The *Arabidopsis BODENLOS* gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning

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Developmental responses to the plant hormone auxin are thought to be mediated by interacting pairs from two protein families: short-lived inhibitory IAA proteins and ARF transcription factors binding to auxin-response elements. *monopteros* mutants lacking activating ARF5 and the auxin-insensitive mutant *bodenlos* fail to initiate the root meristem during early embryogenesis. Here we show that the *bodenlos* phenotype results from an amino-acid exchange in the conserved degradation domain of IAA12. BODENLOS and MONOPTEROS interact in the yeast two-hybrid assay and the two genes are coexpressed in early embryogenesis, suggesting that BODENLOS inhibits MONOPTEROS action in root meristem initiation.

Plant embryogenesis establishes a multicellular body organization that is elaborated postembryonically by the stem-cell systems of the shoot and root meristems to give the species-specific architecture of the adult plant (Jürgens 2001). The two primary meristems themselves originate from distinct regions along the apical-basal axis of the early embryo. Following the asymmetric division of the zygote, the smaller apical daughter cell generates an eight-celled proembryo consisting of two tiers of four cells each. The upper tier, or apical region, gives rise to the shoot meristem and most of the flanking cotyledon primordia. The lower tier, or central region, contributes to the cotyledon primordia but mainly produces the hypocotyl, the embryonic root, and the upper tier of root meristem stem cells. The basal daughter cell of the zygote produces a file of 7–9 initially extraembryonic cells of which the uppermost cell adjacent to the proembryo adopts an embryonic fate. This so-called hypophysis generates the basal region of the embryo that gives rise to the quiescent center and the lower tier of stem cells of the root meristem. Thus, the root meristem is of mixed clonal origin, with the hypophysis initiating root meristem formation during early embryogenesis (Jürgens 2001).

Mutations in two genes, *MONOPTEROS* (*MP*) and *BODENLOS* (*BDL*), affect the formation of the hypophysis in a seemingly noncell autonomous manner. Both *bdl* and *mp* mutations initially affect the orientation of the division plane of the apical daughter of the zygote, resulting in double-octant proembryos (Berleth and Jürgens 1993; Hamann et al. 1999; see Fig. 3K). Subsequently, the presumptive hypophysis fails to undergo the asymmetric division that gives the precursors of the quiescent center and the lower-tier stem cells of the root meristem. The same mutant phenotype was observed in *bdl mp* double mutant embryos, suggesting that the two proteins act in the same developmental pathway (Hamann et al. 1999). The *MP* gene encodes ARF5, a transcription factor of the ARF (auxin response factor) family that activates auxin-responsive target genes (Hardtke and Berleth 1998; Ulmasov et al. 1999a). *bdl* mutant seedlings are insensitive to the auxin analog 2,4-D, implicating BDL in auxin response (Hamann et al. 1999).

The *Arabidopsis* genome encodes 23 ARF transcription factors, of which three have been identified by mutant phenotypes: *ETT/ARF3* in flower development, *MP/ARF5* in embryogenesis, and *NPH4/ARF7* in hypocotyl phototropism (Sessions et al. 1997; Hardtke and Berleth 1998; Harper et al. 2000; Guilfoyle and Hagen 2001). In each case, the mutant phenotypes result from loss-of-function mutations. ARF transcription factors interact with IAA (indole-3-acetic acid) proteins (Kim et al. 1997; Ulmasov et al. 1999b; Ouellet et al. 2001; for review, see Guilfoyle and Hagen 2001; Reed 2001; Rogg and Bartel 2001). The *Arabidopsis* genome encodes ~28 IAA proteins (Reed 2001; Rogg and Bartel 2001). Most of the IAA proteins have recently been shown to repress auxin-responsive gene activation in transient expression assays (Tiwari et al. 2001). Mutations have been identified in eight *IAA* genes, all of which affect exclusively postembryonic development (Rouse et al. 1998; Tian and Reed 1999; Nagpal et al. 2000; Rogg et al. 2001; Fukaki et al. 2002; for review, see Reed 2001; Rogg and Bartel 2001). In each case, the mutant phenotype results from a single amino acid exchange in the conserved domain II that is involved in protein degradation via the ubiquitin-proteasome pathway (Worley et al. 2000; Gray et al. 2001; Oellet et al. 2001; Ramos et al. 2001; Tiwari et al. 2001; Zenser et al. 2001). These observations have led to the idea that IAA proteins form complexes with ARF transcription factors, from which the latter are released in response to auxin. It was hypothesized that specific pairs of ARF/IAA complexes may mediate specific developmental responses to auxin; however, no such pair has been identified to date.

