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Developmental specificity of auxin response by pairs of ARF and Aux/IAA transcriptional regulators

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The plant hormone auxin elicits many specific context-dependent developmental responses. Auxin promotes degradation of Aux/IAA proteins that prevent transcription factors of the auxin response factor (ARF) family from regulating auxin-responsive target genes. Aux/IAAs and ARFs are represented by large gene families in *Arabidopsis*. Here we show that stabilization of BDL/IAA12 or its sister protein IAA13 prevents MP/ARF5-dependent embryonic root formation whereas stabilized SHY2/IAA3 interferes with seedling growth. Although both bdl and shy2-2 proteins inhibited MP/ARF5-dependent reporter gene activation, shy2-2 was much less efficient than bdl to interfere with embryonic root initiation when expressed from the BDL promoter. Similarly, MP was much more efficient than ARF16 in this process. When expressed from the *SHY2* promoter, both shy2-2 and bdl inhibited cell elongation and auxin-induced gene expression in the seedling hypocotyl. By contrast, gravitropism and auxin-induced gene expression in the root, which were promoted by functionally redundant NPH4/ARF7 and ARF19 proteins, were inhibited by shy2-2, but not by bdl protein. Our results suggest that auxin signals are converted into specific responses by matching pairs of coexpressed ARF and Aux/IAA proteins.

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Introduction

The small signaling molecule auxin elicits a multitude of developmental and physiological responses, such as patterning in embryogenesis, apical dominance, cell elongation and gravitropism (Berleth and Sachs, 2001). The cellular response to auxin involves changes in gene regulation. Genes upregulated by auxin contain in their promoters auxin-response elements (AuxRE), which bind transcription factors of the auxin response factor (ARF) family (Ulmasov *et al*, 1997a, 1999). At low auxin concentrations, ARFs are thought to be inhibited by interacting proteins of the Aux/IAA family via domains III and IV that are conserved between the two protein families (Ulmasov *et al*, 1997b). *Aux/IAA* genes were originally identified as genes that are rapidly upregulated in response to auxin (Abel *et al*, 1994). High auxin concentrations promote degradation of Aux/IAA proteins, which would release interacting ARFs from inhibition (Tiwari *et al*, 2001, 2003). Degradation of Aux/IAA proteins involves their conserved domain II, which mediates interaction with the SCF^{TIR1} ubiquitin-ligase complex for targeting of Aux/IAAs to the proteasome (Gray *et al*, 2001). Amino-acid exchanges in conserved residues of domain II affect the interaction with the SCF^{TIR1} ubiquitin-ligase complex, stabilizing mutant Aux/IAA proteins (Ramos *et al*, 2001). Such stabilizing mutations have been reported for 10 *Aux/IAA* genes (Reed, 2001; Hellmann and Estelle, 2002; Tatematsu *et al*, 2004; Yang *et al*, 2004).

How is a generic signal such as auxin converted into specific context-dependent developmental responses? Auxin can increase the affinity between the SCF^{TIR1} ubiquitin-ligase complex and Aux/IAA proteins in a cell-free system without modifying the latter (Dharmasiri *et al*, 2003; Tian *et al*, 2003; Kepinski and Leyser, 2004). This observation suggests that the specificity of response to auxin is generated by interacting Aux/IAA and ARF proteins present in the auxin-responsive cell. The *Arabidopsis* genome encodes 22 ARF and 29 Aux/IAA proteins (Remington *et al*, 2004). Several ARFs have been assigned roles in specific developmental processes on the basis of their loss-of-function mutant phenotypes (Berleth and Jürgens, 1993; Przemeck *et al*, 1996; Sessions *et al*, 1997; Hardtke and Berleth, 1998; Harper *et al*, 2000; Nemhauser *et al*, 2000; Li *et al*, 2004; Tian *et al*, 2004). Although ARFs appear to have unique functions in some contexts, they display overlapping functions in others (Hardtke *et al*, 2004; Li *et al*, 2004). For example, MP/ARF5 is required for embryonic root initiation whereas both MP and NPH4/ARF7 contribute to cotyledon development (Hardtke *et al*, 2004). A larger number of Aux/IAA proteins have been implicated in diverse processes on the basis of their gain-of-function mutant phenotypes (Reed, 2001; Tatematsu *et al*, 2004; Yang *et al*, 2004). The mutant phenotypes are distinct for some Aux/IAA proteins but related for others, suggesting both distinct and overlapping roles in development. For example,

stabilized BDL/IAA12 protein interferes with embryonic root initiation as does the loss of MP/ARF5 protein, suggesting that these two proteins generate a specific developmental response (Hamann *et al*, 2002). In contrast to *ARF* genes, no loss-of-function phenotypes have been reported for *Aux/IAA* genes except *SHY2/IAA3*, which is involved in seedling development (Tian and Reed, 1999). Most of the *Aux/IAA* genes exist as sister genes that appear to have originated by segmental duplications of the genome whereas *ARF* genes are not located in duplicated segments (Remington *et al*, 2004). For example, one pair of sister genes consists of *BDL/IAA12*, which is involved in embryonic root initiation, and *IAA13* (Hamann *et al*, 1999, 2002). It is not known whether IAA13 performs a comparable role to BDL or rather acts in a different process. Furthermore, although mutations in different *ARF* and *Aux/IAA* genes cause distinct phenotypes, it is unclear how these proteins contribute to specificity of action.

Here we address how Aux/IAA and ARF proteins generate specific responses to auxin. The effects of stabilized BDL and SHY2 proteins on embryonic root formation and seedling development were analyzed by swapping their gene promoters. These proteins were also assayed for their ability to inhibit MP-dependent gene activation in the absence of plant-specific accessory factors. Finally, candidate ARF proteins for interaction with BDL or SHY2 were examined for roles in BDL- and SHY2-dependent processes. Our results suggest that transcriptionally regulated optimized pairs of interacting Aux/IAA–ARF proteins generate developmental specificity of auxin response.

Results

IAA13 is a functional paralog of BDL/IAA12

Many *Aux/IAA* genes, including *BDL/IAA12* and its closest homolog *IAA13* (At2g33310), appear in regions of segmental genome duplications (Remington *et al*, 2004). To examine whether IAA13 is functionally related to BDL/IAA12, we first introduced a proline to serine mutation (*iaa13*^{P80S}; Figure 1A) in the conserved domain II of a Myc-epitope-tagged transgenic copy of the *IAA13* gene. The homologous mutation in the *BDL* gene causes semidominant gain-of-function phenotypes, both in the *bdl* mutant and when provided as a transgene (Hamann *et al*, 2002). Plants carrying the *iaa13*^{P80S} transgene resembled *bdl* mutants in all respects. A single transgene copy caused stunted growth (not shown), whereas two copies caused embryonic phenotypes

