Overlapping and non-redundant functions of the *Arabidopsis* auxin response factors *MONOPTEROS* and *NONPHOTOTROPIC HYPOCOTYL 4*

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Summary

Transcription factors of the auxin response factor (ARF) family have been implicated in auxin-dependent gene regulation, but little is known about the functions of individual ARFs in plants. Here, interaction assays, expression studies and combinations of multiple loss- and gain-of-function mutants were used to assess the roles of two ARFs, NONPHOTOTROPIC HYPOCOTYL 4 (NPH4/ARF7) and MONOPTEROS (MP/ARF5), in *Arabidopsis* development. Both MP and NPH4 interact strongly and selectively with themselves and with each other, and are expressed in vastly overlapping domains. We show that the regulatory properties of both genes are far more related than suggested by their single mutant phenotypes. *NPH4* and *MP* are capable of controlling both axis formation in the embryo and auxin-dependent cell expansion. Interaction of MP and NPH4 in *Arabidopsis* plants is indicated by their joint requirement in a number of auxin responses and by synergistic effects associated with the co-overexpression of both genes. Finally, we demonstrate antagonistic interaction between *ARF* and *Aux/IAA* gene functions in *Arabidopsis* development. Overexpression of *MP* suppresses numerous defects associated with a gain-of-function mutation in *BODENLOS* (*BDL*)/*IAA12*. Together these results provide evidence for the biological relevance of ARF-ARF and ARF-Aux/IAA interaction in *Arabidopsis* plants and demonstrate that an individual ARF can act in both invariantly programmed pattern formation as well as in conditional responses to external signals.

Key words: *Arabidopsis*, Auxin response factor interaction, *Aux/IAA* genes, *BODENLOS*, Genetic redundancy, *MONOPTEROS*, *NONPHOTOTROPIC HYPOCOTYL 4*

Introduction

Auxins are involved in many aspects of plant growth and development and have long been known to control diverse responses to external stimuli (Went, 1939; Estelle, 1992; Davies, 1995). More recently, auxin signals have also been implicated in the development of cell patterns, for example, in the early embryo, the vascular tissue and the apical meristems (Sachs, 1991; Sabatini et al., 1999; Reinhardt et al., 2000; Berleth and Chatfield, 2002). At the molecular level, auxin signals affect expression of many genes, among them several classes of rapidly induced auxin-responsive genes (reviewed by Abel and Theologis, 1996; Hagen and Guilfoyle, 2002). Three major gene families, *Aux/IAA*-, *SAUR*- and *GH3*-type genes, have been analyzed most extensively, because many of their mRNAs increase in abundance within minutes after exposure to auxins. These very rapid expression responses suggest that a pre-existing transcriptional machinery is activated by auxin.

Conserved promoter elements that confer rapid auxin-responsive gene expression have been identified and employed in the isolation of trans-acting factors (auxin response factors, ARFs) (reviewed by Guilfoyle et al., 1998; Guilfoyle and Hagen, 2001; Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). All but two ARFs consist of an N-terminal DNA-binding domain (DBD), a central activation domain (AD) or repression domain (RD) and a C-terminal dimerization domain (CTD) (reviewed by Guilfoyle and Hagen, 2001). Within the CTD, two highly conserved motifs are present not only in ARF proteins, but also in another large family of nuclear proteins, the Aux/IAA proteins. Aux/IAA proteins are short lived, and a large body of evidence suggests that the abundance of Aux/IAA proteins is positively regulated through the auxin inducibility of their mRNAs and negatively through auxin-dependent protein degradation (Dharmasiri and Estelle, 2002; Leyser, 2002). In yeast two hybrid studies, ARFs and Aux/IAA proteins are capable of forming homo- and heterotypic interactions through their CTDs, and specific binding of ARF dimers to palindromic AuxRE target sites has been demonstrated in vitro (Kim et al., 1997; Ulmasov et al., 1997a; Ulmasov et al., 1999b). Given the complexity of both *ARF* and *Aux/IAA* gene families, a very high number of combinatorial interactions appear possible (Kim et al., 1997), but it remains

to be determined which interactions can be experimentally confirmed and which of those are reflected in auxin responses of *Arabidopsis* plants. As ARFs can form dimers in vivo (Kim et al., 1997; Ulmasov et al., 1997a), ARF homodimers and certain heterodimers could be biologically relevant. Another potentially important type of interaction appears to involve the negative regulation of ARF activity by Aux/IAA proteins (Gray et al., 2001; Tiwari et al., 2001; Ulmasov et al., 1997b). Auxin has been shown to promote proteasome-mediated degradation of Aux/IAA proteins (Gray et al., 2001; Zenser et al., 2001; Zenser et al., 2003) and could thereby indirectly regulate ARF activity.

Although there is considerable insight into the molecular biology of *ARF* and *Aux/IAA* genes and their products, the actual functions of most of these genes in plant growth and development are unknown (reviewed by Liscum and Reed, 2002). Functions of most *Aux/IAA* genes are defined exclusively by gain-of-function alleles, while loss-of-function alleles are scarce and phenotypically subtle. Within the *ARF* gene family, no gain-of-function alleles have been reported and loss-of-function mutations have been identified in only three out of 22 genes. Of these three genes, *ETTIN (ETT)/ARF3*, is implicated in gynecium development, and encodes an unusual ARF protein lacking the CTD (Sessions et al., 1997; Nemhauser et al., 2000) that functions as a transcriptional repressor in protoplast transfection assays (Tiwari et al., 2003). The remaining two genetically defined *ARF* genes, *MP* and *NPH4*, encode typical members of the ARF family with conserved DBDs and CTDs. *MP* and *NPH4* encode proteins that are highly related in amino acid sequence, especially within their DBDs and CTDs (Ulmasov et al., 1999b) (reviewed by Guilfoyle et al., 2001). These two ARFs contain non-conserved glutamine-rich middle regions and function as transcriptional activators in protoplast transfection assays (Ulmasov et al., 1999a; Tiwari et al., 2003). By sequence similarity, ARF proteins could act in pairwise interactions, with MP and NPH4 being a likely combination (Guilfoyle and Hagen, 2001; Ulmasov et al., 1999b). However, despite their structural similarity, both genes have been implicated in entirely unrelated auxin responses. While *MP* has critical functions in axial cell patterning early in organogenesis (Berleth and Juergens, 1993; Przemeck et al., 1996), *NPH4* mediates auxin-dependent differential cell expansion mainly in the mature hypocotyl (Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Harper et al., 2000).

In this study, we were interested in visualizing further biological functions of *MP* and *NPH4* by assessing their interaction properties and by correlating those to genetic data obtained in *Arabidopsis* plants. To this end, we studied their interaction with ARF and Aux/IAA proteins, their expression profiles in *Arabidopsis* development, and their capacity to trigger downstream events in a variety of genetic backgrounds. We found that the two gene products selectively interact with themselves and with each other, are expressed in overlapping domains, and regulate downstream processes in similar, but quantitatively distinguishable, ways. Depending on their relative expression level, they may act redundantly or non-redundantly, and seem to be negatively controlled by interaction with Aux/IAA proteins.

Materials and methods

Nomenclature

Most genes in this study were identified independently in mutant and molecular searches. We have included all names of a gene at its first appearance in the text and then used the mutant based nomenclature. However, in specific context we made exceptions and used double names to emphasize the molecular identity of the gene product.

Yeast two hybrid assays

Vectors for yeast two hybrid analysis were purchased from BD Biosciences Clontech (Palo Alto, CA). Interaction analysis and β-galactosidase assays were performed as described in the Clontech manual for yeast two hybrid interactions and screening. At least five individual colonies for each interaction were chosen for quantitative β-galactosidase assays. The C-terminal domains [as defined by Ulmasov et al. (Ulmasov et al., 1999b)] of MP/ARF5 and NPH4/ARF7 and full-length BDL/IAA12 were used as bait proteins expressed from pAS2-1. Prey proteins were expressed from pACT2 and consisted of C-terminal domains of ARF1, ARF2, ARF4, ARF5, ARF6, ARF7, ARF8, ARF9 and ARF11 (Ulmasov et al., 1999b; Guilfoyle and Hagen, 2001) and full-length BDL/IAA12. Expression of bait and prey proteins in yeast was confirmed by western blotting using GAL4 AD and BD antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). ARF CTDs, containing domains III and IV (Guilfoyle and Hagen, 2001), were terminated at the stop codon and initiated at the N-terminal amino acid position indicated below: ARF1 (544), ARF2 (717), ARF4 (647), ARF5 (778), ARF6 (778), ARF7 (1031), ARF8 (686), ARF9 (504) and ARF11 (485).