Here, we show that BDL encodes IAA12, which is thus the first IAA protein involved in embryogenesis. Whereas the failure of *mp* mutant embryos to establish the hypophysis is caused by loss-of-function mutations of the ARF5 transcription factor, the similar *bdl* mutant phenotype is due to a gain-of-function mutation in the conserved degradation domain. In addition, BDL and MP proteins interact in yeast, and both genes are expressed

in the proembryo cells but not the presumptive hypophysis. Our results suggest that the hypophysis is established in response to an auxin-dependent signal from the adjacent proembryo.

Results and Discussion

The bodenlos *phenotype is due to a gain-of-function mutation in the auxin response regulator IAA12*

The *BDL* gene was mapped to a genomic interval of ~200 kb that includes the *IAA12* gene involved in auxin response (Fig. 1A). The *IAA12* gene was amplified by PCR from both the parental line, Landsberg *erecta*, and the *bdl* mutant line. Sequence analysis revealed a single G to A transition that results in a predicted amino acid ex-
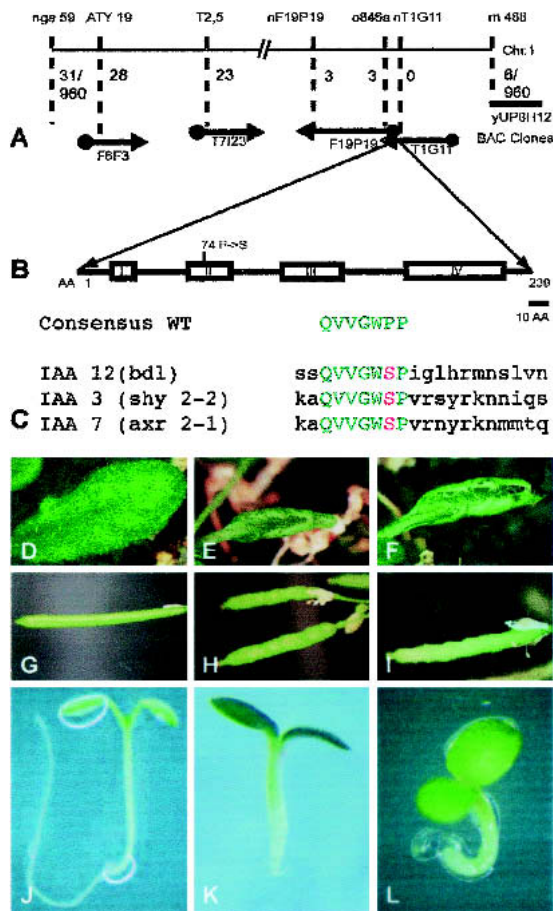

Figure 1. Molecular identification of the *BDL* gene. (*A*) Molecular mapping. Molecular markers and BAC clones are shown at the top and bottom, respectively. Numbers indicate recombinants between markers and the *BDL* gene on BAC clone T1G11 (960 gametes tested). (*B*) BDL protein domain structure showing the four conserved domains of IAA proteins with the Pro 74 → Ser amino acid exchange in bdl mutant protein. (*C*) Homologous amino acid exchanges in the conserved domain II of IAA12 (bdl), IAA3 (shy2-2) and IAA7 (axr2-1) mutant proteins. The consensus sequence of wild-type is shown for comparison. (*D–L*) Phenotypic analysis of transgenic progeny from wild-type plants transformed with the *bdl* mutant genomic fragment. (*D,G,J*) Wild-type (*BDL/BDL*) control; (*E,H,K*) *bdl* heterozygous (*E,H*) and homozygous (*K*) mutant controls; (*F,I,L*) transgenic *BDL/BDL* with one (*F,I*) or two copies (*L*) of the *bdl* transgene. (*D–F*) Leaves, (*G–I*) siliques, (*J–L*) seedlings.

