MONOPTEROS directly activates the auxin-inducible promoter of the Dof5.8 transcription factor gene in Arabidopsis thaliana leaf provascular cells

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Abstract

MONOPTEROS (MP) is an auxin-responsive transcription factor that is required for primary root formation and vascular development, whereas Dof5.8 is a Dof-class transcription factor whose gene is expressed in embryos as well as the pre- and procambial cells in the leaf primordium in Arabidopsis thaliana. In this study, it is shown that MP directly activates the Dof5.8 promoter. Although no apparent phenotype of the single dof5.8 mutants was found, phenotypic analysis with the mp dof5.8 double mutants revealed that mutations within Dof5.8 enhanced the phenotype of a weak allele of mp, with an increase in the penetrance of the ‘rootless’ phenotype and a reduction in the number of cotyledons. Furthermore, interestingly, although mp mutants showed reduced vascular pattern complexity in cotyledons, the mp dof5.8 double mutants displayed both more simplex and more complex vascular patterns in individual cotyledons. These results imply that the product of Dof5.8 whose expression is regulated by MP at least in part might be involved in multiple processes controlled by MP.

Key words: Arabidopsis thaliana, auxin response, Dof transcription factor, embryo development, MONOPTEROS, vascular development.

Introduction

MONOPTEROS (MP), which is also known as auxin response factor 5 (ARF5) of the ARF family, is a key regulator that functions in the establishment of vasculature and body patterns in embryonic and post-embryonic development in Arabidopsis thaliana (Hardtke and Berleth, 1998; Ulmasov et al., 1999a, b). Mutations within the MP gene interfere with the body axis patterning in the early embryo and the formation of vascular strands. Thus, the mp mutants show reduced complexity in vascular patterns in both cotyledons and true leaves, and the mp seedlings are often rootless and have only one cotyledon (Berleth and Jürgens, 1993; Przemeck et al., 1996). Because MP is a transcription factor, direct target genes of MP have been searched for in order to clarify MP-mediated regulations. Consistent with the diverse functions of MP in embryonic root initiation, lateral organ initiation, shoot meristem cell regulation, and vascular patterning in leaves (Berleth and Jürgens, 1993; Przemeck et al., 1996; Hardtke et al., 2004; Schuetz et al., 2008), recent intensive studies revealed that MP activates the expression of DRN involved in cell patterning in embryos (Cole et al., 2009), TMO genes crucial for embryonic root initiation (Schlereth et al., 2010), and LFY for flower initiation (Yamaguchi et al., 2013), and represses the expression of ARR7 and ARR15, negative regulators of cytokinin

Abbreviations: ARF, auxin response factor; BDL, BODENLOS; CIP, chromatin immunoprecipitation; 2,4-D, 2,4-dichlorophenoxyacetic acid; GUS, β-glucuronidase; IAA, indole-3-acetic acid; LUC, luciferase; MP, MONOPTEROS; MS, Murashige and Skoog; qRT–PCR, quantitative reverse transcription–polymerase chain reaction.
signalling, in the shoot apical meristem (Zhao et al., 2010). Furthermore, it has been reported that MP regulates the expression of BRX involved in cross-talk between the auxin and brassinosteroid pathways (Bauby et al., 2007; Beuchat et al., 2010; Scacchi et al., 2010). Athb8 associated with the formation of vascular strands in leaves (Donner et al., 2009), and MP itself (Lau et al., 2011). Target genes of MP probably vary depending on organ, tissue type, or developmental stage, and thus MP functions and MP-induced regulation still remain largely unknown.

Dof transcription factors are a family of transcription factors that harbour a plant-specific Dof DNA-binding domain that recognizes 5′-AAAG-3′ or 5′-CTTT-3′ motifs (Yanagisawa, 2002, 2004). Although the physiological functions of Dof transcription factors are highly diverse, many Dof genes are expressed in the vasculature or during vascular development (Gualberti et al., 2002; Imaiizu et al., 2005; Skirycz et al., 2006; Konishi and Yanagisawa, 2007; Guo et al., 2009; Gardiner et al., 2010; Schlereth et al., 2010; Le Hir and Bellini, 2013). It was shown previously that the promoter of A. thaliana Dof5.8 is specifically active in embryos during the transition and heart stages and the future vasculature of cotyledons at the walking-stick stage, as well as procambial cells (vascular precursors) and pre-procambial cells (cells in the middle of the first stage of vascular development from the ground meristem cells to the procambial cells) in the leaf primordium (Konishi and Yanagisawa, 2007).

