to *35S::CLV3*), suggesting that the wild-type function of these genes is required for the overexpression phenotype (Figure 3.3, A-C). *35S::CLE12* is epistatic to the *clv3-2* mutant phenotype (Figure 3.3, D), implying that CLE12 does not need to form a heterodimer or multimer with CLV3 in order to bind the CLV1 receptor.

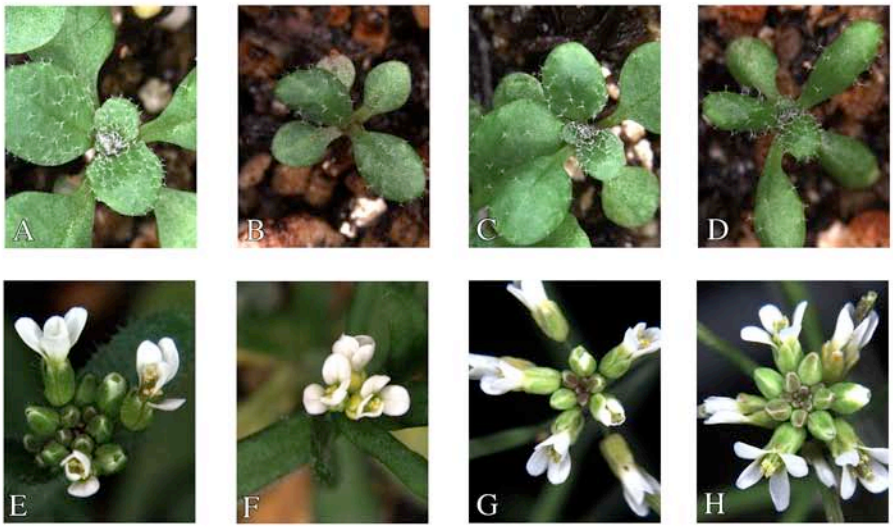


Figure 3.2 Overexpression of several *CLE* genes results in specific phenotypes.
A-D) Vegetative rosettes (17-day-old seedlings).
A) L-*er* (wild type).
B) *35S::CLE12*, like *35S::CLV3*, causes termination of the shoot apical meristem after 1, 2, or 3 true leaves. Axial meristems produce additional leaves, but never an ordered rosette.
C) *35S::CLE13* has no visible effect on vegetative growth.
D) Overexpression of *CLE16* results in smaller and narrower rosette leaves.
E-H) Inflorescences.
E) L-*er*.
F) Strong lines of *35S::CLE12* produce a limited number of flowers before the inflorescence meristem terminates. These flowers have a reduced number of stamens and no central gynoecium. Weaker *35S::CLE12* lines are able to recover sufficiently to make carpels and set seed (data not shown).
G) *35S::CLE13* is indistinguishable from wild type.
H) *35S::CLE16* shows no major defects in the flower, except a slight reduction in female fertility.

