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The AP2 transcription factors DORNRÖSCHEN and DORNRÖSCHEN-LIKE redundantly control *Arabidopsis* embryo patterning via interaction with PHAVOLUTA

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DORNRÖSCHEN (*DRN*) (also known as *ENHANCER OF SHOOT REGENERATION1*; *ESR1*) and *DRN-LIKE* (*DRNL*; also known as *ESR2*) are two linked paralogues encoding AP2 domain-containing proteins. *drn* mutants show embryo cell patterning defects and, similarly to *drnl* mutants, disrupt cotyledon development at incomplete penetrance. *drn drnl* double mutants with weak or strong *drnl* alleles show more highly penetrant and extreme phenotypes, including a *pin*-like embryo without cotyledons, confirming a high degree of functional redundancy for the two genes in embryo patterning. Altered expression of PIN1::PIN1-GFP and DR5::GFP in *drn* mutant embryos places *DRN* upstream of auxin transport and response. A yeast two-hybrid screen with DRN followed by co-immunoprecipitation and bimolecular fluorescence complementation revealed PHAVOLUTA (PHV) to be a protein interaction partner in planta. *drn phv* double mutants show an increased penetrance of embryo cell division defects. DRNL can also interact with PHV and both DRN and DRNL can heterodimerise with additional members of the class III HD-ZIP family, PHABULOSA, REVOLUTA, CORONA and ATHB8. Interactions involve the PAS-like C-terminal regions of these proteins and the DRN/DRNL AP2 domain.

KEY WORDS: *Arabidopsis*, Cotyledon, Embryo

INTRODUCTION

Angiosperm growth results from two developmental growth phases: an embryonic phase whereby the primary body plan is established together with the apical-basal and radial axes of symmetry, and a postembryonic phase, in which new organs are initiated via the shoot and root meristems. Cotyledon development during dicot embryogenesis marks the start of organogenesis and the change from radial to bilateral symmetry at the transition from the globular- to heart-stage embryo. Studies of seedling-lethal *Arabidopsis* mutants have defined three embryonic domains wherein subsequent cell fate is largely determined by position in the embryo (Mayer et al., 1991): (1) the majority of the cotyledons, together with the shoot apical meristem, are derived from the apical domain, recognisable as the derivatives of cell divisions of the upper tier of cells at the octant stage; (2) the central domain mainly gives rise to the hypocotyl and root apical initials; and (3) the basal region, derived from the hypophysis, generates the root quiescent centre (Harada, 1999).

Several *Arabidopsis* genes are important for the development of the apical region of the embryo and give rise to cotyledon phenotypes when mutated. Combined mutations at the *CUP-SHAPED COLYLEDON* (*CUC1*, *2* and *3*) loci result in cotyledon fusion accompanied by an absence of the shoot apical meristem (SAM) (Aida et al., 1997; Vroemen et al., 2003; Hibara et al., 2006). Polar auxin transport essentially contributes to the establishment of both bilateral symmetry (Liu et al., 1993) and apical-basal polarity (Friml et al., 2003), and an abnormal cotyledon number at low penetrance is also observed in plant hormone response mutants (Rashotte et al., 2006; Saibo et al., 2006). In *Arabidopsis*, auxin polarity and embryonic patterning have been extensively studied in mutants of *PIN* gene family members that encode plant-specific proteins involved in auxin efflux. PIN proteins are functionally redundant, and higher-order *pin* mutants reveal defects in embryonic cell division patterns that are reflected postembryonically mostly in cotyledon defects such as fusion or monocotyledony (Friml et al., 2003; Furutani et al., 2004; Vieten et al., 2005). Additionally, *pinoid*, a mutant in a serine/threonine protein kinase that affects localisation of PIN proteins (Friml et al., 2004), or mutations in *MONOPTEROS* (*MP*) and *BODENLOS* (*BDL*), which encode the auxin responsive factor ARF5 and its inhibitor IAA12, respectively, all cause cotyledon defects and/or disrupted embryo domains (Bennet et al., 1995; Hardtke and Berleth, 1998; Hamann et al., 1999). A general feature of auxin signalling and *cuc* mutants is the incomplete penetrance of cotyledon defects, suggesting that pathways leading to bilateral symmetry and cotyledon establishment are considerably redundant.

Redundant control of embryo patterning is also demonstrated by the *Arabidopsis* class III HD-ZIP gene family. *PHAVOLUTA* (*PHV*) and *PHABULOSA* (*PHB*) are well-known representatives of this HD-ZIP subclass and were identified as dominant gain-of-function alleles due to mutations in a highly conserved microRNA target site (McConnell et al., 2001; Bao et al., 2004). Amongst this family, only *REVOLUTA* (*REV*) has a loss-of-function phenotype (Talbert et al., 1995); however, higher-order knockouts reveal redundant functions in embryo and cotyledon patterning (Prigge et al., 2005).

The *DORNRÖSCHEN* (*DRN*) (also known as *ENHANCER OF SHOOT REGENERATION1; ESR1*) gene contributes to *Arabidopsis* meristem organisation (Kirch et al., 2003) and cytokinin-independent shoot regeneration (Banno et al., 2001). *DRN* expression is highly dynamic and is observable from the two- to four-cell stage in the embryo proper, before focussing to the emerging cotyledons and becoming restricted to the SAM at the torpedo stage. During postembryonic development, *DRN* remains

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detectable in the L1 layer of the SAM, from where expression extends into emerging lateral organs (Kirch et al., 2003). A paralogous gene, *DRNL*, is linked to *DRN* on chromosome 1 and has also been named *ESR2* (Ikeda et al., 2006), *SOB2* (Ward et al., 2006) and *BOLITA* (Marsch-Martinez et al., 2006). To elucidate further the role of *DRN* and *DRNL* in *Arabidopsis* development, we characterised loss-of-function mutants and, following a yeast two-hybrid screen with DRN, established that both proteins are capable of heterodimerising with members of the class III HD-ZIP family. In view of the highly redundant control of early embryo patterning involving multiple independent gene pathways, we show here that *DRN* and *DRNL* are two additional factors that control *Arabidopsis* embryogenesis. *DRN* not only acts upstream of auxin polar transport and response, but also functions redundantly with *DRNL* and interacts with *PHV* in planta.

MATERIALS AND METHODS

drn and *drnl* insertion mutants

Two independent insertion mutants in the *DRN* gene (At1g12980) containing a *dSpm* element were obtained from the SLAT lines in Columbia (Tissier et al., 1999): *drn-1* (Kirch et al., 2003) and *drn-2* (SM_3.35017). For the *DRNL* gene (At1g24590), a *drnl-1* mutant in L*er* was identified from an enhancer-inhibitor insertional mutagenesis screen as line ITS75 (Speulman et al., 1999); the *drnl-2* EMS allele in L*er* was a gift from T. Jack (Dartmouth College, Hanover, NH). An insertion line (899_CO2) for the *PHV* gene (At1g30490) was obtained from the SAIL population (Sessions et al., 2002). All gene insertions were confirmed by PCR (primers DRNF and Spm8 for *DRN*; DRNLF and ITIR3 for *DRNL*; PHVIntR and LB3 for *PHV*; Table 1) followed by sequencing of the amplicons. Homozygous mutants were confirmed by the absence of the wild-type gene using primers flanking the insertion (DRNF and DRNR, DRNLF and DRNLR, or PHV and PHVIntR). For *drnl-2*, homozygosity was confirmed using a dCAPS marker with primers DRNLCAPS1 and DRNLCAPS2, which give a wild-type amplicon of 184 bp that is cleaved in the mutant by *Acc*I into two fragments of 159 and 25 bp. *drn-1* and *drnl-1* mutant lines were back-crossed three times.