As the initial steps of vascular development in leaves in dicots are triggered by auxin flow, and then auxin-induced MP activity modulates gene expression for formation of the vascular network (Donner et al., 2009; Ckurchumova et al., 2011), it is known that pre-procambial and procambial cells (hereafter collectively termed ‘provascular cells’) are characterized by expression of the auxin-responsive marker gene, DR5-GUS (Mattsson et al., 2003), or the auxin efflux carrier protein, PIN1 (Scarpella et al., 2006; Wenzel et al., 2007). Based on the expression pattern of Dof5.8 in embryos and provascular cells in the leaf primordium, we speculated that Dof5.8 might be a target of MP and associated with MP-regulated processes. To examine this hypothesis, molecular genetic and biological analyses were performed in this study. The results indicate that MP directly activates the Dof5.8 promoter whereas mutations within Dof5.8 influence multiple phenotypes of the mp mutant, arf5-2.

### Materials and methods

#### Plant materials

Arabidopsis thaliana ecotype Columbia (Col) was used as the wild-type strain in all experiments. Seeds of the mp mutants, arf5-1, SALK_001058 and arf5-2 (also called mp-S319 or SALK_021319), and SALK T-DNA lines of Dof5.8 were obtained from the Arabidopsis Resource Center (Alonso et al., 2003; Okushima et al., 2005; Donner et al., 2009). DR5-GUS (β-glucuronidase) seeds (Ulmasov et al., 1997b) were a gift from Dr Tom J. Guilfoyle. For the analysis of transcript levels of Dof5.8 in mp alleles, selfed seeds from heterozygous mp plants were sown. Seedlings exhibiting the rootless phenotype were collected for quantitative reverse transcription–polymerase chain reaction (qRT–PCR) analysis. To generate the double mutants of arf5-2 and dof5-8-1 or dof5-8-2, the dof5.8 plants that are homozygous for a T-DNA insertion were crossed to heterozygous arf5-2 plants, and F2 plants homozygous for the dof5.8 T-DNA allele and heterozygous for arf5-2 allele were selected by PCR-based genotyping. For phenotypic analysis, rootless F3 seedlings, which are homozygous for the arf5-2 allele (Table 1), were picked for analysis of cotyledon numbers and vascular patterns. For the analysis of the Dof5.8 promoter activity in the arf5-2 background, the Dof5.8pro-GUS line harbouring the GUS reporter gene under the control of the Dof5.8 promoter (Konishi and Yanagisawa, 2007) was crossed to the arf5-2 heterozygous plant. The F2 population that was homozygous for the Dof5.8pro-GUS transgene linked to the glucosamine ammonium resistance gene and heterozygous for the arf5-2 allele was selected by phenotypic analysis of the glucosamine ammonium resistance and rootless phenotype or genotyping using a cotyledon of F3 seedlings.

#### Plant growth conditions

Seeds were sterilized and sown on half-strength Murashige and Skoog (1/2MS) agar plates containing 1% sucrose, as described previously (Konishi and Yanagisawa, 2008). After 3–4 d of stratification, plates were transferred to a chamber set at 23°C with continuous illumination (60 μE m⁻² s⁻¹). For 2,4-dichlorophenoxyacetic acid (2,4-D) treatment, seedlings were grown in liquid 1/2MS medium for 3 d and treated or not with 10 μM 2,4-D for 16 h. For the analysis of the vascular pattern, seeds were plated on 1/2MS agar medium containing 1% sucrose, solidified with 0.3% agar. For protoplast transient assays, ecotype Col plants were grown on peat containing nutrients (Sakatanotane Co., Yokohama, Kanagawa, Japan) at 23°C for 3 weeks under continuous light.

#### Genotyping

DNA extraction was performed according to Konishi and Sugiyama (2003). Primers used in PCR are listed in Supplementary Table S1 available at JXB online.

