

# REVOLUTA regulates meristem initiation at lateral positions

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## Summary

While the shoot apical meristem (SAM) is indirectly responsible for the initiation of all above-ground postembryonic organs, in most plants the vast majority of these organs are directly initiated by lateral meristems. In *Arabidopsis thaliana*, the lateral meristems include flower meristems (FMs), which form on the flanks of the SAM, and lateral shoot meristems (LSMs), which develop in leaf axils. While significant progress has been made on the molecular genetic basis of SAM initiation during embryo development, relatively little is known about the initiation of meristems at lateral positions. Here we have characterized the phenotypic consequences and genetic interactions of mutations in the *REVOLUTA* (*REV*) gene, with an emphasis on the role of *REV* in lateral meristem initiation. Our observations indicate that *REV* is required for initiation of both LSMs and FMs, and likely acts in the same pathway as, and upstream of, known meristem regulators. We identified the *REV* gene and found it encodes a predicted homeodomain/leucine zipper transcription factor that also contains a START sterol-lipid binding domain. *REV* is the same as the *IFL* gene. *REV* was expressed at the earliest stages of LSM and FM formation. Within the inflorescence shoot meristem, *REV* expression appeared to predict 3–5 incipient flower primordia on the flanks of the SAM, and *REV* expression at stage 1 and stage 2 matched that of *WUS* and *STM*, respectively. We propose that *REV* acts at lateral positions to activate the expression of known meristem regulators.

**Keywords:** meristem, axillary, homeodomain, flower.

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## Introduction

In *Arabidopsis* and most other angiosperms, the shoot apical meristem (SAM) is the ultimate source of all above-ground postembryonic organs (e.g. stems, leaves and flowers). However, the majority of these organs are not initiated directly by the SAM, but are instead initiated by lateral meristems. In *Arabidopsis*, the lateral meristems include flower meristems (FM), which form on the flanks of the SAM, and lateral shoot meristems (LSM), which develop in leaf axils. Each *Arabidopsis* FM initiates 16 additional organs and each LSM gives rise to a side branch containing leaves and flowers (Smyth *et al.*, 1990). The overall morphology and reproductive capability of a plant is determined in part by the number, position and growth of lateral meristems.

In recent years, much has been learned about SAM initiation and maintenance (Eshed and Bowman, 2000).

The SAM initiation occurs during embryogenesis, and is a complex process requiring the action of several well-defined genes including *STM* (*SHOOT MERISTEMLESS*), *WUS* (*WUSCHEL*), *ZLL/PND* (*ZWILLE/PINHEAD*), *CUC1* (*CUP SHAPED COTYLEDON*) and *CUC2* (Aida *et al.*, 1997; Barton and Poethig, 1993; Endrizzi *et al.*, 1996; Jürgens *et al.*, 1994; Laux *et al.*, 1996; Long and Barton, 1998; Lynn *et al.*, 1999; Mayer *et al.*, 1998; Moussian *et al.*, 1998). These genes act over a relatively long developmental period, starting with the 16-cell embryo. Post-embryonically, the SAM maintains a nearly constant number of undifferentiated stem cells, and directs flanking progeny cells toward organ formation and eventual differentiation (Clark, 1997; Steeves and Sussex, 1989). Genes important for SAM maintenance include *CLAVATA1* (*CLV1*), *CLV2*, *CLV3*, *STM* and *WUS*. *STM* and *WUS* are required to maintain the

**Table 1.** *Rev* alleles discussed in this paper

| Allele       | Isolate      | Mutagen | Background    | Lesion <sup>a</sup> | Predicted effect <sup>b</sup> | Origin                       |
|--------------|--------------|---------|---------------|---------------------|-------------------------------|------------------------------|
| <i>rev-1</i> | –            | EMS     | No-O          | c                   | splice acceptor loss          | Talbert <i>et al.</i> (1995) |
| <i>rev-3</i> | –            | EMS     | Col           | C1823 to T          | T608 to I                     | Talbert <i>et al.</i> (1995) |
| <i>rev-5</i> | <i>spz-1</i> | EMS     | Col           | C779 to T           | A260 to V                     | Alvarez (1994)               |
| <i>rev-6</i> | <i>vam-1</i> | EMS     | <i>clv3-1</i> | C1036 to T          | R346 to stop                  | Pogany <i>et al.</i> (1998)  |
| <i>rev-7</i> | <i>vam-2</i> | TDNA    | RLD           | nd                  | nd                            | Pogany <i>et al.</i> (1998)  |
| <i>rev-8</i> | tj72         | TDNA    | <i>Ler</i>    | intron 1 insertion  | RNA instability               | Chen <i>et al.</i> (1999)    |

<sup>a</sup>Change in DNA sequence relative to wild-type ecotype, numbered from the start ATG. <sup>b</sup>Change in mRNA structure or predicted protein numbered from the start methionine. <sup>c</sup>See Ratcliffe *et al.* (2000) for description of *rev-1* DNA sequence. nd, not determined.

population of undifferentiated cells (Barton and Poethig, 1993; Laux *et al.*, 1996), and the *CLV* loci are required to regulate the balance of these cells between proliferation and differentiation (Clark *et al.*, 1993; Clark *et al.*, 1995; Kayes and Clark, 1998; Laufs *et al.*, 1998). All of these genes are expressed in specific regions of the SAM: *WUS* is expressed in a small number of centrally located cells in the corpus of the SAM, *CLV1* and *CLV3* are expressed in a central region of the SAM in the corpus and tunica, respectively, and *STM* is expressed in a central region of the shoot meristem in all cell layers (Clark *et al.*, 1997; Fletcher *et al.*, 1999; Long and Barton, 2000; Long *et al.*, 1996; Mayer *et al.*, 1998).

In contrast to the SAM, little is known about lateral meristem initiation. The morphology of FM and LSM formation has been described in *Arabidopsis* (Grbic and Bleecker, 2000; Smyth *et al.*, 1990) and many other species (Steeves and Sussex, 1989), and several mutations affecting FM and LSM formation have been reported. These mutants include the tomato *lateral suppressor (ls)* mutant (Schumacher *et al.*, 1999) and the *Arabidopsis revoluta (rev)* (Talbert *et al.*, 1995), *zwiller/pinhead (zll)* (McConnell and Barton, 1995; Moussian *et al.*, 1998), *caf* (Jacobsen *et al.*, 1999), and *argonaute1 (ago1)* (Bohmert *et al.*, 1997) mutants. Of these, the *ls* and *rev* mutations specifically affect lateral meristem formation (i.e. these mutations affect both LSM and FM formation). *zll* and *ago1* mutations affect the primary SAM in addition to the LSMs. An additional class of mutants, including the recently characterized *axr1* mutant (Stirnberg *et al.*, 1999), initiate LSMs that arrest early in development. Despite the presence of these mutants, and the fact that many of the corresponding genes have been cloned, little is known about the molecular mechanisms of LSM and FM initiation.

Many of the genes expressed in the SAM are also expressed in the lateral meristems. For example, *WUS*, *STM*, *CLV1* and *CLV3*, while not expressed in very young FMs (early stage 1), become re-expressed during early flower development (stages 1 and 2). These data suggest that many of the genes required for SAM formation may also be required for lateral meristem formation.

Furthermore, these data suggest that very young lateral meristems do not retain meristem identity as they are separated from the apex, but instead have meristem identity reactivated shortly after their initiation. This raises the question of how the SAM-expressed genes become re-activated in lateral meristems.