Plants were cultivated on soil in the greenhouse or in sterile culture on 0.5×MS medium supplemented with 1% sucrose under long-day conditions (16 hours light, 8 hours dark) at 22°C.

Histology and in situ hybridisations

Cotyledons were cleared with cold acetone for 20 minutes and decolourised with 100% ethanol overnight. Ovules were dissected from siliques and cleared overnight with Hoyers Solution (2.5 g gum arabic, 100 g chloral hydrate, 5 ml glycerol and 30 ml water). Microscopy was performed using a Zeiss Axiophot microscope equipped with an Axiocam HR CCD camera using differential interference contrast optics.

Non-radioactive in situ hybridisations and the preparation of dioxygenin-labelled RNA probes by T7 RNA polymerase essentially followed the protocols of Kirch et al. (Kirch et al., 2003) or Bradley et al. (Bradley et al., 1993). Probes were as follows: for *DRN*, from nucleotide +377 (relative to the ATG at +1) to the stop codon and including 78 bp of the 3 UTR; for *DRNL*, nucleotide +348 to the stop codon; and for *PHV*, from nucleotide +1585 to +2475.

Confocal imaging

The *DR5::GFPer* and *pPIN1::PIN1:GFP* reporter lines (gifts from J. Friml, ZMBP, Tübingen, Germany) were crossed into the *drn-1* mutant background. Homozygous *drn-1* plants harbouring the *DR5::GFPer* construct or segregating F_2 *drn-1* embryos from a cross between *drn-1* and a *pPIN1::PIN1:GFP* line were monitored for *GFP* expression using a Leica confocal microscope.

Yeast two-hybrid screen

The construction of a meristem-enriched cDNA library has been described by Cole et al. (Cole et al., 2006). As bait, 348 bp of the *DRN* open reading frame (ORF) encoding the N-terminal 116 amino acids of the DRN protein was amplified by PCR, sequenced, directionally cloned into pGBKT7 (Clontech) using *Nco*I and *Bam*HI, and transformed into the yeast strain Y187 (Clontech Palo Alto, CA). The two-hybrid screen was performed by yeast mating, according to the manufacturer's protocol (Clontech PT3024-1), and with quadruple selection (β-glucuronidase, –Leu, –Trp, –Ade).

Table 1. Oligonucleotide primers used for genotyping mutants

Primer	Sequence (5′-3′)
DRNLF	ATGGAAGAAGCAATCATGAGA
DRNLIntR	AACATTCCACCATTTCCGTTC
ITIR3	CTTGCCTTTTTTCTTGTAGTG
DRNF	ATGGAAAAAGCCTTGAGAAAC
DRNR	CTATCCCCACGATCTTCGGCA
Spm8	GTTTTGGCCGACACTCCTTAC
PHVF	ATGATGGCTCATCACTCCATG
PHVIntR	AAGTTTCAAAAGCTTAACAAT
LB3	TAGCATCTGAATTTCATAACCATCTCGATACAC
DRNLCAPS1	TACCGCAAAAGCTGCCTC
DRNLCAPS2	AGCGGCGCAGTCATATGCGCAGTCT

In planta bimolecular fluorescence complementation (BiFC)

The ORFs encoding full-length DRN or DRNL and PHVs (the C-terminal 451 amino acids from amino acid 391 to the end) were cloned in-frame into the *Bam*HI site of pUC-SPYCE or pUC-SPYNE (Walter et al., 2004). For control experiments, GFP fusions were created in the pRT-Ω*Not*I/*Asc*I vector (Überlacker and Werr, 1996). Transient expression in leek epidermal cells was performed according to Cole et al. (Cole et al., 2006). YFP/GFP fluorescence was visualised using a MZFLIII stereomicroscope (Leica) after UV excitation and using a GFP filter. All images were processed using Photoshop software (Adobe).

Co-immunoprecipitation and western blot analysis

Epitope-tagged proteins or peptides were synthesised via the EasyXpress in vitro transcription/translation system (Qiagen) based on the T7 promoter. Templates of the required proteins were obtained via nested PCR reactions on the respective gene ORFs in pUC-SPYCE/NE containing an HA or myc epitope-coding sequence preceding the YFP subdomains. The T7 promoter and 6xHis tag were added to *DRN* and *DRNL* coding regions by nested PCR using primers from the EasyXpress Kit. For co-expression, the relevant amplicons were mixed. Control reactions were performed with single amplicons. Immunoprecipitation (IP), gel electrophoretic analyses and detection of epitope-tagged proteins essentially followed the protocols of Cole et al. (Cole et al., 2006). Peroxidase activity was detected via chemiluminescence and documented on Kodak X-Omat AR film. C-terminal fragments of class III HD-ZIP proteins, the PAS-like domain and the AP2 domain and were amplified by PCR from cDNA derived from various plant tissues, and epitope-coding sequences or the T7 promoter were added by nested PCR using the EasyXpress Kit. Polypeptide termini: PHV, amino acid 624 to end; PHB, amino acid 636 to end; REV, amino acid 644 to end; CNA, amino acid 618 to end; AtHB8, amino acid 619 to end; and PHV PAS-like domain, amino acids 721 to 792. The AP2 domain of DRN and DRNL extended from amino acids 54 and 55 to 115 and 116, respectively.

RESULTS

drn drnl and *phv* mutants

The position of the insertion in the *drn-1* allele is after nucleotide +327 (relative to the ATG) of the *DRN* gene, within the AP2-domain coding region. For *drn-2*, the insertion is after nucleotide +7 of the *DRN* ORF, and for *drnl-1* it is after nucleotide +777. The *drnl-2* allele has a base substitution from C to T at position +278, resulting in an A to V substitution at amino acid 93. This conserved A residue has recently been shown in *Brassica napus* ERF/AP2 proteins to be essential for DNA binding (Liu et al., 2006), suggesting that the *drnl-2* AP2 domain is unable to bind target genes. For the *phv* 899_CO2 allele, the insertion is after nucleotide +84.

Table 2. Phenotypes of *drn*, *drnl*, *phv* and *drn drnl* double mutants

	Wild type	Single or two fused cotyledons	Tricots and various fusions	Cup-shaped cotyledons	*mp*-like phenotype	*pin* embryo phenotype	Percentage phenotype	Embryo cell division defects*
drn-1	1804	92	13	2	15	0	6.34	48/100 (48.0%)
drn-2	2229	53	6	1	4	0	2.79	
drnl-1	3408	44	3	0	0	0	1.36	0/69 (0.00%)
drnl-2	609	46	0	0	0	0	7.6	
phv	317	0	0	0	0	0	0	0/35 (0.00%)
drn-1 phv	2041	138	0	0	28	0	7.55	54/57 (94.7%)
drn-1 drnl-1	718†	271†	0	0	398†	0	48.24	90/96 (93.8%)
drn-1 drnl-2/DRNL‡	492	42	2	0	0	192	28.07	

*Embryo cell division defects refers to the proportion of embryos from cleared ovules showing cellular defects from globular stage (>32 cells) to early heart stage.
†Values are pooled for progeny of eight individual double mutants.
‡*drn-1 drnl-2* double mutant plants are sterile.

drn and *drnl* single mutants are affected in embryonic patterning and cotyledon organogenesis