#### Protoplast transient assays

The DNA fragment from the Dof5.8 promoter was amplified by PCR (Konishi and Yanagisawa, 2007), and used to replace the Cauliflower

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**Table 1. Segregation of the arf5-2 allele among populations derived from plants heterozygous for the arf5-2 allele in the wild-type, dof5-8-1 or dof5-8-2 background**

| Genotype of parental plant | No. of seedlings with the indicated genotype at the MP locus (% of total) | No. of rootless seedlings a |
|---------------------------|---------------------------------------------------------------------|-----------------------------|
|                          | MP/MP                  | MP/arf5-2                 | arf5-2/arf5-2               | Total |                          |
| MP/arf5-2                | 31 (35.2%)          | 38 (43.2%)               | 19 (21.6%)               | 88    | 2                          |
| dof5-8-1/dof5-8-1; MP/arf5-2 | 26 (28.8%)      | 51 (52.6%)               | 20 (20.6%)               | 97    | 14                         |
| dof5-8-2/dof5-8-2; MP/arf5-2 | 23 (19.3%)      | 69 (58.0%)               | 27 (22.7%)               | 119   | 15                         |

a All rootless seedlings were homozygous for the arf5-2 allele.
mosaic virus 35S RNA promoter in pJD301 (Luehrsen et al., 1992) to produce reporter plasmids containing the luciferase (LUC) gene. The deleted versions of the Dof5.8 promoter were generated by digestion of the full-length promoter fragment with SphI for truncation at position –1301 (relative to the translation start site) and EcoRI for truncation at position –1077, whereas mutated Dof5.8 promoters were generated by PCR-based mutagenesis, as described previously (Konishi and Yanagisawa, 2010). Primers used for the mutagenesis are listed in Supplementary Table S1 at JXB online. For the construction of effecter plasmids, MP or the BODENLOS (BDL) cDNA insert was amplified by RT–PCR and inserted in place of EIN3 cDNA in the 35SC4PPDK-EIN3-MYC plasmid (Yanagisawa et al., 2003). All constructs were verified by DNA sequencing. Co-transfection of reporter and effecter plasmids and an internal control plasmid (UBQ10-GUS) into A. thaliana mesophyll protoplasts was carried out according to the method of Yoo et al. (2007). Measurement of LUC and GUS activities and calculations of relative LUC activity levels were performed as described previously (Yanagisawa et al., 2003). For auxin treatment, protoplasts were incubated in the absence or presence of 1 μM indole-3-acetic acid (IAA) after co-transfection.

**qRT–PCR analysis**

RNA preparation and qRT–PCR were performed as described previously (Konishi and Yanagisawa, 2010). The primers used are listed in Supplementary Table S1 at JXB online.

**Construction of binary plasmids and generation of transgenic A. thaliana plants**

To construct binary vectors for GUS staining, the DNA fragments for the truncated or mutated Dof5.8 promoters were excised from the respective reporter plasmids used in protoplast transient assays and then inserted in place of the Dof5.8 promoter in the pCB-Dof5.8pro-GUS construct. The transformations of A. thaliana were carried out using these binary vectors, as described previously (Konishi and Yanagisawa, 2007).

**GUS staining and histological analysis**

Histochemical GUS staining was essentially performed as described previously (Konishi and Yanagisawa, 2007). Samples were fixed in 90% acetone at –20 °C, rinsed four times with 0.1 M sodium phosphate buffer (pH 7.4), and then incubated in X-Gluc solution [0.1 M sodium phosphate (pH 7.4), 3 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.5 g 1 1 - 5-bromo-4-chloro-3-indolyl-β-D-glucuronic cyclohexylammonium salt] at 37 °C. When GUS activity was weak, the concentration of potassium ferricyanide was reduced according to Donnelly et al. (1999). Potassium ferricyanide at 1.75 mM was thus used for the Dof5.8 promoter truncated at –1301 and the promoter containing a 486 bp fragment (from –1558 to –1073) of the Dof5.8 promoter upstream of the 35S minimal promoter, and 0.5 mM potassium ferricyanide was used for the Dof5.8 promoter truncated at –1077. After staining, samples were incubated in methanol to remove chlorophyll and then mounted in the clearing solution (a mixture of chloral hydrate, water, and glycerol in a ratio of 8:2:1). Observation was performed using a stereomicroscope (MZ16F; Leica Microsystems, Germany) or a microscope equipped with Nomarski optics (BX51, Olympus Co., Tokyo, Japan). For the observation of vascular patterns, cotyledons were fixed in a mixture of ethanol and acetic acid in a ratio of 9:1; hydrated through a graded series of ethanol, and then mounted with the clearing solution (Konishi and Sugiyama, 2003).

