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# Transcriptional regulation of epidermal cell fate in the *Arabidopsis* embryo

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How distinct cell fates are specified at correct positions within the plant embryo is unknown. In *Arabidopsis*, different cell fates are generated early on, starting with the two daughter cells of the zygote. To address mechanisms of position-dependent gene activation and cell fate specification, we analyzed the regulatory region of the *Arabidopsis thaliana MERISTEM LAYER 1* (*ATML1*) gene, which is already expressed at the one-cell stage and whose expression is later restricted to the outermost, epidermal cell layer from its inception. A sensitive, multiple GFP reporter revealed a modular organization to the *ATML1* promoter. Each region contributes positively to specific spatial and temporal aspects of the overall expression pattern, including position-dependent but auxin-independent regulation along the apical-basal axis of the embryo. A 101 bp fragment that conferred all aspects of *ATML1* expression contained known binding sites for homeodomain transcription factors and other regulatory sequences. Our results suggest that expression patterns associated with cell fate determination in the plant embryo result from positional signals targeting different regulatory sequences in complex promoters.

**KEY WORDS: *Arabidopsis thaliana*, L1 box, WUSCHEL-binding site**

## INTRODUCTION

Embryogenesis generates distinct cell fates, which correspond to specific gene expression profiles. In the flowering plant *Arabidopsis*, the zygote divides asymmetrically, producing a small apical cell and a large basal cell (Jürgens and Mayer, 1994). These two cells differ in their gene expression profile, suggesting that they have adopted different cell fates (Lu et al., 1996; Friml et al., 2003; Haecker et al., 2004). The apical cell gives rise to most of the embryo, whereas the basal cell generates part of the root meristem and the extra-embryonic suspensor (Scheres et al., 1994). At the eight-cell stage, the apical-basal axis of the embryo is divided into three tiers: an apical region that will mainly give rise to the cotyledons and the shoot apical meristem; a central region that will give rise to the hypocotyl and the root; and a basal region represented by the uppermost suspensor cell called the hypophysis that will give rise to the lower part of the root meristem (quiescent centre and columella cells). Consistent with their distinct fates, these three cell tiers express different sets of genes (Haecker et al., 2004). Subsequently, the eight cells of the proembryo divide tangentially, and the outer daughter cells, which will give rise to the epidermis, start to express different genes than the inner daughter cells (Lu et al., 1996; Abe et al., 2003). Later in embryo development, expression patterns become more complex and many transcriptional domains are formed (Takada and Tasaka, 2002; Weijers and Jürgens, 2005). However, differential gene expression is only a consequence of cell fate specification and thus does not provide information about the underlying mechanism of pattern formation. How a specific cell fate arises at a defined position within the embryo is a central unanswered question in the study of pattern formation. In particular, it is not known what factors provide the necessary positional information.

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Molecular mechanisms of pattern formation have been studied in detail in the *Drosophila* embryo where gradients of maternal transcription factors provide positional cues for the position-dependent activation of the zygotic genes (Lawrence and Struhl, 1996). The regulatory region of the gene plays an important role in the interpretation of these positional signals, acting as a transcriptional switch. Extensive studies in *Drosophila* have demonstrated that the position, number and affinity of the binding sites for maternal transcription factors are important factors for sensing the ratio and/or concentration of activators and repressors in the nucleus, thereby recognizing the position within the embryo, and for activating the gene at a correct position in the embryo (Clyde et al., 2003; Howard and Davidson, 2004; Kulkarni and Arnosti, 2005). Regulatory regions of developmental genes thus contain information about the binding sites of transcription factors that provide positional information, as well as information about the interpretation of the positional signals. Until now, no comparable dissection of regulatory gene regions that function in early plant embryos have been performed.

To gain insight into positional cues that regulate pattern formation in plant embryos, we studied cis-regulatory sequences of the *ATML1* gene, which is expressed in specific cells of the *Arabidopsis* early embryo. *ATML1* encodes an HD-ZIP-type homeodomain protein, and its transcripts are detected in the outermost, or epidermal cell layer of the embryo from the earliest stages onwards (Lu et al., 1996). Interestingly, *ATML1* expression was observed only in the apical daughter cell of the zygote (Lu et al., 1996). ATML1 protein has been shown to bind in vitro to an 8 bp sequence called the L1 box, which is found in the promoter of *ATML1* as well as in other epidermis-specific genes, suggesting a positive-feedback regulation of *ATML1* expression (Abe et al., 2001). However, there is no experimental evidence that the L1 box is required for *ATML1* expression. The *atml1* single mutant shows no obvious mutant phenotype, whereas a double mutant of *ATML1* and its closest homolog, *PROTODERMAL FACTOR2* (*PDF2*), interferes with cotyledon formation and causes partial loss of leaf epidermal tissue, indicating that *ATML1* is required for epidermis specification (Abe et al., 2003). Using a sensitive, multiple GFP reporter gene, we have

identified a 101 bp sequence that contains the L1 box and a putative WUSCHEL-binding site, and is sufficient for all aspects of *ATML1* expression in the embryo. Unexpectedly, the L1 box itself was not sufficient for the activation of *ATML1* expression in all epidermal cells, and several promoter fragments lacking the L1 box sequence were able to activate *GFP* expression in some epidermal cells. Our studies demonstrate that the *ATML1* promoter has a complex modular structure, and that distinct combinations of several promoter regions regulate *ATML1* expression during embryogenesis in space and time.

## MATERIALS AND METHODS

### Plant materials and growth conditions

*Arabidopsis thaliana* ecotype Columbia (Col) was used as wild type. *monopteros-B4149*, *emb30-1* and *atml1;pdf2* in Col background have been described previously (Mayer et al., 1993; Abe et al., 2003; Weijers et al., 2006). Plants were grown in long-day conditions (16 hours light, 8 hours dark) under white fluorescent light at 17°C. Embryos were staged according to Jürgens and Mayer (Jürgens and Mayer, 1994). Expression analyses in mutant backgrounds were performed in embryos from F3 plants homozygous for the transgene and heterozygous for the mutant.

### Plasmid construction and transgenic plants

#### *NLS:3xEGFP* and *35Smini::NLS:3xEGFP* in the pGreen II vector

An *NLS:EGFP* lacking a stop codon, an *EGFP* without a stop codon, and an *EGFP* with a stop codon, were generated by PCR. These three *GFP* fragments and the nopaline synthase terminator (nost) were fused together to generate *NLS:3xEGFP::nost*. *35Smini::NLS:3xEGFP::nost* was generated by ligating the CaMV 35S minimal promoter at the 5′ end of *NLS:3xEGFP::nost*. *NLS:3xEGFP::nost* and *35Smini::NLS:3xEGFP::nost* were cloned into the *Bam*HI site of the pGreen II-0229 vector (Roger et al., 2000).

#### *pATML1::NLS:3xEGFP*

The 3.4 kb region upstream of *ATML1* was amplified from the BAC clone F17L22 by PCR using Hi-fidelity Taq polymerase (Roche, Mannheim, Germany) with primers ATML1-T1 (5′-ATTGATTCTGAACTGTACCC-3′) and ATML1-T2 (5′-TTTAAGCTTAACCGGTGGATTCAGGG-3′). The *ATML1* promoter was fused to the *NLS:3xEGFP* reporter in the pGreen II vector to create *pATML1::NLS:3xEGFP*.