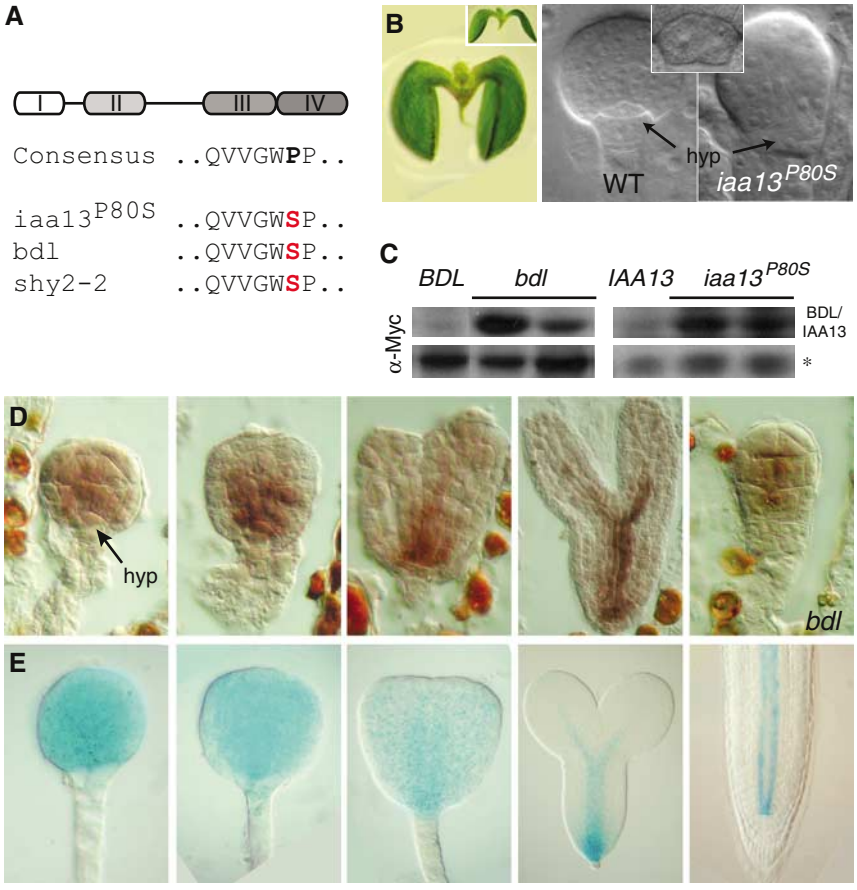

Figure 1 Expression of *IAA13*, and analysis of an *iaa13* stabilizing mutation. (**A**) Domain structure of Aux/IAA proteins. The four conserved domains are depicted. Below is the consensus amino-acid sequence in conserved domain II of the engineered iaa13^{P80S} mutation, of bdl and of shy2-2. (**B**) Rootless seedling homozygous for the *iaa13*^{P80S} mutation (left); inset: *bdl* seedling. Comparison of a wild-type (middle) and homozygous *iaa13*^{P80S} (right) globular stage embryo shows defects in hypophysis (hyp) specification; inset: abnormal division of hypophysis in *bdl* embryo. (**C**) Western blots (Myc antibody) of protein extracts from *pBDL*::*BDL*, *pBDL*::*bdl*, *pIAA13*::*IAA13* and *pIAA13*::*iaa13*^{P80S} seedlings. Individual lanes represent independent transgenics. Asterisk: unspecific crossreacting band demonstrating equal loading. (**D**) mRNA *in situ* hybridization with an *IAA13* antisense probe in wild-type and *bdl* (right) embryos. RNA signals are in red-brown. (**E**) GUS activity in *pIAA13*::*GUS* embryos and seedling root tip (right).

(Figure 1B). Homozygous *iaa13*^{P80S} seedlings had no root, and the origin of this defect could be traced to a failure in the specification of the hypophysis-, the embryonic root meristem precursor- and subsequent abnormal cell division patterns (Figure 1B). Western blot analysis showed that the engineered *iaa13*^{P80S} mutation led to the stabilization of the IAA13 protein (Figure 1C). This effect was quantitatively similar to the stabilization of the BDL protein in *bdl* mutants (Figure 1C), and caused comparable frequencies of embryo phenotypes (Table I).

The phenotypic equivalence of *bdl* and *iaa13*^{P80S} gain-of-function mutations suggests that the two genes are expressed in a similar way. mRNA *in situ* hybridization revealed that the *IAA13* gene is first transcribed specifically in the globular proembryo, but not the hypophysis (Figure 1D). Later, expression extends basally to the lens-shaped apical daughter cell of the hypophysis (Figure 1D). Finally, *IAA13* mRNA accumulation is restricted to the future vascular tissue (Figure 1D). The identical expression pattern was previously detected for *BDL* (Hamann *et al*, 2002). *IAA13* mRNA expression was unchanged in *bdl* mutants (Figure 1D), excluding the possibility that *IAA13* acts downstream of *BDL*. Furthermore, *IAA13* promoter-GUS fusions revealed that the *IAA13* expression pattern is regulated at the level of gene transcription (Figure 1E). In conclusion, the *IAA13* gene is a functional paralog of *BDL*.

Table I Frequencies of rootless phenotypes in genotypes used in this study

Genotype	Line #	Rootless seedlings (% (*N*))	Defective embryos (% (*N*))
Columbia WT		1.4 (141)	
pBDL::*SHY2*	2	1.1 (72)	
	6	0.7 (124)	0.5 (187)
pBDL::*shy2-2*	5	0 (430)	
	19	4.4 (280)	
	20	9.8 (283)	7.1 (70)
	22	8.0 (63)	9.7 (124)
	24	9.1 (212)	
	29	27.6 (134)	
	38	9.4 (112)	
pBDL::*BDL*	38	0 (148)	0 (81)
pBDL::*bdl*	27	19.4 (66)	21.1 (19)
	41	24.6 (61)	32.2 (59)
	73	60.8 (51)	
pBDL::*IAA13*	11	2.5 (115)	2.2 (138)
pBDL::*iaa13*^{P80S}	5	25.2 (283)	25.5 (108)
	28	29.6 (68)	
	38	12.2 (317)	28.3 (53)
pIAA13::*IAA13*	12	0.9 (94)	
	17	0.8 (129)	
pIAA13::*iaa13*^{P80S}	4	32.6 (177)	
	11	23.9 (102)	
	23	29.0 (62)	
pIAA13::*BDL*	5	0 (131)	
	8	1.3 (130)	
pIAA13::*bdl*	1	26.2 (166)	
	7	28.5 (35)	29.4 (68)
	30	23.1 (337)	22.8 (206)
pSHY2::*shy2-2*	11	0 (55)	
pSHY2::*bdl*	4	0 (48)	0.7 (150)
	12	0 (30)	
pSHY2::*iaa13*^{P80S}	7	2.5 (38)	1.9 (161)
	11	5.0 (20)	
bdl		22.3 (264)	

Aux/IAA specificity in embryogenesis is transcriptionally regulated

To assess the relative contributions of transcriptional regulation of *Aux/IAA* genes and Aux/IAA protein determinants in specificity of action in the embryo, a promoter-swap strategy was adopted. The promoters of *BDL* or *IAA13* were fused to Myc-epitope-tagged genomic coding regions of the *SHY2/IAA3*, *BDL* or *IAA13* genes. Homologous stabilizing proline to serine domain II mutations (SHY2^{P69S}—*shy2-2* (Tian and Reed, 1999); BDL^{P72S}—*bdl* (Hamann *et al*, 2002); IAA13^{P80S}) were introduced into each construct, and wild-type versions of the transgenes were analyzed as controls.

In accordance with the similar *BDL* and *IAA13* gene activities, *pBDL*::*iaa13*^{P80S} and *pIAA13*::*bdl* plants showed embryonic and postembryonic phenotypes comparable to the *bdl* and *iaa13*^{P80S} mutants (Figure 2A; Table I; not shown). Notably, however, whereas *shy2-2* mutants have no embryonic phenotypes (Tian and Reed, 1999; Table I), *pBDL*::*shy2-2* plants showed a rootless phenotype similar to that of *bdl* (Figure 2A). In addition, *pBDL*::*shy2-2* plants showed *bdl*-like postembryonic growth abnormalities (Figure 2B). Although phenotypes were qualitatively similar in all genotypes, the frequency of embryonic phenotypes was significantly lower in *pBDL*::*shy2-2* plants (Table I). However, Western blot analysis showed that shy2-2, bdl and iaa13^{P80S} proteins accumulated to comparable levels (Figure 2C). These results suggest that the specificity of BDL and IAA13 action in embryogenesis is mainly regulated at the level of gene transcription, but other Aux/IAA proteins may also affect root formation when expressed in the embryo.

The ARF transcription factor that likely acts in concert with BDL and IAA13 in the embryo is MONOPTEROS (MP)/ARF5 (Hardtke and Berleth, 1998; Hamann *et al*, 2002). To examine whether the ability to inhibit MP activity reflects the activity of bdl and shy2-2 in the embryo, we developed a heterologous assay for ARF and Aux/IAA activity. A direct repeat of eight ARF-binding *DR5(rev)* repeat sequences (Ulmasov *et al*, 1997b) was placed upstream of a minimal promoter for expression in yeast, and this *yDR5* (*y*east-*DR5*) promoter was fused to the *lacZ* gene for β-galactosidase. When HA-epitope-tagged *MP* cDNA was expressed in *yDR5*::*lacZ* yeast cells, the activity of the reporter was induced several-fold (Figure 3A). Next, HA-epitope-tagged cDNAs of *SHY2* or *BDL* were introduced on the same plasmid as *MP:HA*. Coexpression of SHY2:HA or BDL:HA with MP:HA nearly completely repressed MP:HA activity (Figure 3A), showing that both proteins can inhibit MP activity. Western blot analysis consistently showed that MP:HA, SHY2:HA and BDL:HA were expressed in yeast, indicating that SHY2 and BDL did not interfere with MP expression. However, SHY2:HA consistently accumulated to higher levels than