change of Pro 74 to Ser in the highly conserved domain II (Fig. 1B). Identical mutations have been identified in dominant mutant alleles of *IAA3* (*shy2-2*; Tian and Reed 1999) and *IAA7* (*axr2-1*; Nagpal et al. 2000) (Fig. 1C). Comparable mutations affecting the same or the adjacent proline residue are known for *IAA14* (*slr-1*; Fukaki et al. 2002), *IAA17* (*axr3-1*; Rouse et al. 1998), *IAA19* (*msg2-1*; Nagpal et al. 2000) and *IAA28* (*iar-1*; Rogg et al. 2001). Recent analyses of half-life times for axr3 mutant protein in vitro and in vivo indicate that these amino acid replacements render the mutant proteins several-fold more stable than the wild-type protein (Worley et al. 2000; Ouellet et al. 2001; Ramos et al. 2001; Zenser et al. 2001). Furthermore, the IAA7 protein from the *axr2-1* mutant line, in contrast to the wild-type protein, does not interact with the SCFTIR1 complex involved in IAA protein degradation (Gray et al. 2001).

The *bdl* mutant has a semidominant phenotype during postembryonic development but a recessive embryonic phenotype (Hamann et al. 1999). To analyze the effect of the *bdl* mutant allele, we performed reciprocal plant transformation experiments, using homologous *BDL* 4-kb genomic fragments from wild-type and *bdl* mutant lines. The *BDL* wild-type transgene did not rescue the *bdl* mutant phenotypes (Table 1). In contrast, nine wild-type plants independently transformed with the *bdl* mutant transgene gave transgenic progeny that displayed the *bdl/BDL* heterozygous plant phenotypes (Fig. 1D–I). Three of these transgenic plants were analyzed further. Upon selfing, they produced about 22% *bdl* mutant seedlings, a proportion comparable to that of selfed *bdl/BDL* heterozygous plants (Fig. 1J–L; Table 1). Reciprocal crosses between *bdl/BDL* heterozygous plants and wild-type plants carrying the *bdl* mutant transgene also resulted in a similar proportion of *bdl* mutant seedling progeny (Table 1). These results suggest that the copy number of the *bdl* mutant allele is critical, both in the absence and in the presence of wild-type alleles.

BODENLOS/IAA12 *interacts with* MONOPTEROS/ARF5, *and the two genes are coexpressed in early embryogenesis*

To determine its subcellular localization, we transiently expressed BDL protein fused to green fluorescent protein (GFP) in parsley protoplasts (Kircher et al. 1999). The GFP-BDL protein strongly accumulated in the nucleus in a compartmentalized manner (Fig. 2A–F), whereas BDL proteins truncated at amino acids 66 or 72 were equally partitioned between nucleus and cytoplasm (data not shown). Domains III and IV of IAA proteins, which are also present at the C terminus of ARFs, mediate homomeric and heteromeric interactions with other IAAs and ARFs in the yeast two-hybrid system (Kim et al. 1997; Ulmasov et al. 1999b). In this assay, BDL interacted both with itself and with the C terminus of MP/ARF5 (Fig. 2G). Thus, BDL can interact directly with MP.

A prerequisite for in vivo interaction to occur is coexpression of the interacting partners. We compared the spatial and temporal accumulation patterns of *BDL* and *MP* mRNAs during embryogenesis (Fig. 3). At the octant stage, *BDL* and *MP* mRNAs accumulated in the proembryo cells derived from the apical daughter cell of the zygote (Fig. 3A,E). Subsequently, both mRNA species were gradually confined to subepidermal cells, eventually being restricted to the vascular precursor cells (Fig.

Table 1. *Analysis of progeny from transgenic plants bearing bdl alleles*

	Seedling progeny phenotypes[b]				
Parental genotypes[a]	No. analyzed	bdl (R)[c]	bdl (s)[d]	WT (R)[c]	WT (s)[d]
bdl/BDL; T(*BDL*)/– (selfing)	630	18%	5%	57%	20%
BDL/BDL; T(*bdl*)/– (selfing)	3,672	22%	0	53%	25%
bdl/BDL;–/– × *BDL/BDL*; T(*bdl*)– (reciprocal crosses)	295	29%	0	26%	45%
bdl/BDL[e] (selfing)	1,203	0	18%	0	82%

[a]Endogenous alleles at *BDL* locus and transgenic alleles (T): (*bdl*) mutant; (*BDL*) wild type.
[b]See Figure 1. Phenotypes: (bdl) mutant; (WT) wild type.
[c](R) Resistant to Basta due to transgene.
[d](s) Sensitive to Basta due to lack of transgene.
[e]Data from Hamann et al. (1999).