To understand the mechanisms of lateral meristem initiation, we carried out an analysis of the *rev* mutant. The original description of *REV* detailed pleiotropic effects of loss-of-function mutations in this gene (Talbert *et al.*, 1995). These defects included a reduction in LSM and FM initiation, as well as leaf and flower organ defects. *Rev* alleles were subsequently described as *variable meristem (vam)*, a modifier of the *clv* mutant phenotype (Pogany *et al.*, 1998). Based on our prior analysis of *rev/vam* mutants, we hypothesized that *REV* was required to reactivate meristem activity in lateral shoot and flower meristem positions. To test this we have characterized the meristem defects of *rev* mutants and the genetic interactions with other meristem regulators. We present findings that indicate that *REV* is required for lateral meristem initiation, and that *REV* acts upstream of the *CLV* loci, as well as in the same pathway as *STM* in lateral meristem initiation. We isolated the *REV* gene and show that it encodes a group III homeodomain-zip (leucine-zipper) protein. This gene was previously isolated as *IFL1*, which is required for proper lignification and differentiation of cortical cells in the stem (Zhong and Ye, 1999). Our results are consistent with the proposed role of *REV* in activating meristem activity as lateral positions.

## Results

### *Isolation of rev mutants*

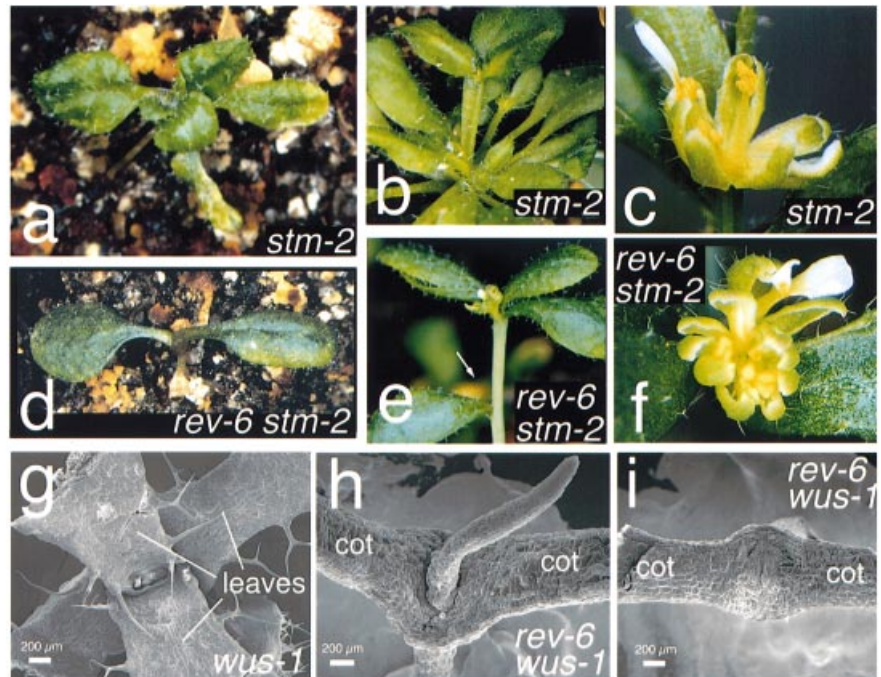
We isolated three new *rev* alleles that have been named *rev-6*, *rev-7* and *rev-8* (Table 1). *Rev-6* was isolated as a modifier of the *Clv3-1* phenotype. *Rev-7* was identified in the RLD background. *Rev-6* and *rev-7* were previously named *vam* mutations (Pogany *et al.*, 1998). *Rev-8* was previously described as an enhancer of *filamentous flower*

**Figure 1.** *Rev* mutants exhibit lateral meristem defects. The majority of cauline leaf axils of *rev-6* mutant plants were bare (a, arrows). Rarely, a filamentous structure (b, arrow) or leaf (c, arrow) was observed. In contrast to wild-type flowers (d), *rev* mutant flowers sometimes lacked stamens and carpels (e,f). A sepal and petal were removed from the flower in panel (e).



**Figure 5.** *Rev stm* and *rev wus* double mutants.

*Rev-6* enhances *stm-2* vegetative but not flower phenotypes. During vegetative growth, *rev-6 stm-2* plants (d,e) developed fewer lateral meristems than *stm-2* plants (a,b), and often failed to initiate lateral meristems in axils of leaves (e, arrow). *Rev-6 stm-2* flowers (f) initiated the same complement of organs as *stm-2* flowers (c). While *wus-1* plants continually generated adventitious meristems, which form multiple leaves (g, leaves), *rev-6 wus-1* double mutants usually initiated either no organs (i) or a single filamentous structure (h). Samples shown were collected at 25 days after germination. cot, cotyledon. Scale bars are indicated.



(*fil*) (Chen *et al.*, 1999). *Rev-6* was chosen for further analysis and appears to encode a null allele (see below). *Rev-6* was backcrossed to Landsberg *erecta* (*Ler*) three times before phenotypic and genetic characterization.

#### The *rev-6* mutation affects lateral meristem formation

*Rev-6* mutants exhibit reduced lateral meristem formation. Most dramatically affected were the LSMs (axillary meristems) of rosette and cauline leaves (Figure 1). In *rev-6* mutant plants, approximately 70% of cauline leaves lacked a lateral meristem (Table 2), and 16% failed to produce meristems in the axils of any rosette leaves. Decapitation

of the primary shoot meristem (SAM) failed to stimulate axillary meristem formation (data not shown). In the place of LSMs, the axils of affected leaves were usually bare, but occasionally developed a differentiated structure that was a leaf or a filamentous structure (Figure 1b,c).

*Rev-6* mutant plants also exhibited a reduction in flower meristem activity. While the majority of the flowers developed normally, 12% of the flowers initiated only a limited set of flower organs and appeared to have a defective flower meristem (Figure 1e,f). The inner organs were preferentially absent from *rev-6* flowers with defective meristems (Table 3). This pattern of reduction in flower organ number is similar to that seen in *wus* and *fil* mutants

(Chen *et al.*, 1999; Laux *et al.*, 1996). In affected flowers, the floral meristem was reduced in size or nearly absent by stage 3 of flower development (Figure 2d). Other flowers on *rev-6* plants were identical to wild-type. Although lateral meristems were dramatically affected in *rev-6* mutants, SAM size and the phyllotaxy of flanking primordia appeared normal (Figure 2c).

#### The *rev-6* phenotypes are affected by polygenic modifiers

The original *rev-1* allele, which was isolated from the Nossen ecotype, exhibited pleiotropic defects in FM activity, LSM activity, leaf morphology (size, shape, color and senescence), and floral organ morphology (Talbert *et al.*, 1995). In contrast, the *rev-6* allele in the *Ler* ecotype showed none of the non-meristematic defects. These dramatic differences in allele severity could either be due to differences between the effect of the mutations on gene function or to modifiers in the different ecotypes. To test this, we crossed *rev-6* in the *Ler* ecotype with the Columbia ecotype, and scored the phenotypes segregating in the F2 generation. A range of *Rev*<sup>-</sup> phenotypes were observed, including those described for the original *rev-1* allele. Statistical analysis indicated a correlation between the

percentage of absent LSMs and the severity of leaf morphology and color. The severity of leaf morphology and color was also correlated with the percentage of meristem-defective flowers; however, the percentage of absent LSMs was independent of the percentage of meristem-defective flowers (see Supplementary Material text at end of paper). These data suggest that the Columbia ecotype contains multiple modifiers of the *Rev*<sup>-</sup> phenotype.