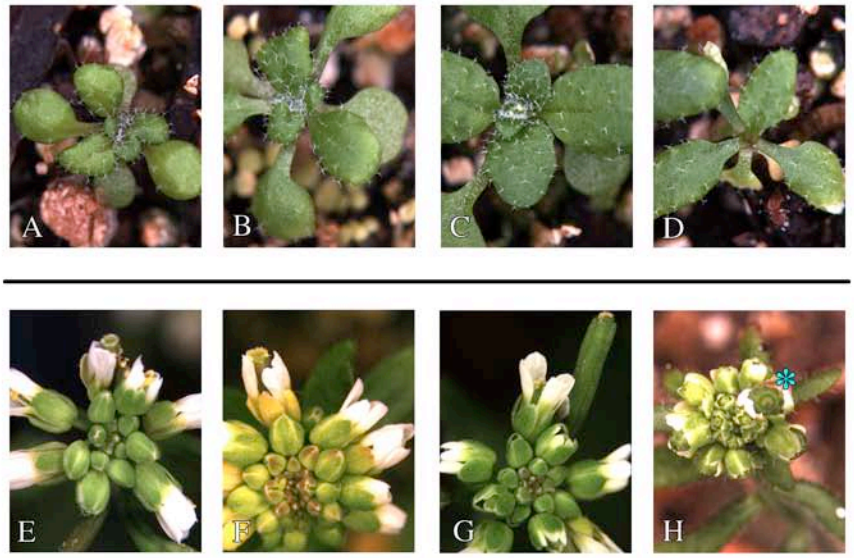


Figure 3.3 The *35S::CLE12* phenotype requires wild-type CLV1 activity, but not CLV3.
A) *35S::CLE12*; *clv1-1*
B) *35S::CLE12*; *clv1-6*
C) *35S::CLE12*; *clv2-1*
D) *35S::CLE12*; *clv3-2*. Note that *clv1-1*, *clv1-6*, and *clv2-1* are epistatic to *35S::CLE12*, but *35S::CLE12* is epistatic to *clv3-2*, as demonstrated by the terminated meristem in D.
E) *clv1-1* F) *35S::CLE12*; *clv1-1*
G) *clv1-6* H) *35S::CLE12*; *clv1-6*. Carpel number in *clv1-1* is unchanged with the addition of *35S::CLE12*, but is increased in *35S::CLE12*; *clv1-6* relative to *clv1-6* alone (see asterix in H).

There is a curious allele-specific effect of *35S::CLE12* on *clv1* mutants: the overexpression of *CLE12* enhances *clv1-6* dramatically in the flower (Figure 3.3, G and H), but has little influence on *clv1-1* and *clv1-4* (Figure 3.3, E and F). The *clv1-6* mutation is a one-base pair deletion which causes a frameshift and an early stop (causing much of the CLV1 kinase domain to be missing as a result), while the *clv1-1* and *clv1-4* alleles have missense mutations in the kinase domain and extracellular domain, respectively (Clark et al., 1997).

Physiological levels of CLE12 can substitute for CLV3

To determine whether the apparent function of these proteins was an artificial by-product of their overabundance, we set out to determine whether they could functionally replace CLV3 at physiologically relevant levels. For this experiment, we used the *CLV3* promoter to drive expression of *CLV3*, *CLE12*, and *CLE13* in a *clv3-2* mutant background. CLV3 rescues the *clv3-2* phenotype completely, while CLE12 shows nearly complete rescue (Figure 3.4, B and C, respectively). CLE13 has no effect.

The *CLE* genes are expressed in diverse but overlapping patterns in the inflorescence

To gain a better understanding of the endogenous functions of these proteins, we analyzed their expression patterns by generating beta-glucuronidase (GUS – EC# 3.2.1.31) reporter lines for each gene. We subcloned a given length of 5′ sequence for each gene (between 1.3 and 3.0 kb, depending on the proximity and orientation of the nearest 5′ gene). Transformants were stained for GUS activity to determine where each gene was expressed.

Figure 3.4 CLE12 can functionally replace CLV3 in the meristem.
A) *clv3-2* has enlarged shoot and floral meristems, resulting in fasciation and extra floral organs.
B) The expression of *CLV3* from its native promoter is sufficient to rescue the *clv3-2* phenotype.
C) *pCLV3::CLE12* shows partial rescue of *clv3-2*. Some fasciation is still evident.
D) CLE13, though highly similar to CLV3, CLE11, and CLE12 at the level of protein sequence, cannot rescue *clv3-2* when expressed from the *CLV3* promoter.