3B–D,F–H). At the basal end of early embryos, the hypophysis adjacent to the proembryo did not express either mRNA species (Fig. 3A,B,E,F, arrows). After the asymmetric division of the hypophysis, both *BDL* and *MP* mRNAs were observed in its smaller lens-shaped daughter cell that gives rise to the quiescent center of the root meristem (Fig. 3C,G, arrows). Thus, there was a coordinated basal shift in the expression domains of both genes at the globular stage. Late in embryogenesis, *MP*

Figure 2. Nuclear localization and interactions of BDL protein. (*A–F*) Transient expression of *35S::GFP-BDL* in parsley protoplasts. (*A–C*) GFP fluorescence, (*D–F*) corresponding bright-field illumination images of (*A,D*) GFP-BDL, (*B,E*) GFP with nuclear localization signal, and (*C,F*) GFP with nuclear export signal. Note strong accumulation of BDL-GFP in patches within the nucleus. (*G*) Yeast two-hybrid interaction assays. The strength of interaction is shown in activity units of the β-galactosidase reporter (β-Gal). BDL (IAA12) interacts with itself and with the C terminus of MP (ARF5) comprising domains III and IV (ARF5ct). Negative controls: BDL tested against the empty prey vector (IAA12/–), and GNOM tested against the C terminus of MP (GN/ARF5ct).

but not *BDL* was expressed in the central root cap, which was the only conspicuous difference between the two gene expression patterns (Fig. 3I,J, arrowheads). Since the *bdl* stabilizing mutation mimics the *mp* early-embryo phenotype, we examined the possibility that *bdl* might interfere with *MP* transcription. However, *MP* was expressed in the proembryo cells of *bdl* mutant early embryos (Fig. 3K,L), indicating that the *bdl* mutation does not abolish expression of the *MP* gene.
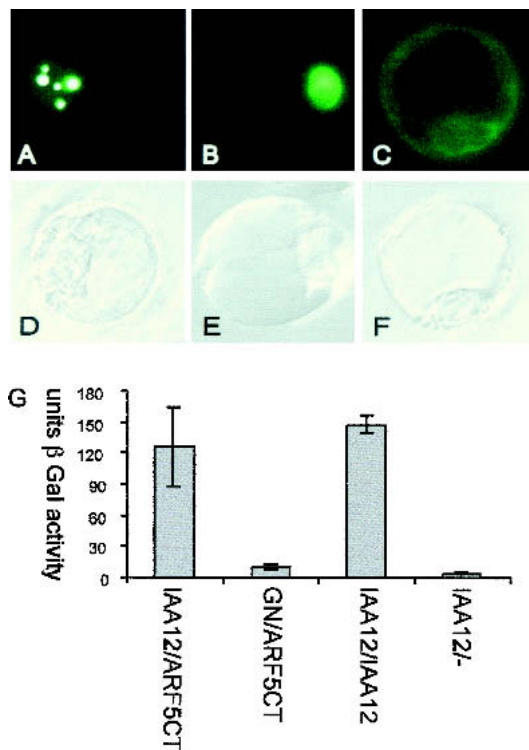
Promoter sequences of BODENLOS/IAA12 *and* SHY2/IAA3 *confer different expression patterns*

The *bdl* mutation causes an embryonic phenotype, whereas the identical mutation in the *IAA3* gene only leads to a postembryonic defect that was opposite to its loss-of-function phenotype (Tian and Reed 1999). This raised the possibility that transcriptional regulation of *IAA* genes may limit their in vivo interactions with ARF proteins. We therefore compared the expression patterns of *BDL* and *IAA3* promoter sequences that were transcriptionally fused to the β-glucuronidase (GUS) reporter (Reintanz et al. 2001). In contrast to *BDL::GUS* (Fig. 4A–D), *IAA3::GUS* expression was not detectable before the mid-torpedo stage of embryogenesis (Fig. 4E–H). Different expression patterns were also observed in seedlings (Fig. 4, cf. I and J; see also Tian et al. 2002 for *IAA3::GUS* expression). Thus, transcriptional regulation can account for the phenotypic difference between identical mutations in two different *IAA* genes by limiting the choices of potential interaction between IAA and ARF proteins.