#### *pATML1::CYCB1-N::NLS:3xEGFP* in the pGreen II vector

A DNA fragment encoding the N-terminal region (amino acid residues 1-184) of CYCLINB1;2 (At5g06150) was cloned using PCR and fused to the 5′ end of *NLS:3xEGFP* in *pATML1::NLS:3xEGFP* in pGreen II to create *pATML1::CYCB1-N::NLS:3xEGFP*.

#### Deletions of promoter regions A-D

A region from –3318 to –1468, a region from –1463 to –664, and a region from –667 to –219, were deleted from the 3.4 kb promoter to produce ΔA, ΔB, and ΔC promoters, respectively. For the ΔD promoter, a region from –213 to +66 was deleted using PCR. Regions from –3318 to –1463, from –1467 to –214, from –663 to +66, and from –218 to +66, were cloned and used as ΔBCD, ΔAD, ΔAB, and ΔABC fragments, respectively.

#### Small internal deletions in region D

Regions from –213 to –181, from –180 to –155, from –154 to –131, from –130 to –80, from –79 to +66, from –40 to +66, and from –170 to –164, were deleted from region D to generate Δ33bp, Δ26bp, Δ24bp, Δ51bp, Δ145bp, Δ106bp, and ΔWUS constructs, respectively.

#### Hexamer constructs

The regions indicated in the Results section were amplified by PCR with specific forward primers containing an *Xba*I site, and reverse primers containing an *Spe*I site. Amplified fragments were cloned into the pGEM-T vector (Promega, Mannheim, Germany), and hexamer constructs were generated by sequentially inserting five *Xba*I-*Spe*I fragments at the *Xba*I site. Base substitutions in the WUS-binding site and in the L1 box were made using PCR.

All of the PCR-derived clones were sequenced. Wild-type *Arabidopsis* plants were transformed using the floral dip method (Clough and Bent, 1998), and T1 plants were selected on soil with BASTA.

### Confocal laser scanning microscopy analysis

The embryos were excised from the ovules in 4% paraformaldehyde and 5% glycerol solution. GFP signals were observed using a confocal laser scanning microscope (Leica) by excitation at 488 nm and by collection at 504-526 nm (green). The background autofluorescence was collected in the range 613-648 nm (red).

## RESULTS

### Visualization of *ATML1* promoter activity with a nuclear-localized triple GFP reporter

It has been reported that a 3384 bp region upstream of the *ATML1* open reading frame is sufficient to activate reporter gene expression in the epidermis of globular-stage embryos (Sessions et al., 1999). However, *ATML1* promoter activity has not been analysed at earlier stages (Sessions et al., 1999). To improve the sensitivity and spatial resolution for detecting *ATML1* promoter activity in the embryo, we generated an *NLS:3xEGFP* reporter gene that consists of the SV40 nuclear localization signal (NLS) and three tandem enhanced green fluorescence protein (*3xEGFP*) sequences (Fig. 1A). Nuclear localization of GFP was expected to increase the concentration of GFP locally. Indeed, this *NLS:3xEGFP* reporter gene enabled us to visualize the 3.4 kb *ATML1* promoter activity in the one-cell stage embryo (in 12 independent transgenic lines) (Fig. 1C). Unexpectedly, GFP signals were detected in both the apical and the basal daughter cell of the zygote (Fig. 1C, see below). During the 32- to 64-cell stages, *pATML1::NLS:3xEGFP* expression disappeared from the inner cells and was restricted to the suspensor and the outer cell layer of the embryo proper (Fig. 1D,E). After the division of the hypophyseal cell, GFP signals disappeared from the inner daughter cells by the heart stage (Fig. 1F,G). In the mature embryos, GFP signals were still detected in the outermost cell layer (Fig. 1H). In summary, the *ATML1* promoter was active only in the cells that were located at the surface of the embryo.

### The 3.4 kb promoter of *ATML1* is active in the suspensor

As mentioned above, *pATML1::NLS:3xEGFP* expression was detected in the basal cell and in the suspensor, in contrast to previous reports of *ATML1* promoter activity in the apical lineage only (Lu et al., 1996; Sessions et al., 1999). This inconsistency might be due to the stable inheritance of the NLS:3xEGFP protein from the zygote, given that *ATML1* promoter activity was detected in the dividing zygote (data not shown). To examine potential effects of NLS:3xEGFP protein stability on the expression pattern, we generated an unstable version by fusing an N-terminal destruction box-containing fragment of CYCLINB1;2 (CYCB1-N) to the N-terminus of the NLS:3xEGFP reporter (Fig. 1B). Because CYCLINB1 is degraded during anaphase and its N-terminal region confers the same instability to proteins fused to it, only newly synthesized NLS:3xEGFP should be detected after cell division (Glotzer et al., 1991; Colon-Carmona et al., 1999). In the *pATML1::CYCB1-N:NLS:3xEGFP* lines, GFP signals disappeared from the inner cells at the 16-cell stage, and thus earlier than in the *NLS:3xEGFP* lines, suggesting that *ATML1* promoter activity is downregulated in the inner cells as early as the 16-cell stage (in 4 of 4 lines) (Fig. 1J). By contrast, GFP signals were still detected in the suspensor and in the basal cell (in 5 of 5 lines that showed expression), which indicates that the *ATML1* promoter is active in these cells (Fig. 1I). In support of this conclusion, some promoter-deletion lines displayed GFP signals in the suspensor but only from the eight-cell stage (see below). Our data

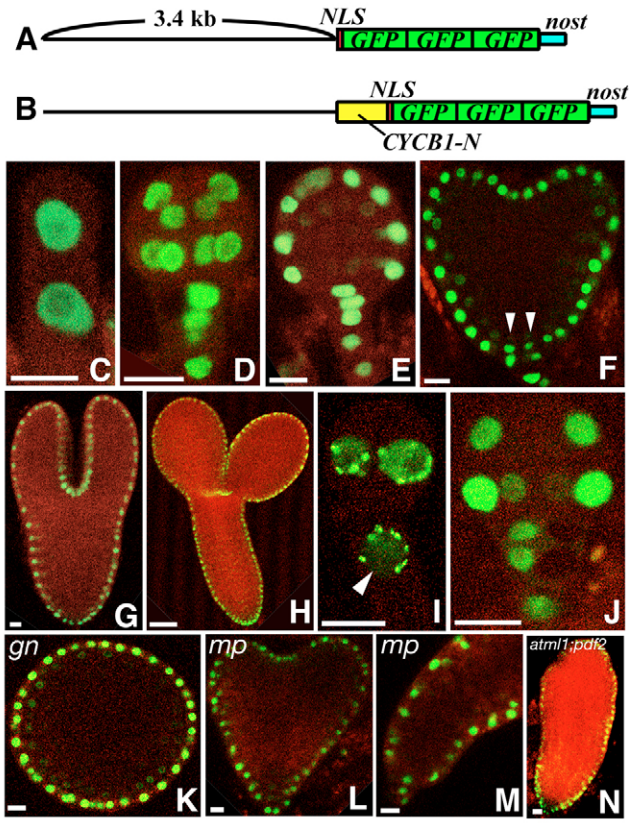


**Fig. 1. *ATML1* promoter activity in wild-type and mutant embryos.** (**A**,**B**) *ATML1* promoter-GFP reporter fusion constructs *pATML1::NLS:3xEGFP* (A) and *pATML1::CYCB1-N:NLS:3xEGFP* (B). *NLS*, SV40 nuclear localization signal sequence; *GFP*, enhanced green fluorescence protein sequence; *nost*, nopaline synthase terminator; *CYCB1-N*, a sequence encoding an N-terminal fragment of CYCLINB1;2, including the destruction box. (**C-H**) *pATML1::NLS:3xEGFP* expression in wild-type embryos at successively older stages: (C) one-cell; (D) 16-cell; (E) 32-cell; (F) mid-heart (arrowheads indicate quiescent center cells lacking GFP signal); (G) torpedo; (H) bent-cotyledon. (**I**,**J**) *pATML1::CYCB1-N:NLS:3xEGFP* expression in wild-type embryos at different stages: (I) two-cell (arrowhead indicates *GFP* expression in nucleus of suspensor cell), and (J) 16-cell (compare with D). (**K-N**) *pATML1::NLS:3xEGFP* expression in mutant embryos: (K) ball-shaped *gn*; (L) heart-stage *mp*; (M) basal peg of *mp*; (N) *atml1;pdf2*. Green, GFP signals; red, chlorophyll autofluorescence. Scale bars: 10 μm in C-G,I-N; 50 μm in H.