The major phenotype of the *drn* mutant is abnormal cell division, observable from the globular embryo stage onwards (Table 2). The wild-type globular embryo contacts the single file of cells known as the suspensor through the hypophysis, which divides asymmetrically in the globular embryo to give: (1) an upper lens-shaped cell (see Fig. 1A,B), which is the progenitor of the quiescent centre; and (2) a subtending daughter cell, which will generate the columella stem cells. It is at the transition from globular to heart stage that cell divisions parallel to the surface at lateral marginal positions give rise to the emerging cotyledon lobes (West and Harada, 1993). *drnl* mutants showed no embryo development defects. By contrast, approximately half (48.0%) of homozygous *drn-1* embryos exhibited a phenotype consisting of abnormal development in the hypophysis region, or no obvious distinction between embryo proper and suspensor. In a few percent of *drn* mutants, the hypophyseal cell divided periclinally at an early stage (Fig. 1C), resulting in the absence of the lens-shaped cell and a subsequent haphazard cellular organisation of this region (Fig. 1D,F). At a similar frequency, suspensors of *drn* mutants had double or triple cell files (Fig. 1E), presumably resulting from supernumerary cell divisions perpendicular to the normal plane of division. Both *drn* and *drnl* single mutants showed defective cotyledon development phenotypes at incomplete penetrance, including monocotyledonous seedlings, seedlings with partially fused cotyledons, tricots or various tricot fusion combinations (Table 2 and Fig. 1G-I). Completely fused or cup-shaped cotyledons were occasionally observed (<1%) in both *drn* mutant alleles, and these plants produced a functional SAM and developed normally (Table 2 and Fig. 1J,K). Also rare (<1%) were plants with a single cotyledon-like structure, no hypocotyl and a rudimentary root (Fig. 1L,M), reminiscent of *mp* (Berleth and Jürgens, 1993) and *bdl* (Hamann et al., 1999) seedlings, which are mutants in *ARF5* (Hardtke and Berleth, 1998) and its *IAA12* partner protein (Hamann et al., 2002). The lower penetrance in *drn* mutants of cotyledon defects as compared with cell division defects indicates that embryo cell patterning defects are not manifested in post-germination development.

Inappropriate cotyledon development in *drn* or *drnl* mutants resulted in subsequent alterations of leaf phyllotaxy: a single leaf initiated opposite single fused cotyledons (Fig. 1N), or, in the case of tricots, three leaf primordia initiated between the cotyledons (Fig. 1O). Cleared monocotyledonous *drn-1* and *drnl-1* cotyledons either showed a wild-type vasculature pattern (Fig. 1P) with a single mid-vein (Fig. 1Q), or two or more mid-veins (Fig. 1R), showing that the cotyledon derived from either a single cotyledon primordium or from the fusion of two discrete primordia. Both alternatives suggest the improper recruitment of cells into developing cotyledon primordia, and the improper establishment or maintenance of organ boundaries during organogenesis.

In view of the *drn* embryo mutant phenotype, we reinvestigated the *drn-D* overexpression mutant (Kirch et al., 2003) for more subtle phenotypes. The main phenotype is an enlarged SAM and vegetative meristem arrest coupled with the initiation of radialised leaves (Kirch et al., 2003). However, ectopic *DRN* expression also affects embryonic development; polycotyledony was occasionally observed (>1%; see Fig. S1A in the supplementary material). Postembryonic developmental phenotypes were also observed, including the fusion of leaf margins, fusion between stems and leaves and variable floral organ number (>2-3% of plants; see Fig. S1B-E in the supplementary material), implying that *DRN* contributes to organogenesis and patterning throughout development.

DRN and *DRNL* are functionally redundant

The incomplete penetrance and similarity of *drn* and *drnl* single-mutant cotyledon phenotypes suggest redundant gene functions. We therefore created double mutants between *drn-1* and either *drnl-1* or the stronger allele *drnl-2*. Embryonic cell patterning defects were observed in essentially all (90 of 96) *drn-1 drnl-1* double-mutant embryos (Fig. 1S,T), considerably higher than the frequency for *drn-1* single-mutant embryos (Table 2). *drn-1 drnl-1* double mutants also showed pleiotropic cotyledon phenotypes, including those observed in the single mutants (Table 2). However, the most significant feature of the *drn-1 drnl-1* double mutant was a large increase in phenotypic penetrance: the *mp*-like phenotype was observed in 20% of mutants, and cotyledon defects in 30%, raising the total penetrance of plants with a phenotype to about 50% (Table 2). Double-homozygous *drn-1 drnl-2* plants are sterile, but approximately a quarter of the progeny of *drn-1 drnl-2/DRNL* plants had *pin*-like embryos, with a complete absence of cotyledons (Fig. 1U). These plants were genotyped as double-homozygous mutants and directly initiated leaves from a functional SAM (Fig. 1V).

Expression patterns of *DRN* and *DRNL*

In view of the genetic redundancy observed between *DRN* and *DRNL*, we investigated whether the expression patterns of these genes overlapped in *Arabidopsis* embryos using RNA in situ hybridisation. *DRN* is expressed from the four-cell stage (Kirch et al., 2003) and throughout the globular embryo (Fig. 2A). At the transition stage, *DRN* expression was localised to the apical cell tiers (Fig. 2B), and throughout the heart stage it became increasingly restricted to the lobes of the developing cotyledons (Fig. 2C). From the mature heart stage throughout the torpedo