#### REV is required for ectopic LSM formation in *stm*, *wus* and *ap1* mutants

Several mutants such as *stm*, *wus* and *apetala1* (*ap1*) produce ectopic meristems. Within *stm* and *wus* mutants, ectopic meristems are produced in the axils of cotyledons, and within *ap1* mutants, ectopic flower meristems are produced in the axils of whorl 1 leaf-like organs. To test whether *REV* is required for formation of these ectopic meristems, we analysed *rev stm*, *rev wus* and *rev ap1* doubly mutant strains.

Mutations in the *STM* gene result in a failure to initiate an embryonic SAM (Barton and Poethig, 1993). Thus, postembryonic growth in these mutants is entirely dependent on ectopic organogenesis. In the weak *stm-2* allele, ectopic shoot meristems are formed in the axils of the cotyledons (Clark *et al.*, 1996). Because *STM* is also required to maintain the undifferentiated cells of shoot and flower meristems, the ectopic meristems in *stm-2* plants terminate after initiating a limited number of organs. Further growth is dependent on additional LSMs that are initiated in the axils of existing leaves. To determine whether the ectopic lateral meristems in *stm-2* plants required *REV* activity, we analysed *rev-6 stm-2* double mutants. In contrast to *stm-2* plants, in which all plants produced ectopic lateral meristems and the majority did so by 10 days after germination, 13% of *rev-6 stm-2* plants never formed shoot meristems, and those that did were delayed in meristem initiation (Figure 4). Furthermore, for those *rev-6 stm-2* plants that did undergo postembryonic development, there was a dramatic reduction in the

**Table 2.** *Rev-6* reduces frequency of meristem initiation in cauline leaf axils

| Genotype            | Frequency of cauline leaves with |      |          |          | <i>n</i> |
|---------------------|----------------------------------|------|----------|----------|----------|
|                     | Bare axils                       | Leaf | Filament | Meristem |          |
| <i>rev-6</i>        | 0.64                             | 0.05 | 0.02     | 0.29     | 342      |
| <i>rev-6 clv1-4</i> | 0.76                             | 0.05 | 0        | 0.20     | 66       |
| <i>rev-6 clv2-1</i> | 0.59                             | 0.07 | 0        | 0.35     | 121      |
| <i>rev-6 clv3-2</i> | 0.72                             | 0.08 | 0        | 0.16     | 85       |

All cauline leaves on the primary inflorescence stems of the indicated genotype were assessed for development within their axils of a meristem, filamentous structure (filament), leaf, or no development (bare axils). *n* indicates the number of cauline leaves analysed.

**Table 3.** *Rev-6* is epistatic to *clv* mutations in meristem defective flowers

| Genotype            | Organ number per flower <sup>a</sup> |            |            |         | <i>n</i> | % of total <sup>b</sup> |
|---------------------|--------------------------------------|------------|------------|---------|----------|-------------------------|
|                     | Sepals                               | Petals     | Stamens    | Carpels |          |                         |
| <i>rev-6</i>        | 2.7 ± 0.13                           | 1.9 ± 0.25 | 0.3 ± 0.11 | 0       | 42       | 12%                     |
| <i>rev-6 clv1-4</i> | 2.3 ± 0.11                           | 1.5 ± 0.26 | 0.2 ± 0.07 | 0       | 33       | 41%                     |
| <i>rev-6 clv3-2</i> | 2.6 ± 0.09                           | 1.7 ± 0.16 | 0.1 ± 0.05 | 0       | 69       | 24%                     |

<sup>a</sup>Mean plus standard error for *n* flowers. Only flowers lacking normal meristematic activity were used for these means (all others are presented in Figure 3). <sup>b</sup>Percentage of all flowers counted that lacked normal meristematic activity. *n* indicates the number of flowers analysed.

number of LSMs and FMs generated (Figure 5). These data suggest that *REV* is required for postembryonic development of shoot meristems in *stm-2* plants.

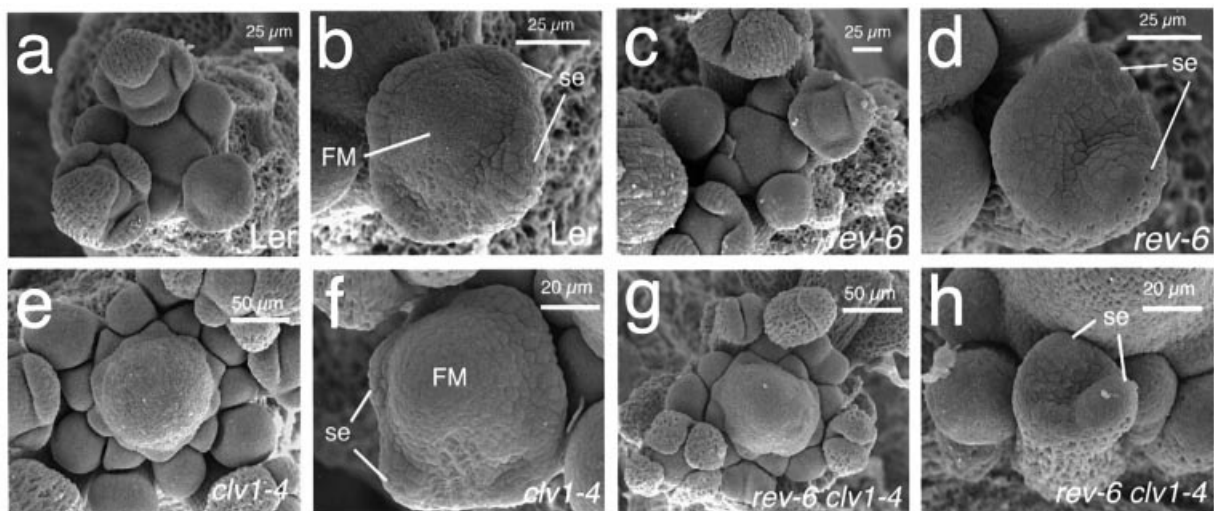
The strong *stm-1* allele is phenotypically different from the *stm-2* allele in that *stm-1* plants never form morphologically identifiable shoot meristems (Barton and Poethig, 1993). In *stm-1* plants, leaves are occasionally initiated in the axils of the cotyledons. Later leaves are initiated in the axils of existing leaves (Clark *et al.*, 1996). The origin of postembryonic *stm-1* leaves is unclear. It has been hypothesized that *stm-1* LSMs become entirely committed to the formation of a differentiated leaf (Clark *et al.*, 1996). An alternative possibility is that these leaves are initiated in a meristem-independent manner. We attempted to distinguish between these possibilities by determining if *REV* was required for the formation of the postembryonic *stm-1* leaves. *Rev-6 stm-1* plants were generated and the frequency and timing of the postembryonic initiation of leaves was measured and compared to *stm-1* single mutants (Figure 4). *Rev-6 stm-1* plants exhibited both a delay and a reduction in the total frequency of plants capable of postembryonic organ formation compared to *stm-1* plants, indicating a role for *REV* in this process.

Mutations in the *WUS* gene result in phenotypes similar to partial-loss-of-function mutations in the *STM* gene (e.g. *stm-2*), namely, the failure to initiate a shoot meristem in the embryo, but the ability to form adventitious shoots postembryonically (Laux *et al.*, 1996). To determine if the adventitious shoot formation in *wus* plants required *REV* activity, *rev-6 wus-1* double mutant plants were compared

to *wus-1* plants for the ability to initiate postembryonic organs. As shown in Figure 4, all *wus-1* plants initiated a visible adventitious shoot by 17 days after germination. By contrast, over 50% of *rev-6 wus-1* plants never initiated postembryonic organs (Figures 4 and 5i). Among the *rev-6 wus-1* plants that did initiate organs, most only formed a single filamentous structure between the cotyledons (Figure 5h).