A model for BDL/MP action in root meristem initiation

Our results suggest that BDL and MP are interacting IAA and ARF proteins that are required for root meristem initiation in early embryogenesis. They act genetically in the same pathway, can interact physically, and are expressed in the same cells. Most IAA proteins, including IAA12, were shown to repress auxin response in the carrot protoplast transient expression assay (Tiwari et al. 2001). Furthermore, mutations in domain II that stabilize IAA proteins resulted in the inhibition of auxin-responsive reporter gene expression (Worley et al. 2000; Ouellet et al. 2001; Ramos et al. 2001; for review, see Rogg and Bartel 2001). Conversely, ARF5 encoded by the *MP* gene acts as an activator of auxin-responsive genes in carrot protoplasts (Hardtke and Berleth 1998; Ulmasov et al. 1999a), suggesting that loss-of-function
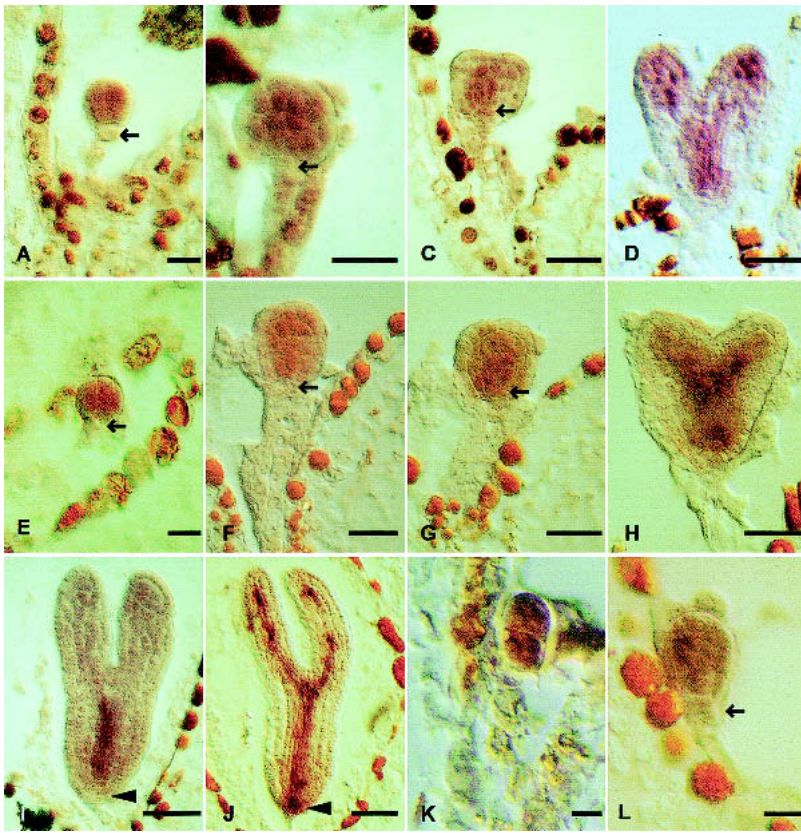
Figure 3. mRNA in situ expression analysis of *BDL* and *MP* in developing embryos. (*A–D,I*) *BDL*, (*E–H,J–L*) *MP* mRNA accumulation in wild-type (*E–J*) and *bdl* mutant (*K, L*) embryos. Developmental stages (Jürgens and Mayer 1994): (*A,E*) octant; (*B,F,L*) early-globular; (*C,G*) globular with lens-shaped cell; (*D,H*) heart; (*I,J*) torpedo; (*K*) four-cell. Arrows mark the hypophysis in (*A,B,E,F,L*) and the lens-shaped cell in (*C,G*), arrowheads mark the central root cap in (*I,J*). Bar, 20 μm.

model for their developmental action in early embryogenesis. BDL and MP may form a complex that prevents MP from activating target genes. BDL protein would be degraded in response to auxin, thus releasing MP.