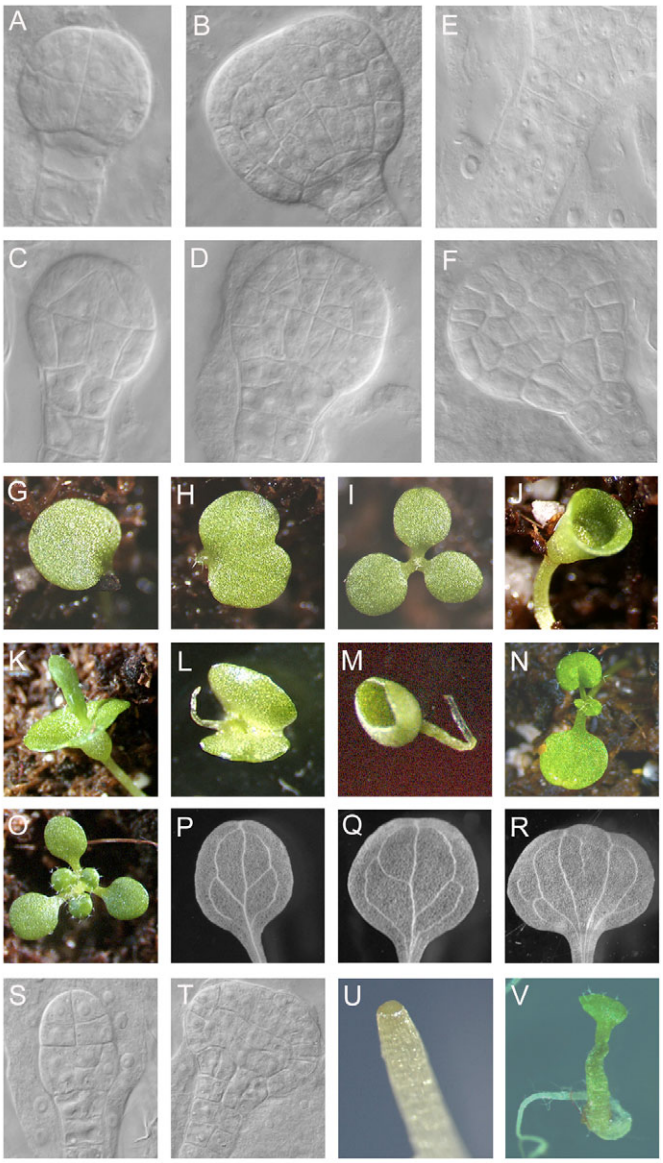

Fig. 1. *drn* mutant and *drn drnl* double-mutant phenotypes. Wild-type embryos at early (**A**) and late (**B**) globular stages. Abnormal hypophyseal cell divisions in *drn-1* mutant embryos at early (**C**) and late (**D**) globular stages. (**E**) Multiple cell files in the suspensor of a *drn1* mutant; the embryo proper is towards the top. (**F**) *drn-1* mutants lacking basal embryo organisation. (**G-K**) Pleiotropy of cotyledon phenotypes for the *drn-1* mutant showing mono- (G) and polycotyledony (I), cotyledon fusion (H) and cup-shaped cotyledons (J). The SAM is active despite complete cotyledon fusion (K). (**L**,**M**) *mp*-like phenotype of *drn* (L) and *drn-1 drnl-1* (M) mutants. (**N**,**O**) Altered phyllotaxis of first leaves in monocotyledonous (N) or polycotyledonous (O) *drn-1* mutants. (**P-R**) Cleared cotyledons of wild type (P) and a monocotyledonous *drn-1* mutant showing single (Q) or fused cotyledon (R) ontogeny. (**S**,**T**) *drn-1 drnl-1* double-mutant embryos showing asymmetric cell divisions at early (S) or late (T) globular stages. (**U**,**V**) *pin*-like hypocotyl of *drn-1 drnl-2* double-mutant plant lacking cotyledons (U) and directly producing leaves as evidenced by trichome formation (V).

stage and until embryo maturity, *DRN* expression was confined to the SAM (Fig. 2D). *DRNL* expression began in the early globular embryo (Fig. 2E) and was then restricted to the apical portion of the globular embryo (Fig. 2F). During the heart stage, the *DRNL* expression domain pre-patterned that of the emerging cotyledons, and at the elaborated heart stage, transcripts were confined to the sub-epidermal cells at the tip of the cotyledons (Fig. 2G); *DRNL* transcription ceased after the heart stage (Fig. 2H). These data show that the expression of *DRN* and *DRNL* overlap in the apical hemisphere of the globular-stage embryo and in sub-epidermal cells of the developing cotyledons.

DRN affects auxin responsiveness and expression in the embryo

The anomaly between the expression domains of *DRN* and *DRNL* in the central and apical embryo region and in the hypophysis region, where defective cell division phenotypes are observed in *drn-1* and *drn-1 drnl-1* mutants, suggests that both genes have non cell-autonomous functions. Although movement of DRN and DRNL proteins cannot be excluded, given the similarities between *drn* and *drnl* mutant phenotypes and those of many auxin-signalling mutants, auxin is a good candidate for mediating *DRN* and *DRNL* function. To test this, we crossed the *DR5::GFP* reporter construct, which indirectly measures auxin distribution and response, into the *drn-1* mutant background. In wild type, a *DR5::GFP* maximum was established in the hypophysis and upper suspensor cell at about the 32-cell stage (Fig. 2I). In *drn-1* mutants, *DR5::GFP* showed a more centralised, diffuse expression (Fig. 2J), and a subsequent maximum in the basal domain often appeared that was asymmetrical and not confined to the hypophysis and upper suspensor cells (Fig. 2K). In wild-type heart-stage embryos, *DR5::GFP* maxima were observed at the base of the embryo and at the tips of the developing cotyledons in the L1 cell layer (Fig. 2L). Heart-stage *drn* embryos showed either a similar *DR5::GFP* maximum to that of wild type in normal cotyledons, or an absence of expression in phenotypically abnormal cotyledons (Fig. 2M). The abnormal *DR5::GFP* expression pattern in *drn* mutants demonstrates that *DRN* functions upstream of auxin-mediated responses necessary for root and cotyledon specification.

We used PIN1 as an additional marker to address auxin transport in the *drn* mutant embryo. We analysed F_2 embryos from a cross between *drn* and a *PIN1::PIN1-GFP* transgenic line, segregating *drn* and wild-type embryos and allowing a simultaneous comparison of PIN1 expression to be made in both genotypes. From a total of 149 embryos analysed, 21 showed a *drn* mutant phenotype. At about the 32-cell stage, PIN1 in the embryo centre was laterally localised, whereas in the wild type it was basally localised (compare Fig. 2N with 2O). Slightly later, the disorganised cells in the hypophysis region of the *drn* mutant also expressed PIN1 with a variable cellular localisation, including significant lateral concentration, whereas in the wild type the distribution was basal (compare Fig. 2P with 2Q). The altered expression of both *DR5::GFP* and *PIN1::PIN1-GFP* in the *drn* mutant unequivocally places *DRN* function upstream of auxin transport and response in the early embryo.

PHAVOLUTA (PHV) is an interaction partner of DRN

To elucidate further the role of DRN in embryo development, we isolated putative interacting protein partners in a yeast two-hybrid screen using the N-terminal 116 amino acids of the DRN protein, including the AP2 domain, as bait. The C-terminal part of the DRN protein was excluded as it demonstrated weak auto-activation activity. Amongst 177 positive clones sequenced from a total of 1576 following quadruple selection for Trp, Leu, Ade and the β-glucuronidase reporter gene, were sequences encoding several kinases, metabolic enzymes and proteins of unknown function. Since DRN belongs to a plant-specific family of transcription factors, we

focussed our analyses on potential transcription factor partners. Of note was PHV (AtHB9; At1g30490), which was independently isolated eight times, based on cDNA termini sequences. All isolated PHV clones encoded C-terminal parts of the protein, extending maximally from amino acid 754 to the last amino acid, 841.

The *PHV* expression pattern is described elsewhere (Prigge et al., 2005), but to support the functional significance of the putative protein-protein interactions between *PHV* and *DRN* or *DRNL*, we demonstrated that *PHV* is temporally and spatially co-expressed with *DRN* and *DRNL* in the proembryo, and is concentrated apically in the developing globular embryo before becoming localised to the adaxial side of the developing cotyledons (Fig. 2R-U).

We used two methods to substantiate the affinity of the protein-protein interaction between DRN and PHV shown by the yeast two-hybrid screen. Firstly, we performed co-immunoprecipitation (CoIP) experiments using epitope-tagged full-length DRN, DRNL and PHV. These results (Fig. 3A,B) confirmed the protein-protein interaction between DRN and PHV and between DRNL and PHV in vitro. DRNL could not be co-immunoprecipitated by DRN (Fig. 4B), demonstrating that DRN and DRNL are not capable of heterodimerisation.

Additionally, we used bimolecular fluorescence complementation (BiFC) (Walter et al., 2004) in a transient assay in leek epidermal cells to verify the observed biochemical interaction between DRN or DRNL and PHV, in vivo, using full-length DRN and PHVs as the C-terminal part of PHV was sufficient to sustain interaction with DRN in the two-hybrid screen. GFP expression was observed in control experiments (Fig. 3C-E) and YFP expression was reproducibly obtained in multiple independent experiments when the PHVs BiFC construct was co-bombarded with DRN or DRNL BiFC constructs (Fig. 3F,G). A series of negative controls were performed, including co-bombardment of empty YFP vectors, and no evidence for DRN heterodimerisation with DRNL was observed (data not shown). More importantly, no YFP fluorescence was observed following co-bombardment of DRN in pUC-SPYCE and SHOOTMERISTEMLESS (STM) in pUC-SPYNE, nor in the reciprocal cloning combination (data not shown). These results not only demonstrate that that both DRN and DRNL can form stable heterodimers with the PHV protein in planta, but that these interactions are specific.