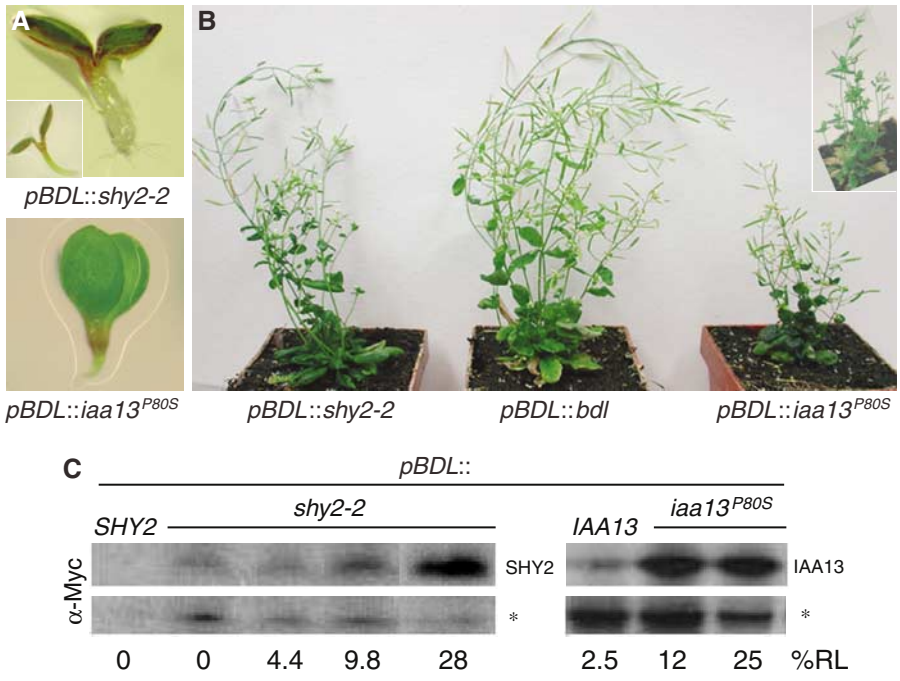

Figure 2 *BDL* promoter-swap experiments. (**A**) Rootless *pBDL*::*shy2-2* and *pBDL*::*iaa13*^{P80S} homozygous seedlings; inset: *pBDL*::*bdl* seedling. (**B**) Flowering plants (4 weeks old) are bushy and short; inset: heterozygous *bdl* plant. (**C**) Western blots of protein extracts from *pBDL*::*SHY2*, *pBDL*::*shy2-2*, *pBDL*::*IAA13* and *pBDL*::*iaa13*^{P80S} seedlings. Asterisk: unspecific crossreacting band demonstrating approximately equal loading. Percentage of rootless seedlings (%RL) is indicated for each line.

BDL:HA (Figure 3B), presumably because of cleavage of the BDL:HA protein (not shown). This assay shows that both SHY2 and BDL can directly inhibit MP transcription factor activity in the absence of plant-specific accessory factors, and BDL may be more potent than SHY2.

Aux/IAA specificity in hypocotyl and shoot is transcriptionally regulated

Elongation of the seedling hypocotyl involves SHY2-dependent auxin responses (Tian and Reed, 1999; Tian *et al*, 2002). In contrast, *bdl* mutants show normal hypocotyl elongation (Hamann *et al*, 1999). SHY2 is predominantly expressed in cotyledons and hypocotyl including peripheral cell layers whereas BDL expression is largely confined to the central vascular strands (Hamann *et al*, 2002; Tian *et al*, 2002). To assess if the specificity of SHY2 action in the hypocotyl is also subject to transcriptional regulation, we expressed Myc-epitope-tagged bdl or iaa13^{P80S} proteins from the *SHY2* promoter, and compared their phenotypes with *pSHY2*::*shy2-2* seedlings.

The *pSHY2*::*shy2-2* construct induced hypocotyl elongation defects in both light- and dark-grown seedlings (Figure 4A), and the severity of defects correlated well with the level of mutant protein accumulation (Figure 4B). By comparison, *pSHY2*::*bdl* and *pSHY2*::*iaa13*^{P80S} seedlings showed a slightly stronger inhibition of hypocotyl elongation (Figure 4A) although mutant proteins accumulated to lower levels than in *pSHY2*::*shy2-2* seedlings (Figure 4B). These results suggest that, as in embryonic root formation, bdl and iaa13 mutant proteins are more effective than the shy2-2 protein in inhibiting auxin responses. Subsequent shoot development in *pSHY2*::*shy2-2*, *pSHY2*::*bdl* and *pSHY2*::*iaa13*^{P80S} plants resembled that of *shy2-2* mutants (Figure 4C; Tian and Reed, 1999). However, the phenotypes were again quantitatively different between the genotypes. Plants from different *pSHY2*::*shy2-2* lines showed different phenotypic strengths, ranging from those seen in *shy2-2* heterozygotes to those of *shy2-2* homozygotes, whereas the phenotypes of the other two transgenic genotypes strongly resembled *shy2-2* homozygotes (Figure 4C).

Aux/IAA proteins act primarily at the level of auxin-dependent gene expression (Tiwari *et al*, 2001). To test whether this process is similarly affected in *pSHY2*::*shy2-2* and *pSHY2*::*bdl* seedlings, we analyzed auxin-dependent *pSHY2*::*GUS* activity. In wild-type hypocotyls, *pSHY2*::*GUS* was induced by auxin in the outer cell layers (Figure 4D). This auxin-induced expression was almost completely lost in the hypocotyl of both *pSHY2*::*shy2-2* and *pSHY2*::*bdl* seedlings (Figure 4D). Thus, SHY2 activity feeds back on *SHY2* gene expression, and this function can be taken over by BDL protein, indicating functional equivalence of the two Aux/IAA proteins in this specific auxin response.

At similar protein concentrations, bdl appeared to have a stronger effect than did shy2 on the hypocotyl and shoot phenotypes, just as it did for embryo phenotypes. Taken together, these results suggest that specificity of SHY2 action in hypocotyl and shoot is determined by the activity of its promoter, with protein determinants affecting only the extent to which auxin responses are inhibited.

Aux/IAA protein specificity in auxin-mediated root development

The *shy2-2* mutation not only affects auxin responses in hypocotyl and shoot, but also in the root (Tian and Reed,

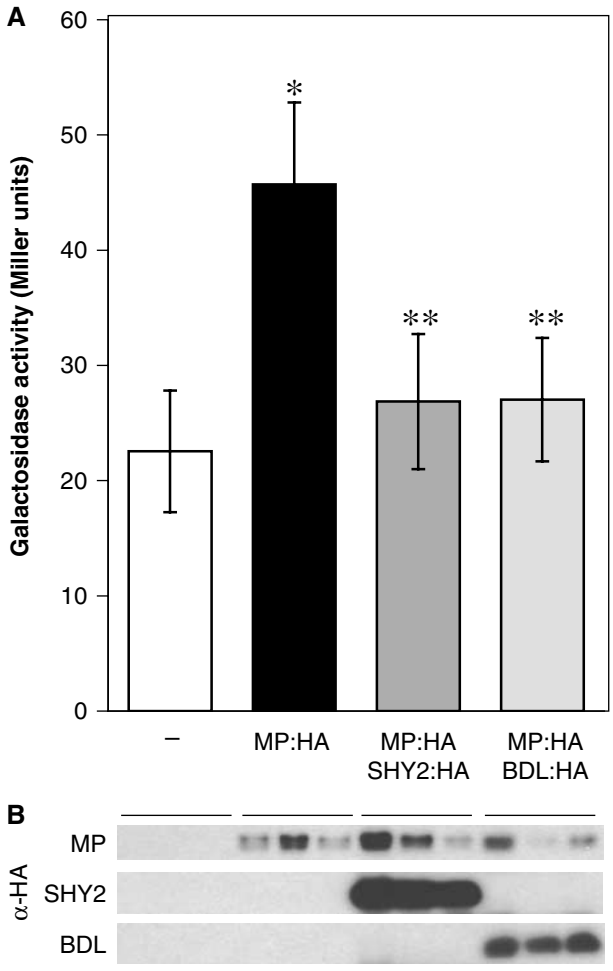

Figure 3 Repression of MP activity by SHY2 and BDL in yeast. (**A**) Galactosidase activity in *yDR5* yeast cells expressing the empty vector (−), MP:HA (MP), MP:HA and SHY2:HA (MP + SHY2) or MP:HA and BDL:HA (MP + BDL). Values (±s.d.) are the average of 12 independent transformants. Asterisks represent statistically significant difference between MP and – (*$P<0.001$, two-tailed Student's *t*-test), MP + SHY2 and MP (**$P<0.001$, two-tailed Student's *t*-test), and MP + BDL and MP (**$P<0.001$, two-tailed Student's *t*-test). (**B**) Western blot (HA antibody) with equal amounts of protein extracts from three independent yeast transformants for each plasmid. The regions from the same blot that represent MP:HA (105 kDa), SHY2:HA (25 kDa) and BDL:HA (29 kDa) are depicted. Note that expression levels of MP:HA protein vary between different colonies of the same genotype.