Both *BDL* and *MP* are expressed in the proembryo cells but not in the adjacent hypophysis which normally initiates root meristem formation. This observation suggests that an auxin-dependent signal needs to be relayed from the proembryo to the adjacent extraembryonic cell, which in response switches to the hypophyseal cell fate (Hamann et al. 1999). It remains to be determined whether this apparent noncell autonomous effect of *MP* involves activation of auxin-response target genes that would then mediate signaling to the adjacent cell. Alternatively, MP protein may be transported into the presumptive hypophysis, which would be analogous to the movement of the SHORT ROOT transcription factor from the provascular cells to the adjacent ground tissue layer (Nakajima et al. 2001).

Materials and methods

Plant material and map-based cloning of the BDL *gene*

Plant growth conditions and the *bdl* mutant allele have been described (Hamann et al. 1999). From a cross between *bdl/BDL* (L*er*) and wild type (Nd), 480 *bdl* mutant F₂ seedlings were selected to map the gene against CAPS or SSLP markers (www.arabidopsis.org). After the interval was narrowed down to a small region at the top of chromosome I, wild-type and mutant alleles of the candidate gene were sequenced. Genomic fragments from 2.4 kb upstream of the translation start to 0.75 kb downstream of the *BDL* gene were amplified by PCR from wild-type and *bdl* mutant genomic DNA, using a Roche High Fidelity amplification kit (primer sequences, 5'-GGTTGGACTTGC TAAGCACTG-3' and 3'-CCATGTGGTAGTGTCGAGAAGG-5'). Fragments were subcloned in pGEM (Promega), sequenced, and transferred in pBAR vector by cutting with *Eco*52I, blunting and ligating via the *Sma*I site of the vector (Völker et al. 2001). Constructs were introduced into *A. tumefaciens* strain GV 3101 (Clough and Bent 1998). Plants were transformed using the floral-dip method, and transgenic seedlings were selected on plates (2.1 g MS salts per L [Duchefa], 1% bactoagar, 1% sucrose) or soil, using 4 mg/L phosphinotricin (Clough and Bent 1998).

mutations in the *MP* gene eliminate the capacity of ARF5 to activate auxin-responsive genes (Hardtke and Berleth 1998). Thus, failure to initiate the root meristem in early embryogenesis results from either of two genetic alterations: a stabilizing mutation of the auxin-response inhibitor BDL, or loss of the auxin-response activator MP. These observations suggest a simple

In situ hybridization

RNA in situ hybridization was performed as described, except that Western Blue (Promega) was used for staining (Mayer et al. 1998). The fragment for the *BDL* antisense probe was amplified by PCR, and restriction sites for *Hin*dIII/*Xho*I were introduced (primer sequences, 5'-TTCT CGAGGAATTTCGAGAGCCATTGAGTGG-3' and 3'-AACAAGCTTAGTCCACTAGCTTCT GAGGTTCC-5'). The fragment was inserted in pBssk⁻(MBI). Linearization of the antisense probe was done with *Bsp*120I and transcription with T3 RNA polymerase (hydrolysis time 37 sec; Promega transcription kit). Clones for *MP* antisense probes were transcribed as described (Hardtke and Berleth 1998).
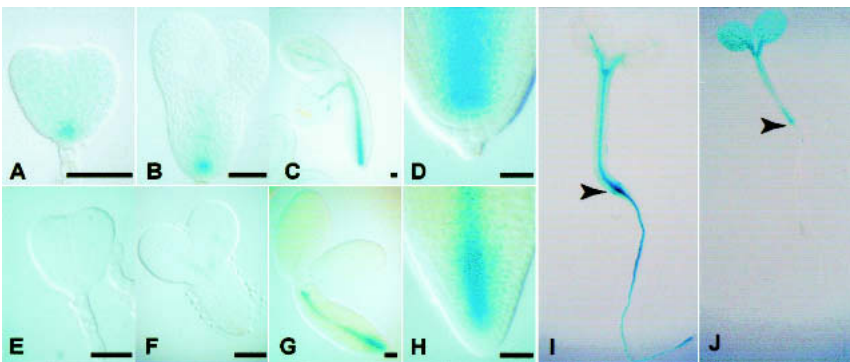

Figure 4. *IAA* gene promoter-GUS reporter expression in embryos and seedlings. (*A–D,I*) *pBDL::GUS*, (*E–H,J*) *pIAA3::GUS*. Developmental stages (Jürgens and Mayer 1994): (*A,E*) triangular; (*B,F*) early-torpedo; (*C,G*) bent-cotyledon. (*D,H*) Root end of bent-cotyledon embryos shown at higher magnification. (*I,J*) Four-day-old seedlings; arrowheads mark the boundary between hypocotyl and root. Bar for *A–H*, 20 μm.