indicate that the *ATML1* promoter is active in both daughter cells of the zygote and, thus, that *ATML1* expression cannot be used as a marker for apical cell fate in the one-cell stage embryo.

## pATML1::NLS:3xEGFP is expressed normally in auxin-related mutant embryos

We examined whether the plant hormone auxin is necessary for *ATML1* expression in the embryo, as auxin may provide positional information (Sabatini et al., 1999; Friml et al., 2003). We introgressed a *pATML1::NLS:3xEGFP* line into two auxin-related mutants, *gnom* (*gn*) and *monopteros* (*mp*) (Berleth and Jürgens, 1993; Mayer et al., 1993). Auxin distribution is abnormal in *gn* mutant embryos, and auxin response is defective in *mp* mutant embryos (Friml et al., 2003).

*gn* embryos fail to form the root, and in extreme cases the embryo is ball-shaped and does not exhibit any apical-basal polarity (Mayer et al., 1993). *mp* is defective in organising the basal region of the embryo (Berleth and Jürgens, 1993). Although these two embryo mutants display clear auxin-related defects, *pATML1::NLS:3xEGFP* was expressed in the epidermis of both ball-shaped *gn* mutant embryos (Fig. 1K) and *mp* embryos, including their abnormal basal end (Fig. 1L,M). These observations suggest that the pattern of *ATML1* expression is determined independently of auxin distribution or signaling. Moreover, this implies that the epidermis is specified independently of apical-basal patterning.

## Promoter regions required for early activation and apical expression

In order to define regulatory sequences that control *ATML1* expression in the embryo, we generated promoter-deletion constructs. The *ATML1* promoter was divided into four regions denoted A, B, C and D, that correspond to nucleotide positions –3318 (*Hin*dIII) to –1468 (*Nco*I), –1467 to –664 (*Pst*I), –663 to –219 (*Xba*I) and –218 to +66, respectively, relative to the transcription initiation site (Fig. 2). Initially, regions A, B or C were deleted from the 3.4 kb promoter, whereas region D was replaced by a minimal promoter derived from the cauliflower mosaic virus (CaMV) 35S rRNA promoter (–53 to +4) (Benfey et al., 1990). Deletion of region D (ΔD) abolished reporter expression in the early embryo (12 of 12 lines that showed expression), whereas other deletions did not show the same effect, indicating that region D is necessary for early expression (Fig. 2). Further deletion studies revealed that regions C and D (ΔAB) rarely activated *GFP* expression in the early embryo (1 of 9 lines), and region D alone (ΔABC) was not sufficient for the expression in the early embryo (0 of 11 lines). However, the combinations B+C+D (ΔA) and A+B+D (ΔC) conferred *GFP* expression in the early embryo (12 of 12 and 7 of 7 lines, respectively), indicating that either region AB or (B)C is also required for region D-mediated early expression (Fig. 2).

In the *ΔD* lines, GFP signals were first detected in the suspensor at the eight-cell stage and then in the epidermis from the 32-cell stage onwards (Fig. 3J,K). Interestingly, deletion of region D abolished *GFP* expression in the apical half of the embryo proper until the late-heart stage (12 of 12 lines that showed expression) (Fig. 3K,L). Thus, although not essential for expression in the epidermis, region D is necessary for expression in the apical half of the embryo proper. By contrast, other promoter deletions still gave *GFP* expression in the apical half of globular-stage embryos, although it was reduced in a subset of the lines (see below). Deletion of both regions A and D (ΔAD) abolished *GFP* expression in the epidermis (0 of 11 lines), whereas region A alone (ΔBCD) was still sufficient for the expression in the central region of the embryo (13 of 13 lines that showed expression) (Fig. 3I). Thus, regions A and D seem to contain functionally redundant cis-regulatory elements for epidermis-specific expression (Fig. 2). However, in contrast to region A (ΔBCD), region D alone (ΔABC) was able to activate expression in wider regions at the heart stage (10 of 10 lines examined at this stage) (Fig. 2; Fig. 3E,F), although GFP signals were not detected in the apical half of the embryo proper until the mid-heart stage (Fig. 3E), and *GFP* expression was reduced in the adaxial side of the cotyledons even at later stages (Fig. 2; Fig. 3F). Thus, region D plays a major regulatory role in *ATML1* expression during embryogenesis.

## Specific promoter fragments stabilize expression in different regions of the embryo

Deletion of region B abolished expression in the cells of the basal lineage in one out of ten independent transgenic lines from early stages onwards, and in one of ten lines from the late-globular stage (Fig. 3H). In *ΔAB*, this ‘apical lineage’ expression was also observed in three out of nine independent lines from early stages onwards, and