DRN and *PHV* genetically co-regulate embryo patterning

Considering the biochemical dimerisation between DRN and PHV in vitro and in planta, we asked whether genetic evidence would support the hypothesis of an active DRN-PHV protein dimer by constructing a *drn-1 phv* double mutant. The *phv* mutant showed wild-type embryo development. However, the *drn-1 phv* mutant showed embryo cell division defects similar to those observed in the *drn-1 drnl-1* double mutant and at almost complete penetrance as compared with that of the *drn* single mutant (Table 2). Additionally, the penetrance of *drn-1 phv* cotyledon defects was higher than that of *drn* single mutants, with slightly more plants with an *mp* phenocopy (Table1). The increased penetrance of embryo cell defects of *drn-1 phv* plants over *drn* single mutants suggests that both genes contribute to the same embryo developmental pathways.

DRN and DRNL can interact with all class III HD-ZIP family members

The *Arabidopsis* class III HD-ZIP protein family also contains the closely related homologue of PHV, PHABULOSA (PHB), in addition to REVOLUTA (REV), CORONA (CNA; also known as ATHB-15 – The *Arabidopsis* Information Resource) and ATHB8 (Sessa et al., 1994; Prigge et al., 2005). As these family members act redundantly (Prigge et al., 2005), we investigated whether DRN and DRNL could interact with other class III HD-ZIP members apart from PHV. We used equivalent C-terminal parts of all five

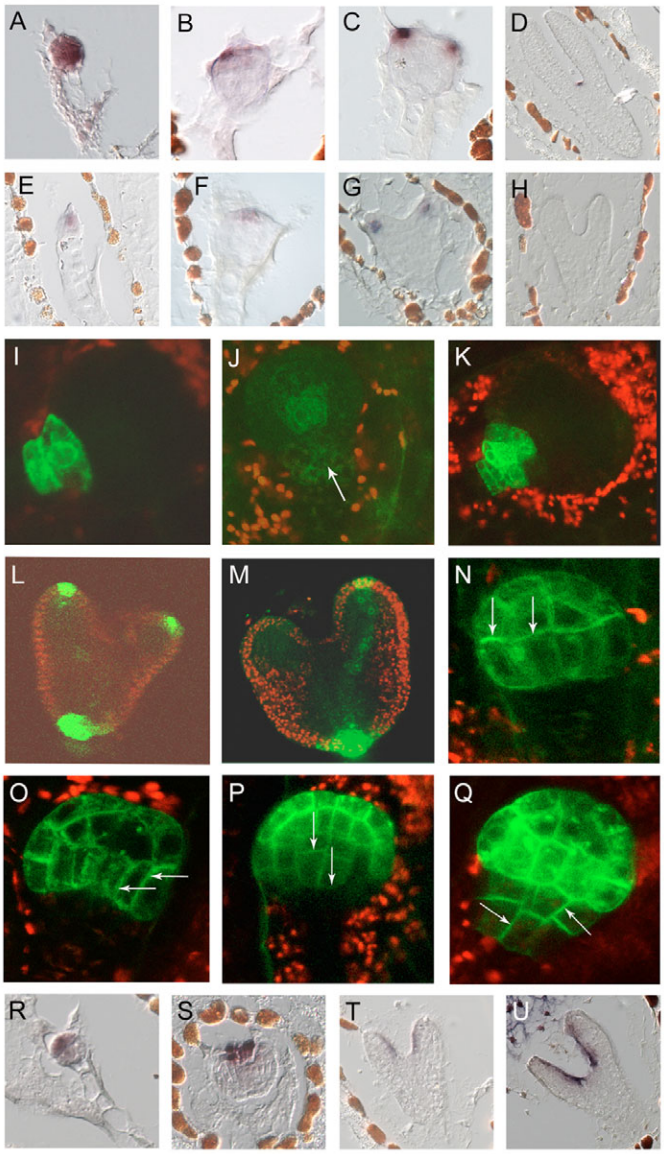

Fig. 2. RNA in situ hybridisations and alteration of *DR5* and *PIN1* expression in *drn* mutants. (**A-D**) *DRN* expression on median longitudinal sections of wild-type embryos in early globular (A), late globular (B), early heart (C) and torpedo (D) stages. (**E-H**) *DRNL* expression in wild-type embryos in early globular (E), late globular (F), early heart (G) and late heart (H) stages. (**I-K**) *DR5::GFP* expression in globular wild-type (I), globular *drn-1* mutant (J) and *drn-1* transition-stage (K) embryos. The arrow in J marks the hypophysis region. Note supernumery cell files in the suspensor (K). (**L**,**M**) *DR5::GFP* expression in wild-type (L) and *drn-1* (M) heart-stage embryos. Note the normal and mutant cotyledons in M. (**N-Q**) PIN1 expression in wild-type (N) and *drn* (O) 32-cell-stage embryos, and in wild-type (P) and *drn* (Q) globular embryos. Arrows show basal PIN1 polarity (N,P) and abnormal lateral PIN1 expression (O,Q). (**R-U**) RNA in situ hybridisation for *PHV* on median longitudinal sections of wild-type embryos at early globular (R), late globular (S), heart (T) or late heart (U) stage.

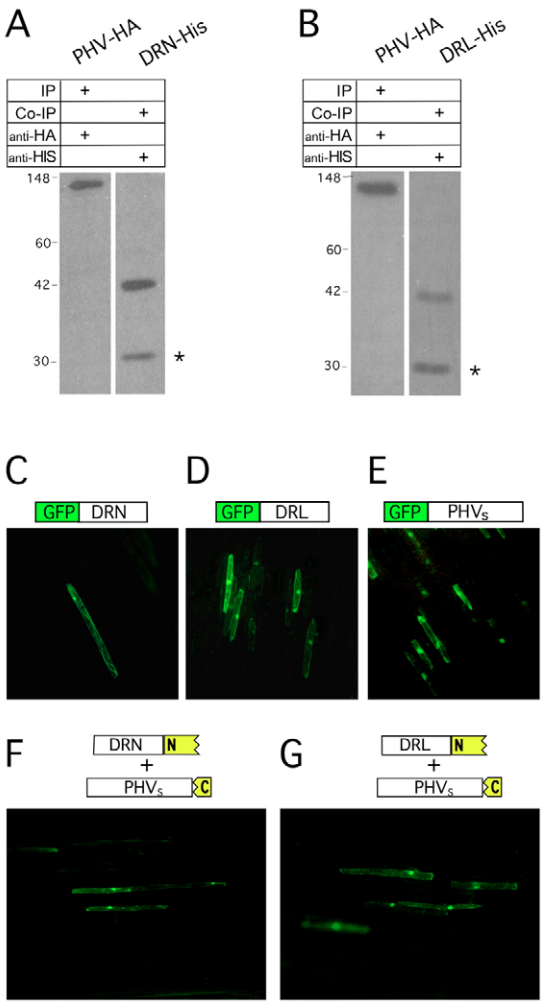

Fig. 3. CoIP and BiFC analysis for the interaction between DRN or DRNL and PHV. (**A**,**B**) Full-length PHV protein can precipitate full-length DRN (A) or DRNL (B). Protein mixtures were precipitated via PHV-HA. The grid summarises the IP and Co-IP lanes and the antibodies used. Asterisks mark the co-eluted IgG light chain. (**C-E**) Cellular fluorescence of GFP-DRN (C), GFP-DRNL (D) or GFP-PHVs (E) in leek epidermal cells. (**F**,**G**) Bimolecular fluorescence complementation showing YFP expression observed with a GFP filter in leek epidermal cells following co-bombardment of full-length DRN and PHVs proteins (F) or full-length DRNL and PHVs proteins (G) fused to complementary YFP subdomains.