Protoplast transfection assays

The G4M(4X)-GUS reporter gene containing four yeast GAL4 DNA binding sites upstream of a minimal –46 Cauliflower Mosaic Virus (CaMV) 35S promoter has been described previously (Ulmasov et al., 1995; Tiwari et al., 2003). ARF effector genes consisted of a yeast GAL4 DBD fused in-frame with the MP/ARF5 middle activator region (MR) and C-terminal domain CTD [GAL4DBD-MP/ARF5(MR+CTD)] and the NPH4/ARF7 MR and CTD [GAL4DBD-NPH4/ARF7(MR+CTD)] (Ulmasov et al., 1999a; Tiwari et al., 2003). *IAA4*, *IAA9* and *BDL/IAA12* effector constructs encoding full-length Aux/IAA proteins have been described elsewhere (Tiwari et al., 2001). All effector genes were placed under control of the CaMV 35S double enhancer promoter followed by a translational enhancer from the tobacco mosaic virus 5′ leader (Skuzeski et al., 1990) and contained a 3′ nopaline synthase untranslated region (Ulmasov et al., 1995). Isolation of carrot protoplasts, transfections and GUS activity assays were performed as described previously (Ulmasov et al., 1995; Tiwari et al., 2003).

In situ hybridization to tissue sections

DIG-labeled single-stranded RNA probes were generated by in vitro transcription and hybridized to tissue sections as previously described (Beeckman et al., 2002). As *NPH4* probe, a 650 bp fragment from the *NPH4* cDNA without significant homologies to other *Arabidopsis* sequences in BLAST searches was amplified using GATGAAA-GACCCTTCGAGTAC and ACCATTGTAAAGCTGATTCTG as primers, subcloned in both orientations into pBluescript (Stratagene) and transcribed from the T7 promoter. In situ hybridization probes used for visualizing *MP* transcripts have been described previously (Hardtke and Berleth, 1998).

Plant growth conditions and plant material

Plants on soil were grown under long-day (16 hour) light cycles in growth chambers (Conviron) at 22°C. For the analysis of seedlings, seeds were surface-sterilized in 15% commercial bleach, washed in distilled water and stratified in growth medium at 4°C for 4 days. Light-germinating seeds were germinated on growth medium (ready-to-use

0.5× Murashige and Skoog (MS) salt mixture, vitamins, 1.5% sucrose, buffered to pH 5.7, Gibco) at 22°C and placed under continuous fluorescent white light (80 μM $m^{-2}s^{-1}$). We refer to days after germination (dag) as days after exposure of imbibed seeds to light. For germination in the dark, seeds were plated on the same medium without sucrose and exposed to fluorescent white light (80 μM m^{-2} s^{-1}) for 2 hours to synchronize germination. Subsequently, plates were wrapped in three layers of aluminum foil and incubated at 25°C.

We refer to the *Columbia-0 (Col-0*) line as 'wild type' in all experiments. Mutant plants were obtained from K. Yamamoto (*nph4-103*, *-105*, *-106*) and E. Liscum (*nph4-1*), from R. Z. Sung (*mpG12*), and from G. Juergens, U. Mayer and T. Hamann (*bdl*). The molecular lesions in these mutants have been described (Harper et al., 2000; Hardtke and Berleth, 1998; Hamann et al., 2002).

Generation of transgenic lines

To generate plants expressing *MP* antisense RNA, a fragment of ~2 kb (primer sequences: ACAAGGTCATCTCGAGCAGGTT and TTGGCGAGAGAATTCCTGTGAGTC) was amplified from the *MP* cDNA (AF037229) (Hardtke and Berleth, 1998) by PCR using Vent DNA polymerase (New England Biolabs). The maximum sequence similarity to any other *Arabidopsis* sequence in small (100 bp) intervals is below 30%. After digestion with *Xho*I and *Eco*RI the fragment was cloned in antisense orientation into the respective sites adjacent to the CaMV 35S promoter in binary vector pEGAD (Cutler et al., 2000). *Arabidopsis* Col-0 plants were transformed with this construct (*35S::MPAS*) in *Agrobacterium tumefaciens* strain GV3101pMP90 (Koncz and Schell, 1986) by the floral dip method (Clough and Bent, 1998). Progeny of 28 transformants with ~3:1 segregation ratios of resistance to L-Phosphinotricin (BAR marker) were phenotypically characterized in the T2 and T3 generation and three lines (*35S::MPAS1-3*) with invariant *mp*-specific inflorescence defects (Przemeck et al., 1996), but residual fertility, were selected for detailed analysis. Because of the inactivity of the CaMV 35S promoter in early embryos (Volker et al., 2001), transgenic plants have normal appearance at the seedling stage, but resemble weak *mp* mutants at all subsequent stages.

The abundance of *MP* and *NPH4* transcripts in *35S::MPAS* plants was determined in RNA extracted from the fourth rosette leaf of T3 plants 14 days after germination using RNeasy Plant Mini Kit (Qiagen). RT-PCR analysis of total RNA was performed using One Step RT-PCR Kit (Qiagen). Primers were as follows: MPFL (CCCGGAATTCATGATGGCTTCATTG, CAATGGTGGAAATAGCTTCTCT); NPH4 (GATGAAAGACCCTTCGAGTAC, ACCATTGTAAAGCTGATTCGT); and ACT7 (GGTGAGGATATTCAGCCACTTGTCTG, TGTGAGATCCCGACCCGCAAGATC). The amplification fragment from the *ACT7* transcript served as expression standard and, as the PCR product spans an intron, as a tool to detect possible amplification of genomic DNA. Linearity of PCR amplification was controlled as described (Beeckman et al., 2002). In two independent experiments per line, *MP* transcript levels were reduced to <5% of the wild-type level, while *NPH4* transcript abundance remained unchanged.

35S::NPH4 and *35S::MP* transgenic lines: full-length *MP* cDNA or regions of it, as well as full-length *NPH4* cDNA were amplified with Pfu DNA polymerase from the respective full-length cDNA clones isolated from a library (Kieber et al., 1993) and control sequenced. Binary plasmids expressing the cDNA fragments under control of the CaMV 35S promoter were then constructed in the vector pTCSH1 (Hardtke et al., 2000) and transformed into *Arabidopsis* plants as described for *35S::MPAS* T-DNA above. Transgenic lines for each type of construct were produced in three independent batches, using three independently cloned plasmids. The phenotypic range of transgenics for a particular construct was similar in all cases. All plants were genotyped for presence of the respective transgene(s) by PCR using a *35S*-specific and a gene-specific oligonucleotide. Between seven and 21 transgenic lines were obtained for each construct. Stability and reproducibility of phenotypic traits was established in more than 10 independent *35S::MP* and *35S::NPH4* lines. Plants overexpressing *NPH4* and *MP* full-length or partial cDNA fragments were derived from crosses between primary transformants. Progeny from two to four crosses between at least two individual primary transformants of each genotype, derived from independent transformations, was analyzed for each combination. Relative transcript abundance in wild type, *35S::MP* and *35S::NPH4* lines was quantified as described (Mattsson et al., 2003).

Double mutant analysis

To determine the seedling phenotypes of *mp; nph4* double mutants, plants homozygous for *nph4* and heterozygous for *mp* were identified in the F2 of a cross of the respective single mutants by the appearance of *nph4*-specific leaf traits and by the appearance of rootless seedlings in their F3 progeny. Homozygosity for *nph4-1* was assessed by PCR (below). In the progenies of these plants, rootless individuals were observed at frequencies close to 25% and the remaining seedlings displayed hypocotyl elongation properties indistinguishable from *nph4* homozygous seedlings of the respective *nph4* allele. The rootless F3 individuals were considered *mp; nph4* double mutants.

Genotypes of other multiple mutant plants were confirmed by the following PCR assays. Presence of transgene constructs in all genetic combinations was monitored by PCR reactions using a *35S*-specific and a gene-specific oligonucleotide. Homozygosity for *nph4-1* (in double mutants with *mp* alleles or in *35S::MPAS* lines) was verified by the absence of a wild-type specific amplification product in an analytical PCR using TCCTGCTGAGTTTGTGGTTCCTT and GGGGCTTGCTGATTCTGTTTGTTA as primers in combination with an unrelated control PCR reaction. The genotype at the *BDL* locus was monitored by PCR amplification of an 800 bp *BDL* fragment (primers GCTCAAATCTTGTGATGTGAGTG and AGTCCACTAGCTTCTGAGGTTCCC) followed by an analytical, wild-type-specific *Hae*III digest.