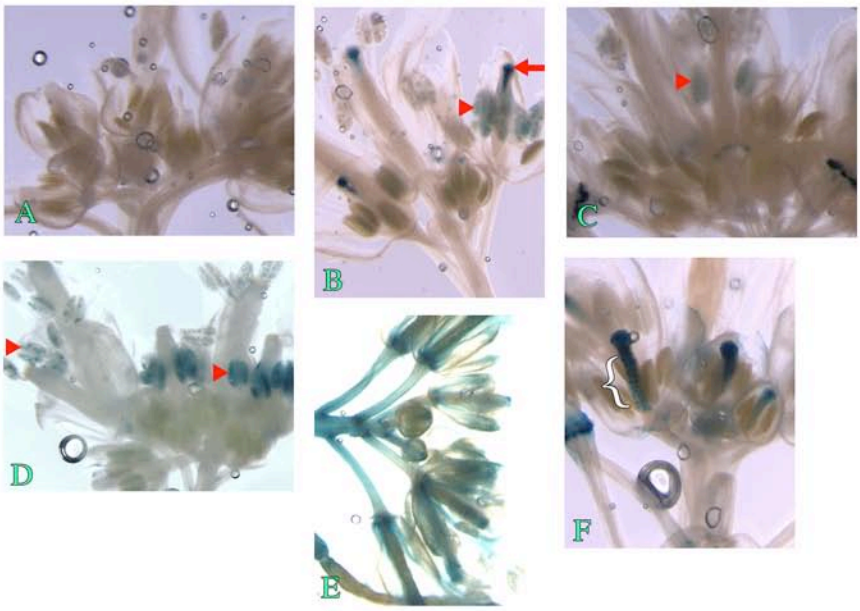


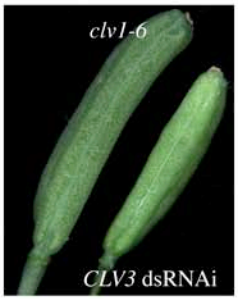
Figure 3.5 The *CLE* genes have discrete but overlapping expression patterns in the inflorescence.
A) L-*er* (wild type).
B) *pCLE11::GUS*. Note expression in developing pollen (arrowhead) and stigmae (arrow).
C) *pCLE12::GUS*. *CLE12* is expressed at low levels in stamens.
D) *pCLE13::GUS*, showing staining in stamens. The GUS activity is restricted to pollen in older stamens.
E) *pCLE16::GUS*
F) *pCLE17::GUS*. *CLE17* is expressed in stigmatic tissue and briefly in ovules (white bracket).

None of the five genes tested are expressed in the inflorescence meristem or floral meristems, suggesting that the overexpression phenotypes of *CLE11* and *CLE12* do not represent their true role *in vivo*. However, there are some specific yet overlapping expression patterns. In particular, *CLE11*, *12*, and *13* are expressed in stamens (Figure 3.5, B, C, and D), whereas *CLE11* and *CLE17* signal is found in stylar and early stigmatic tissue (Figure 3.5, B and F). *pCLE17::GUS* also shows temporally restricted but robust staining in ovules (Figure 3.5, F).

RNA interference of the *CLE* genes

We next set out to investigate whether these overexpression phenotypes (for CLE11, 12, 16, and 17) represented the endogenous function of these genes. Since there are no known point mutations or T-DNA insertions in any of these genes, we decided to use double-stranded RNA interference (dsRNAi). The constructs used contained the specific gene in the sense and antisense orientation, so that the resulting RNA would form a double-stranded structure. Such structures are known to be processed into siRNAs, which can destabilize the mRNA of the endogenous gene.

Plants carrying a *CLV3* dsRNAi construct had phenotypes similar to those of *clv3* and *clv1* loss-of-function mutants (Figure 3.6, A). Similar constructs made for the *CLE* genes had no visible effect under normal growing conditions (data not shown).



	T1	T2
35S::CLV3(AS)-intr-CLV3(S)	7/11	4/5
35S::CLE11(AS)-intr-CLE11(S)	0/26	0/10
35S::CLE12(AS)-intr-CLE12(S)	0/11	0/10
35S::CLE13(AS)-intr-CLE13(S)	0/14	0/10
35S::CLE16(AS)-intr-CLE16(S)	0/8	0/3
35S::CLE17(AS)-intr-CLE17(S)	0/14	0/9

Figure 3.6 Double-stranded RNA interference (dsRNAi) of *CLV3* has a *clv*-like phenotype, but no phenotypes are observed after dsRNAi of *CLE11-13*, *16*, or *17*.
The panel on the left shows green siliques from *clv1-6* and a typical *CLV3* dsRNAi line. The table at right lists the number of lines showing a phenotype over the total number of lines screened, in the T1 and T2 generations. For the *CLV3* dsRNAi T2 analysis, progeny of 3 strong lines, 1 intermediate line, and one wt-like line were grown.

3.3 Discussion

In the course of these experiments, we have gained insight the functions some of these CLE proteins are capable of performing. In particular, CLE11 and CLE12 can mimic CLV3 when overexpressed, and ectopic CLE16 and 17 activity can alter leaf shape. However, the total absence of RNA interference phenotypes leaves their true *in vivo* function a mystery. The failure of dsRNAi to cause mutant phenotypes for the *CLE* genes could suggest at least two possible scenarios. First, the reduction of function in one CLE protein could be compensated for by another. This is conceivable because (a) more than one *CLE* gene is expressed in a given tissue type (stamens, style) and (b) it is clear from this and other published work that many CLE proteins are capable of substituting for CLV3. A second scenario assumes that these five proteins are kept inactive under normal conditions, by mechanisms affecting such processes as post-translational modification, sub-cellular localization, or abundance of a co-factor and/or receptor.