**Chromatin immunoprecipitation analysis**

Chromatin immunoprecipitation (ChIP) analysis was carried out to examine the binding of MP to the Dof5.8 promoter, as described previously (Donner et al., 2009).

**Results**

**Regulation of the provascular expression of Dof5.8 by MP**

It was shown previously that the Dof5.8 promoter is active in provascular cells in leaf primordia (Konishi and Yanagisawa, 2007). Because the synthetic auxin response element, DR5, induces a similar expression pattern in leaves (Ulmasov et al., 1997b; Mattsson et al., 2003; Scarpellia et al., 2006), the expression patterns produced by a GUS reporter gene under the control of either the Dof5.8 promoter or DR5 during the development of the first leaf primordia were compared (Fig. 1A). The Dof5.8 promoter initially directed strong GUS expression deep inside the bulges of the leaf primordia (Fig. 1A, ‘1d’). This expression then expanded vertically with the upward extension of the primordia. From day 2.5 onwards, reporter expression was localized to the provascular network in a pattern similar to that produced by DR5. This suggested that the activity of the Dof5.8 promoter is regulated at least in part by auxin that accumulated during pre-procambium formation. Consistently, a treatment with the synthetic auxin 2,4-D significantly strengthened expression of the GUS reporter gene under the control of the Dof5.8 promoter (Fig. 1B).

Among the ARFs, MP is known to be involved in vascular development. mp mutants show reduced vascular pattern

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**Fig. 1.** Provascular activity of the Dof5.8 promoter and its enhancement by auxin. (A) Time course analysis of GUS expression under the control of the Dof5.8 promoter (Dof5.8pro) or the DR5 element (DR5) during development of the primordia of the first true leaves. Two primordia flanking the shoot apical meristem (1 d and 2 d) and individual leaf primordia (2.5–5 d) are shown. Scale bars=20 μm in 1 d and 2 d, 50 μm in 2.5 d and 3 d, 100 μm in 4 d and 5 d. (B) Effect of auxin treatment on the activity of the Dof5.8 promoter. Seedlings were treated or not with 10 μM 2,4-D for 16 h. Scale bar=100 μm.
complexity and sometimes form a disconnected vasculature (Berleth and Jürgens, 1993; Przemeck et al., 1996; Hardtke and Berleth, 1998). Thus, it was hypothesized that the Dof5.8 promoter is activated by MP. To examine this possibility, transactivation assays were performed in A. thaliana protoplasts using a reporter plasmid containing the LUC gene under the control of the Dof5.8 promoter (Dof5.8pro-LUC) and an effector plasmid directing the constitutive high level expression of MP (Fig. 2A). The results indicated that MP increases Dof5.8 promoter activity by ~3-fold and that the MP-induced activation was hampered by the co-expression of BDL/IAA12 (Fig. 2B), a cognate repressor protein of MP (Hamann et al., 2002). Furthermore, the addition of a natural auxin, IAA, into the incubation buffer of the transfected protoplasts enhanced the effect of MP. This could be due to the activation of MP through the degradation of repressor proteins of MP, namely endogenous Aux/IAA proteins or co-expressed BDL, by auxin (Mockaitis and Estelle, 2008). These findings suggested that MP could transactivate the Dof5.8 promoter in response to auxin.