Characterization of seedling phenotypes and leaf vascular defects

Histological preparation and microscopic inspection of seedlings and leaves was performed as described (Berleth and Juergens, 1993). Mutant seedlings were cleared at 7 dag. Vascular system features were analyzed in one of the two first rosette leaves (from more than 20 plants) taken at 14 dag. *35S::MPAS* transgenes were maintained hemizygously in either wild-type or *nph4* homozygous background and plants carrying the transgene were selected by germination on 0.5×MS medium supplemented with 10 μg/ml L-Phosphinotricin prior to transfer to soil and leaf analysis.

Hypocotyl elongation and cotyledon expansion auxin response assays

Seeds were germinated in the dark as described above. For auxin response assays the medium was supplemented with 20 μM IAA (Sigma-Aldrich) unless otherwise indicated. After 5 days at 25°C in the dark, the lengths of hypocotyls were measured on digitally captured images. Auxin sensitivity in this assay correlated with phototropic response in all genotypes, but hypocotyl length could be quantified at higher resolution than hypocotyl bending. For hemizygously maintained genotypes (*35S::MPAS* and certain *35S::MP* lines), individually measured seedlings were subsequently grown to maturity and genotyped by adult phenotype traits and the inheritance of resistance to L-Phosphinotricin. Variability of hypocotyl length on hormone-free media was not correlated to any of the genotypes in standard *t*-tests.

Cotyledon expansion in light germinated seedlings was measured as described previously (Mattsson et al., 2003).

Auxin-induced gene expression

Quantification of auxin-induced gene expression on northern blots

was determined in seedlings 7 days after exposure to light as previously described (Mattsson et al., 2003). All northern hybridizations resulted in single bands. Probe specificity was ensured by BLAST searches of the *Arabidopsis* genome sequence.

Results

ARF-ARF interactions are highly selective

The *MP* and *NPH4* gene products are highly related in both the DBD and the CTD (Guilfoyle and Hagen, 2001; Ulmasov et al., 1999b). Both proteins could redundantly regulate common downstream targets or, alternatively, both could interact with each other as necessary components of common regulatory complexes. We first assessed the propensity of the two *ARF* gene products to interact with themselves and with each other in yeast two hybrid assays. To this end, cDNA sections encoding C-terminal regions (i.e. including domains III and IV of the dimerization domain) (see Ulmasov et al., 1999b; Guilfoyle and Hagen, 2001) of a representative sample of nine previously characterized ARFs (Ulmasov et al., 1999a; Ulmasov et al., 1999b) were cloned into an activation domain vector (pACT2). The ARFs used for analysis were selected because they had been previously experimentally tested for functionality (i.e. functioning as either an activator or repressor) in transient transfection assays (Ulmasov et al., 1999a; Tiwari et al., 2003). These constructs were then introduced into yeast cells expressing MP or NPH4 C-terminal domains fused to a DNA-binding domain vector (pAS2-1), and the response of the β-galactosidase reporter gene was quantified fluorimetrically. As shown in Fig. 1A, interaction of MP was essentially restricted to itself and NPH4 when MP was tested as the bait plasmid. NPH4 interacted strongest with MP, and less strongly with itself and ARF6 when NPH4 was tested as the bait plasmid. Weaker interactions with NPH4 bait were observed with ARF8 and ARF4. Of the ARFs tested in two hybrid assays, ARF6 and ARF8 are more closely related in amino acid sequence to MP and NPH4 than the other ARFs assayed. These four ARFs function as transcriptional activators, while the remainder of the ARFs tested function as transcriptional repressors in protoplast transfection assays (Ulmasov et al., 1999a; Tiwari et al., 2003). It is worth noting that neither MP nor NPH4 interacted strongly with any of the transcriptional repressors tested (i.e. ARF1, ARF2, ARF4 and ARF9). However, we have observed that ARFs 1 and 2 interact strongly with each other in yeast two hybrid assays, but fail to interact or interact only weakly with the ARF transcriptional activators (Ulmasov et al., 1997a) (S.B.T., G.H. and T.J.G., unpublished).

In summary, our results suggest that ARF-ARF interactions are selective and that MP and NPH4 constitute a subgroup of strongly interacting proteins within the ARF family (see Discussion).

ARF-Aux/IAA interaction interferes with activation by MP and NPH4

We next asked whether the similarities of the CTDs in MP and NPH4 imply that both proteins interact similarly with a given protein of the Aux/IAA family. A potentially biologically relevant interaction of MP and BODENLOS (BDL)/IAA12 has previously been demonstrated in yeast two hybrid assays (Hamann et al., 2002). As shown in Fig. 1A, MP and NPH4 interact equivalently with BDL in parallel yeast two hybrid assays.

In an additional set of experiments, we asked whether BDL could interfere with MP- and NPH4-mediated gene activation in carrot protoplast transfection assays (Ulmasov et al., 1997b; Tiwari et al., 2001) and, if so, whether this interference was specifically associated with expression of *BDL* or could also be observed upon expression of other *Aux/IAA* genes. IAA4 and IAA9 were selected as representative Aux/IAA repressors along with BDL/IAA12 (see Tiwari et al., 2001). Full-length cDNAs of *IAA4*, *IAA9* and *BDL/IAA12* were co-expressed in carrot protoplasts with chimeric *MP* and *NPH4*, in which the natural DBDs were replaced by the DBD of yeast GAL4. Downstream gene activation was monitored through the expression of a GUS reporter gene coupled to UAS, the GAL4 target sequence as described (Ulmasov et al., 1999a; Tiwari et al., 2001). As shown in Fig. 1B, expression of both chimeric MP and NPH4 leads to high reporter gene expression, further enhanced by the application of 1-naphthalene acetic acid (1-NAA). Consistent with the results of yeast two hybrid assays, co-expression of BDL/IAA12, a potential negative regulator of both ARF proteins, results in a dramatic reduction of reporter gene expression, which is partially restored upon auxin application. Co-expression of either IAA4 or IAA9, along with either chimeric MP or NPH4, resulted in a similar level of reporter gene repression as that observed with BDL/IAA12. We have also tested IAA7, IAA17 and IAA19 with chimeric MP and observed a similar amount of reporter gene repression as documented in Fig. 1B with IAA4, IAA9 and BDL/IAA12 (Tiwari et al., 2003) (S.B.T., G.H. and T.J.G., unpublished).

In conclusion, these results indicate negative regulation of both MP and NPH4 activity by interaction with Aux/IAA co-regulators in carrot suspension cell protoplasts and support the notion that auxin promotes ARF-dependent gene activation by reducing the amount of these negative regulators that dimerize with ARF activators on AuxREs (see also Tiwari et al., 2001; Tiwari et al., 2003). In the plant cell protoplast system, little specificity of repression by Aux/IAA proteins is observed when effector genes encoding Aux/IAA and MP or NPH4 are co-transfected into prototoplasts (Tiwari et al., 2003) (Fig. 1B), suggesting that additional parameters, such as the specific expression profiles of the interacting proteins, enhance interaction specificity in the plant (see Discussion). A similar lack of specificity between Aux/IAA and ARF activator interactions is seen in yeast two hybrid assays (S.B.T., G.H. and T.J.G., unpublished).

MP and NPH4 expression domains overlap

Both scenarios, the redundant action of NPH4 and MP, e.g. as homodimers, as well as their heterodimeric interaction in common transcriptional complexes, depend on overlapping expression domains of both genes. To determine whether there is overlap between the two expression domains, we visualized *MP* and *NPH4* mRNA at various stages of *Arabidopsis* development by in situ hybridization to tissue sections.