1999). To examine whether the specificity of SHY2 action in the root is also transcriptionally regulated, we studied root-specific auxin responses in *pSHY2*::*shy2-2* and *pSHY2*::*bdl* seedlings. Alignment of the root tip with a changing gravity vector requires auxin response, and this response is strongly diminished in *shy2-2* as well as in *pSHY2*::*shy2-2* roots (Tian and Reed, 1999; Figure 5A). Surprisingly, roots of *pSHY2*::*bdl* seedlings responded almost normally to gravity although their hypocotyls displayed strong inhibition of elongation (Figure 5A, compare with Figure 4A). Similarly, although root growth was comparably reduced in both *pSHY2*::*shy2-2* and *pSHY2*::*bdl* seedlings under normal conditions, the two genotypes differed in their response to the growth-inhibiting effects of auxin (Figure 5B). Whereas *pSHY2*::*bdl* root growth was sensitive, *shy2-2* and *pSHY2*::*shy2-2* root growth was partially resistant (Figure 5B). This difference in auxin response between *pSHY2*::*shy2-2* and *pSHY2*::*bdl* roots must lie in the shy2-2 and bdl proteins themselves because both are expressed in the root (Figure 5C).

As in the hypocotyl, auxin responses in the root involve changes in gene expression. The *shy2-2* mutation and *pSHY2*::*shy2-2* prevented the auxin-induced expression of *pSHY2*::*GUS* and *pDR5(7x)*::*GUS* (Figure 5D; Tian *et al*, 2002). In contrast, *pSHY2*::*bdl* roots showed normal auxin-induced expression of both reporters (Figure 5D). The non-induced expression in the root vascular tissues and the distal tip was similarly affected in both *pSHY2*::*shy2-2* and *pSHY2*::*bdl* (Figure 5D). In summary, bdl did not regulate auxin-induced gene expression or gravitropism in roots, even when present at similar protein levels to levels of shy2-2 that have a strong effect on these phenotypes. These results indicate that shy2-2 is effective in inhibiting auxin-mediated root development whereas bdl is not, which is in contrast to the stronger activity of bdl in embryo development, auxin response in the hypocotyl, and shoot and root growth.

ARF7 and ARF19 as targets of Aux/IAA inhibition in auxin-mediated root development

Aux/IAA proteins inhibit auxin responses through interactions with ARF transcription factors (Tiwari *et al*, 2003). Hence, a plausible explanation for the differential effects of *pSHY2*::*shy2-2* and *pSHY2*::*bdl* on auxin-dependent root development would be that shy2-2, but not bdl, interacts with a yet unidentified ARF that regulates these auxin responses. To date, no *arf* mutant has been reported to have root phenotypes that resemble *shy2-2*. We took a candidate approach to identify ARF protein(s) involved in auxin-dependent root development. Initially, we analyzed double mutants for *ARF10* and *ARF16*, two closely related genes (Remington *et al*, 2004) that are highly expressed in elongating root epidermis cells (Birnbaum *et al*, 2003). These double mutants showed normal gravitropism and auxin response in the primary root (not shown). Thus, ARF10 and ARF16 are unlikely to be targets of shy2-2 inhibition.

The *NPH4/ARF7* gene is required for shoot tropisms, but the *nph4* mutant has no root gravitropism defect (Liscum and Briggs, 1996; Watahiki and Yamamoto, 1997; Tatematsu *et al*, 2004; Figure 6A). However, the *ARF19* gene is highly related to *NPH4* (Remington *et al*, 2004), and it has recently been shown that ARF19 acts redundantly with NPH4 in plant growth, including gravi- and phototropism in seedlings (Okushima *et al*, 2005). To test whether ARF19 regulates the same responses that are disturbed in *shy2-2* mutants, two mutant alleles of *ARF19* were tested. One of these, *arf19-4*, has a weak but significant phenotype in auxin-mediated root development. Gravitropic response as well as growth sensitivity to 2,4-D is impaired (Figure 6A and B). An *nph4-1 arf19-4* double mutant, however, was severely impaired in gravitropism (Figure 6A) and also showed nearly complete auxin-resistant root growth (Figure 6B). Correspondingly, auxin-induced *SHY2* gene expression was partially impaired in each single mutant, and nearly completely lost in the *nph4-1 arf19-4* double mutant (Figure 6C). These results suggest that NPH4 and ARF19 act redundantly in auxin responses in the primary root tip, and are therefore good candidates for targets of inhibition by shy2-2.

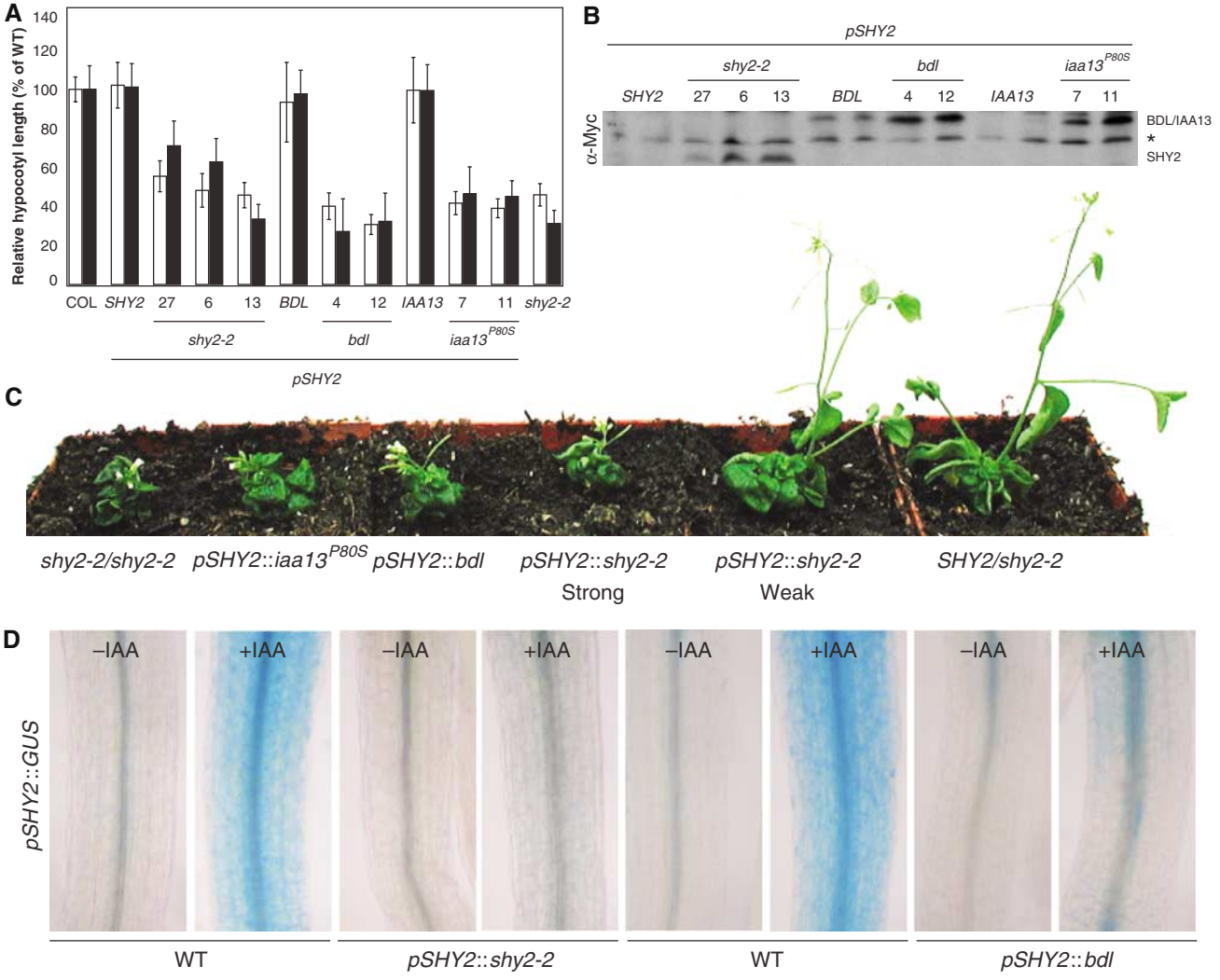

Figure 4 Inhibition of auxin responses in the shoot by stabilized Aux/IAA proteins. (**A**) Hypocotyl length in light-grown (white bars) or dark-grown (black bars) seedlings of wild type (COL), *pSHY2*::*SHY2*, *pSHY2*::*shy2-2* (lines #27, #6 and #13), *pSHY2*::*BDL*, *pSHY2*::*bdl* (lines #4 and #12), *pSHY2*::*IAA13*, *pSHY2*::*iaa13*^{P80S} (lines #7 and #11) and *shy2-2*. Hypocotyl length (±s.d.) is represented as percentage of COL. (**B**) Western blot of protein extracts from light-grown seedlings in (A). Blot was probed with anti-Myc antibodies; asterisk: unspecific crossreacting band demonstrating equal loading. (**C**) Phenotypes of flowering plants. (**D**) GUS activity in hypocotyl of seedlings hemizygous for *pSHY2*::*GUS* and *pSHY2*::*shy2-2*, *pSHY2*::*bdl* or wild-type controls from same cross. Seedlings were treated with IAA for 5 h and stained for GUS activity.