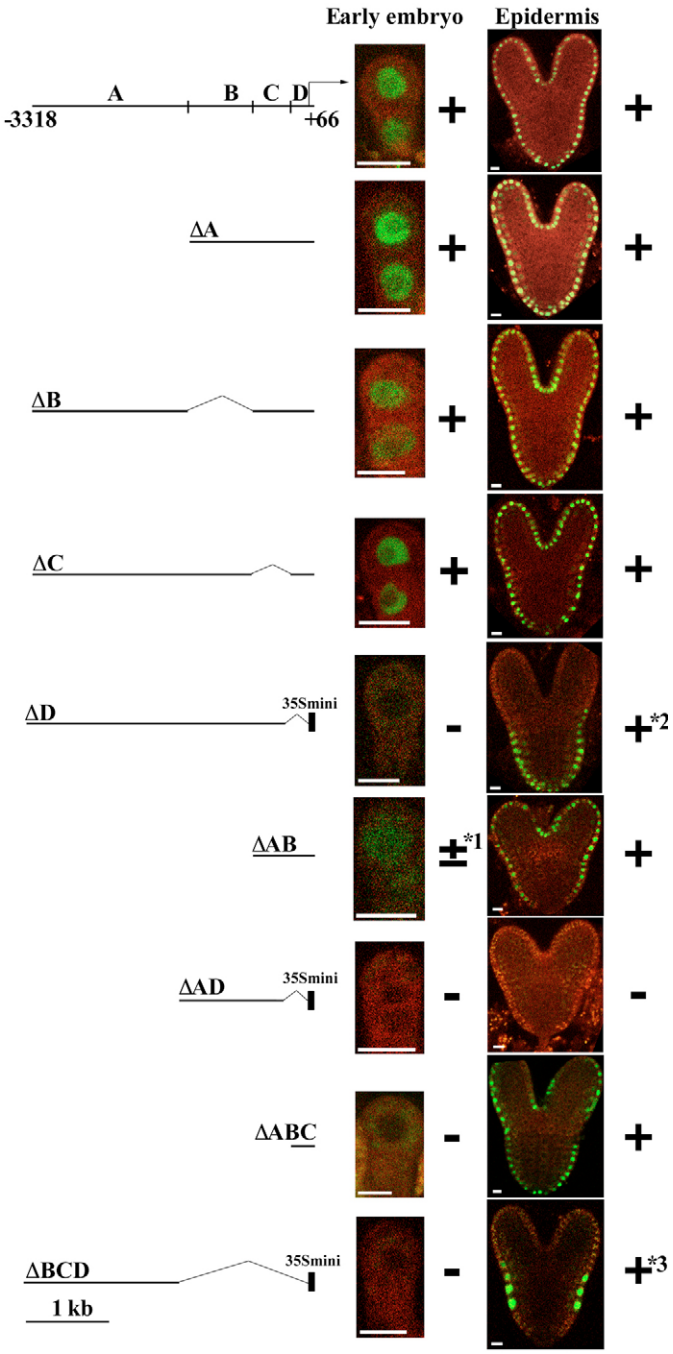


**Fig. 2. Expression analysis of *ATML1* promoter regions.** Deletions of promoter regions A, B, C and D are illustrated to the left; corresponding *GFP* expression patterns at the one-cell stage (early embryo) and heart stage (epidermis) are shown to the right. Presence or absence of *GFP* expression (green or yellow) are indicated by + or –, respectively. *1, one of nine lines showed expression at the one-cell stage; *2, no *GFP* expression in the apical half of the embryo proper; *3, *GFP* expression only in the central region of the embryo. In the schematic, the arrow (top diagram) indicates the transcription start site (+1); 35Smini, minimal promoter derived from the cauliflower mosaic virus 35S promoter. Scale bars: 10 μm.

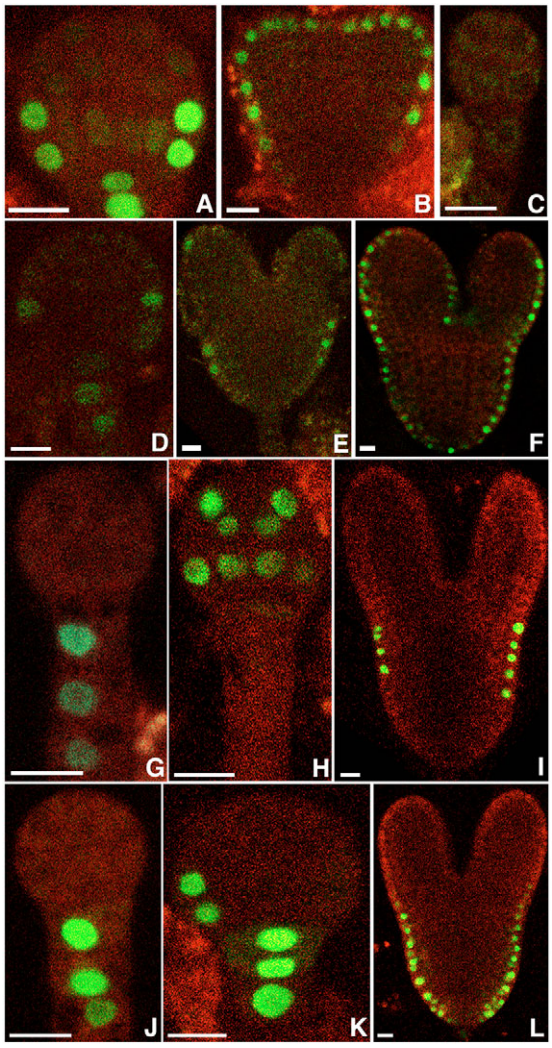


**Fig. 3. Position-dependent *GFP* expression along the apical-basal axis conferred by *ATML1* promoter deletions.** (**A**) 'Basal half' expression at the 32-cell stage in *ΔA*. (**B**) 'Apical lineage' expression at the early-heart stage in *ΔAB*. (**C-F**) *GFP* expression in *ΔABC* at the eight-cell (C), 32-cell (D), mid-heart (E) and late-heart (F) stages. (**G**) 'Basal lineage' expression at the eight-cell stage in *ΔAD*. (**H**) 'Apical lineage' expression at the 16-cell stage in *ΔB*. (**I**) 'Central' expression at the late-heart stage in *ΔBCD*. (**J-L**) *GFP* expression in *ΔD* at eight-cell (J), 32-cell (K) and late-heart (L) stages. Scale bars: 10 μm.

in three of nine lines from the heart stage (Fig. 3B). One explanation for these variable expression patterns is that region B and the other regions might contain functionally redundant regulatory elements that are responsible for gene activation in the suspensor. Consistent with this idea, deletion of regions A+B+C completely abolished *GFP* expression at the basal pole from the heart stage onwards (10 of 10 lines) (Fig. 3E). In addition, regions B and C alone (ΔAD) were able to activate weak *GFP* expression only in the suspensor until the globular stages (9 of 9 lines that showed expression) (Fig. 3G), suggesting that region B might indeed regulate *ATML1* expression in the suspensor. Other deletion lines and the full-length *ATML1* promoter lines never showed this 'apical lineage' expression (Table 1).

Surprisingly, in a subset of the deletion lines including *ΔA*, *ΔB* and *ΔC* lines, GFP signals were not detected in the apical half of the embryo proper at the globular stages (Fig. 3A, Table 1). In these lines, the apical expression was recovered by the early-heart stage, although GFP signals were sometimes weak in the adaxial side of

**Table 1. Frequency of expression patterns observed in globular-stage embryos**

| Promoter construct | Deleted base-pairs | 'Apical lineage' expression | 'Basal half' expression | Normal expression | *n* |
|---|---|---|---|---|---|
| pATML1 (3.4 kb) | – | 0 | 0 | 11 | 11 |
| ΔA | –3318 to –1468 | 0 | 8 | 4 | 12 |
| ΔAB | –3318 to –664 | 3 | 5 | 1 | 9 |
| ΔB | –1463 to –664 | 1 | 5 | 4 | 10 |
| ΔC | –667 to –219 | 0 | 1 | 7 | 8 |
| ΔD | –213 to +66 | 0 | 12 | 0 | 12 |
| Δ33bp | –213 to –181 | 0 | 1 | 6 | 7 |
| Δ26bp | –180 to –155 | 0 | 6 | 2 | 8 |
| Δ24bp (3.4 kbΔL1) | –154 to –131 | 0 | 7 | 1 | 8 |
| Δ51bp | –130 to –80 | 0 | 2 | 4 | 6 |
| Δ145bp | –79 to +66 | 0 | 4 | 3 | 7 |
| Δ106bp | –40 to +66 | 0 | 2 | 7 | 9 |
| 3.4 kbΔWUS | –170 to –164 | 0 | 7 | 4 | 11 |

The number of lines that showed the above expression patterns is indicated for each construct, along with the total number of independent transgenic lines that showed *GFP* expression (*n*). The position of deleted nucleotides is indicated relative to the transcription initiation site. 'Apical lineage' refers to the proembryo derived from the apical daughter cell of the zygote. 'Basal half' expression refers to that in the basal half of the proembryo.

the cotyledons until the late-heart stage (data not shown). This 'basal half' expression pattern was also observed in a subset of the lines with small deletions within region D (Table 1). These results suggest that all regions are necessary for the stable expression in the apical half of the embryo proper at the globular stages.