Although the defects in *nph4* mutants are restricted to sharply defined stages, *NPH4* turned out to be expressed throughout the *Arabidopsis* life cycle. Already in heart stage embryos, *NPH4* transcripts are present in all major tissue types, often somewhat more abundant in cotyledons than in the basal part of the embryo (Fig. 2A). Transcripts of *NPH4* are detected

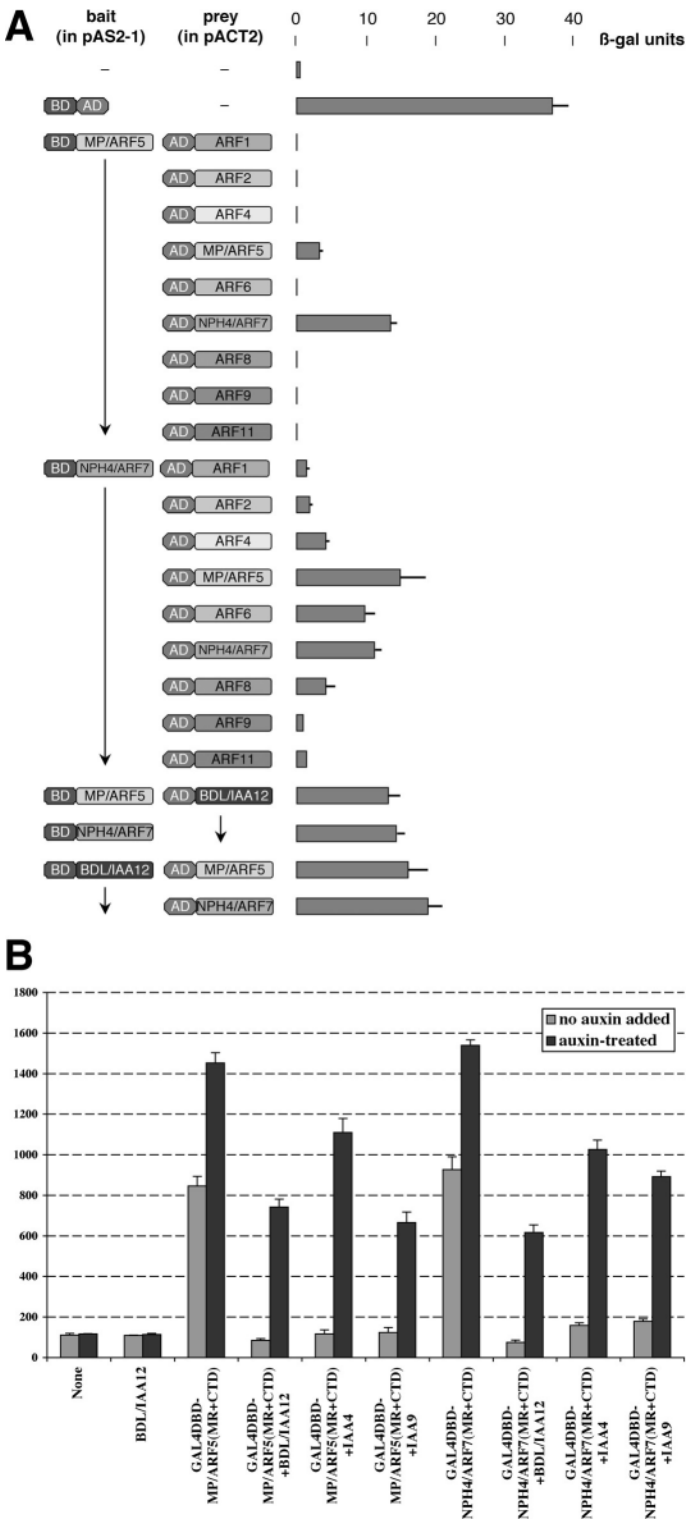

Fig. 1. ARF protein interactions. (A) ARF-ARF and ARF-Aux/IAA protein interaction in yeast two hybrid assays. Relative reporter gene expression levels (β-galactosidase units, right) in yeast cells co-expressing C-terminal regions of the indicated ARF gene-coding sequences (left) fused to DNA binding (BD) and activation (AD) domains. Line one shows β-galactosidase expression levels in yeast cells harboring both empty vectors (pAS2-1, bait; pACT2, prey). Line two shows β-galactosidase expression in response to the CLONTECH™ positive control. Bars represent the mean±s.e. (B) Auxin-regulated interference of Aux/IAA expression with ARF transcriptional activation in carrot suspension cell protoplasts. Quantification of GUS reporter gene activity after transfection of the reporter gene and indicated effector genes in the absence (gray) and presence (black) of 10 μM 1-NAA. Columns represent the mean±s.d. Both ARF effector genes (*MP/ARF5* and *NPH4/ARF7*) comprise the DNA-binding domain (DBD) of yeast GAL4 (GAL4DBD) and the middle region (MR), and C-terminal domain (CTD) of the respective ARF. *IAA* effector genes encode full-length wild-type IAA4, IAA9, IAA19 or BDL/IAA12 proteins. Expression of effector genes was driven by the CaMV 35S promoter (Tiwari et al., 2003). The GUS reporter gene is under control of the GAL4 response element and contains a –46 minimal CaMV 35S promoter with four GAL4-binding sites fused just upstream (see Ulmasov et al., 1995; Tiwari et al., 2003).

throughout the embryo at all subsequent embryonic stages (Fig. 2B,C). Post-embryonically, *NPH4* expression remains ubiquitous, but is generally stronger in the small, non-vacuolated cells of young organs. In vegetative leaf primordia, for example, *NPH4* expression is strongest in the developing lamina as opposed to weaker expression in the outer layers of the midrib region, which are comprised of larger cells (Fig. 2D). Low-level uniform expression is also observed in the organs of all floral whorls, but there is a distinctly higher expression level in pollen, tapetum and ovules (Fig. 2E).

Expression of *MP* has previously been shown to be initially uniform in sub-epidermal tissues of early embryos, apical meristems and organ primordia. As the organ matures, expression becomes restricted first to a central domain and then to individual vascular strands (Hardtke and Berleth, 1998) (Fig. 2F). We extended this analysis to precisely compare *MP* and *NPH4* transcript distribution. In bent-cotyledon stage embryos, *MP* mRNA is present at low levels throughout the embryo, but is more abundant in the vascular tissues both in the hypocotyl and in the cotyledons (Fig. 2H). In vegetative leaf primordia, *MP* is expressed throughout the lamina, but is only extremely weakly expressed in the vacuolated cells of the midrib region (Fig. 2I). Unlike *NPH4*, however, *MP* is expressed at far higher levels in the most central regions of leaf organs (Fig. 2I). In floral organs, *MP* expression is very weak or possibly absent outside of the central region. In carpels, *MP* expression is strong not only in the central domain, but also in the developing ovules (Fig. 2J).

In summary, the expression domains of *NPH4* and *MP* overlap extensively. Both genes are expressed at low levels in nearly identical domains, except that *MP* is additionally expressed at higher levels specifically in a central region and eventually in the vascular system.

Redundant functions of MP and NPH4

If NPH4 and MP act as homodimers (or in any other kind of transcriptional complex comprising only one of the two ARF proteins), they may redundantly regulate target genes. Recognizable abnormalities in *nph4* mutants are restricted to defects in hypocotyl elongation and leaf shape (Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998), but regulatory potential of *NPH4* at other stages might be masked by *MP* activity. This potential should then become apparent in the background of reduced *MP* activity.

To see whether phenotypes of *mp; nph4* double mutants are

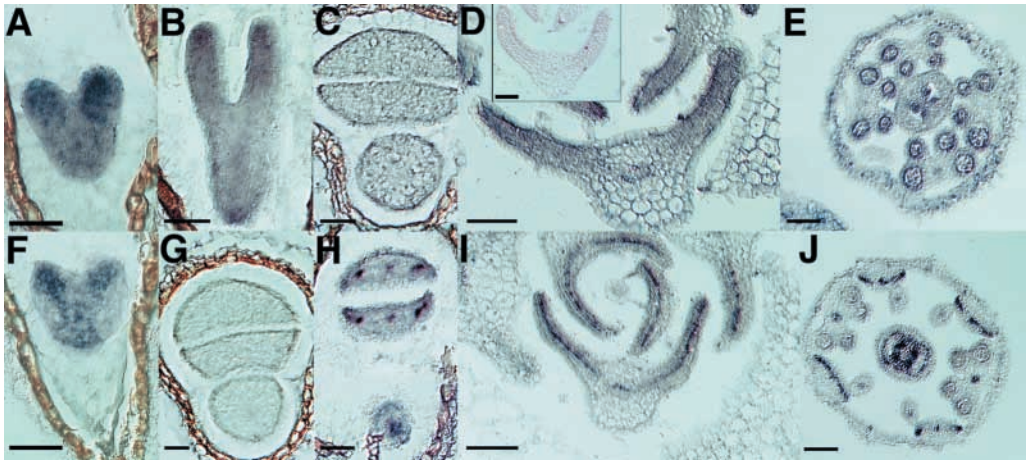

Fig. 2. Expression pattern of *NPH4* and *MP* mRNA in wild-type plants. In situ hybridization with *NPH4* (A-E) and *MP* (F,H-J) antisense probes and with *NPH4* sense probe (inset in D, G). Embryos at early heart (A,F), torpedo (B) and bent cotyledon (C,G,H) stages, vegetative leaf primordia (D,I) and flower primordia (E,J). Median longitudinal sections (A,B,F), cross-sections (C-E,G-J). Scale bars: 50 μm in A-C,F-G; 250 μm in D,E,I,J.