Yeast two-hybrid assays were performed to test whether ARF7 and ARF19 can be targets for SHY2 action in the root, and whether these could be the effectors that discriminate between shy2-2 and bdl. Because ARF19 seems to contribute most strongly to the physiological responses that are inhibited by shy2-2, we tested the interaction between full-length SHY2 or BDL proteins and C-terminal regions corresponding to domains III and IV of ARF19. As a control, interactions between SHY2 or BDL and domains III and IV of MP were tested.

Both SHY2 and BDL interacted with MP as well as with ARF19 (Figure 6D). Unfortunately, although preferential interactions between MP and BDL, on the one hand, and SHY2 and ARF19, on the other, were seen in several independent experiments, the resolution of yeast two-hybrid assays was too coarse to substantiate differential Aux/IAA–ARF affinities. Nonetheless, the ability of SHY2 to interact with ARF19, combined with the fact that mutant phenotypes of *shy2-2*, *arf19-4* and *nph4-1 arf19-4* roots are very similar, suggests that NPH4 and ARF19 are targets for SHY2 activity. Whether differential Aux/IAA–ARF interactions underlie specificity of SHY2 in the root tip remains unresolved.

Specific ARF activity in embryogenesis involves protein determinants

To address the relative contributions of transcriptional control and protein determinants in specificity of embryonic ARF action, we performed similar promoter-swap experiments to those described above for Aux/IAAs. We fused the cDNAs of two distantly related ARFs, *MP/ARF5* and *ARF16*, to the *BDL* *cis*-regulatory sequences, and assessed to what extent these transgenes can complement the rootless phenotype of *mp* mutant seedlings. The *mp* mutation prevents the formation of the embryonic root in approximately 25% of the progeny of heterozygous plants (Berleth and Jürgens, 1993). When *pBDL*::*MP* was transformed into *mp* heterozygotes, 11 out of 20 plants that carried the *mp* mutation had a strongly reduced frequency of rootless seedlings (Figure 7A), indicating

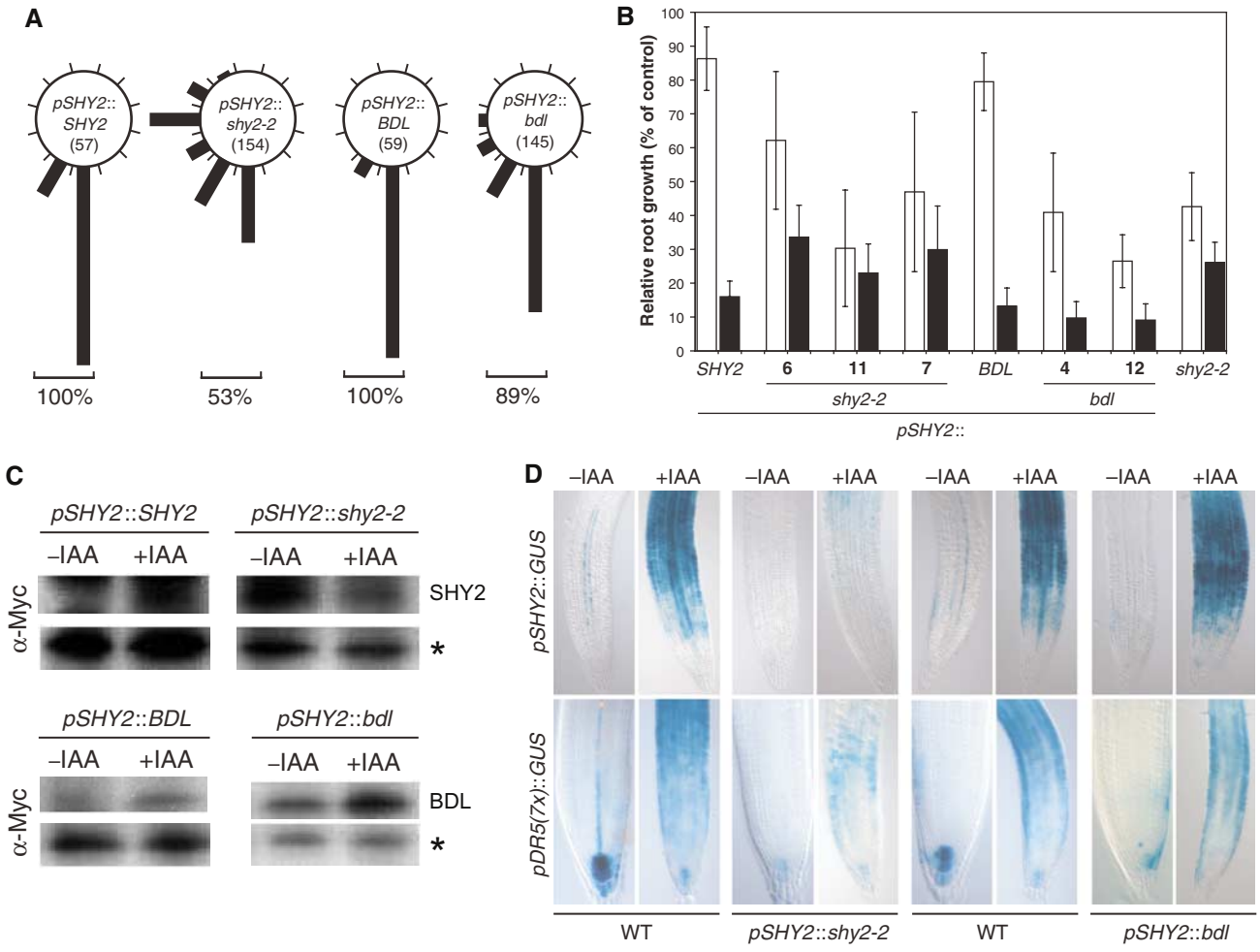

Figure 5 Inhibition of auxin responses in the root by shy2-2, but not by bdl. (**A**) Gravitropic response of *pSHY2*::*SHY2*, *pSHY2*::*shy2-2*, *pSHY2*::*BDL* and *pSHY2*::*bdl* seedlings after reorientation by 90° (data from two to five independent transgenic lines for each genotype). The percentages represent the fraction of seedlings (numbers analyzed in parentheses) with normal gravitropic response. (**B**) Inhibition of root growth by 2,4-D. Root length (±s.d.) was measured after 3 days of vertical growth on medium with (black bars) or without (white bars) 0.1 μM 2,4-D. Growth is represented as percentage of root length in wild type (COL) on control media. (**C**) Western blots of protein extracts from IAA-treated (10 μM for 5 h) or untreated roots of *pSHY2*::*SHY2*, *pSHY2*::*shy2-2*, *pSHY2*::*BDL* and *pSHY2*::*bdl* seedlings. Asterisks: unspecific crossreaction as loading control. Note that SHY2 or BDL accumulation is induced by auxin in *pSHY2*::*SHY2*, *pSHY2*::*BDL* and *pSHY2*::*bdl*, but not in *pSHY2*::*shy2-2*. (**D**) GUS activity in root tips of F1 seedlings from crosses between hemizygous *pSHY2*::*shy2-2* or *pSHY2*::*bdl* lines and homozygous *pSHY2*::*GUS* or *pDR5(7x)*::*GUS* lines. Controls (WT) are wild-type segregants from the same cross. −IAA, untreated; +IAA, 10 μM IAA for 5 h. GUS staining time was 3 h for untreated and 1.5 h for IAA-treated roots.

complementation of the embryonic phenotype. In contrast, the *pBDL*::*ARF16* transgene reduced the frequency of rootless seedlings in only two out of 11 lines (Figure 7B). Thus, ARF16 protein can potentially complement for the absence of MP. However, MP is much more effective than ARF16, suggesting that features specific to MP protein are required for effectiveness, although not strictly for specificity of action in embryogenesis.