Another intriguing aspect of this set of results is that the plants overexpressing these genes did not show defects in the tissues in which the genes are actually expressed. This phenomenon can be accounted for by at least two possible explanations. First, the CLE proteins may act under normal growth conditions to repress responses to external stimuli, such as heat, cold, salinity, or pathogen attack. In this situation, overexpression of a *CLE* gene will cause a phenotype (suppressed response) only in the presence of the appropriate external stimulus. Another explanation is similar to the second scenario described above for understanding the lack of RNAi phenotypes; namely, that

the activity of the CLE proteins is heavily regulated and restricted at the protein level, rather than at the level of transcription. One caveat is that this regulation, if at the level of protein stability or general receptor-binding activity, would have to be restricted to the tissues in which each gene is normally expressed; otherwise, there would be no overexpression phenotype at all. Stringent receptor-specific regulation would not necessarily have to be restricted to the tissues expressing each gene. If it was, however, aspects of the overexpression phenotype elsewhere in the plant could be explained by activation of the proper receptor in the wrong places. In other words, the effects of *35S::CLE16* and *35S::CLE17* on leaf shape may occur because the proper receptor(s) for these proteins are not under as tight regulation in leaves as they are in the tissues where *CLE16* and *CLE17* are normally expressed. The more conservative explanation for all of the overexpression phenotypes, however, is that the CLE proteins are binding receptors with which they do not interact under normal conditions—either because they are expressed ectopically, or because they are accumulating at such high levels that variables such as low binding affinity become irrelevant.

Although we have not deciphered much about the *in vivo* functions of these five proteins, the overexpression results may provide insight into the structural or sequence-level basis for the specificity of this family of genes. Particularly powerful analysis can be performed on the sequence requirements for successful activation of the CLV1 receptor. We know (from published work, as mentioned above) that CLE19 and 40 can activate CLV1, and now CLE11 and 12 can be added to the list. The overexpression phenotypes of CLE16 and 17 may represent useful negative results. Computational analysis, followed by judicious

domain-swapping and site-directed mutagenesis, should allow identification of exact domains or even residues required for binding to and activating CLV1.

One surprising result to emerge from these experiments is the curious enhancing effect of *35S::CLE11* and *35S::CLE12* on *clv1-6*, and the lack of an effect on the other alleles tested, including *clv1-1* and *clv1-4*. It has been suggested that although *clv1-6* is weaker than these other two alleles, it is closest to a true null allele, whereas the others are more complicated, possibly compromising the function of related receptors (Clark et al., 1997; Dievart et al., 2003). This would be particularly relevant if there is another LRR-RLK which can form functional heterodimers with CLV1, and which can also form homodimers with at least a limited CLV3-binding capability. For example, if region(s) missing in the *clv1-6* allele are required for robust dimerization, then the primary opportunity for CLV3 signaling would be through the homodimer of the other RLK. If the *clv1-1* and *clv1-4* versions of the protein are still able to form CLV3-binding heterodimers, but can transduce only a fraction of the normal signal, then the phenotype may be more severe.

It is not yet known whether *35S::CLV3* has the same effect on *clv1-6* (this experiment is underway), but if it does not (particularly if *35S::CLV3*; *clv1-6* resembles *35S::CLV3* in L-*er*), then the following model is possible, given the scenario described above: 1) CLE11 and CLE12 can bind both CLV1 and the other RLK, but can only produce signal through CLV1; 2) the overexpression of CLE11 or CLE12 in the *clv1-1* or *clv1-4* meristem has little effect on the CLV1/RLK heterodimers (particularly if the problem is in the propagation of the signal, rather than ligand binding); and 3) high levels of CLE11 or CLE12 in the *clv1-6* meristem bind the homodimers of the other RLK non-productively, out-

competing (by greater abundance) the endogenous, functional CLV3. This might be tested by co-overexpressing *CLV3* and *CLE12* (or *CLE11*) in *clv1-6*; if overexpressed CLE12 is drowning out effective signal from CLV3, adding high levels of CLV3 should negate the enhancing effect of *35S::CLE12* on *clv1-6*.

If, on the other hand, *35S::CLV3*; *clv1-6* resembles *35S::CLE12*; *clv1-6*, then the picture gets even more complicated. This result would raise the possibility that there are two opposing pathways, both of which can receive the CLV3 signal (and, in artificial situations, CLE11 or CLE12).

3.4 Materials and Methods

Plant growth conditions

Seeds were sown on a 4:3:2 mixture of potting soil, vermiculite, and perlite. Each flat was given sufficient water 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).