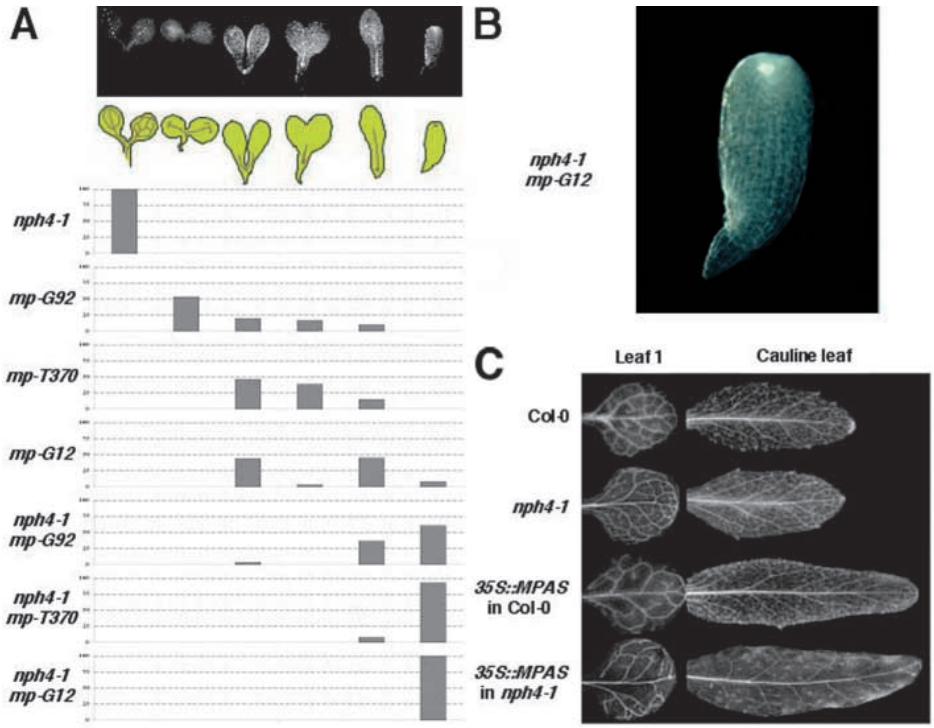

Fig. 3. Overlapping functions of *MP* and *NPH4* in embryos. (A) Two upper panels (dark-field optics, top; schematic, bottom) illustrate phenotype classes (from left): dicotyledonous with normal vasculature, dicotyledonous with reduced secondary vein lobes, solitary midvein, fused cotyledons, single cotyledons and club-shaped seedlings. Panel below shows the percentage of each phenotype class in single and double mutants for *nph4* in various double homozygous allelic combinations with weak (*mpG92; nph4-1*), intermediate (*mpT370; nph4-1*) and strong (*mpG12; nph4-1*) *mp* alleles. Columns represent the proportion of each phenotype class in % (broken lines indicate 25, 50, 75 and 100%). Approximately 100 double mutants were evaluated for each genotype; 290 double mutants were evaluated for *mpG12; nph4-1*. (B) Phenotype of a 'club-shaped' seedling (*mpG12; nph4-1*). Neither cotyledons nor any other lateral outgrowth is visible and only very few disorganized vascular cells are being produced (dark-field optics). (C) Leaf venation (xylem strands) in the first rosette leaf (left) and first cauline leaf (right) of the genotypes: Col-0 wild type, *nph4-1* homozygous, *35S::MPAS* in wild-type background and *35S::MPAS* hemizygous in *nph4-1* homozygous background. Note that the venation pattern in *nph4-1* single mutants is unaffected, displaying primary (midvein), secondary (first order branches), tertiary and some quarternary veins in the area enclosed by the secondary vein arches. Tertiary and quarternary venation is strongly reduced in *35S::MPAS* plants. In *35S::MPAS; nph4-1* plants, tertiary and quarternary venation is further reduced and the pattern of secondary vein lobes is disrupted (dark-field optics).

more severe than those of the corresponding single mutants, the precise quantification of phenotype strengths is necessary. All *mp* mutants are rootless, but a classification scheme based on vascular defects has been used to quantify residual gene activity in *mp* mutant alleles (Berleth and Juergens, 1993). This scheme was found to be consistent with the characteristics of molecular lesions in mutant alleles (Hardtke and Berleth, 1998). For example, premature stop codons in the C-terminal part of the activation domain are associated with a spectrum of phenotypes, ranging from dicotyledonous seedlings with some ramified vascular strands to single cotyledonous seedlings with no more than a short midvein (*mpG92* in Fig. 3A), while stop codons in more N-terminal positions are associated with more extreme reductions of the vascular system (*mpG12, mpT370* in Fig. 3A).

The phenotype classification scheme can be used to quantitatively relate double mutant phenotypes to those of *mp* single mutants. As shown in Fig. 3A, *mpG92; nph4-1* double mutants are phenotypically stronger than *mpG92* single mutants as the spectrum of observed phenotypes is shifted towards stronger phenotype classes. Weaker phenotypes, such as seedlings with two cotyledons and branched (secondary vein forming) vasculature are not observed in *mpG92; nph4-1*. Instead, a large proportion of seedlings is single cotyledoneous with vascular systems reduced to short cotyledon midveins and a substantial portion does not form cotyledons at all. Mutant seedlings

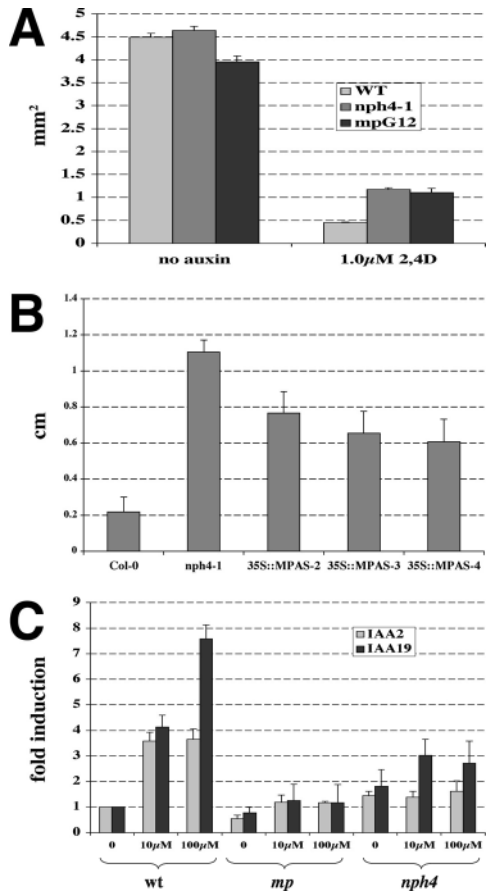

Fig. 4. Non-overlapping functions of *MP/ARF5* and *NPH4/ARF7*. (A) Quantification of cotyledon area of light-germinated seedlings of the indicated genotypes at 10 dag, grown in the presence of 0 and 1 μM 2,4-D. Sample sizes are 45 to 50 cotyledons. (B) Hypocotyl length of seedlings of the indicated genotypes germinated and grown in the dark for 5 days in the presence of 20 μM IAA. Sample sizes are 10 to 50 seedlings. On hormone-free media, hypocotyls of all genotypes elongated to ~1.2 cm. (C) Transcript abundance of the *Aux/IAA* genes *IAA2* and *IAA19* in light-germinated seedlings of the indicated genotypes exposed to 0, 10 and 100 μM IAA at 7 dag for 30 minutes. Transcript abundance from three independent experiments is displayed as multiples of the wild-type level at 0 μM IAA. Columns represent the mean±s.e. (A,C) and s.d. (B).

completely devoid of cotyledons and continuous vascular strands ('club shaped' seedlings in the following) are rare even in the strongest *mp* mutant lines (below) and are not at all observed in *mpG92* lines. In *mpG92; nph4-1* double mutants, by contrast, this class of 'club'-shaped individuals amounts to 60% of the double mutant seedlings. This shift in the severity of mutant defects in *mpG92; nph4-1* double mutants is purely quantitative, as the same phenotype classes are also observed in mutants of strong *mp* alleles. This confirms that the *NPH4* wild-type product confers an activity that is functionally equivalent to the residual *MP* gene activity present in *mpG92* mutants.

Double mutants comprising strong *nph4* and *mp* alleles displayed further enhanced phenotype spectra (Fig. 3A). Most strikingly, double mutants comprising the phenotypically strongest alleles, *nph4-1* and *mpG12*, failed to initiate the outgrowth of cotyledons or to align vascular cell differentiation (Fig. 3A,B). The entirely homogenous phenotype of the *nph4-1*; *mpG12* double mutant indicates that in the absence of both gene activities the formation of new lateral organs and of continuous vascular strands is no longer possible and that therefore the limited extent of oriented differentiation in the apical region of *mp* single mutant embryos can be attributed to *NPH4* function.