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Nuclear localization studies

Full-length cDNA was amplified from the *BDL* cDNA by PCR, and *Bam*HI/*Xba*I restriction sites were introduced at the ends (5′-CGG GATCCATGCGTGGTGTGTCAGAATTG-3′ and 3′-GCTCTAGACTA AACAGGGTTGTTTCTTTGT-5′). The fragment was then inserted in frame in the transfection vector RT103 and sequenced. Nuclear (*NLS-GFP*) and cytosolic (*NES-GFP*) controls were provided by K. Harter (University of Freiburg, Germany). Parsley protoplasts were isolated and transformed as described (Kircher et al. 1999).

Yeast interaction assays

The cDNA encoding the C terminus of MP (amino acids 753–902) was amplified from a flower/young silique cDNA library (primer sequences, 5′-CCGCTCGAGTTATGAAACAGAAGTCTTAAG-3′ and 3′-CCGGA ATTCGATTTTGATGATTGTAGTCTG-5′), subcloned in pGEM (Promega) and sequenced before in-frame insertion in yeast two-hybrid vectors pJG4-5 or pEG202 via *Eco*RI/*Xho*I restriction sites (Grebe et al. 2000). *BDL* was amplified from a full-length *BDL* cDNA clone by PCR (primer sequences, 5′-CGGAATTCATGCGTGGTGTGTCAGAATTG-3′ and 3′-GCCTCGAGCTAAACAGGGTTGTTTCTTTGT-5′), and *Eco*RI/ *Xho*I sites were introduced. The fragment was subcloned in pGEM and sequenced before in-frame insertion in pJG4-5 or pEG202 via *Eco*RI/*Xho*I sites. The GNOM (amino acids 1–246) control construct has been described (Grebe et al. 2000). For yeast transformation, cultures, and assays, standard techniques were employed as described (Ausubel et al. 2000).

Analysis of GUS expression

Promoter GUS fusions were generated by replacing the 35S promotor of pVKH35sGUSpA with PCR-amplified 1.76 kb or 1.96 kb fragments upstream of the translational start sites of *IAA3* and *IAA12* genes (*IAA3* 5′-CGCGAGCTCTCGTTGGTTAAGTGCTAAAAAT-3′, 3′-CGC GGATCCTTCTTCAAGAATTGCAGGAGAA-5′; *IAA12* 5′-CGCGAG CTCTATGCCGTATAGTTCGTATCAA-3′, 3′-CGCGGATCCGTCAAT AACAAAACCCTAGACT-5′; Reintanz et al. 2001). A *Bam*HI site was introduced at one end while the other was cleaved with either *Pvu*II (*IAA 12*) or *Hinc*II (*IAA3*). The promoter fragment was cloned in the plant transformation vector pVKH35sGUSpA via *Bam*HI/*Ecl*136I (*IAA12*) or *Bam*HI/ *Hinc*II (*IAA3*). Agrobacterium and plant transformations were performed as described above. Transgenic embryos and 4-day-old seedlings were stained with variable concentrations of potassium ferricyanide/ferrocyanide (0.5, 1, 2 mM each) for up to 20 h (Malamy and Benfey 1997). Stained embryos were cleared in a chloralhydrate:water:glycerol solution (8:2:1), stained seedlings were cleared as described (Malamy and Benfey 1997).

Microscopic analysis, image processing, and computer work

Microscopic analysis was done on a Zeiss Axiophot or a Leica confocal laser scanning microscope with TCS-NT imaging software (Leica); pictures were taken with a Nikon Coolpix 990 camera. Primer design and sequence analysis were performed using Mac-Vector (Kodak Imaging Systems) and Vector NTI (Informax). Database searches were done using the BLAST algorithm (Altschul et al. 1990). Images were processed with Adobe Photoshop and Adobe Illustrator (Adobe Systems).

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