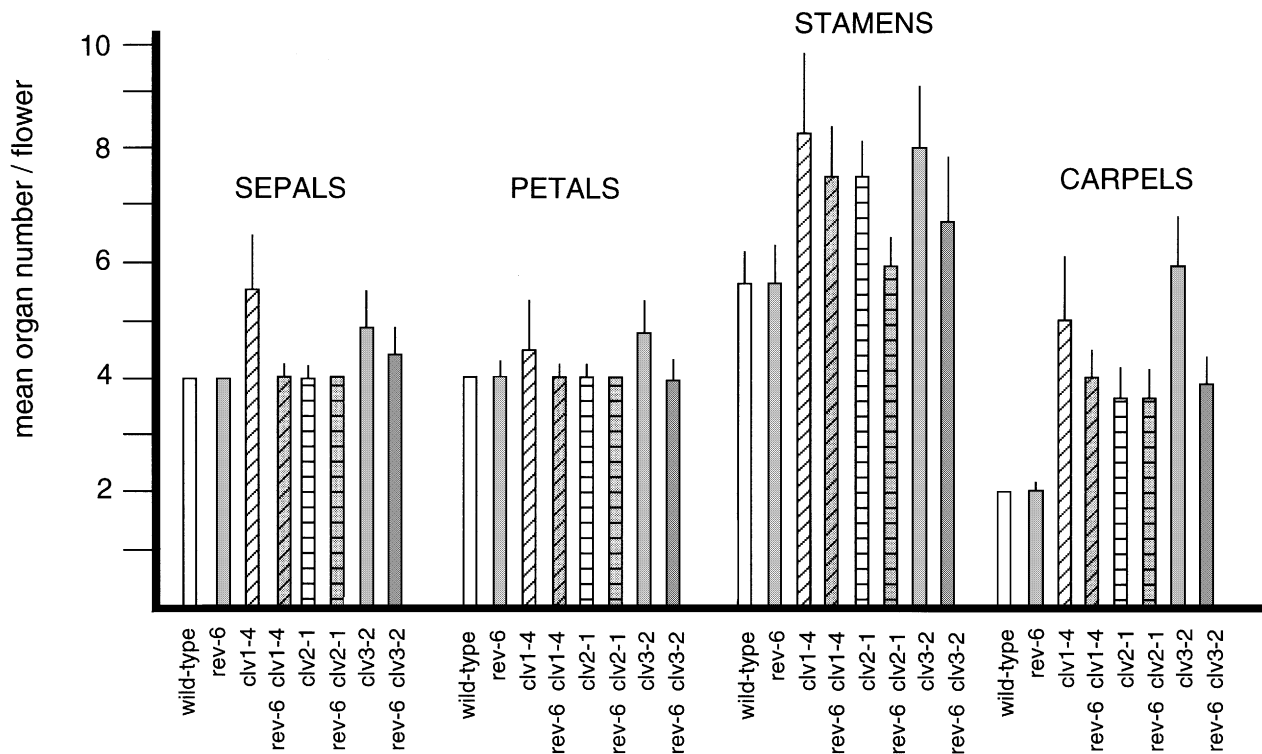
*Ap1* flowers develop leaf-like sepals in whorl 1 that often develop flower meristems in their axils (Bowman *et al.*, 1993). To determine if *REV* was required for the ectopic flower meristems in *ap1* mutants, *rev-6 ap1-1* double mutant plants were generated and the percentage of flowers containing axillary flowers was compared with *ap1* mutant plants. 36.2% of *ap1-1* flowers ( $n = 958$ ) produced ectopic flower meristems. By contrast, only 2.8% of *rev-6 ap1-1* flowers ( $n = 782$ ) produced ectopic flower meristems, which represents a reduction of over 90%. These data indicate that *REV* activity is required for the formation of FMs in the axils of whorl 1 bracts in *ap1* flowers.

*Ap1 cauliflower (cal)* double mutant plants exhibit even more dramatic ectopic lateral meristem formation. The shoot meristem of these plants initiates lateral meristems, that each in turn initiate lateral meristems, in a reiterative process that leads to the formation of hundreds of meristems (Bowman *et al.*, 1993; see Supplementary Material at end of paper, Figure S1). The *rev-6 ap1-1 cal-1* triple mutant plants exhibited a range in reduction of lateral meristem formation, likely



**Figure 2.** *Rev* is epistatic to *clv1* and *clv3* in reduced flowers.

Sixteen-day-old wild-type *Ler* (a,b), *rev-6* (c,d), *clv1-4* (e,f), and *rev-6 clv1-4* (g,h) plants were collected and analysed by scanning electron microscopy (SEM). While *rev* shoot apical meristems were indistinguishable from wild-type (a,c), stage 3 *rev* flowers occasionally lacked flower meristems (FM) interior to the sepals (se) (all stages according to Smyth *et al.*, 1990). While *rev-6* had no effect on the shoot apical meristems of *clv1* mutants (compare e to g), reduced stage 3 flowers of *rev-6 clv1* plants (h) lacked the enlarged flower meristems of *clv1* stage 3 flowers (f) and were indistinguishable from *rev-6* reduced flowers (d). Scale bars are indicated.



**Figure 3.** *Rev-6* partially suppresses *clv* mutant flower phenotypes.

The number of flower organs in wild-type *Ler*, *rev-6*, *clv1-4*, *rev-6 clv1-4*, *clv2-1*, *rev-6 clv2-1*, *clv3-2*, and *rev-6 clv3-2* plants were counted. Only complete flowers were included in the calculations of the mean and standard deviation for each genotype (see text). At least 100 flowers were counted for each mean, and only the first 10 flowers on any given plant were counted.