Redundant post-embryonic functions of MP and NPH4

The limited development of *mp; nph4* double mutants precludes direct tests of *NPH4* function in cell patterning during post-embryonic organogenesis. To downregulate *MP* activity post-embryonically, we expressed *MP* antisense RNA under control of the CaMV 35S promoter (*35S::MPAS*) in wild-type and *nph4* mutant background. All experimental results involving *35S::MPAS* lines are based on observations in three selected lines (*35S::MPAS1-3*, see details in Materials and methods).

The basic outline of *Arabidopsis* rosette and cauline leaf venation comprises a series of secondary vein lobes extending from the (primary) midvein and a system of higher order veins (tertiary and quarternary) within these lobes. In *nph4* mutant plants, no significant vein pattern irregularities are observed. By contrast, lines carrying the *35S::MPAS* construct have generally fewer higher order veins in rosette leaves. Their cauline leaves are elongated, but have an essentially normal density of higher order venation (Fig. 3C). In the *nph4* mutant background, however, secondary vein lobes of all *35S::MPAS* lines are drastically reduced in rosette leaves and nearly absent in cauline leaves (Fig. 3C).

In conclusion, the enhancement of *35S::MPAS* phenotypes in *nph4* mutant background suggests overlapping functions of both genes also in post-embryonic development.

Both NPH4 and MP are required for auxin-regulated cell expansion

In an alternative scenario, NPH4 and MP proteins could form heterodimers, implying that a downstream process could be equally affected by loss of either of the two gene activities. We have previously shown that MP is involved in an auxin-controlled cell expansion process (Mattsson et al., 2003). When light-germinated wild-type seedlings are exposed to auxin, the expansion of their cotyledons is dramatically reduced (Fig. 4A). This response to auxin is not only compromised in *mp* mutants (Mattsson et al., 2003), but also in *nph4* mutants (Fig. 4A), suggesting that both genes are involved in auxin-controlled cell expansion.

The *NPH4* gene has further been implicated in the auxin-mediated control of hypocotyl cell elongation (Watahiki and Yamamoto, 1997; Stowe-Evans et al., 1998; Harper et al., 2000). In this process, an equivalent role of *MP* would not have been detected, because *mp* mutants fail to initiate the hypocotyl in the early embryo (Berleth and Juergens, 1993). To bypass the embryonic defects in *mp* mutants, we used again *MP* antisense lines (*35S::MPAS1-3*) to downregulate *MP* selectively at a post-embryonic stage. When germinated in the dark on media containing 20 μM IAA, wild-type seedling hypocotyl expansion is strongly inhibited, whereas in *nph4-1* mutant hypocotyl elongation is nearly unaffected (Fig. 4B). Remarkably,

expression of *MP* antisense RNA dramatically reduces auxin responsiveness in the hypocotyl, indicating that not only *NPH4*, but also *MP* is required for the inhibition of auxin-controlled hypocotyl elongation. Hypocotyls of *35S::MPAS* seedlings remain more auxin sensitive than *nph4-1* mutants, but it has to be considered that all *35S::MPAS* lines had been selected for residual levels of *MP* activity in order to be maintained as fertile lines. Their phenotypes are generally weaker than those of any characterized *mp* allele. Therefore, it seems possible that complete loss of *MP* function would lead to auxin insensitive hypocotyls similar to those in *nph4* mutants.

Finally, we used gene expression profiles of rapidly auxin-responsive genes to identify regulatory events that are dependent on both transcription factors. Both *MP* and *NPH4* have been shown to be required for auxin-dependent gene regulation in *Arabidopsis* plants (Stowe-Evans et al., 1998; Mattsson et al., 2003). As shown in Fig. 4C, auxin-induced expression of two primary auxin response genes, *IAA2* and *IAA19*, crucially depends on both *MP* and *NPH4* gene activity and could therefore reflect the joint regulation by both ARFs.

Functional divergence of MP and NPH4 proteins

To determine the extent to which both gene products could substitute for each other irrespective of their relative expression profiles, we expressed full-length cDNAs of both genes individually under control of the full-length CaMV 35S promoter, which resulted in transcript levels 30-50 fold higher than the normal expression levels of each gene (see Materials and methods).

High level expression of each gene normalizes the respective mutant phenotype, verifying the functionality of each of the overexpressed cDNAs. When *35S::NPH4* is expressed in the strong *nph4-1* mutant, hypocotyl cell elongation is strongly responsive to auxin and vegetative leaf shape is similar to wild