proteins corresponding to the PHV sequences obtained from the two-hybrid screen (see Materials and methods). His-tagged full-length DRN and DRNL proteins were detected following CoIP via the HA-tagged C-terminal fragments of all five members of the class III HD-ZIP family (Fig. 4B). DRN and DRNL can thus dimerise with all *Arabidopsis* class III HD-ZIP proteins, a finding supported by their overlapping expression patterns in the embryo (Prigge et al., 2005) (Fig. 2). We also tested the specificity of PHV interactions by investigating whether the full-length PHV protein could co-precipitate STM, another protein whose expression domain overlaps that of PHV in the embryo. A full-length STM-GFP fusion protein could not be co-precipitated via the HA tag of PHV (Fig. 4C), confirming that PHV specifically interacts with DRN and DRNL.

The interaction between DRN and PHV is dependent on the AP2 domain and the C-terminal conserved PAS-LIKE domain

The C-terminal region of class III HD-ZIP members of *Arabidopsis* is highly conserved (Fig. 4A) and therefore indicative of conserved function. Using the SMART protein motif search of EMBL (http://smart.embl-heidelberg.de), putative homology was found to several characterised domains, including the PAS domain – named after *Drosophila* period (PER), vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT) and *Drosophila* single minded (SIM) – which is found in a superfamily of more than 1000 eukaryotic proteins directly or indirectly involved in signal transduction (Mukherjee and Bürglin, 2006). Homology to the PAS domain resides between amino acids 722 and 792 of the PHV protein, within the sequence we have shown to dimerise with DRN and DRNL. Although this homology is weak, probably representing the low sequence identity between PAS domains (Gilles-Gonzalez and Gonzalez, 2004), this region spans five conserved α-helices of the PAS domain. For this reason, we investigated whether this region of the PHV protein was sufficient to maintain high affinity binding to the full-length DRN protein. In pull-down assays, DRN could dimerise with the 70-amino-acid PHV PAS fragment (Fig. 4D) and, reciprocally, the PHV PAS fragment was able to precipitate DRN or DRNL (Fig. 4D), identifying a functional protein-protein interaction domain within this region.

To establish which region of the DRN protein is responsible for dimerisation with PHV, we performed CoIP experiments with subfragments of the N-terminus of DRN used for the two-hybrid screen: (1) the N-terminal polypeptide (amino acids 1-53) excluding the AP2 domain; and (2) the conserved 61 amino acid AP2 consensus domain (Kim et al., 2006) (DRN amino acids 54-115). The N-terminal polypeptide of DRN was not able to co-precipitate PHV (data not shown), whereas the AP2 domain could (Fig. 4F), confirming that it is sufficient to mediate heterodimer formation with PHV. The same experiment was performed using the DRNL AP2 domain (amino acids 55 to 116), which was also able to precipitate the PHV polypeptide (Fig. 4F). The AP2 domain is generally considered to comprise a GCC-box DNA-binding motif (Riechmann and Meyerowitz, 1998; Sakuma et al., 2002) composed of three-stranded β-sheets and an α-helix, occurring either in tandem repeats such as in the founding family member APETALA2 (Okamuro et al., 1997), or as a single domain as in DRN or DRNL. The 3D structure of the *Arabidopsis* ERF1 AP2 domain (Allen et al., 1998; Marchler-Bauer et al., 2005) shows that the DNA-binding domain resides in the N-terminal β-sheet region of the domain, leaving residues within the C-terminus to orientate on the opposite face to the DNA-binding face so as to be sterically potentially able to interact with other proteins. The cysteine and serine residues in DRN and DRNL AP2 domains (Fig. 4E; marked in yellow Fig. 4G) are noteworthy: the cysteine conserved in both DRN and DRNL is unique amongst plant AP2 domain proteins (see Kim et al., 2006) and falls within the α-helix of the RAYD domain, considered to be a conserved structural motif relevant to the function of all AP2 proteins (Okamuro et al., 1997).

DISCUSSION

DRN and *DRNL* are functionally redundant

Two independent *drn* or *drnl* mutant alleles share similar pleiotropic cotyledon phenotypes at low penetrance; however, *DRN* has a more pronounced role in patterning of the hypophysis and suspensor regions because *drnl* mutants were unaffected in this aspect of embryo development. The *drnl-2* allele showed the highest cotyledon phenotype penetrance and is presumably a stronger allele