To substantiate that the expression of Dof5.8 is under the control of MP in planta, Dof5.8 transcript levels were first analysed in three mp alleles by qRT–PCR. The expression levels of Dof5.8 in all mp mutants, including arf5-1 (Alonso et al., 2003; Okushima et al., 2005) and arf5-2 (Alonso et al., 2003; Donner et al., 2009), were reduced to ~30% of those in the wild-type A. thaliana (Fig. 2C). Furthermore, when the GUS gene fused to the Dof5.8 promoter was introduced into the arf5-2 mutant by crossing, the GUS expression was found to be lower in leaf primordia of the arf5-2 seedlings than in those of the wild-type seedlings (Fig. 2D). These results indicate that Dof5.8 expression is regulated by MP activity, at least in part.

Identification of the MP-binding sites required for provascular activation of the Dof5.8 promoter

To determine the region within the Dof5.8 promoter that is required for provascular expression, increasing segments of this promoter were deleted from its 5’ end (Fig. 3A). Deletion of the region from –1558 to –1301 in the Dof5.8 promoter diminished GUS expression in provascular cells (Fig. 3B). In addition, a synthetic promoter in which the 486 bp fragment from –1558 to –1073 was fused to the 35S minimal promoter (the 486f promoter) was able to direct provascular GUS reporter expression (Fig. 3A, B), indicating that this 486 bp region is sufficient to confer Dof5.8 expression in provascular cells.

The relationship between MP-mediated activation and expression of Dof5.8 in provascular cells was examined using the series of truncated Dof5.8 promoters. The results revealed that MP transactivated only the 486f synthetic promoter (Fig. 3C, D), indicating that the region from –1558 to –1301 is required, whilst the region from –1558 and –1073 is sufficient for both provascular cell expression and activation by MP (Fig. 3B–D). These data further indicate that an intimate relationship exists between MP-mediated activation and expression of Dof5.8 in provascular cells.

ARFs are known to recognize and bind to 5’-TGTCCG-3’ sequences (Ulmov et al., 1999a, 1999b). Four putative ARF-binding sequences were identified in the region between positions –1558 and –1073 of the Dof5.8 promoter (Fig. 3A). A mutation in site 1 (M1) or simultaneous mutations in sites 2 and 3 (M23) reduced the magnitude of activation by MP (Fig. 3A, E). Combination of these mutations (M123) led to an enhanced reduction in reporter enzyme activity, whereas disruption of the fourth site (M1234) had no apparent additional effect. The effects of these mutations on provascular expression in leaf primordia were also assessed (Fig. 3F). The mutated Dof5.8 promoters still directed provascular expression in leaf primordia, but mutations significantly decreased the GUS expression levels. These results suggest that MP
recognizes multiple sites in the Dof5.8 promoter that are involved in provascular expression of Dof5.8, although other sites in addition to these putative ARF-binding sites analysed are probably involved in provascular expression of Dof5.8, as is argued in detail in the Discussion.

The binding of MP to the Dof5.8 promoter in vivo was investigated using ChIP analysis of transgenic A. thaliana plants expressing a functional cyan fluorescent protein (CFP)-tagged MP under the control of its own promoter (Donner et al., 2009). As shown in Fig. 3G and H, the results indicated that the binding of MP to the Dof5.8 promoter is comparable with (amplicon ‘a’) and even stronger than (amplicon ‘b’) that to the Athb8 promoter, a known target of MP during vascular development (Donner et al., 2009). Collectively, these results suggest that the Dof5.8 promoter is a direct target of MP in provascular cells and that the entire region from –1558 to –1077 contributes to provascular expression of Dof5.8.