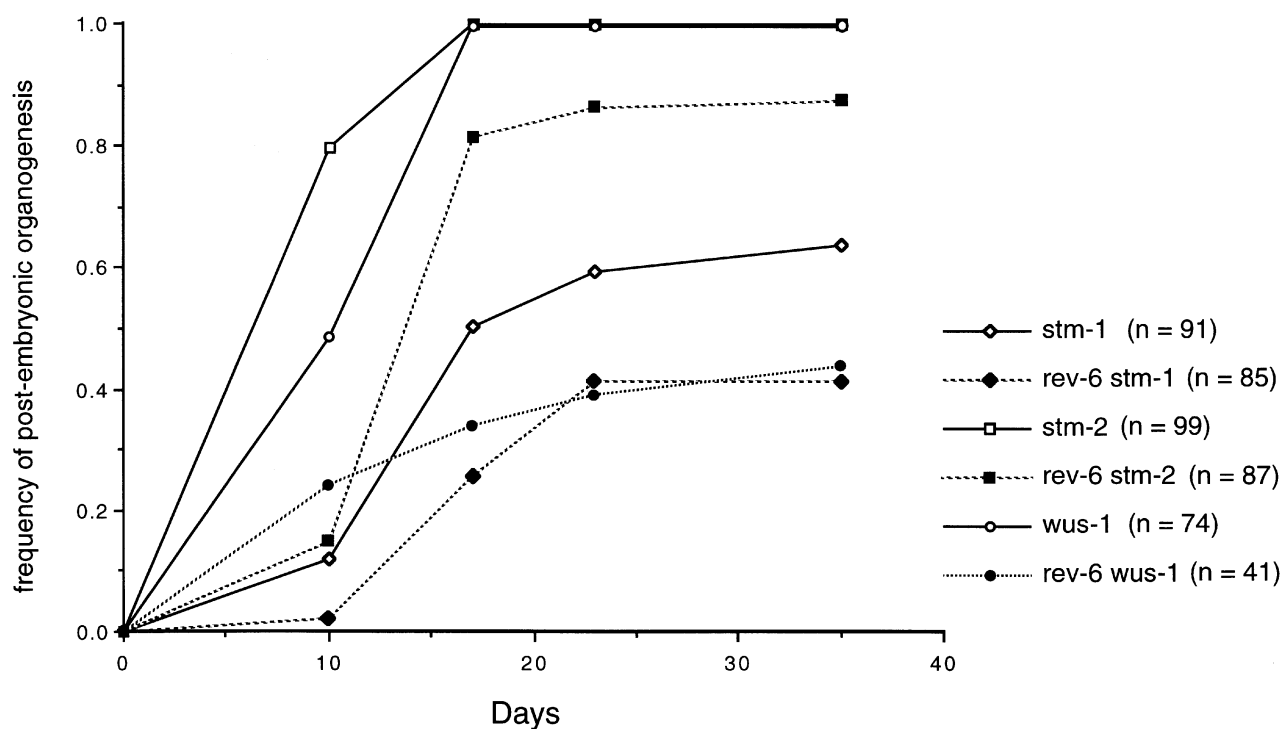
due to the mixed Landsberg/Wassilewskija ecotype of the *ap1-1 cal-1* double mutant. At the extreme end of the phenotypic range, no lateral meristems were formed on the flanks of the triple mutant plants (see Supplementary Material at end of paper, Figure S1). The only lateral organs produced were small bract-like or filamentous structures. Other triple mutant plants exhibited a strong reduction in lateral meristem formation, but still initiated a number of lateral shoots, that each initiated small bract-like structures (see Supplementary Material at end of paper, Figure S1). Taken together, these data suggest that *REV* is required for the initiation of all postembryonic shoot and flower meristems.

#### *CLV* and *STM* activity appear reduced in *rev* mutants

*Rev-6* plants exhibited a variably expressive reduced meristem activity at lateral positions. The most severe examples of this were the reduced flowers (flowers with no stamens or carpels) and bare leaf axils, both of which appeared to completely lack meristem activity. If this is indeed the case, the reduced flowers and bare axils should

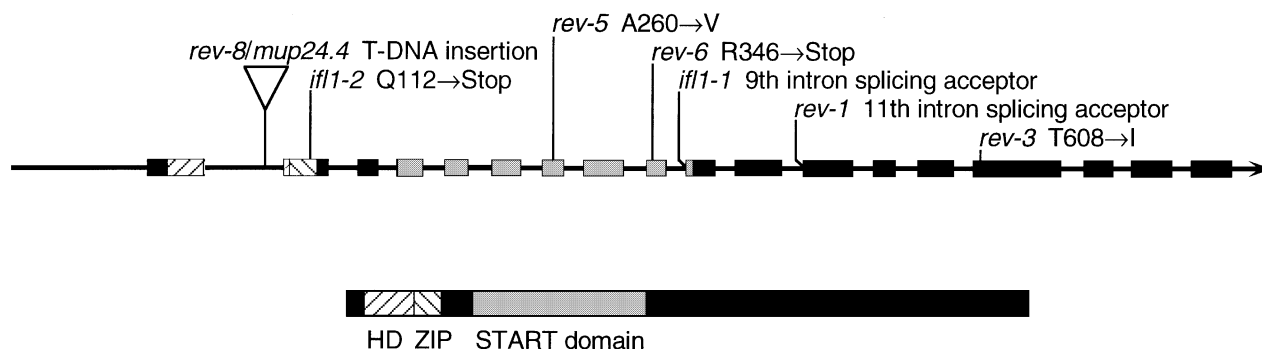
be unaffected by mutations in genes that modulate meristem activity, such as *clv*, *stm* and *wus* mutations. Furthermore, the *rev-6* mutation should attenuate the phenotype of mutants with increased meristem activity such as *clv* mutants. To test this, we analysed *rev-6 clv* and *rev-6 stm* doubly mutant strains. As discussed above, *rev-6 wus* double mutants failed to undergo postembryonic growth, preventing us from assessing the effect of *rev-6* mutation on *wus* flower development.

We generated double mutant plants carrying *rev-6* with the *clv1-4*, *clv2-1* or *clv3-2* mutations. In these double mutants, the frequency of cauline leaves with bare axils (Table 2) and the phenotype of reduced flowers (Table 3) was similar to that in *rev-6* single mutants. A higher percentage of reduced flowers was found in the double mutant plants in comparison to *rev-6* single mutant plants (Table 3). These flowers exhibited clear reductions in floral primordia size at stage 3 (Figure 2h, data not shown), and initiated a nearly identical number of floral organs compared to the reduced flowers of *rev-6* single mutants (Table 3). The 'complete' flowers (flowers with all organ types) exhibited a *Clv<sup>-</sup>* phenotype, but the severity was reduced in comparison to the respective *clv* single mutant.



**Figure 4.** *Rev-6* limits postembryonic development of *stm* and *wus* mutants.

The ability of *stm-1*, *rev-6 stm-1*, *stm-2*, *rev-6 stm-2*, *wus-1* and *rev-6 wus-2* plants to initiate postembryonic organs was measured in tissue culture as described in Experimental procedures. Shown are the proportion of plants for each genotype that had developed postembryonic organs. The numbers of plants monitored for each genotype are indicated (*n*).