Discussion

Auxin response is mediated by ARF transcription factors and their Aux/IAA inhibitors. Here, we examined Aux/IAA protein functions in their physiological contexts, by using promoter-swapping experiments with stabilized variants. As controls we used wild-type versions of the same proteins. These accumulated to barely detectable levels and did not cause physiological changes, unlike the stabilized forms, which is in line with previous studies on ARX3/IAA17 (Worley *et al*, 2000). For this reason, we focused our study on qualitative and quantitative differences between stabilized proteins expressed in the same developmental context. In different transgenic lines expressing either bdl or shy2-2 protein, the amount of detectable Aux/IAA protein correlated quantitatively with the strength of the resulting phenotypes. However, bdl had a stronger effect on embryo and hypocotyl phenotypes, whereas shy2-2 had a stronger effect on root phenotypes. Taken together, the results indicate that the specificity of auxin response is generated by both transcriptional regulation and protein function.

Regulation of auxin response in embryogenesis by the MP and BDL or IAA13 pairs

Embryonic root initiation is promoted by MP/ARF5 and inhibited by stabilized BDL/IAA12 (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; Hamann *et al*, 1999, 2002;

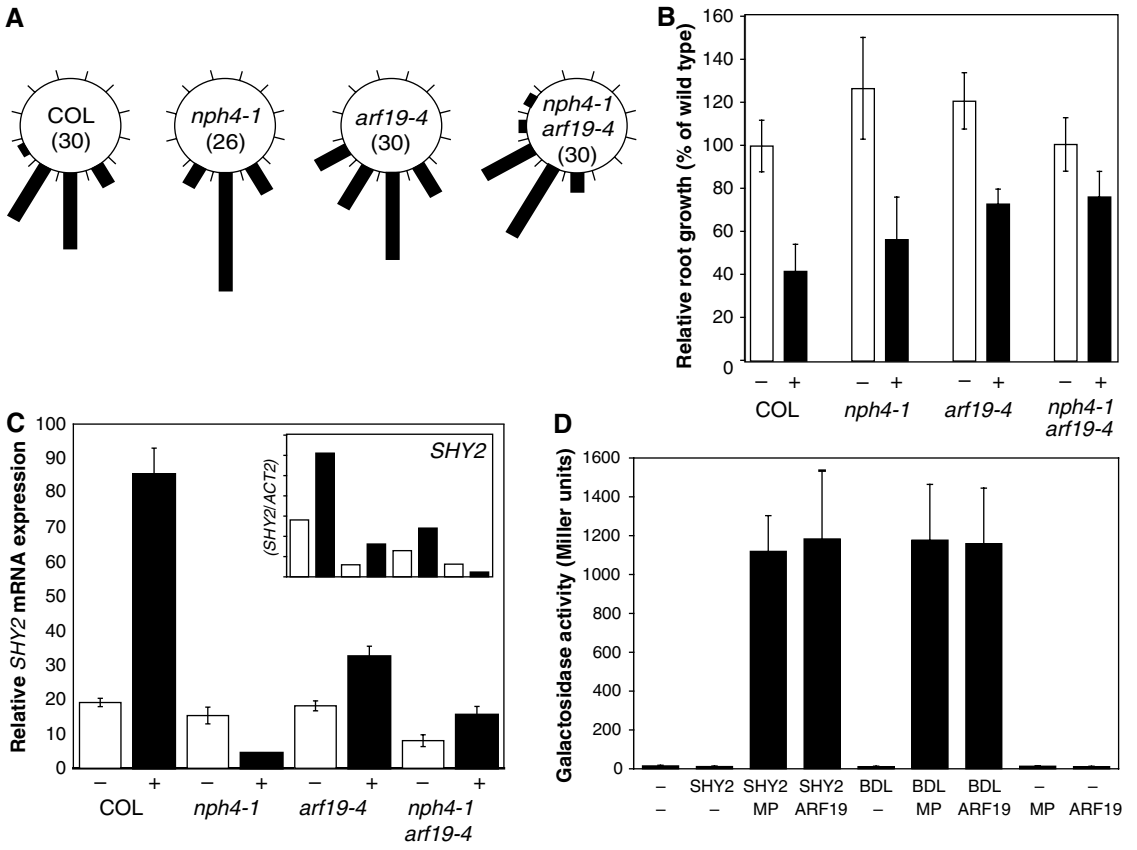

Figure 6 NPH4 and ARF19 as targets for shy2 inhibition in the root. (**A**) Gravitropic response of wild-type (COL), *nph4-1*, *arf19-4* and *nph4-1 arf19-4* seedling roots (numbers analyzed in parentheses) upon reorientation by 90°. (**B**) Inhibition of root growth by 2,4-D. Root length (±s.d.) was measured after 3 days of vertical growth on medium with (black bars) or without (white bars) 0.1 μM 2,4-D. Growth is represented as percentage of root length in wild type (COL) on control media. (**C**) *SHY2* mRNA expression in COL, *nph4-1*, *arf19-4* and *nph4-1 arf19-4* seedlings treated with unsupplemented medium (white bars) or with medium containing 50 μM IAA (black bars) for 3 h. Average values (±s.d.) are taken from five to six replicate real-time PCR reactions (quantitative RT–PCR); inset: semiquantitative RT–PCR experiment on dissected roots from three replicates. Expression is relative to *ACT2* expression in the same cDNA samples. (**D**) Interaction of SHY2 or BDL with MP and ARF19 in yeast two-hybrid assays. Galactosidase activity (±s.d.) was measured in at least 12 independent colonies expressing each of the depicted plasmids (−, empty vector control).

this study). *MP* and *BDL* mRNAs accumulate in the same cells of the young embryo, and the two proteins interact in the yeast two-hybrid assay (Hamann *et al*, 2002). In addition, MP-mediated activation of *DR5*::*LacZ* expression in yeast is inhibited by BDL in the absence of accessory plant-specific factors, as shown here. Overexpression of MP rescues *bdl* mutant plants and conversely, *bdl* suppresses the floral defects caused by overexpression of MP (Hardtke *et al*, 2004). These results suggest that BDL counteracts MP in auxin response. Here we identified *IAA13* as the functionally equivalent sister gene of *BDL*. *IAA13* displayed the same expression pattern as *BDL*, and a genomic fragment of *IAA13* carrying a *bdl* homologous mutation gave rootless seedlings at comparable frequencies. It is thus likely that both BDL and IAA13 need to be degraded in early embryogenesis for MP to promote root initiation (Figure 8).

To examine whether transcriptional regulation determines the specificity of auxin response in embryogenesis, we replaced BDL and MP by their distant relatives, SHY2 and ARF16, respectively. When expressed from the *BDL* promoter, the stabilized shy2-2 protein also caused rootless seedlings. However, syh2-2 was much less efficient than bdl. Similarly, ARF16 was much less effective than MP to rescue *mp* mutant embryos when expressed from the *BDL* promoter. Thus, specificity of auxin response in embryogenesis not only requires transcriptional regulation but also features of both ARF and Aux/IAA proteins, suggesting that MP and BDL or IAA13 are optimized ARF–Aux/IAA pairs.

Regulation of auxin response in the root by the SHY2 and NPH4 or ARF19 pairs

SHY2 plays a role in auxin response in the seedling root (Tian and Reed, 1999). However, no corresponding ARF has been identified. Here, we showed that SHY2 acts through inhibiting the redundant action of the previously known NPH4/ARF7 and the newly identified ARF19. Like *shy2-2*, double mutants lacking NPH4/ARF7 and ARF19 were defective in auxin-dependent root growth and root gravitropism, and also failed to activate the auxin-inducible *SHY2* gene. To address whether, like BDL and MP in the embryo, SHY2 forms pairs with NPH4 and ARF19 for auxin responses in the root, we expressed stabilized bdl protein from the SHY2 promoter. In contrast to shy2-2, bdl did not interfere with root gravitropism, auxin responses and auxin-induced gene expression (Figure 8). Thus, BDL cannot replace SHY2 in the root at comparable expression levels. Hence, the contribution of

protein determinants to auxin response specificity in the root is even more pronounced than in the embryo. This is also supported by the finding that heat shock promoter-driven expression of two stabilized Aux/IAA proteins, shy2-2 and axr3-1, in the root tip caused different responses of the same cell (Knox *et al*, 2003). Our findings imply that SHY2 forms optimized pairs with NPH4/ARF7 and ARF19 in the root. Furthermore, different optimized ARF–Aux/IAA pairs appear to regulate different developmental auxin responses.