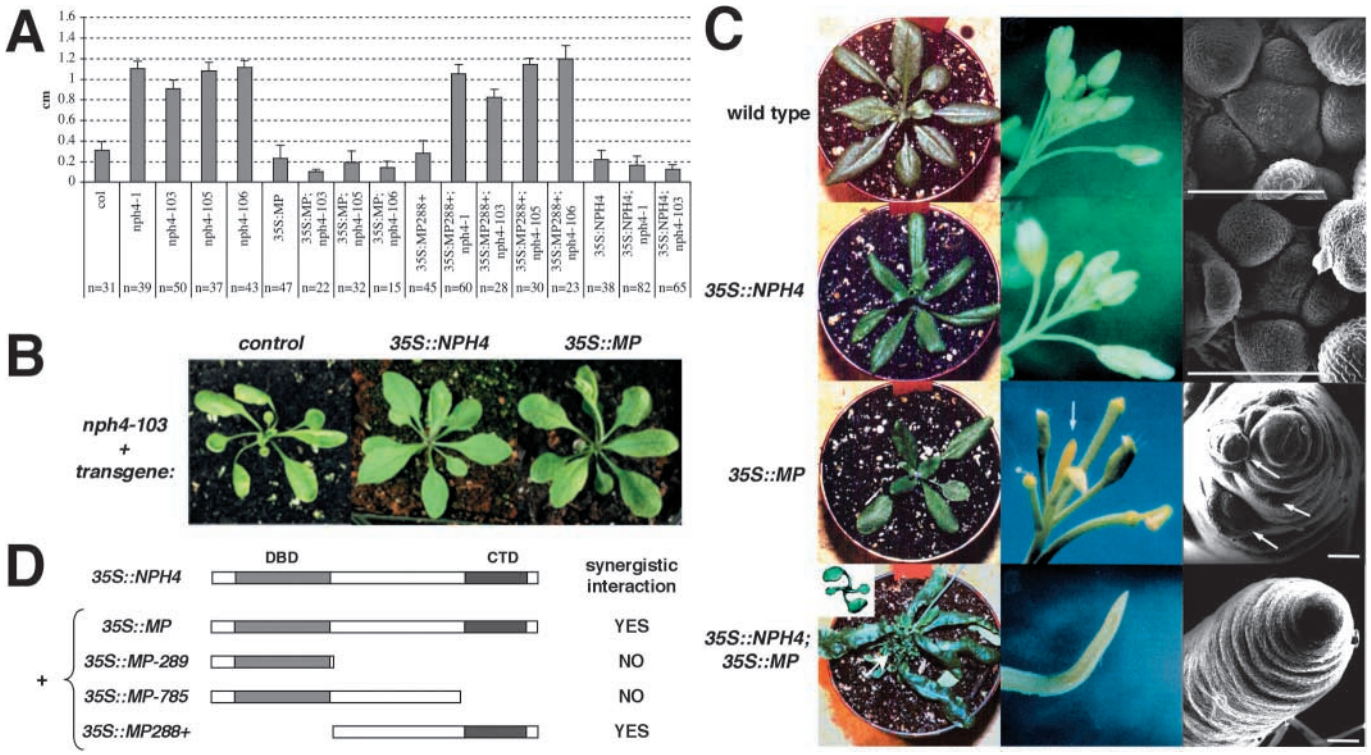

Fig. 5. Ubiquitous overexpression of *MP* and *NPH4*. (A) Hypocotyl length of seedlings of the indicated genotypes germinated and grown in the dark for 5 days in the presence of 20 μM IAA. Sample sizes are 15 to 82 seedlings. Columns represent the mean±s.d. Note that both *35S::NPH4* and *35S::MP* restore auxin sensitivity. (B) Normalization of the vegetative leaf shape defects (*nph4-103*, left) by expression of *35S::NPH4* (center) and *35S::MP* (right) in the *nph4-103* background. (C) Vegetative and inflorescence phenotypes of wild-type, *35S::MP*, *35S::NPH4* and *35S::NPH4; 35S::MP* plants. Left column contains rosettes at 21 dag, represented by the insert in the bottom panel, because of the delayed development of *35S::NPH4; 35S::MP* plants. Large rosette in this panel is at 35 dag and has bolted. Note the formation of extremely twisted leaves in *35S::NPH4; 35S::MP* plants of all stages and the appearance of numerous small leaves late in vegetative development (white arrow in *35S::NPH4; 35S::MP* rosette). Middle column indicates inflorescence tips at 40 dag. Inflorescences of *35S::NPH4* plants are indistinguishable from wild type, while inflorescences of *35S::MP* plants produce distorted, usually sterile, flowers and may terminate in pin-shaped inflorescence tips (white arrow). Inflorescences of *35S::NPH4; 35S::MP* plants do not produce any flowers. (Right column) Upper two images are scanning electron micrographs of pre-bolting inflorescence meristems. Note that there is no recognizable effect of *35S::NPH4* expression on meristem organization or size. (Lower two images) Post-bolting inflorescence meristems of *35S::NPH4; 35S::MP* plants invariantly lack flower primordia, which are produced at variable density in the inflorescence meristems of *35S::MP* plants (white arrows). Scale bars: 100 μm. (D) Synergistic phenotype effects associated with co-expression of *NPH4* together with regions of the *MP* coding sequence, both under control of the CaMV *35S* promoter. Synergistic effects are indistinguishable from the phenotype shown in the lower panel of C and were observed in all (yes) or none (no) of at least seven double overexpressing plants. Constructs refer to amino acid residues of the *MP*-coding sequence (Hardtke and Berleth, 1998): MP-289, residues 1-289; MP-785, residues 1-785; MP288+, residues 288-902.

type (Fig. 5A,B). Expression of *35S::MP* normalizes the defective vasculature in *mp* mutant vegetative leaves (data not shown).

Next, we tested whether high level expression of each gene could normalize the reciprocal mutant phenotype. While expression of the *35S::NPH4* gene fails to normalize vascular defects in *mp* mutants (data not shown), expression of the *35S::MP* gene results in highly auxin-responsive hypocotyl elongation and restoration of leaf morphology in *nph4* mutants (Fig. 5A,B). The capacity of MP to control hypocotyl cell elongation in the absence of NPH4 depends on the presence of a DBD in the overexpressed MP protein (Fig. 5A) and may therefore involve direct binding of MP to DNA.

We conclude that the joint requirement of both NPH4 and MP in the control of hypocotyl elongation in the wild-type auxin response as described above is dependent upon wild-type levels of *MP* expression. In *35S::MP* plants, by contrast, *NPH4* does not seem to be required either in the control of hypocotyl elongation or in the control of leaf shape.

Co-overexpression of MP and NPH4 results in synergistic effects

In vivo interactions of transcription factors may become apparent as synergistic phenotypes of gain-of-function alleles, for example, as a consequence of the physical association of specific DNA binding and activation domains. We expressed functional cDNAs of both *MP* and *NPH4* individually and in combination in transgenic plants and assessed their phenotypes in at least seven independent transformants (for details see Materials and methods).

Plants carrying the *35S::NPH4* transgene are indistinguishable from wild-type plants during both vegetative and reproductive stages of development (Fig. 5C). By contrast, upregulation of *MP* in *35S::MP* plants is associated with a number of abnormalities primarily in the inflorescences, which produce fewer, predominantly sterile flowers and eventually terminate in pin-shaped tips (Fig. 5C). This observation is interesting, because *35S::MP* plants have been shown to enhance auxin responses, whereas *mp* mutants display diminished auxin responses (Mattsson et al., 2003). The fact that both genotypes are associated with pin-shaped inflorescences suggests that lateral organ formation in the inflorescence meristem requires differential auxin signaling and is obstructed by constitutively elevated as well as reduced levels of auxin signaling. Most strikingly, co-overexpression of both *MP* and *NPH4* invariably results in new, extreme phenotype traits (16/16 plants derived from crosses between two individual primary transformants for each construct), which also severely affect vegetative development (Fig. 5C). In plants co-overexpressing both transgenes, the formation of rosette leaves is extremely delayed. Rosette leaves have hyponastic leaf blades and short petioles (Fig. 5C). Both leaf blades and petioles of rosette leaves are often twisted and very small irregular leaves are formed in older rosettes (Fig. 5C). Inflorescence meristems retain normal dimensions but never produce flower primordia, which results in exclusively pin-shaped inflorescences (Fig. 5C). Because of the normal phenotype of *35S::NPH4* plants, the new phenotype cannot be explained as the superimposition of defects, but as a synergistic enhancement of the effects associated with the expression of the individual transgenes.

We mapped the origin of the synergistic interaction of both transgenes by co-overexpressing under control of the CaMV 35S promoter regions of the *MP* cDNA along with the full-length *NPH4* cDNA in transgenic plants (Fig. 5D). Co-overexpression of NPH4 along with a fragment comprising the MP AD and CTD but excluding the DBD (*35S::MP288+*, amino acids 288-902), results in plants indistinguishable from those overexpressing both full-length products (9/9plants). By contrast, no phenotypic change is associated with the co-overexpression of NPH4 together with a large proportion of the MP-coding region encompassing the DBD and the AD (*35S::MP-785*, amino acids 1-785) (7/7plants). These results indicate that interaction of the MP-AD with overexpressed NPH4 is essential, while MP DNA binding is dispensible for generating synergistic phenotypic effects in plant growth. (see Fig.1 and Discussion).

MP overexpression suppresses the bdl mutant phenotype

Finally, we addressed the question whether there is genetic evidence in *Arabidopsis* plants for the negative interaction between an ARF and an Aux/IAA protein. Phenotype similarity has been observed between mutants carrying a gain-of-function mutation in *BDL/IAA12*, which is expected to lead to enhanced stability and presumably abundance of the protein product, and the phenotype of loss-of-function mutations in *MP*, suggesting that BDL could negatively regulate MP activity (Hamann et al., 2002). If overabundant *BDL* product inhibits MP function, this effect might be suppressed by compensatory *MP* overexpression. The *bdl* mutant is semi-dominant and homozygous mutants invariably display severe defects during vegetative and inflorescence development. These mutants are often also rootless and then cannot be grown on soil. To compare all genotypes under similar growth conditions, we used only homozygous *bdl* mutants that had formed roots (see Materials and methods). Rosettes of homozygous *bdl* mutants consist of small, curled leaves and are not larger than 20 mm in diameter (number of rosettes greater than 20 mm in size is 0/25). Inflorescences, if produced at all (12/25), are extremely short and do not produce fertile flowers (fertile plants: 0/25) (Fig. 6A). By contrast, homozygous *bdl* mutants carrying one or two copies of the *35S::MP* transgene are typically of normal morphology, form large rosettes (number of rosettes greater than 60 mm is 110/129) and are usually fertile (94/129; Fig. 6B,D). The latter aspect is particularly interesting, because it demonstrates mutual suppression of *bdl* and *35S::MP*, as inflorescences of *35S::MP* plants in wild-type background are semi-sterile and phenotypically abnormal (Fig. 6C). The mutual suppression of two gain-of-function genetic disorders as opposed to the superimposition of their phenotypic defects is remarkable as it indicates a highly specific antagonistic interaction of both proteins in a variety of developmental contexts (see Discussion).