**Figure 6.** *REV* encodes a putative transcription factor.

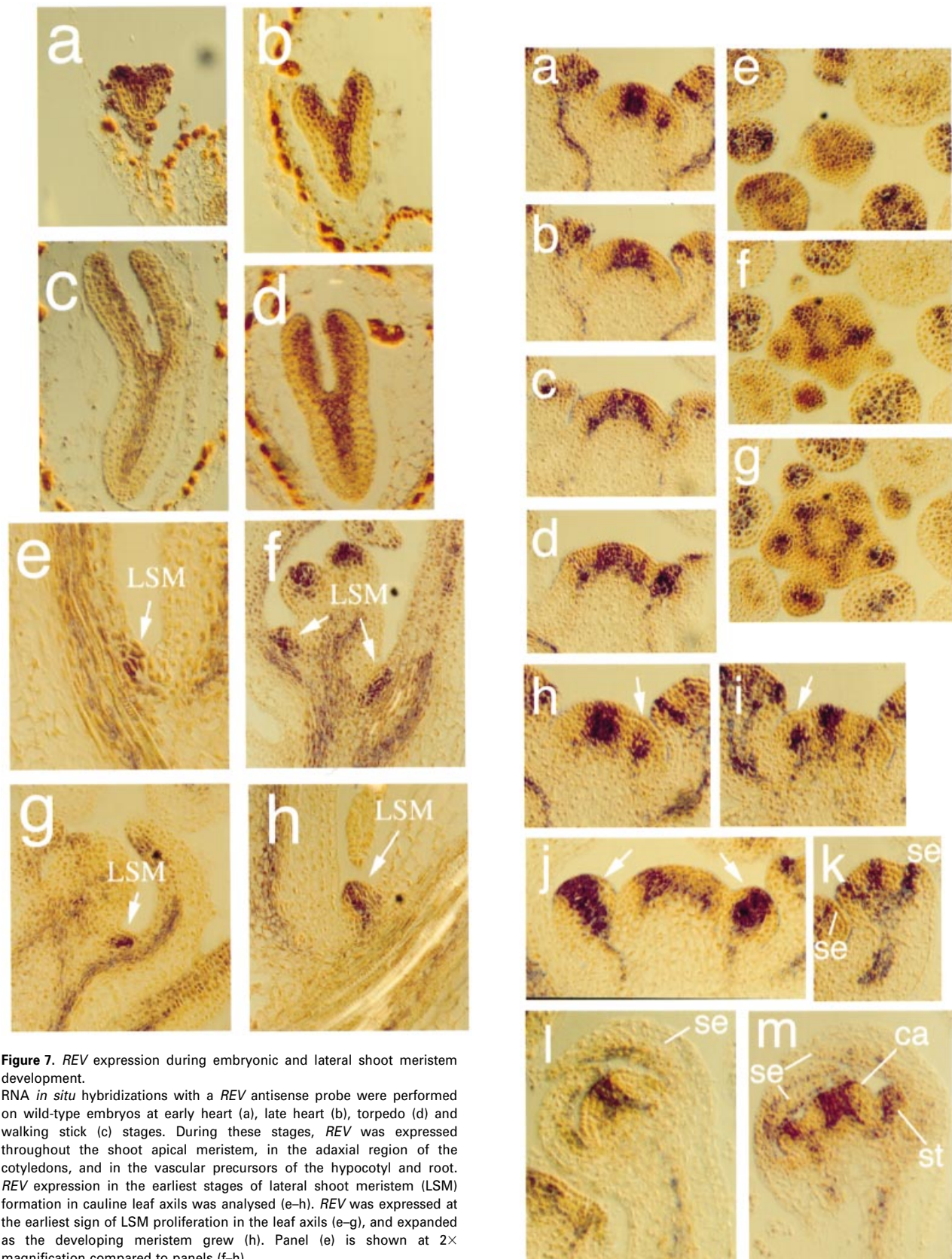
The genomic and mRNA structures for the *REVOLUTA* gene are shown. Domains encoding the predicted homeodomain (HD), leucine zipper (ZIP) and START domain (START) are indicated. The locations of lesions in *rev* alleles are shown.

This was especially true in the *rev-6* double mutant plants with the strong *clv1-4* and *clv3-2* alleles (Figure 3). Taken together, these data suggest that the *REV* gene is required to promote meristem formation and/or activity at lateral positions.

We generated double mutant plants carrying *rev-6* with the weak *stm-2* mutation. *Stm-2* mutants form shoot meristems that give rise to floral meristems. *Stm-2* flowers exhibit limited meristem activity and produce a reduced

number of flower organs, especially in the inner whorls of stamens and carpels (Clark *et al.*, 1996). In *rev-6 stm-2* double mutant plants, there was a dramatic reduction in the number of floral meristems generated compared to *stm-2* single mutants. Those flowers that were initiated contained sepals, petals and stamens (Figure 5f). Thus, the flower meristem phenotype of *rev-6 stm-2* was not additive, suggesting a common pathway for *REV* and *STM* in floral meristem development.





**Figure 7.** *REV* expression during embryonic and lateral shoot meristem development. RNA *in situ* hybridizations with a *REV* antisense probe were performed on wild-type embryos at early heart (a), late heart (b), torpedo (d) and walking stick (c) stages. During these stages, *REV* was expressed throughout the shoot apical meristem, in the adaxial region of the cotyledons, and in the vascular precursors of the hypocotyl and root. *REV* expression in the earliest stages of lateral shoot meristem (LSM) formation in cauline leaf axils was analysed (e–h). *REV* was expressed at the earliest sign of LSM proliferation in the leaf axils (e–g), and expanded as the developing meristem grew (h). Panel (e) is shown at 2× magnification compared to panels (f–h).



### REV encodes a homeodomain-containing protein

To further analyse *REV* function, we isolated the *REV* gene. We localized *REV* to BAC MUP24 using a fine structure recombination mapping strategy with F2 progeny of *rev-1* (No-0 ecotype) crossed to Ler (data not shown). One ORF (MUP24.4) failed to produce a PCR product in *rev-8* due to a T-DNA insertion in intron 1. To verify that MUP24.4 corresponded to the *REV* gene, we determined the DNA sequence of this gene in wild type and three additional *rev* alleles. As summarized in Table 1 and Figure 6, we observed a lesion in each *rev* allele, indicating that ORF MUP24.4 corresponds to the *REV* gene.

The *REV* gene corresponds to the recently cloned *INTERFASCICULAR FIBERLESS1 (IFL1)* gene, which is required for normal vascular tissue differentiation in stems (Ratcliffe *et al.*, 2000; Zhong *et al.*, 1997; Zhong and Ye, 1999). The *REV/IFL1* gene contains 18 exons and encodes a predicted protein of 842 amino acids (Figure 6). The predicted *REV* protein contains both a homeodomain and a leucine zipper. *REV* is a member of the *Arabidopsis* HD-ZIP III subfamily of HD-ZIP genes that includes *ATHB8*, *ATHB9* and *ATHB14* (Sessa *et al.*, 1998). These genes all share a sterol-lipid binding domain, known as a START domain (Ponting and Aravind, 1999). The role of this domain in a putative transcription factor is unclear.

### REV expression predicts sites of lateral meristem formation

If *REV* is required for the initiation of meristem activity at LSMs and FMs, its expression should precede the establishment of meristems in leaf axils and floral primordia. Specifically, its expression should precede the expression of the meristem regulators *WUS*, *STM*, *CLV1* and *CLV3*. In addition, the pleiotropic phenotypic defects of *rev/iff1* mutants imply that *REV* is also expressed during organ development and in vascular tissue. To test these predictions, we carried out RNA *in*

*situ* hybridization experiments with embryo, leaf and floral tissue.

During embryo development, *REV* expression was detected as early as the heart stage (earlier stages were not tested). *REV* expression encompassed the adaxial portion of the cotyledon primordia, the SAM and the vascular precursor cells of the hypocotyl and root (Figure 7a–d). This expression pattern was maintained through at least the 'walking stick' stage, although at comparably lower levels.

Because LSM formation is delayed until the transition to flowering (Grbic and Bleecker, 2000), we examined *REV* expression within developing cauline leaves (Figure 7e–h). In addition to expression on the adaxial portion of the leaf and within the developing leaf vasculature, *REV* expression was detected at the earliest signs of LSM formation. At this point, *REV* was expressed in a small number of subepidermal cells. *REV* expression continued as the LSM was initiated, eventually taking on the expression pattern seen within the SAM (see below). Thus, *REV* was expressed at the earliest stages and throughout LSM initiation and development.

Within the SAM, *REV* exhibited a very complex expression pattern (Figure 8). When inflorescence SAMs were sectioned longitudinally and used for *in situ* hybridization, *REV* was detected in the center of the SAM in an inverted-cup-shaped population of cells (Figure 8a–d). The cells in the very center of the meristem in the lower L3 cells did not express *REV*. These should correspond approximately to the *WUS*-expressing cells (Mayer *et al.*, 1998). Above these cells, *REV* was expressed in L2 and the topmost L3 cells, but on the flanks of the meristem *REV* was expressed only in L3 cells. SAMs sectioned transversely were also used for *in situ* hybridization (Figure 8e–g). These again revealed *REV* expression in L2 and topmost L3 cells in the center of the SAM. Interestingly, *REV* expression on the flanks of the meristem predicted sites of flower primordia formation. Using the existing organ primordia to establish the phyllotactic pattern, *REV* expression appeared to predict the next 3–5 sites of flower primordia initiation, making *REV* the earliest marker to date for organ anlage (Figure 9). No previous gene expression studies have identified primordia this early in development; however, the position of gene expression matches the predicted position of the incipient organs. Most significantly, these data show that *REV* was expressed prior to *WUS*.