## A 179 bp promoter fragment is sufficient for *ATML1* expression in the embryo

The initial deletion studies suggested the existence of a regulatory sequence within region D for *ATML1* activation in the early embryo. However, a series of small deletions covering region D did not abolish *GFP* expression (see Table 1 for the numbers of lines examined) (Fig. 4), indicating that several regions regulate *ATML1* expression in the early embryo. In addition, these deletions did not cause ectopic *GFP* expression in the inner cells (Fig. 4), which suggests that there might not be a simple, negative-regulatory sequence that represses *ATML1* expression in the inner cells.

Region D alone conferred expression in the epidermis of globular-stage embryos, but not earlier. This raised the possibility of an early promoter activity of region D that, however, was below the detection limit owing to the absence of general enhancers in the other regions. To enhance the signal, we made a construct that contains six tandem repeats of a 179 bp fragment (–219 to –41) from region D, fused to the 35S minimal promoter and the NLS:3xEGFP coding sequence (Fig. 5). This artificial promoter generated GFP signals from the one-cell stage onwards in a manner indistinguishable from the full-length promoter (6 of 6 lines) (Fig. 5A-E, compare with Fig. 1C-F). This result suggests that the 179 bp fragment of region D can mediate all aspects of *ATML1* expression in the embryo.

## Mutational analysis of the L1 box and WUS-binding site reveals composite regulation of *ATML1* expression

The 179 bp fragment of region D contains two known cis-regulatory elements; a WUSCHEL (WUS)-binding site and an L1 box (Abe et al., 2003). The WUS-binding site was identified in the regulatory region of the floral homeotic gene *AGAMOUS*, which is positively regulated by *WUS* in the center of the floral meristem (Lohmann et al., 2001). Although *WUS* is expressed in the inner cells in the apical half of the embryo proper, but not in the *ATML1* expression domain (Mayer et al., 1998), there is a family of WUS-related (WOX) transcription factors, some of which are expressed in the *ATML1*-expressing cells of the embryo (Haecker et al., 2004), suggesting that one or more of them might bind to the WUS-binding site of the *ATML1* gene. The L1 box was first identified in the promoter of the *PROTODERMAL FACTOR1* (*PDF1*) gene and was shown to be essential for *PDF1* expression in the outermost cell layer of the shoot apical meristem (SAM) (Abe et al., 2001). As ATML1 binds directly to the L1 box in vitro, the L1 box may be involved in positive-feedback regulation (Abe et al., 2001). However, there was no evidence that the WUS-binding site and the L1 box are required for *ATML1* expression in the embryo. To examine the role of these putative binding sites, we deleted either the WUS-binding site or a 24 bp region including the L1 box from the ΔABC construct and named the derivatives DΔWUS and DΔL1, respectively (Fig. 4). With these constructs, GFP signals were detected in the epidermis at a low level in only a few transgenic plants (4 of 22 transgenic lines for DΔL1, and 6 of 20 lines for DΔWUS), in contrast to the construct without deletions (12 of 18 lines). This suggests that the L1 box and the WUS-binding site are necessary to enhance the expression level of *ATML1* (Fig. 4).

To examine the role of the L1 box and the WUS-binding site at earlier stages, we analyzed how mutations in these binding sites affected the activity of the 6x179bp construct (Fig. 5). Surprisingly, both mutations had similar effects on the *GFP* expression pattern. The mutations in the L1 box (from TAAATGCA to GCCCGTAC; 6x179bpmL1) and the WUS-binding site (from TTAATGG to GGCCGTT; 6x179bpmWUS) did not abolish the early activation of *GFP* in the one-cell stage (11 of 12 and 7 of 8 lines, respectively) (Fig. 5F,K). However, GFP signals were downregulated by the eight-cell stage (7 of 7 and 7 of 7 lines, respectively) (Fig. 5G,L). *GFP* expression was reactivated in the epidermis by the late-globular or early-heart stage, although GFP signals were not detected in the apical half of the embryo proper nor at the basal pole (14 of 14 and 12 of 12 lines, respectively) (Fig. 5H,M). Expression in the columella root cap cells and in the abaxial side of the cotyledons was recovered by the late-heart stage in *6x179bpmWUS* (11 of 11 lines) but at best rarely in *6x179bpmL1* [0 of 10 lines (columella), 3 of 10 lines (abaxial)] (Fig. 5I,J and 5N,O). Deletion of the WUS-binding site or the 24 bp region in the context of the 3.4 kb promoter [3.4 kbΔWUS and 3.4 kbΔL1(Δ24bp)] also reduced the expression in the apical half of the globular-stage embryo proper at a high frequency (7 of 11 and 7 of 8 lines, respectively), although *GFP* expression was detected normally at the basal pole (11 of 11 and 8 of 8 lines, respectively) (Fig. 4).

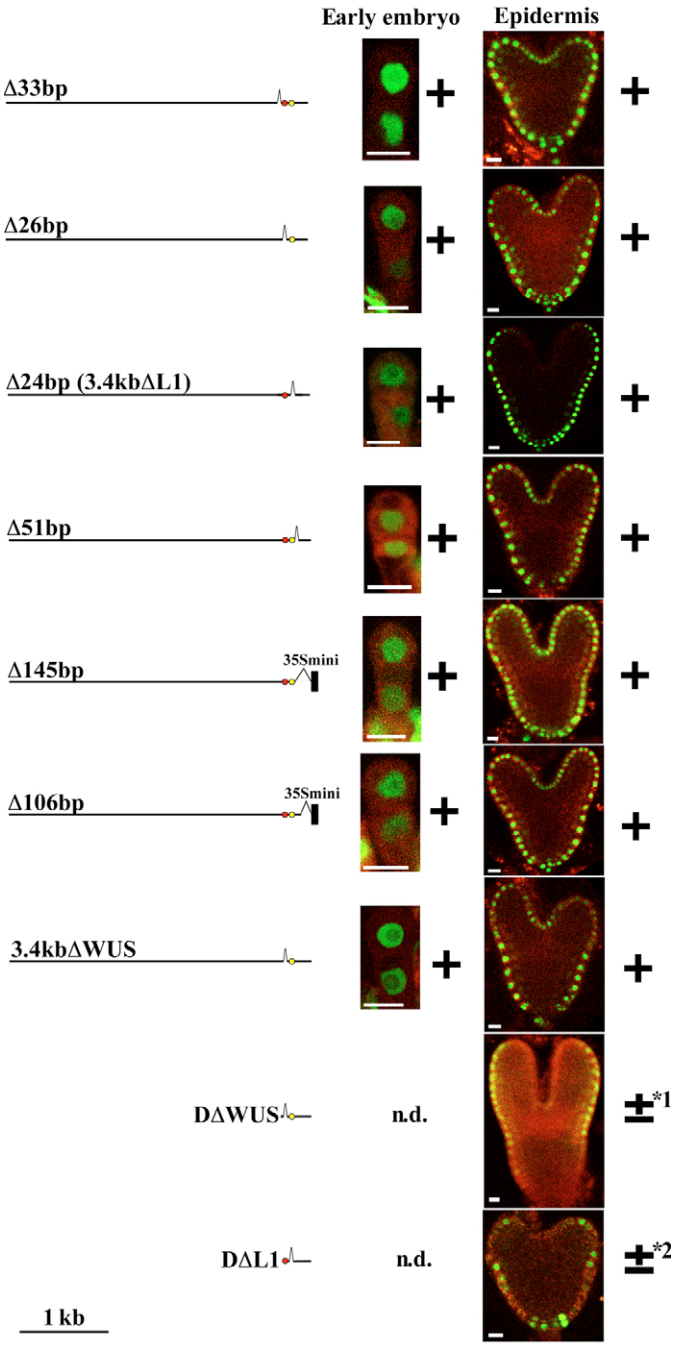


**Fig. 4. Effects of small deletions within region D on *ATML1* promoter activity.** To the right of each deletion construct, presence or absence of promoter activity at the one-cell stage (early embryo) and heart stage (epidermis) are indicated by + or –, respectively. 35Smini, 35S minimal promoter; n.d., not done; *1, only six of 20 transformants showed expression; *2, only four of 22 transformants showed expression. Scale bars: 10 μm.