Loss of Dof5.8 affects the root and cotyledon phenotypes of the arf5-2 mutant

To explore the role of Dof5.8, two T-DNA insertion lines, dof5.8-1 (SALK_002536) and dof5.8-2 (SALK_022708), were analysed. Because T-DNA was inserted into the region...
encoding the Dof DNA-binding domain in the *dof5.8*-1 allele, this allele is a null allele. In *dof5.8*-2, T-DNA was inserted into the C-terminal region flanking the N-terminal Dof domain, suggesting that the product of this allele may retain its DNA-binding activity (Fig. 4A). However, because of the reduced transcript level in *dof5.8*-2, it is probably a loss-of-function allele (Fig. 4A). Although neither allele exhibited an apparent phenotype, it was found that they enhanced the phenotypes of a weak allele of *mp*, *arf5*-2, including abnormal root and cotyledon development. Seedlings of the *mp* mutants are rootless and often have only one cotyledon (Berleth and Jürgens, 1993). The penetrance of both phenotypes is low in the *arf5*-2 allele (Donner et al., 2009; Rademacher et al., 2011). In an experiment using a population from the MP*/arf5*-2 parent plant, 3.4% of the *arf5*-2 seedlings were rootless, as 21 seedlings out of 614 showed the phenotype (Fig. 4B). Considerably larger numbers of seedlings were found to be rootless when populations from the parent plants that are homozygous for the *dof5.8* mutation and heterozygous for the *arf5*-2 allele were investigated: 17.1% of the *dof5.8*-1 *arf5*-2 population and 9.7% of the *dof5.8*-2 *arf5*-2 population were rootless. Since the result of genotyping indicated that the percentage of *arf5*-2 homozygotes was mostly the same (~20%) in these three populations (Table 1; Supplementary Fig. 1 at JXB online), it was concluded that *dof5.8* mutations increased the penetrance of the rootless phenotype of *arf5*-2.

Another effect of *dof5.8* mutations in the *arf5*-2 mutant was also found. Most of the rootless *arf5*-2 seedlings (95%) possessed two cotyledons, while far fewer seedlings of rootless *arf5*-2 *dof5.8*-1 (10.7%) and *arf5*-2 *dof5.8*-2 (18.2%) seedlings possessed two cotyledons (Fig. 5A). Furthermore, considerable numbers of the double mutant seedlings had no cotyledons, although such a phenotype was rarely seen in the single *mp* mutants. The cotyledon-less seedlings of the double mutants always had a fat hypocotyl-like structure, which was topped with true leaves with trichomes but did not include developed vascular elements (Fig. 5D, E). This result suggests that an interaction between *arf5*-2 and *dof5.8* mutations influenced embryonic development and thus formation of cotyledons.
Effects of dof5.8 mutations on vascular patterning in the arf5-2 mutant

Vein patterns of the double mutants were also analysed, and it was found that the dof5.8 mutation modulates the vascular pattern of arf5-2 cotyledons. Vascular patterns in the cotyledons of wild-type *A. thaliana* are relatively simple and invariant, with the secondary veins delimiting the two upper aeroles and the zero to two lower aeroles (Fig. 6A). The majority of arf5-2 cotyledons showed vascular patterns similar to those of the wild type (Fig. 6A). However, a small portion (7.9%) had no aeroles due to incomplete formation of the secondary veins (Fig. 6B). The dof5.8 mutations increased the ratio of cotyledons lacking aeroles (18.6% in arf5-2 dof5.8-1 and 25.9% in arf5-2 dof5.8-2; Fig. 6B). However, at the same time, some arf5-2 dof5.8 cotyledons showed more complex vascular patterns with extra aeroles (10.4% in arf5-2 dof5.8-1 and 3.7% in arf5-2 dof5.8-2; Fig. 6A, B). The number of branch points in arf5-2 dof5.8 cotyledons also showed a broader distribution (Fig. 6D). The formation of extra aeroles and branch points was not observed in the wild type, or in arf5-2 or dof5.8 single mutants.

Discussion

In this study, it was shown that MP regulates the expression of the Dof5.8 transcription factor gene through its direct binding to the Dof5.8 promoter sequence. It was also shown that two Dof5.8 mutations (dof5.8-1 and dof5.8-2) influence abnormal root and cotyledon development and vascular patterning in the arf5-2 mutant, although the effects of dof5.8 mutations alone were not recognizable in the wild-type genetic background. These phenotypes, together with the evidence that the expression of Dof5.8 is regulated by MP, suggest a genetic interaction between MP and Dof5.8 in the developmental programme in *A. thaliana*.