In developing flowers, *REV* expression was detected prior to stage 1 in the L3 layer. In early stage 1 flowers, *REV* expression was detected in the center of the L3 layer, which is a pattern similar to that of *WUS* (Figure 8h,i). During late stage 1, *REV* expression expanded to the central region of the L2 and L1 layers (Figure 8j). By stage 2, *REV* expression was at its highest and found in a pattern very similar to that for *STM*, namely, in all cell

**Figure 8.** *REV* is expressed in a complex manner in the shoot and flower meristem.

RNA *in situ* hybridizations with a *REV* antisense probe were performed on wild-type shoot apical meristems sectioned longitudinally (a–d) and transversely (e–g). See text for discussion of expression pattern. In early stage 1 flowers, *REV* was expressed in a central group of L3 cells (h,i, arrows). By the stage1/stage2 boundary (j, right arrow), *REV* expression expanded to include L1 and L2 cells and continued in this pattern through stage 2 (j, left arrow). At stage 3, *REV* expression continued in the center of the meristem, but was also expressed on the adaxial face of sepal primordia (se) as well as internal vascular precursor cells (k). Central meristem expression continued through stage 5 (l), eventually comprising the adaxial face of carpel (ca) primordia at stage 6 (m). st, stamen primordium.

layers but excluded from presumptive sepal anlage (Long *et al.*, 1996; Figure 8j). By stage 3, *REV* expression became more complex, with expression in the center of the flower meristem retained, but additional expression on the adaxial side of sepal primordia, as well as internal tissue connecting the base of the sepal primordia to the center of the flower stem (Figure 8k). *REV* expression was maintained in the center of the flower meristem through stages 4 and 5 (Figure 8l). By stage 6, carpel primordia are initiated in the center of the flower meristem (Smyth *et al.*, 1990), and *REV*-expressing cells in the center constituted the adaxial side of the carpel primordia (Figure 8m). *REV* continued to be expressed on the adaxial carpel face, eventually being expressed in the placenta and ovules (data not shown). Within other organs, *REV* expression was largely on the adaxial portion of the primordia (data not shown).

*REV* is a member of a closely related gene family, and the family members most closely related to *REV* are *ATHB9*, and *ATHB14*. This raised the possibility that the *REV* expression pattern might, in part, represent cross hybridization to the *ATHB9* and *ATHB14* genes. Using *in vitro* translated RNAs and hybridization and wash conditions identical to those used in *in situ* hybridization experiments, we found that the *REV* antisense probe hybridized at least 1000 times more strongly with the *REV* RNA than *ATHB14* or *ATHB9* RNA (see Supplementary Material at end of paper, Figure S1). Thus, under the conditions used for *in situ* hybridization, the *REV* probe specifically detects *REV* mRNA.

## Discussion

We have investigated the phenotype and genetic interactions of mutations in the *REV* gene, focusing on the role of *REV* in lateral meristem formation. Our observations indicate that *REV* is required for lateral meristem initiation, and that *REV* likely acts in the same pathway as, and upstream of several known meristem regulators. *REV* encodes a predicted transcription factor whose expression is consistent with an early role in meristem initiation.

### *REV is required for lateral meristem activity*

Several lines of evidence indicate that *REV* is required to establish meristems in leaf axils and floral primordia. First, phenotypic analysis indicates that many leaves and flowers in *rev* mutants lack meristem activity. In leaf axils this is characterized by a complete absence of growth in the lateral position. Floral primordia lacking meristem activity develop into flowers containing sepals and petals but lacking stamens and carpels, and this is the floral

phenotype most commonly observed in *rev* mutants. While it may be unclear how a flower lacking meristem activity would be able to develop multiple organs, the *wus* mutant, which appears to lack meristem activity within the flower as well, develops a similar pattern of flower organs (Laux *et al.*, 1996). Floral primordia in *rev* mutants also occasionally develop into filamentous structures, which may represent a more severe loss of cell division activity.

Second, although *clv* mutations dominantly restore meristem formation and activity within the severely affected *stm-1* mutant plants, they have no effect on the development of LSMs or the number of organs initiated by reduced flowers in a *rev* mutant background. This suggests that *rev* mutants lack meristem activity, and hence *CLV* function.

Third, *rev* does not display additive interactions with the partial-loss-of-function *stm-2* allele. The flowers of *rev stm-2* plants develop a similar complement of organs as either single mutant, suggesting that *REV* and *STM* act in a common pathway. This is consistent with a role for *REV* in activating *STM* activity, and is supported by gene expression studies (see below).

Finally, additional genetic studies indicate that *REV* is required for ectopic and adventitious meristem formation in *wus*, *stm*, *ap1* and *ap1 cal* mutant plants. Thus, all postembryonic shoot and flower meristems, and all ectopic meristems of this sort (i.e. non-root meristems) require *REV* activity.

### *Rev expressivity and ecotype modifiers*

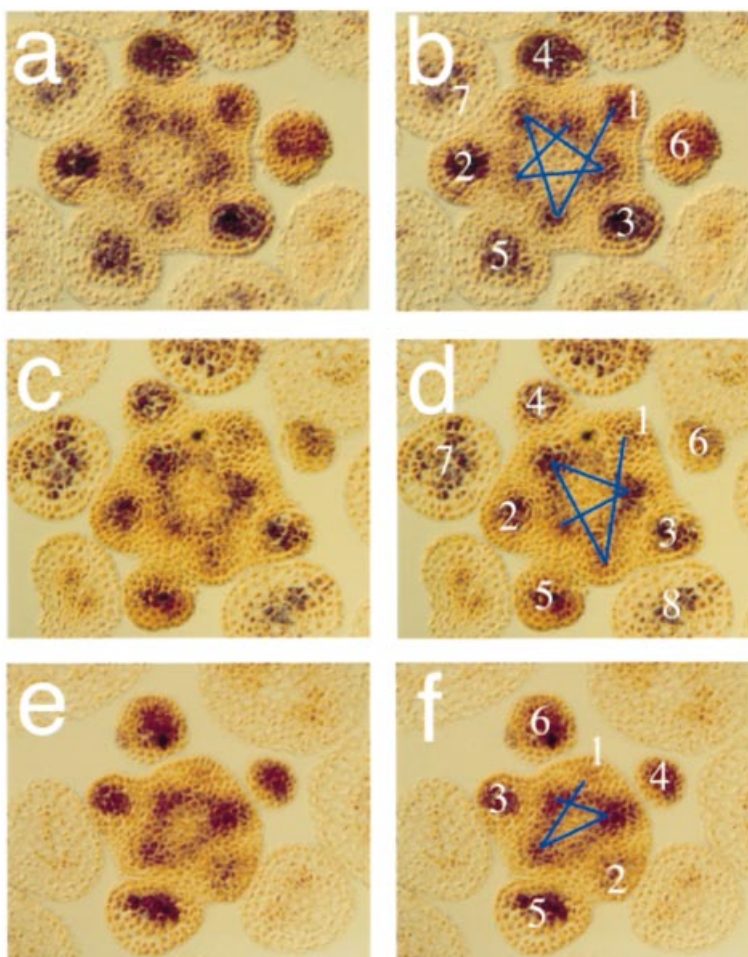
Despite a fairly clear role for *REV* in LSM and FM activity, many LSMs and FMs develop normally within *rev* mutants. This implies the existence of redundant factors that are capable of carrying out lateral meristem initiation in the absence of *REV* activity. One possibility is that the very closely related *ATHB9* and *ATHB14* genes are capable of acting redundantly with *REV*. A lower or different expression pattern for these two genes could account for the *rev* phenotype and expressivity. An interesting corollary hypothesis would be that the modifiers of the *Rev*<sup>-</sup> phenotype in the various ecotypes could represent variability in *ATHB9* and *ATHB14* expression.