In summary, the L1 box and the WUS-binding site are required for *ATML1* expression in the apical half and the basal pole of the embryo from the globular stage, and for the maintenance of the expression during the globular stages, but are not necessary for the initial activation nor for expression in the basal half of the embryo from the late-globular stage. Interestingly, the L1 box is not necessary for the expression in some epidermal cells. More importantly, the L1 box, which might be involved in the autoregulation of *ATML1*, is not sufficient for the expression in all epidermal cells in the absence of the WUS-binding site.

To further assess the role of autoregulation, *pATML1::NLS:3xEGFP* expression was examined in the *atml1;pdf2* double mutant. Severe mutant embryos lack cotyledon primordia, indicating that epidermal cells are required for the formation of organ primordia, as suggested by laser ablation experiments in the tomato SAM (Abe et al., 2003; Reinhardt et al., 2003). Weak GFP signals were detected in the outer cell layer of *atml1;pdf2* embryos (Fig. 1N). This indicates that autoregulation plays a minor role in determining the expression pattern and suggests that *ATML1* itself is not necessary for its own expression in the outermost cell layer, although it has a role in maintaining the expression level.

## The 179 bp region has a modular organization

To further narrow down the region sufficient for *ATML1* expression in the epidermis, four overlapping fragments [89 bp (from –219 to –131), 101 bp (from –180 to –80), 114 bp (from –154 to-41), and 90 bp (from-130 to –41)] derived from the 179 bp region were used to drive the *NLS:3xEGFP* reporter gene (Fig. 6). Six copies of each fragment were cloned in front of the 35S minimal promoter fused to the *NLS:3xEGFP* reporter (6x89bp, 6x101bp, 6x114bp and 6x90bp). Among these four constructs, only 6x101bp showed normal expression (15 of 15 lines), indicating that six copies of the 101 bp region can confer all aspects of *ATML1* expression in the embryo (Fig. 6F-J).

6x89bp (9 of 9 lines) and 6x114bp (8 of 11 lines) constructs were able to activate *GFP* expression in the one-cell stage embryo (Fig. 6A,K), whereas 6x90bp was not sufficient for early expression (0 of 19 lines) (Fig. 6P), indicating that a 24 bp fragment (from –154 to –131) contains a sequence required for the early activation. Also, in *6x114bp*, early *GFP* expression was weak and sometimes undetectable (3 of 11 lines), suggesting that a 26 bp region (from –180 to –155) is also necessary for stable expression in the early embryo.

During the globular stages, *6x114bp* expression was not detected in the apical half of the embryo proper (13 of 14 lines) nor at the basal pole (12 of 13 lines) (Fig. 6M), whereas the expression in the apical half of the embryo was not affected in *6x89bp* (11 of 11 lines) or *6x101bp* (10 of 10 lines) (Fig. 6C,H), indicating that the 26 bp region is necessary for the expression in the apical half of the embryo proper at the globular stage. Since *Δ24bp* and *6x179bpmL* were also defective in the expression in the apical half, both the 26 bp and 24 bp regions are required for the expression in the apical half.

During the heart stages, *6x114bp* was defective in reporter expression in the very apical region, including the adaxial side of the cotyledons and the presumptive SAM region between the two cotyledon primordia (12 of 12 lines) (Fig. 6N). Expression at the basal pole was recovered by the late-heart stage in *6x114bp* (11 of 13 lines) (Fig. 6O). These expression patterns are similar to those in *6x179mWUS*, implying that the activity of the 26 bp region is largely dependent on the WUS-binding site.

*6x89bp* showed normal expression until the 16-cell stage. However, at the 32-cell stage, GFP signals started to disappear from the basal pole (6 of 10 lines examined). Moreover, in seven of eleven lines that showed GFP signals, *GFP* expression was downregulated in the SAM region (Fig. 6D,E). Since *6x179bpmL1* and *6x114bp* were also defective in reporter expression in the SAM and the suspensor, we conclude that the 26 bp region (or the WUS-binding

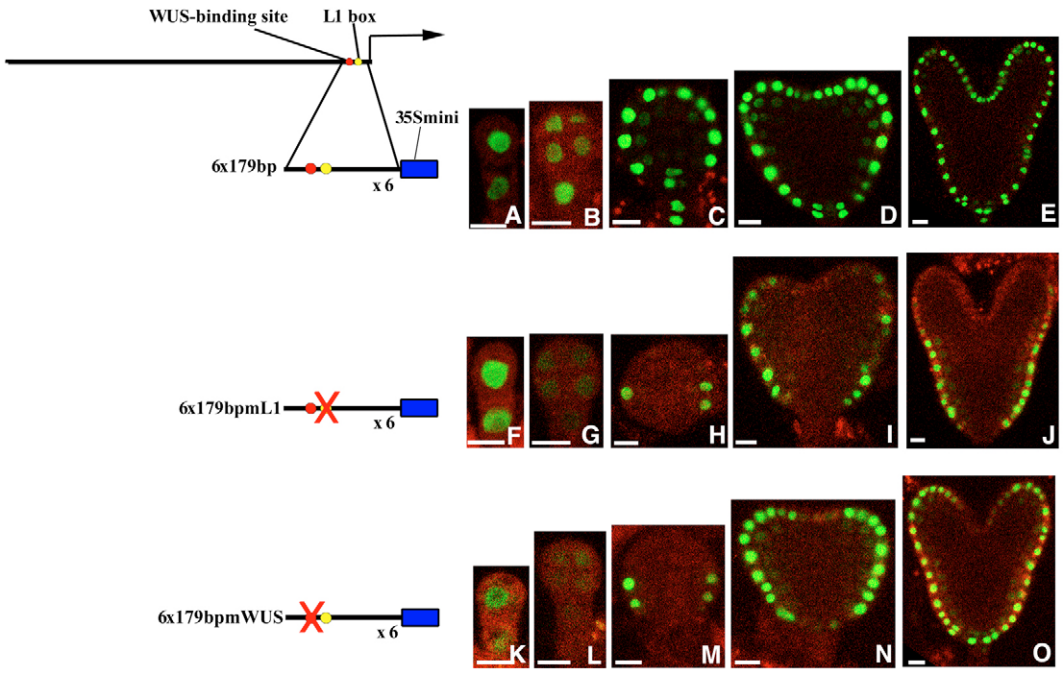


**Fig. 5. *GFP* expression driven by six tandem repeats of the 179 bp promoter fragment.** The diagrams to the left indicate the position of the 179 bp fragment in the *ATML1* promoter, the WUS-binding site (red circle) and the L1 box (yellow circle). The arrow indicates the transcription start site. Red crosses indicate mutations in cis-regulatory sequences. All fragments consisted of six tandem copies (x 6) fused to the 35S minimal promoter (35Smini). (**A-E**) *6x179bp* expression at one-cell (A), eight-cell (B), 32-cell (C), early-heart (D) and late-heart (E) stages. (**F-J**) *6x179bpmL1* expression at one-cell (F), eight-cell (G), late-globular (H), early-heart (I) and late-heart (J) stages. (**K-O**) *6x179bpmWUS* expression at one-cell (K), eight-cell (L), 32-cell (M), early-heart (N) and late-heart (O) stages. Scale bars: 10 μm.

site), the 24 bp region (or the L1 box), and a 51 bp region (from –130 to –80), are required for stable expression in the SAM and the suspensor, although the 26 bp region plays a minor role in the expression at the basal pole at the late-heart stage.