Constructing vectors for transgenics – starting materials

PCR-amplified *CLE11-13*, *16*, and *17* products were cloned into pCR2.1 in at least one of three sets: complete coding sequence (plasmids **A-E**), coding sequence without stop codon (plasmids **A′**, **B′**, **D′**, and **E′** – not done for *CLE13*), and complete coding sequence with an extra BamHI site added at the 5′ end, for greater cloning flexibility (plasmids **F-I**; *CLE13* not included). A clone containing

the coding sequence of CLV3 in the BamHI site of pBS–SK was provided by F. Wellmer (plasmid **J**, orientation T7 5′ 3′ T3).

The *CLE* coding sequences were moved into pBS-SK to generate more restriction site options: plasmid **K** – BamHI fragment from F, orientation T7 5′ 3′ T3; plasmid **L** – EcoRV/HindIII fragment from B′, orientation T7 5′ 3′ T3; plasmid **M1** – EcoRI fragment from C, orientation T7 3′ 5′ T3; plasmid **M2** – EcoRI fragment from C, orientation T7 5′ 3′ T3; plasmid **N** – HindIII fragment from D′, orientation T7 5′ 3′ T3; plasmid **O** – EcoRV/HindIII fragment from E′, orientation T7 3′ 5′ T3; plasmid **P** – EcoRV/SpeI fragment from E′ into pBS-SK, orientation T7 5′ 3′ T3.

Some of the shuttle vectors included a modified pBJ36 (BaR), containing the 35S promoter from pHANNIBAL as well as the octopine synthase (OCS) terminator (plasmid **Q**, from J. Long), pBJ36 with the *CLV3* 5′ and 3′ sequences flanking the multiple cloning site (MCS) (plasmid **R**, from J. Long), and pRITA, which contains the GUS coding sequence following the MCS (plasmid **S**, from J. Long). Plasmid Q was modified for use as a basic RNAi vector by adding in the catalase intron (from pCATIN, J. Long) into the XbaI/BamHI site, to generate plasmid **T**.

We used pMLBART as a binary vector in all cases. Fragments were cloned into the NotI site of pMLBART; this is the last step for making all of the finished products detailed below.

Overexpression constructs

For *35S* overexpression constructs, we used pBJ36/*35S* (plasmid Q). The digests and source plasmids for each coding sequence are as follows: *CLE11*, BamHI, F;

CLE12, EcoRI, B; *CLE13*, SmaI/ClaI, M1; *CLE16*, EcoRI, D; *CLE17*, EcoRI, E; and *CLV3*, BamHI, J.

Expression from CLV3 promoter

All constructs with the *CLV3* promoter made use of plasmid R, one of the modified versions of pBJ36 (see above). The *pCLV3::CLE11*, *16*, *17*, and *pCLV3::CLV3* constructs were made with the BamHI fragment of plasmids F, H, I, and J, respectively. For *pCLV3::CLE13*, the SmaI/ClaI fragment from plasmid M1 was used, and for *pCLV3::CLE12*, we used the SmaI/XhoI fragment from the *35S::CLE12* construct.

dsRNA interference lines

For the *CLE11*, *12*, *16*, and *CLV3* dsRNAi lines, we ligated the BamHI fragment from plasmids F, G, H, and J (respectively) into plasmid T. Resulting clones with the correct orientation were subsequently linearized with XbaI and SalI. The XbaI/SalI fragments from plasmids K, L, N, and J were ligated into these vectors, to create antisense-linker-sense constructs.

For the *CLE13* and *17* dsRNAi lines, the SalI/SmaI fragments from plasmids M1 and O, respectively, were ligated into the XhoI/SmaI sites of plasmid T. The resulting clones were linearized with XbaI and SalI, and the XbaI/SalI fragment from plasmid M2 and P (respectively) were ligated into these vectors, again creating antisense-linker-sense constructs.

GUS reporter constructs

The 5' upstream region of each *CLE* gene was PCR-amplified and ligated into pCR2.1. For *pCLV3::CLE11*, the EcoRI fragment from the pCR2.1 clone was ligated into plasmid S, and resulting clones were checked for proper orientation. For *pCLV3::CLE12*, *13*, *16*, and *17*, the KpnI/XhoI fragment from the corresponding pCR2.1 clone was ligated into plasmid S.

Plant transformations

Plasmids were transformed into the ASE strain of *Agrobacterium tumefaciens* (kan chlor), and L-*er* plants were transformed by the floral dip method (Clough and Bent, 1998). Seeds were sown directly on soil, and T1 plants were sprayed with BASTA several times, starting about 6 days after germination.

GUS staining

Tissues were fixed in ice-cold 90% acetone for 30 minutes, then washed with rinse solution (34 mM Na_2HPO_4, 15.8 mM NaH_2PO_4, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4FE(CN)_6$, 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.

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