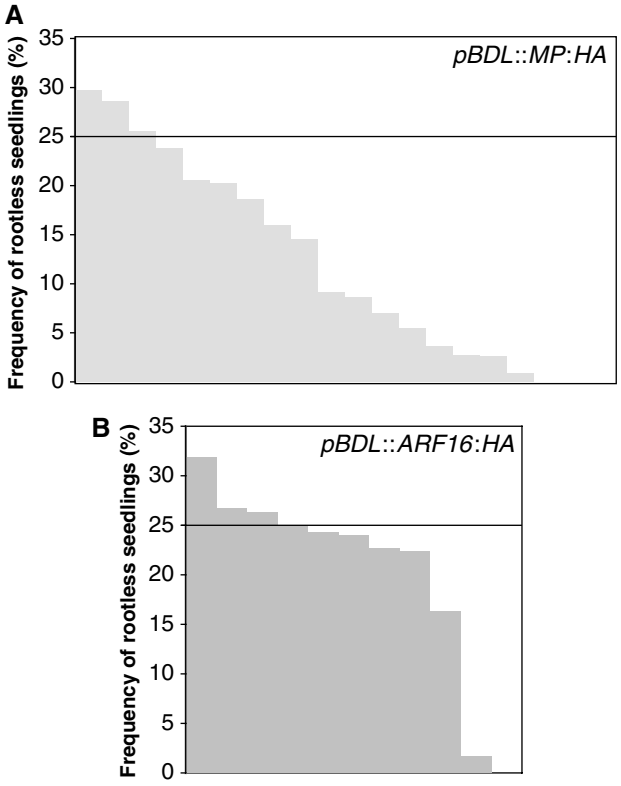

Figure 7 Specificity of MP action during embryogenesis. Percentage of rootless seedlings in (**A**) 20 independent *pBDL*::*MP* transgenic lines and (**B**) 11 independent *pBDL*::*ARF16* transgenic lines, all carrying the *mp* mutation. The line at 25% marks the frequency of rootless seedlings in the untransformed *mp* mutant.

Possible mechanisms of specificity

Auxin directly stimulates the interaction between Aux/IAA proteins and SCF^{TIR1} E3 ubiquitin-ligase complexes, resulting in the degradation of Aux/IAA proteins (Dharmasiri *et al*, 2003; Tian *et al*, 2002; Kepinski and Leyser, 2004). In this way, ARF transcription factors are released from inhibition and can regulate the expression of auxin-responsive target genes (Weijers and Jürgens, 2004). Thus, ARFs and interacting Aux/IAA inhibitors are prime candidates for converting the generic signal into a specific response. If any ARF could interact with any Aux/IAA protein, there would be more than 600 possible pairwise combinations. One way to reduce this complexity is to regulate *ARFs* and *Aux/IAA* gene transcription. Our results suggest that transcriptional regulation of *ARF* and *Aux/IAA* genes indeed contributes to specifying developmental responses to auxin. However, when expressed in the same developmental context, different members of the ARF and Aux/IAA protein families are functionally diverse and thus protein determinants also contribute to the specificity of response to auxin.

Auxin-dependent regulation of a target gene is the outcome of the activity of a DNA-bound ARF transcription factor, the activity of the associated Aux/IAA inhibitor and the affinity between the two. Transcriptional regulation by ARFs has been studied in cell cultures, using artificial reporter genes, but nothing is known about their natural targets in plant development. Their DNA-binding specificities for these target genes may differ, but this has not been addressed experimentally. The cell-culture studies suggest that ARFs differ in their activation potential, which has been related to sequences in the middle region (Tiwari *et al*, 2003). Similar to ARFs, Aux/IAA proteins could have differential effects. For example,

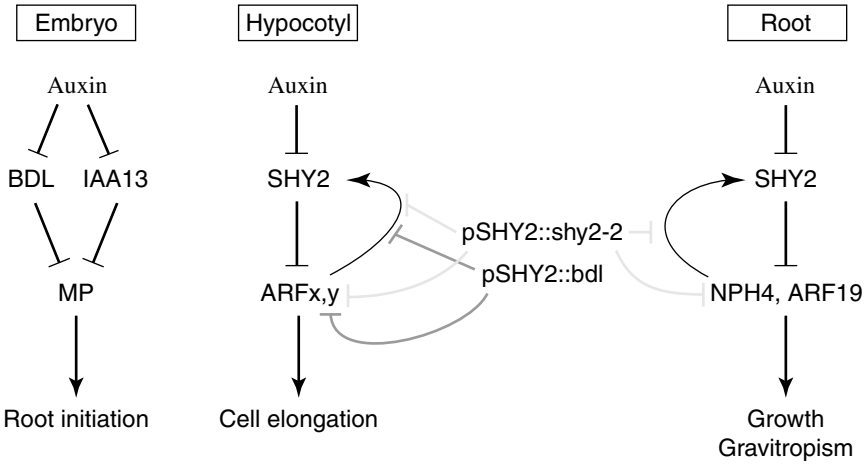

Figure 8 Model for Aux/IAA- and ARF-mediated auxin responses. In all tissues, auxin promotes the degradation of Aux/IAA proteins. In the embryo, BDL and IAA13 likely inhibit MP. When released from inhibition, MP promotes root initiation. In hypocotyl, SHY2 inhibits yet unknown ARF(s) (ARFx,y). When released from inhibition, ARFx,y promote cell elongation and enhance the transcription of *SHY2*. In root tips, SHY2 inhibits NPH4 and ARF19, which, when released, promote root growth and gravitropic response. NPH4 and ARF19 activity also stimulates *SHY2* transcription. When expressed from the *SHY2* promoter, shy2-2 mutant protein (*pSHY2*::*shy2-2*) constitutively inhibits ARFx,y activity in the hypocotyl and NPH4 and ARF19 activity in the root, including feedback regulation of *SHY2* transcription. Mutant bdl protein, expressed from the *SHY2* promoter (*pSHY2*::*bdl*), inhibits ARFx,y activity in the hypocotyl, but not NPH4 and ARF19 activity in the root.

their domain I has an LxLxL motif that, when fused to a DNA-binding domain, confers transcriptional repression of a reporter gene, and this motif is conserved between BDL and IAA13 but different in SHY2 (Tiwari *et al*, 2004).

Regarding interactions of ARFs with Aux/IAAs, all currently available data are from yeast studies, in particular two-hybrid studies. ARFs can activate auxin-responsive genes in yeast and their activity can be counteracted by Aux/IAAs, as shown here. Yet, none of these two-hybrid studies revealed differential interactions (Ouellet *et al*, 2001; Hamann *et al*, 2002; Hardtke *et al*, 2004; Tatematsu *et al*, 2004; this study). Although this might be taken to indicate that there are no differential interactions between ARFs and Aux/IAA proteins in the plant, there are caveats to yeast two-hybrid experiments that make this possibility unlikely. For example, the high levels of protein expression used in the yeast assays may allow for indiscriminate interactions between any protein of one family and any protein of the other family. Furthermore, yeast may lack factors that increase or prevent particular ARF–Aux/IAA interactions in the plant. Thus, direct ARF–Aux/IAA interaction needs to be analyzed *in planta*, which cannot be carried out at present. Considering the genetic evidence for optimized ARF–Aux/IAA pairs requiring both a specific ARF and a specific Aux/IAA, differential interactions are plausible.

In summary, we propose that the specificity of response to the generic signal auxin in different developmental and physiological contexts is generated at the level of interacting ARF–Aux/IAA proteins by two layers of control. First, transcriptional regulation of *ARF* and *Aux/IAA* genes limits the options of the responsive cell. Second, optimized pairs of interacting ARF and Aux/IAA proteins increase the specificity of response. The same ARF may be inhibited by one or more Aux/IAA proteins, depending on functional redundancy of duplicated *Aux/IAA* genes. It remains to be determined whether a single cell is capable of responding to auxin in several specific ways by expressing different optimized pairs of interacting ARF and Aux/IAA proteins.

Materials and methods

Plant material

bdl, *shy2-2* and *nph4-1* mutants have been described (Hamann *et al*, 1999; Tian and Reed, 1999; Harper *et al*, 2000). T-DNA insertion lines *arf10-1* (SALK_143232), *arf10-2* (SALK_087247), *arf16-1* (SALK_021448), *arf16-2* (SALK_021432), *arf19-3* (SALK_021481) and *arf19-4* (SALK_009879) were obtained from the Arabidopsis Biological Resource Center (Alonso *et al*, 2003). Double homozygotes for both alleles of *arf10* and *arf16* were identified in the F2 generation. Construction of *nph4-1 arf19-3* and *nph4-1 arf19-4* double mutants will be described elsewhere (JC Wilmoth and JW Reed, unpublished results).