Interestingly, some of the *6x90bp* lines (7 of 19 lines) still showed weak expression from the mid-heart stage in some epidermal cells (Fig. 6S,T), indicating that epidermis-specific expression can be activated independently of the L1 box and the WUS-binding site.

In summary, we found that the combination of the 26 bp, 24 bp and 51 bp regions (a 101 bp fragment from –180 to –80) is necessary for expression in the SAM and at the basal pole of the embryo, and can mediate all aspects of *ATML1* expression in the embryo.

## DISCUSSION

### The *ATML1* promoter is active not only in the epidermis of the embryo proper (apical lineage) but also in the basal cell lineage

The activity of the 3.4 kb *ATML1* promoter was previously detected in the apical cell and its progeny but not in the basal cell and its derivatives, as monitored by mRNA hybridization of a *uidA* reporter (Sessions et al., 1999). By contrast, our data clearly reveal activity of the same 3.4 kb *ATML1* promoter fused to nuclear-localised GFP in both apical and basal daughter cells of the zygote as well as their progeny. One possible explanation for this discrepancy is that mRNAs are difficult to detect in the basal cell, which is highly vacuolated and less cytoplasm-rich than the apical cell. Moreover, the basal cell is larger than the apical cell, which would reduce the signal intensity if transcripts accumulate to the same level in both apical and basal cells. By contrast, the nuclear-localized triple GFP used in this study is very sensitive and does not face the same problem because the sizes of the nuclei do not vary much in early embryogenesis.

### Stage-dependent activation of *ATML1*

Region D of the promoter is required for the early activation of *ATML1*. Although region D on its own was not able to activate a detectable level of expression in the early embryo, six copies of a 179 bp fragment derived from region D were able to do so, suggesting that region D contains a cis-regulatory sequence that mediates *ATML1* expression in the early embryo. The 179 bp fragment contains two known regulatory motifs, an L1 box and a WUS-binding site. Mutation of one or other motif did not affect the early activation of *ATML1*. Rather, both motifs seem to be necessary for the maintenance of early expression, as *GFP* reporter expression was downregulated before the globular stage in the absence of the L1 box or the WUS-binding site. However, GFP signals were reactivated by the early-heart stage, indicating that an additional later-acting transcriptional mechanism activates *ATML1* expression independently of the L1 box or the WUS-binding site. Collectively, these results suggest that *ATML1* expression is regulated by at least three different mechanisms at different stages of embryogenesis: initial activation, subsequent maintenance of expression, and reactivation at later stages. Our deletion studies also revealed that the 90 bp fragment of region D activates the expression only at later stages, and that the adjacent 50 bp fragment is required for stable expression in the early embryo, implying that stage-specific factors might act through distinct cis-regulatory sequences.

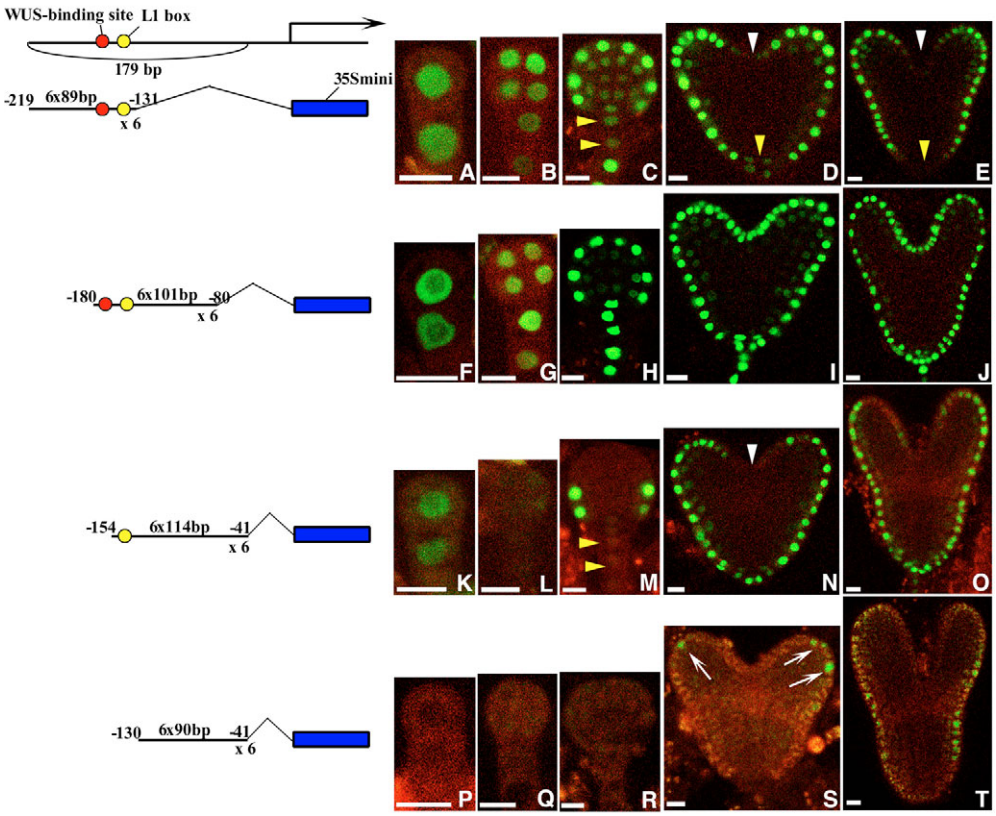


**Fig. 6. *GFP* expression driven by four overlapping subfragments of the 179 bp promoter fragment.** The constructs are shown to the left, the corresponding *GFP* expression patterns in embryos to the right. All the promoter subfragments were hexamerized (x 6) and fused to the 35S minimal promoter (35Smini). Sequence numbering refers to the distance in bp from the transcription start site (+1; arrow in top diagram). Circles indicate the WUS-binding site (red) and L1 box (yellow). *GFP* expression in (**A-E**) *6x89bp*, (**F-J**) *6x101bp*, (**K-O**) *6x114bp*, (**P-T**) *6x90bp*. Embryo stages: (A,F,K,P) one-cell; (B,G,L,Q) eight-cell; (C,H,M,R) 32-cell; (D,I,N,S) mid-heart; (E,J,O,T) late-heart. White arrowheads (D,E,N) indicate reduced expression in the presumptive SAM region; yellow arrowheads (C-E,M) indicate reduced expression at the basal pole; white arrows (S) indicate patchy expression in epidermis. Scale bars: 10 μm.