Discussion

The *Arabidopsis* ARF family (22 genes) and the family of Aux/IAA nuclear co-regulators (29 genes) can serve as examples for the difficulties associated with assigning biological roles to individual transcriptional regulators within large families of potentially redundantly acting proteins. Only

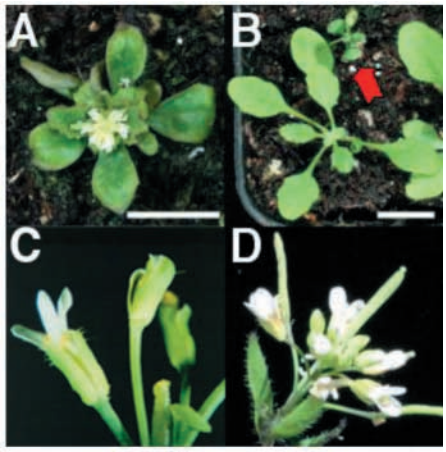

Fig. 6. Mutual suppression of *35S::MP* and *bdl*. (A) Homozygous *bdl* mutant grown in soil for 5 weeks. The rosette diameter is ~20 mm and a short inflorescence has been produced. (B) Homozygous *bdl* mutant (red arrow) and homozygous *bdl* mutant hemizygously carrying the *35S::MP* transgene (bottom left) grown in soil for 3 weeks. (C) Inflorescence tip of a hemizygous *35S::MP* plant. (D) Inflorescence tip of a homozygous *bdl* mutant hemizygously carrying the *35S::MP* transgene. Scale bars: 1 cm.

two out of 20 canonical ARFs (comprising DBD, activation or repression domain, and CTD), MP/ARF5 and NPH4/ARF7, are defined by mutation and *Aux/IAA* gene functions have nearly exclusively been deduced from gain-of-function alleles (Liscum and Reed, 2002; Reed, 2001). Given the size of both gene families, huge systematic efforts will be required to gradually elucidate the in vivo functions of these genes. In this study, we have employed interaction and expression assays as well as an expanded genetic analysis involving multiple loss- and gain-of-function genotypes to learn more about the roles of *MP* and *NPH4* in *Arabidopsis* development. We demonstrate selective self and inter se interactions of both proteins in yeast two hybrid assays. These are matched by unexpectedly similar biological roles, which had been obscured by overlap of both gene functions and by the early lethality of previously characterized *mp* mutants. We found that both genes act redundantly in oriented cell differentiation and jointly control auxin-responsive cell expansion. When ubiquitously expressed, however, *MP* can substitute for *NPH4* activity even in those processes where *NPH4* is normally strictly required. We finally found genetic support for antagonistic interaction between an ARF and an Aux/IAA protein at various stages of development.

MP and NPH4 functions in axial patterning

Auxin signals have been genetically implicated in the formation of the apicobasal axis of the embryo (reviewed by Juergens, 2001; Paquette and Benfey, 2001; Berleth and Chatfield, 2002), the organized outgrowth of lateral organs (Reinhardt et al., 2000), cell patterning in the root meristem (reviewed by Scheres, 2000) and continuous vascular differentiation (reviewed by Sachs, 1991). These four patterning processes, to which we refer here as axial patterning, seem to have a common derivation in defective responses to polar auxin signals and are collectively disturbed in previously characterized auxin-insensitive embryo mutants (Hardtke and Berleth, 1998; Hamann et al., 1999; Hobbie et al., 2000). The overlap of *MP* and *NPH4* gene functions in axial patterning is asymmetrical. Both genes have the capacity to relay auxin signals that are required for proper patterning at early stages of organ development, but the corresponding regulatory potential of *NPH4* is masked in the presence of *MP* wild-type activity. The phenotype distributions in all allelic combinations show that the contributions of the *MP* and *NPH4* genes to embryo patterning differ quantitatively, but clearly affect the same process. No new phenotype classes are observed in double mutants. Instead, the spectra of phenotypes of all *mp* mutant alleles are shifted towards more severe distributions, when analyzed in double mutant combination with *nph4* mutants. With the strong *mp; nph4* double mutant, we describe a genotype in which all four processes are nearly obstructed. Moreover, this genotype is associated with a remarkably invariant seedling phenotype, indicating that residual axial patterning and phenotype variability in strong *mp* mutants is largely due to *NPH4* activity.

The nearly obstructed axial differentiation in *mp; nph4* double mutants leaves very little room for potential further redundant functions of other ARF genes in embryo axis formation. Their contribution could be extremely subtle. Alternatively, they could act non-redundantly with associated strong phenotypes, which for some reason have not been identified in saturating screens for seedling pattern mutants (Mayer et al., 1991). Finally, the functions of most other ARFs could be restricted to stage-specific processes rather than affecting general patterning properties of plant cells.

MP and NPH4 functions in auxin-responsive cell expansion

Although *NPH4* acts redundantly with *MP* in vascular development, *NPH4* has non-redundant functions in other processes, such as the regulation of auxin-inducible genes or in auxin-controlled cotyledon and hypocotyl expansion. Interestingly, the fact that *MP* cannot substitute for *NPH4* activity in these processes does not mean that *MP* is not involved in their regulation. Instead, MP and NPH4 similarly regulate a number of auxin-inducible genes and cell expansion processes. The joint requirement for both MP and NPH4 (Fig. 4), in conjunction with evidence for physical interaction between the two proteins in yeast cells (Fig. 1), could reflect their integration into heteromeric complexes regulating these auxin responses.

It is important to recognize that the evidence for a joint requirement of *MP* and *NPH4* in the control of cell expansion is entirely based on loss-of-function data and therefore reflects the genuine activities of both genes. Observations in gain-of-function genotypes confirm the potential of *MP* to function in auxin-controlled cell expansion, but further show that, when overexpressed, *MP* acquires the capacity to autonomously control a process that it normally controls in conjunction with *NPH4*. In normal development, therefore, the level of *MP* expression must be strictly limited to provide a role for *NPH4*. As the ARF gene family seems to be remarkably conserved (Sato et al., 2001), it will be interesting to see whether the same functional relationship between the two genes is observed in other species. Evolutionary conserved dimerization of MP and NPH4 in cell expansion responses could make regulatory

sense. In a possible analogy to the recently proposed 'potentiation' mechanism (Tiwari et al., 2003), MP and NPH4 could form a heterodimer, in which the obligatory involvement of NPH4 would allow for certain regulatory inputs, while MP could potentiate the auxin response through its strong, autonomous gene activation properties. Several observations suggest a strong, autonomous gene activation capacity of MP. First, *MP* overexpression leads to enhanced auxin-responsive gene expression of downstream genes, suggesting that the *MP* product is the limiting factor in their regulation (Mattsson et al., 2003). Second, *MP* overexpression, but not *NPH4* overexpression leads to morphological abnormalities. Third, overexpression of only the MP AD-CTD along with NPH4 leads to dramatic morphological abnormalities, suggesting that MP AD, when associated with NPH4 can trigger gene expression that is not triggered by NPH4 overexpression alone.

Interaction with Aux/IAA genes

ARF activity is believed to be negatively regulated by interaction with nuclear co-regulators of the Aux/IAA class (reviewed by Hagen and Guilfoyle, 2002; Liscum and Reed, 2002; Leyser, 2002). Mutations in *Aux/IAA* genes with marked phenotypes are usually dominant mutations in the distinct, conserved domain II, and as far as investigated in detail are associated with increased protein stability and thereby increased abundance of the gene product (Worley et al., 2000; Ouellet et al., 2001). Presently, it is not clear to what extent these dominant mutations reflect the wild-type activities of the respective genes. A domain II gain-of-function mutation in a single *Aux/IAA* gene, *BDL/IAA12*, has been reported to interfere with pattern formation in the early embryo (Hamann et al., 1999). This may indicate that *BDL* negatively regulates *MP* or genes in the same pathway, and it has recently been demonstrated that BDL interacts with MP in yeast (Hamann et al., 2002). Experimentally confirmed interaction, however, leaves unresolved the issue of whether *MP* and *BDL* interact antagonistically throughout development and whether *MP* is a predominant target of negative regulation by *BDL* at all stages. The fact that the elevated expression of MP combined with the expression of mutant, probably overabundant BDL protein, leads to a striking restoration of normal morphology documents that both proteins interact with remarkable precision at several stages during *Arabidopsis* development. The restoration of normal morphology in the seedling, in vegetative development and in the complex organization of the inflorescence is not what one would normally expect as the result of the superimposition of two gain-of-function genetic distortions, because it implies the exact compensation of the abnormal regulatory influences in many processes, suggesting that the antagonism of both proteins is part of their normal function.

How can this precise compensatory interaction of *MP* and *BDL* (Fig. 6) be reconciled with the apparent lack of selectivity in ARF-Aux/IAA interactions (Fig. 1B)? One possibility is that the selectivity of interaction in the phenotypically relevant sites in the plant is higher than in the transient expression system. Alternatively or additionally, the interaction may in fact not be exclusive. MP may interact with several Aux/IAA proteins and BDL with several ARF proteins. However, all interactions would involve only *ARF* and *Aux/IAA* genes that act redundantly during the patterning stages early in organ development. For example, BDL may negatively interact also with NPH4 in embryo patterning, which would become apparent only under conditions of reduced MP activity. This seems to be indeed the case, as *mp; bdl* double mutants have been reported to cause an enhancement of the *mp* phenotype, including the formation of cotyledon-less seedlings (Hamann et al., 1999). These defects are reminiscent of those in *mp; nph4* double mutants, consistent with a negative regulation of NPH4 by BDL (Fig. 1). It remains to be seen how many Aux/IAA proteins are expressed in early organ primordia, but there is the possibility that many or all of those could interfere with axial patterning as interchangeable, purely quantitative regulators of *MP* and *NPH4* activity.

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