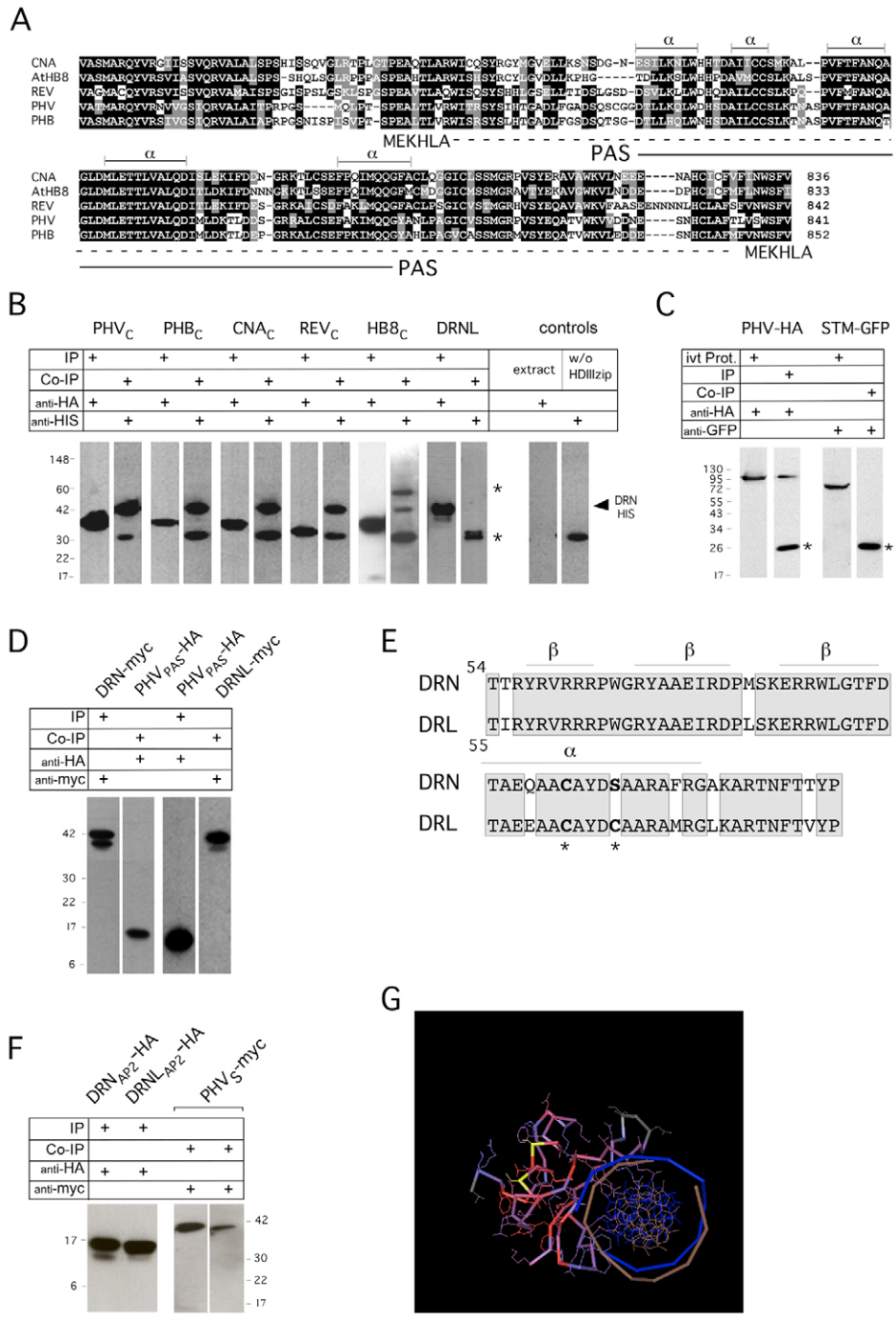

Fig. 4. The interaction between DRN and class III HD-ZIP family proteins involves the AP2 and PAS-like domains, respectively. (**A**) A comparison of the C-terminal regions of the *Arabidopsis* class III HD-ZIP proteins. Accession numbers for the proteins are as follows: CNA, AAW88440; AtHB8, CAD29660; REV AAF42938; PHV CAD29544; PHB, NP_181018. Homology is compared over the region from PHV amino acid 654 to the C-terminus. The MEKHLA domain, homology to the PAS domain from http://smart.embl-heidelberg.de and the α-helices within the PHV PAS domain are marked. (**B**) Full-length DRN is precipitated by C-terminal regions of all class III HD-ZIP members, but not by DRNL. HA-tagged class III HD-ZIP proteins or DRNL were used for precipitation of DRN. The grid shows IP and Co-IP lanes and which antibodies were used. The position of the DRN protein is marked with an arrowhead. Asterisks represent the IgG heavy or light chains. The control lanes show IPs in the absence of either or both in vitro transcribed/translated products, confirming the identity of the lower IgG band. (**C**) Full-length PHV cannot co-precipitate full-length STM-GFP. The first and third lanes contain in vitro translated proteins before precipitation. PHV-HA was used for precipitation. (**D**) Full-length DRN can co-precipitate the 71 amino acid PHV$_{PAS}$ domain (first and second lanes) and, reciprocally, PHV$_{PAS}$ can co-precipitate full-length DRNL (third and fourth lanes). (**E**) An alignment of the AP2 domains of DRN and DRNL. Identical amino acids are shaded and the position of the unique cysteine and serine residues within the α-helix are in bold. (**F**) The DRN AP2 domain (IP) can precipitate PHVs (Co-IP) (first and third lanes) and the DRNL AP2 domain can precipitate PHVs (second and fourth lanes). (**G**) 3D crystal structure of the AP2 domain of the *Arabidopsis* ERF1 protein (Allen et al., 1998; Marchler-Bauer et al., 2005) binding a DNA helix shown on the right, viewed from above into the helix. Cysteine/serine residues of the DRN/DRNL proteins are highlighted in yellow.

than *drnl-1*. The mutated Ala residue has been shown in *Brassica napus* ERF/AP2 proteins to be essential for DNA binding (Liu et al., 2006), suggesting that the mutated *drnl-2* protein is unable to bind target genes. Importantly, the penetrance of hypophysis or cotyledon phenotypes significantly increased in *drn-1 drnl-1* double mutants, thereby demonstrating a role for *DRN* and *DRNL* in both the apical and basal domain of the *Arabidopsis* embryo and that the genes act redundantly. The penetrance of *drn-1 drnl-1* double-mutant phenotypes was still incomplete compared with that of *drn-1 drnl-2* double mutants, probably reflecting different *drnl-1* allele strengths. The complete lack of cotyledons at almost complete penetrance in *drn-1 drnl-2* mutants demonstrates that both genes together are essential for cotyledon initiation.

Functional contributions of *DRN* and *DRNL* can be separated in the apical and basal embryo domains: loss of function of either gene in the apical domain is manifest in the cotyledon phenotype observed in single and double mutants. This correlates with the expression pattern of both genes in apical cell tiers concomitant with cotyledon initiation. No cell patterning defects were observed in the basal embryo domain up to the 16-cell stage of *drn-1 drnl-1* mutants, despite transcriptional activity of both genes throughout the proembryo. However, about 50% of *drn* single-mutant embryos and essentially all *drn-1 drnl-1* double-mutant embryos exhibited subsequent cell division defects in the hypophyseal region and subtending suspensor cells in the absence of either *DRN* or *DRNL* transcription in these cells. The spatial separation of gene expression and hypophyseal phenotype, therefore, must involve either mobile DRN or DRNL proteins or other interdomain signaling components. This is similar to the situation observed for *BDL* and *MP* (Berleth and Jürgens, 1993; Hamann et al., 1999), which are expressed in proembryo cells but not in the hypophysis, which in *mp* and *bdl* mutants fails to undergo the asymmetric division that gives rise to the quiescent centre precursors and columella initials. *MP* and *BDL* therefore affect the hypophysis in a non-cell-autonomous manner and signalling between embryo proper and underlying hypophysis is essential for normal root development. A transient indirect response to auxin together with a postulated additional factor operating downstream of MP and BD underlies this cell-to-cell signalling (Weijers et al., 2006). Expression of *DR5::GFP* in *drn* mutants is more informative in explaining the spatial anomaly between *DRN*/*DRNL* gene expression domains and phenotype. In wild type, an auxin maximum is established in the hypophysis and upper suspensor cell at the late globular stage and is necessary for hypophysis cell fate specification (Friml et al., 2003). This maximum as reported by *DR5::GFP* is absent in *drn* mutant globular embryos, similar to the situation in *bdl* mutants (Weijers et al., 2006), and might explain the cell division defects in the hypophysis region. The absence of local *DR5::GFP* response at the cotyledon tips and in provascular strands in *drn* mutant cotyledons correlates with phenotypic defects in cotyledon initiation.

PIN1, an additional auxin marker, marks all cell boundaries up to the 16-cell stage in wild type, before the polarity in expression is established that concentrates PIN1 basally in the provascular cells facing the hypophysis (Steinman et al., 1999; Friml et al., 2003). The variable polarity of PIN1 in cells of the hypophysis region of *drn* mutants suggests an alteration in directed auxin flow, which might explain the absence of DR5::GFP accumulation here at an appropriate temporal phase. Although variable PIN1 polarity correlates with the abnormal cell divisions in the hypophysis region, it is distinct from the domain of *DRN* transcription. This strengthens our conclusion that *drn* cell division phenotypes arise either from movement of the DRN protein or that *DRN* functions upstream of auxin transport and involves additional interdomain signaling components. The altered PIN1 distribution in *drn* embryos unequivocally places *DRN* function upstream of the auxin transport fundamental for embryo apical-basal patterning (Friml et al., 2003; Weijers et al., 2005), a conclusion that could not have been derived from altered DR5 activity alone. This is supported by the *pin*-like phenotype of *drn-1 drnl-2* embryos, which phenocopies *pin* mutants deficient in polar auxin transport.