## Epidermis-specific expression of *ATML1* is controlled by several regulatory sequences

Regions A and D contain regulatory sequences for the expression in the epidermis. Notably, multiple copies of a 101 bp sequence from region D, which includes the L1 box and the WUS-binding site, were sufficient for expression in all epidermal cells of the embryo.

Some epidermis-specific expression can be activated independently of the L1 box, as six copies of the 179 bp fragment containing a mutated L1 box can still confer transcriptional activity in the epidermis. Moreover, region A, and a 90 bp fragment of region D, both of which lack an L1-box sequence, can activate reporter gene expression in the epidermis, indicating that the epidermis-specific expression of *ATML1* is regulated by several independent inputs through different cis-regulatory elements.

Importantly, the L1 box was not sufficient for the expression in all epidermal cells in the *6x179bpmWUS*, *6x89bp* and *6x114bp* lines. These observations indicate that the function of the L1 box is context-sensitive and that other regulatory sequences (e.g. the WUS-binding site) are required for L1 box-mediated transcription in the embryo. This idea is supported by the fact that the *SCARECROW* promoter, which contains an L1 box sequence, is active in the epidermal cells of the postembryonic shoot meristem, but not in those of the embryo (Wysocka-Diller et al., 2000; Abe et al., 2001).

## *ATML1* expression is differentially regulated along the apical-basal axis of the embryo

Our analysis indicates that different combinations of regulatory sequences regulate *ATML1* expression in different regions of the developing embryo (Fig. 7). *ATML1* expression in the globular embryo can be broken down into three distinct domains – apical and basal halves of the embryo, and the basal pole of the embryo plus the suspensor (Fig. 7B). A 50 bp sequence in region D (D50), encompassing the WUS-binding site and the L1 box, makes a major contribution to *ATML1* expression in the apical half of the embryo (Fig. 7A,B). In addition, our deletion experiments indicate that all promoter regions (A, B, C and D) are required for stable expression in the apical half (Table 1). These findings might suggest that the apical half, in which cells divide more frequently than in the basal half (Jürgens and Mayer, 1994), needs relatively more transcriptional enhancers to maintain the expression level during rapid cell divisions, and is thus sensitive to the deletion of general enhancer(s). In the basal half of the embryo, *6x89bp*, *6x101bp* and *6x114bp* are all sufficient for expression, indicating that the overlapping 24 bp fragment in region D (D24) is important. However, there appear to be other functionally redundant elements in D, as *DΔL1* still showed some expression in the basal half of the embryo (Fig. 4). In the absence of D, the combination of regions A, B and C is also sufficient for the expression in the basal half of the embryo, suggesting even more redundancy. At the basal pole, *ATML1* expression is regulated by the 101 bp fragment of region D (D101) encompassing the WUS-binding site and the L1 box. In addition to D101, a combination of B and C is sufficient for expression in the basal pole, reflecting the redundant organization of the *ATML1* promoter.

Heart-stage embryos display six domains of *ATML1* expression (Fig. 7B). At the apical end of the embryo, D50 and D101 are required for the expression at the adaxial side of the cotyledon primordia and in the SAM region, respectively. These expression domains are dependent on the WUS-binding site and the L1 box. The D24 fragment is involved in the expression in a wider lateral region of the embryo at the heart stage than at the earlier globular stage. The 90 bp fragment of region D (D90) can also activate *ATML1* expression, though weakly, in epidermal cells located

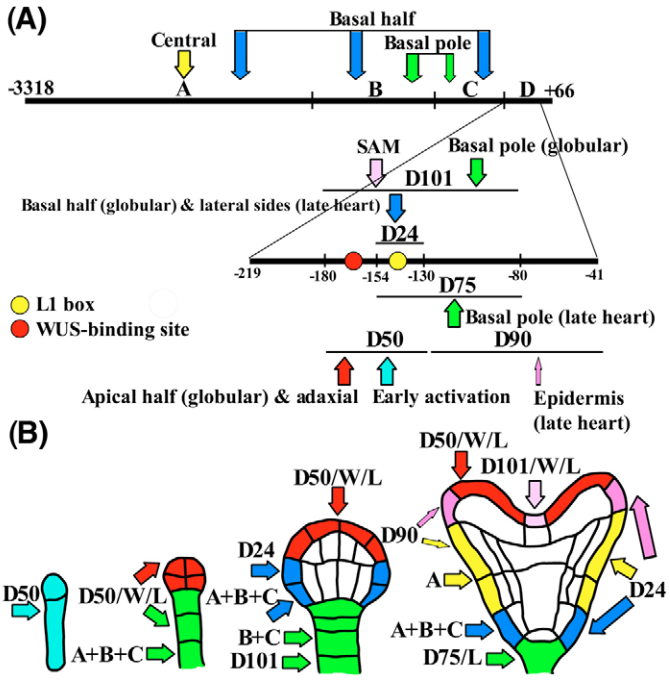


**Fig. 7. Model for the transcriptional regulation of *ATML1* in embryogenesis.** (**A**) Regulatory organization of the *ATML1* promoter. Target regions of putative positional signals (arrows) are indicated. Sequence numbering refers to the distance in bp from the transcription start site (+1). (**B**) Regulation of *ATML1* expression in embryogenesis. Each color represents a different expression domain. The regulatory sequences that mainly contribute to the expression in each domain are indicated (see text for details). Successive embryo stages are depicted (from left to right): one-cell stage, eight-cell stage, globular stages, heart stage.

apically within the D24 domain. At the basal pole, the 26 bp fragment and the WUS-binding site are not necessary for the expression at the late-heart stage. Instead, D75 (D101 minus the 26 bp fragment) and the L1 box mediate the expression at the basal pole. Promoter region A alone can confer activity in the central region of the embryo, whereas the combined regions A+B+C can activate expression in the basal half of the embryo proper.

## Positional cues that restrict the expression of *ATML1* to the epidermis

The plant hormone auxin has been implicated in apical-basal axis specification of the embryo (Friml et al., 2003). Although the expression of *ATML1* in the epidermis is differentially regulated along the apical-basal axis, we observed no evidence for its auxin-dependent regulation. Thus, position-dependent gene activation in embryogenesis might also involve other, unknown signaling molecules that provide positional information.

With the formation of the epidermal cell layer by periclinal divisions of the octant-stage proembryo cells, *ATML1* expression is discontinued in the newly-formed inner cells. Our deletion analysis makes it highly unlikely that a negative regulatory sequence represses *ATML1* expression in the inner cells, suggesting instead that *ATML1* expression in the epidermis is regulated by positive regulators. It has been proposed that cell wall components of the zygote may provide positional cues for epidermal cell specification in the embryo because only the outermost cells of the embryo retain the cell walls derived from the zygote (Laux et al., 2004). This idea is consistent with *pATML1::NLS:3xEGFP* expression, which is detected in the cells located at the surface of the embryo and is downregulated in cells that have lost the cell walls derived from the zygote (e.g. quiescent center cells). It is tempting to speculate that *ATML1* expression is positively regulated by as yet unknown transcription factors that are directly activated by ligands available only in the outermost cell layer. Indeed, some transcription factors can directly bind to lipid or sterol ligands and could thus convey positional cues provided by these molecules (Schrick et al., 2004; Alvarez-Venegas et al., 2006). The identification of several promoter fragments that regulate specific aspects of *ATML1* expression in the embryo can now be used to isolate trans-acting factors that receive positional cues for epidermis specification, which will eventually lead to the elucidation of mechanisms by which transcription factors convey positional cues in the embryo.

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