Cloning of promoter::GUS fusions and promoter swapping

pSHY2::GUS was described previously (Hamann *et al*, 2002). Similarly, the *IAA13* promoter was amplified by PCR as a 2.11 kb fragment upstream of the translational start (sense primer 5′CGCGAGCTCCTCCATCATTTATCTTCAACCA3′, antisense primer 5′CGCGGATCCCAGAGAGACCACAACAACAACA3′) and cloned in the plant transformation vector pVKH35sGUSpA (Hamann *et al*, 2002) using S*ac*I/B*am*HI to result in pVKHIAA13GUSpA. The constructs/vectors pVKHSHY2GUSpA, pVKHBDLGUSpA (Hamann *et al*, 2002; contain 1.76 kb and 1.96 kb of sequence upstream of the ATG of *SHY2* and *BDL*, respectively) or pVKHIAA13GUSpA were used to generate promoter fusions with genomic fragments spanning the complete coding sequence for *SHY2*, *BDL* or *IAA13* genes, respectively, with 1.4 kb (*SHY2*), 1.95 kb (*BDL*) or 1.99 kb (*IAA13*) fragments downstream of ATG obtained by PCR amplification. *SHY2-2*, *BDL* and *IAA13* coding sequences were amplified from genomic DNA of *bdl*, *shy2-2* and Col-0, respectively. A sequence coding for an N-terminal c-Myc tag was added to each forward primer. A P80S mutation was introduced into *IAA13* by PCR (primers: 5′ATAGGAGACCATCCAACAACTTG3′, 5′CAAGTTGTTGGATGGTCTCCTAT3′). Coding sequences were cloned in the plant transformation vectors pVKHIAA3GUSpA, pVKHIAA12GUSpA or pVKHIAA13GUSpA, respectively, by replacing the GUS coding sequence using B*am*HI/*Sal*I restriction sites. *Agrobacterium* and *Arabidopsis* Col-0 ecotype plant transformations were performed as described (Hamann *et al*, 2002). Transgenic plants were selected using 15 mg/l hygromycin.

Growth conditions and phenotypic analysis

Plants were grown on a 16 h light/8 h dark cycle at 18 or 23°C. Exogenous drug application was performed by incubation of 7-day-old seedlings in liquid minimal salt media (2.1 g/l MS salts, 1% sucrose) supplemented with 10 μM IAA for 5 h.

For hypocotyl measurements, seedlings were grown on minimal salt media (0.7% agar) along the surface of vertical agar plates on a 16 h light/8 h dark cycle or in constant darkness. Hypocotyl lengths were measured from digital images taken after 7 or 5 days of growth.

For auxin resistance assays, seedlings grown for 5 days as described above were transferred to MS medium or MS medium supplemented with 0.1 μM 2,4-D. Root elongation was measured 3 days after transfer.

For gravitropism assays, seedlings were vertically grown for 7–9 days in a light/dark cycle, and then transferred to darkness and reoriented by 90°. The angle between the root tip and gravity vector was determined from digital images taken 24 h after reorientation.

Analysis of GUS expression and in situ hybridization

GUS expression and RNA *in situ* hybridization were performed as described (Hamann *et al*, 2002). The fragment for the *IAA13* probe was amplified from cDNA by PCR using primers IAA13S (5′TCTGATCGATATGCTGGTTCATCTCCTCCTCG3′) and IAA13AS (5′GTCTCTCTAGAGGTTCTTGATTTCGAGCAGCGA3′) and subcloned in pBluescript SK+.

RT–PCR

For analysis of *SHY2* expression, seedlings were incubated in control medium or medium containing 50 μM IAA at room temperature for 2 h. In one experiment, entire 3-day-old seedlings were used for RNA isolation using an RNeasy kit (QIAgen) according to the manufacturer's instructions. Poly(A)$^+$-RNA was isolated using a Dynal mRNA DIRECT kit (Dynal, Oslo, Norway). cDNAs were synthesized using MMuLV-RT (Invitrogen), and real-time quantitative RT–PCR reactions were performed on an icycler machine (BIO-RAD). *ACT2* (sense: 5′ATTCAGATGCCCAGAAGTCTTGTTC3′; antisense: 5′GCAAGTGCTGTGATTTCTTTGCTCA3′) and *SHY2* (sense: 5′GTCGACGAATTCATGGGGGAGCAAAAGCTTATTTCTGAGGAGGATGATGAGTTTGTTAACC; antisense: 5′GAATTCGGATCCTCATACACCACAGCC3′) PCRs were performed on three different serial dilutions of each cDNA and each reaction was performed at least five times. In a separate experiment, cDNAs were synthesized from poly(A)$^+$-RNA isolated from roots of 5- to 10-day-old seedlings as above, and PCR reactions for *ACT2* and *SHY2* were performed using different dilutions of cDNA. Intensities of bands on ethidium bromide-stained gels were quantified from scans using the ImageJ program, and normalized *SHY2*/*ACT2* values are represented. RT–PCRs were performed five times on two independent RNA batches, and results were comparable in all experiments.

Analysis of protein expression

Protein extracts from 7-day-old seedlings or roots of 12-day-old seedlings were prepared by grinding and boiling in Laemmli buffer (Laemmli, 1970). Proteins were separated on polyacrylamide gels and transferred to PVDF membranes by Western blotting. Membranes were incubated with monoclonal anti-Myc antibody (9E10) at 1:600 dilution and then with alkaline phosphatase-conjugated anti-mouse secondary antibody at dilution 1:5000, and signals were detected with the Western Star detection kit (Tropix). For Western blot analysis of yeast cells, crude protein extracts were prepared by boiling pelleted yeast cells in 4 volumes of Laemmli buffer. Proteins

were separated, blotted and incubated with 1:1000 diluted horseradish peroxidase (POD)-coupled anti-HA monoclonal antibody (Sigma), and washed and detected according to the manufacturer's recommendations.

Yeast assays

Yeast (*Saccharomyces cerevisiae*) strain YPH500 was transformed with plasmid *yDR5*::*LacZ* containing a direct (8 ×) repeat of the *DR5-rev* sequence (Ulmasov *et al*, 1997b), fused to a minimal *CYC1* promoter and the *lacZ* coding sequence in *pLacZi* (Clontech). Two to four independent *yDR5*::*LacZ* transformants were used in all experiments. *yDR5*::*LacZ* strains were transformed with pESC-TRP (Stratagene) plasmids containing either C-terminal HA-epitope-tagged cDNAs of *ARF5/MP* alone, or in combination with C-terminal HA-epitope-tagged cDNAs of *IAA3/SHY2* or *IAA12/BDL*. All cDNAs were amplified by PCR and sequenced. Approximately 12 yeast colonies per plasmid were grown overnight in minimal medium lacking tryptophan and containing 2% glucose. Cultures were then diluted to a final OD_{600} of 0.2 in the same medium now containing 2% raffinose and 2% galactose for *GAL1/10* promoter induction. Galactosidase activity was measured after 16 h induction according to Meijer *et al* (2000). All experiments were repeated at least three times and gave comparable results. For yeast two-hybrid experiments, entire *SHY2* or *BDL* open reading frames were amplified from wild-type cDNA. C-terminal regions of *MP* (aa 777–902) and *ARF19* (aa 948–1086) were PCR-amplified from cDNA libraries (Grebe *et al*, 2000). PCR products were subcloned into pGEM-T and subsequently cloned in-frame with LexA in pEG202 and with B42 in pJG4-5. Yeast strain EGY48 was first transformed with plasmid pSH18-34, and subsequently cotransformed with all possible combinations of pEG and pJG plasmids (in both directions). Experiments with Aux/IAA proteins as LexA fusions gave more consistent results, and are presented here. Galactosidase activity was quantified in cultures from at least 12 colonies for each plasmid combination in each of five experiments.

Complementation of monopteros

MP was C-terminally HA epitope tagged by exchanging a 3′ region of the cDNA (*Mun*I–*Afl*II) with a corresponding PCR fragment containing a C-terminal HA tag (primers: MpcMunI-S, 5′GATCAATT GATGTCACAAGCTTTAAAGAC3′ and MpcHAAflII-AS, 5′GCTTAAG AGCATAATCAGGAACATCATAAGGATAATCGTTAATGCCTGC3′). The HA-tagged cDNA was then used to replace the *BDL* coding region in a 4.5 kb genomic *BDL* fragment in pGreenII0229 (Roger *et al*, 2000) with *Bam*HI and *Spe*I sites generated at start and stop codons, respectively. The *ARF16* cDNA was amplified from a cDNA library with primers ARF16-CDS-S (5′CCGGATCCGAATTCATGATAAATG TGATGAATCC3′) and ARF16-CDS-HA-AS (5′CCACTAGTTTAAGCAT AATCAGGAACATCATAAGGATATACTACAACGCTCTCAC3′), the latter containing a C-terminal HA tag. The PCR fragment was used to replace the *BDL* coding region in pGreenII *BDL* as described above. Constructs were transformed into mp^{B4149} (Columbia ecotype; a gift from B Scheres) heterozygotes, and frequencies of rootless seedlings were determined in T2 seedlings from transgenics carrying the mp^{B4149} allele.

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