### *REV has multiple functions*

*REV* functions can be separated into three categories: (1) *REV* is required for lateral meristem activity, as described above; (2) *REV* is required for normal organ development, as described in detail previously (Talbert *et al.*, 1995); (3) *REV* is required for proper differentiation of vascular-associated elements within the stem, as described for *if1* mutants (Zhong *et al.*, 1997). The data

**Figure 9.** *REV* expression marks incipient flower primordia.

RNA *in situ* hybridizations with a *REV* antisense probe were performed on transversely sectioned inflorescence shoot apical meristems. For each of three different meristems, expression is shown on the left (a,c,e). On the right, flower primordia are numbered from the youngest, and the predicted pattern of incipient flower primordia based on the phyllotaxy of existing flowers is indicated with a blue line (b,d,f). Note that *REV* was expressed in patches of cells in the next 3–5 primordia.



we present here suggest that these activities can be functionally separated. *Rev* mutant alleles in the *Ler* ecotype exhibit lateral meristem defects without any apparent organ defects. This suggests that the lateral meristem defects are not an indirect result of organ defects. In addition, there is no absolute correlation between organ defects and lateral meristem defects when segregating populations of *rev* in *Ler* crossed into Columbia were examined. In other words, the plants with the most severely affected organs did not always have the most severe reduction in lateral meristem activity. While there was a general correlation, we suggest this is due to common redundant elements affecting both organ and meristem phenotypes. Finally, expression patterns are consistent with each of these activities constituting a separate function (see below).

#### *REV* expression matches predicted function

Each of the hypotheses of *REV* function based on genetic and phenotypic analysis makes specific predictions about

the expression pattern of the *REV* gene. If *REV* is required for LSM and FM activity, *REV* should be expressed at the earliest stages of lateral meristem development. If *REV* has multiple separate functions within lateral meristems, organs and vasculature, then *REV* should be expressed separately within each of these tissues.

Within the SAM, the complex *REV* expression pattern matches the function predicted by phenotypes. *REV* is the earliest marker to date for flower primordia. Based on phyllotaxy of existing organs, *REV* appears to mark the next three to five flower primordia forming on the flanks of the shoot meristem. Thus *REV* expression precedes that of all other FM regulators. Curiously, *REV* expression appears in the same position within stage 1 flowers as the earliest known regulator of FM activity, *WUS*. *WUS* is expressed in a central group of L3 cells within the stage 1 FM (Mayer *et al.*, 1998), and *REV* is found in a similar pattern. The next meristem regulator expressed is *STM* at stage 2, which is found throughout the cell layers everywhere except the sepal anlage (Long *et al.*, 1996). *REV* is found in a coincident pattern by stage 2. We propose that *REV* acts either indirectly to establish meristem identity, or directly to

activate the expression of other meristem regulators. The observations that *REV* expression matches that of *WUS* and *STM* at their initiation and that *REV* encodes a putative transcription factor may make direct activation a plausible hypothesis.

Within developing LSMs, *REV* appears to be expressed coincident with the earliest morphological establishment of an axillary meristem. *REV* and *STM* (Long and Barton, 2000) appear to be the earliest markers for these developing meristems. We hypothesize that within these cells *REV* acts either indirectly to establish meristem identity, or directly to activate the expression of other meristem regulators.

Within organs, *REV* is expressed largely on the adaxial portion of the organ. A number of genes are expressed specifically on one 'face' (i.e. abaxial or adaxial) of leaf and flower organs, including *CRABS CLAW* (Bowman and Smyth, 1999), *INNER NO OUTER* (Villanueva *et al.*, 1999), *FIL*, *YABBY2* (*YAB2*), *YAB3* (Siegfried *et al.*, 1999), *ZWILLE/PINHEAD* (Lynn *et al.*, 1999; Moussian *et al.*, 1998), and *PROLIFERA* (Springer *et al.*, 2000). Many of these genes have been shown to be required for abaxial or adaxial fate. By analogy, *REV* may be involved in establishing adaxial fate in cotyledons, leaves and floral organs. In this regard, the loss of axillary meristems in *rev* mutants may be an indirect consequence of loss of adaxial cell fate in leaves, which has been proposed to promote axillary meristem formation (McConnell and Barton, 1998; Siegfried *et al.*, 1999).

*REV* is also expressed in developing vasculature. Shortly after the initiation of sepal primordia within the flower, *REV* appears to mark vascular precursor cells that will link the sepals to the main vascular cells in the center of the flower pedicel. Additional expression of *REV* is observed within vascular precursors within the inflorescence stem and developing flower organs. A detailed analysis of *rev/if1* mutants has already established the role of *REV* within the developing stem (Zhong *et al.*, 1997).

A critical question raised is how *REV* can act in three different tissue types to carry out distinct developmental programs. Perhaps *REV* regulates a single common process (e.g. cell division) in each tissue. Alternatively, *REV* may interact with different transcription factors in each tissue.

## Experimental procedures

### Plant growth and tissue processing

The isolation of the *rev-6* (*vam-1*), *rev-7* (*vam-2*), and *rev-8* (*tj-72*) were previously described (Chen *et al.*, 1999; Pogany *et al.*, 1998). All plants were in the Landsberg *erecta* background except those specifically mentioned in the text.

Seeds were sown on a 1:1:1 mix of top soil: perlite: vermiculite and imbibed for 7 days at 4°C. Plants were grown at 22°C under approximately 800-foot-candles of constant cool white fluorescent light. Plants were fertilized once a week.

Tissue and image processing for scanning electron microscopy (SEM) was carried out as described previously (Yu *et al.*, 2000).

Post-embryonic organ formation for the genotypes indicated in Figure 4 was performed as follows. The description is for *stm-1*, and is applicable to all genotypes analysed. Progeny of a heterozygote *stm-1/+* plant were sterilized and germinated in 0.5 × MS media supplemented with 1% sucrose. After 7 days' treatment at 4°C, plates were moved to 20°C at ~150-foot-candles of cool white fluorescent light. At regular intervals, plates were opened under sterile conditions and examined with a stereo microscope. All plants not homozygous for *stm-1* were identified within 8–10 days and removed. Remaining plants were monitored for any postembryonic organ formation. Plants that did not survive the entire 35-day period were not included in any calculations.

### RNA in situ hybridization

Nonradioactive *in situ* hybridization experiments were carried out as previously described (Klucher *et al.*, 1996) except that tissue was fixed and embedded as described by (Vielle-Calzada *et al.*, 1999). *REV* antisense probes were made from cDNA clone Col#19. The probe contained nucleotides 680 (relative to the ATG; Eae I recognition sequence) to the end of the mRNA.

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