Interdomain signalling in *Arabidopsis* embryos is also suggested by analyses of embryo-lethal mutants, in which normal embryo development involves the inhibition of the embryonic potential of the suspensor by the embryo proper (Marsden and Meinke, 1985). Aberrant hypophyseal cell divisions in *drn* single mutants rarely lead to a defective root phenotype, which suggests an ability of the lower hypophysis cell to generate columella stem cells or organise the RAM, independent of the number of precursor cells. This supports the hypothesis that additional and partially redundant pathways are activated as cell number increases during embryogenesis, and that these can compensate for early developmental defects (Laux et al., 2004). It should be remembered that stereotypic cell division patterns of *Arabidopsis* embryos are not representative of plants in general and a single row of suspensor cells is not the rule in dicots; for example, *Phaseolus multiflorus* has a massive suspensor consisting of multiple cell files merging into the embryo proper (Wardlaw, 1955).

The vasculature of monocotyledonous *drn* mutants shows that cotyledons may be single or arise from cotyledon fusion, presumably via disruption of cell recruitment into correctly initiated cotyledons and disruption in the maintenance of cotyledon boundaries during organogenesis. This, together with supernumery cotyledons in *drn* and *drnl* mutants, shows that both genes have roles in both cotyledon initiation and boundary maintenance. Leaf primordia initiate at auxin concentration maxima via the dynamic expression of PIN1, which acts as an instructive signal for organ initiation (Reinhardt et al., 2003). In *drn* and *drnl* mutants, disruption of leaf phyllotaxis suggests that cotyledon misdevelopment at least temporarily reprogrammes the phyllotactic inductive signals for leaf initiation, perhaps via further disruption of auxin responses.

DRN and DRNL form heterodimers with class III HD-ZIP proteins via a C-terminal PAS-LIKE domain

We have demonstrated biochemically and via transient in planta assays that DRN and DRNL can heterodimerise with the C-termini of all members of the class III HD-ZIP family. The in vivo relevance of these interactions is supported by the expression patterns of the gene family members: all except *ATHB8* are co-expressed with *DRN* and *DRNL* in the apical embryo domain (Emery et al., 2003). In the absence of evidence for heterodimerisation between DRN and DRNL, both proteins may individually compete for the same class III HD-ZIP interaction partners, which also act redundantly (Prigge et al., 2005).

The conserved C-terminus region is characteristic for class III HD-ZIP proteins, although a function has yet to be assigned to it. Mukherjee and Bürglin (Mukherjee and Bürglin, 2006) identify a MEKHLA domain within eukaryotic PAS-containing proteins, specific to class III HD-ZIP genes of higher plants and *Chlamydomonas reinhardtii*. It contains the PAS domain and an additional 150 amino acids and is hypothesised to represent a discrete functional unit involved in a signalling pathway (Mukherjee and Bürglin, 2006). Our data unequivocally show that a 71 amino acid region containing five α-helices within the PAS-like domain is sufficient to mediate interactions between DRN and PHV. These α-helices and the associated five- to six-stranded antiparallel β-barrels form a pocket that may contain various prosthetic groups (Mukherjee and Bürglin, 2006). PAS domains have been reported to mediate protein-protein interactions (Taylor and Zhulin, 1999; Card et al.,

2005) and contain two highly conserved S1 and S2 regions (Zhulin et al., 1997). Bacterial PAS domains sense oxygen via a bound heme molecule and propagate a signal via the His kinase pathway (Hao et al., 2002). Plant PAS proteins possibly function similarly, or the MEKHLA domain might provide a docking structure to recruit other proteins into a transcriptionally functional complex. Although plant HD-ZIP proteins efficiently bind DNA as homo- or heterodimers (Sessa et al., 1993; Johannesson et al., 2001), it can be envisaged that higher order protein complexes containing DRN and/or DRNL and possibly other proteins are formed and co-ordinately act as a transcriptional unit in the control of embryo patterning. In vitro CoIP experiments demonstrate heterodimerisation between full-length DRN and PHV proteins, and BiFC experiments performed with the PHV C-terminus and full-length DRN further confirm the interaction in planta. *PHV* and *PHB* are involved in adaxial/abaxial leaf patterning (McConnell et al., 2001; Emery et al., 2003) and are also expressed in the SAM and during early stages of leaf development where *DRN* remains active postembryonically (McConnell et al., 2001; Kirch et al., 2003), suggesting that class III HD-ZIP proteins and AP2-class transcription factors such as DRN or DRNL might be partners throughout the plant life cycle.

DRN and *PHV* act in a common embryonic patterning pathway

We have supported DRN-PHV protein interaction data with genetic data showing a combined genetic effect of *PHV* and *DRN* in embryonic patterning: enhanced embryo cell division defects of the *drn-1 phv* double mutant show that both genes contribute to the same embryonic patterning pathways. This might reflect partial redundancy between both gene functions; but if DRN and PHV act in a common protein complex, and as both DRN and DRNL are functionally redundant and can interact with other partially redundant class III HD-ZIP family members, it is more likely to reflect redundancy involving other heterodimer combinations between different members of both protein families.

The AP2 domain is responsible for protein-protein interactions

Our finding that the DRN and DRNL AP2 domain alone is sufficient to mediate heterodimerisation with PHV is to the best of our knowledge the first experimental evidence that plant AP2 domains have a role in protein dimerisation as well as in DNA binding. AP2 proteins are plant-specific and with 144 AP2/ERF members, comprise one of the largest transcription factor families in *Arabidopsis* (Sakuma et al., 2002). They are key regulators in diverse developmental processes such as flower formation [AP2 (Jofuku et al., 1994)], ovule development [AINTEGUMENTA (Elliott et al., 1996; Klucher et al., 1996)] and abiotic stress [TaDREB1 (Shen et al., 2003)]. Both the tandem repeat unit and the single AP2 domain comprise functional DNA-binding motifs. The cysteine and serine residues in the DRNL and DRN AP2 domains, unique among *Arabidopsis* AP2 domains, reside within the core α-helix of the RAYD element, which has been proposed to mediate protein-protein interactions (Okamuro et al., 1997). Two alternative effects of dimerisation which would affect the regulation of target genes are that either the interaction between DRN or DRNL and class III HD ZIP proteins sterically interferes with the DNA-binding activity of the AP2 domain, or it contributes to the DNA-binding specificity/affinity of the AP2 domain, as is known for protein-DNA interactions mediated by the homeodomain (Moens and Selleri, 2006). Based on overlapping transcription patterns, a heterodimeric complex between DRN-PHV or DRNL-PHV or related HD-ZIP III proteins could control the transcription of target genes required for normal cotyledon development. PHV and DRN do not promiscuously interact with other proteins, such as STM, that are co-expressed in the early embryo, supporting the specificity of complexes involving DRN and PHV.

We show in this paper that two *Arabidopsis* AP2-domain containing paralogues control embryo development in specific and early embryo expression domains. Both proteins can form protein-protein interactions with class III HD-ZIP proteins and with PHV via a PAS-like domain in the C-terminal region of PHV and PHB, and the AP2 domain of DRN and DRNL. Our data suggest that transcriptional complexes involving DRN and DRNL act redundantly with their class III HD-ZIP partners to control embryo organogenesis and patterning. The robustness of embryonic patterning as suggested by the low penetrance of mutant phenotypes therefore finds a biochemical basis in the promiscuity of transcription factor interactions. The identification of DRN and DRNL as partners of class III HD-ZIP proteins therefore enables biochemical access to signal transduction cascades in the embryo on the basis of genetic pathways.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/9/1653/DC1

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