![Fig. 6](https://academic.oup.com/jxb/article-abstract/66/1/283/2893431/2893431)

Fig. 6. The vascular patterns of arf5-2 dof5.8 cotyledons. (A) Representative images of the vascular pattern of cotyledons of wild-type (Col), arf5-2 mutant, and arf5-2 dof5.8-1 double mutant seedlings. Scale bars=1 mm. (B–D) The percentage of cotyledons with the indicated number of aeroles (B), freely ending veins (C), and branch points (D). Seeds from plants heterozygous for arf5-2 in the wild-type, dof5.8-1 homozygous or dof5.8-2 homozygous background were sown, and rootless seedlings were used in this analysis. (This figure is available in colour at JXB online.)
Provascular expression of Dof5.8

The results of the deletion and mutation analyses of the Dof5.8 promoter and a ChIP analysis indicated the contribution of MP to the activity of the Dof5.8 promoter in provascular cells (Figs 2, 3). Nevertheless, there are still questions as to the observed phenomenon. The early activity of the Dof5.8 promoter was observed inside leaf primordia where DR5 activity is absent. Because MP mRNA is present within primordia at this stage (Wenzel et al., 2007), the lack of DR5 activity may reflect the lack of the activity of MP protein and the regulation of the Dof5.8 promoter by other transcription factors, although there is another possibility that this phenomenon is due to the limitation of using this synthetic reporter. Furthermore, the results of deletion and point mutational analyses in protoplasts and in planta were not perfectly consistent with each another: the effect of deletion of the region from –1558 to –1301 was stronger than that of the M1 mutation (Fig. 3). On the other hand, the result of a ChIP analysis suggests that binding to the region from –1558 to –1301 is weaker than that to the region from –1301 to –1077. Therefore, cis-elements for other transcription factors that act co-operatively with or independently of MP may be present in this region. Alternatively, MP might bind to two 5′-TGTC-3′ sequences in this region in addition to the first putative MP-binding site (site 1 in Fig. 3A), since the TGTC sequence, a part of the consensus sequence for MP binding (5′-TGTCNC-3′), functioned as an MP-binding site in the Athb8 and TM07 promoters (Donner et al., 2009; Schlereth et al., 2010). Moreover, the mutations on four putative MP-binding sites (M1–M4) affected the Dof promoter activity differently in mesophyll protoplasts and leaf primordia. For instance, the M1 mutation appeared to reduce MP-dependent activation in mesophyll protoplasts and provascular cells to different extents (Fig. 3E, F). This fact also implies the possibility that other transcription factors besides MP are involved in the regulation of the expression of the Dof5.8 promoter in provascular cells. Taken together, although the results indicated that the activity of the Dof5.8 promoter in provascular cells is modulated by MP through the interaction with the region from –1558 to –1077, further analysis focused on this region would be necessary for complete understanding of the molecular mechanisms underlying the expression of Dof5.8 in provascular cells.

The role of Dof5.8 in cotyledon and root formation, and vein patterning

The dof5.8 mutations enhanced the effect of the mp mutation on embryonic root and cotyledon development, consistent with the expression of Dof5.8 in embryos as well as in the immature veins of young leaves (Konishi and Yanagisawa, 2007). The dof5.8 mutations increased the penetration of the rootless phenotype of arf5-2, suggesting that, when the activity of MP is compromised, Dof5.8 becomes critical for embryonic root formation. Such an auxiliary role to that of MP was also reported for ARF6 (Rademacher et al., 2011). The dof5.8 mutations also produced a synergistic effect with the mp mutation on cotyledon development, which resulted in cotyledon-less seedlings (Fig. 5). Although such a severe phenotype has rarely been reported for any single mp alleles, the combination of mp with a gain-of-function allele of BDL, or with the nph4 mutation produces cotyledon-less seedlings (Hamann et al., 1999; Hardtke et al., 2004). BDL is an inhibitor of ARFs including MP, and NPH4 encodes ARF7. Therefore, a more severe defect in auxin response during embryonic development could cause such cotyledon-less seedlings. The fact that dof5.8 mp double mutants showed such a severe phenotype implies that Dof5.8 is associated with the auxin- and MP-induced developmental programme during embryogenesis.

In contrast to the effects in embryonic development, dof5.8 mutations exert both positive and negative effects on vascular formation in leaf primordia of the arf5-2 mutant. The vein patterns of cotyledons in both the wild type and the arf5-2 mutant are relatively invariant, whereas the vein patterns of the arf5-2 dof5.8 mutants exhibited larger variation, with both reduced and increased complexity in vein pattern (Fig. 6). Although this phenomenon is interesting, it is difficult to explain it if Dof5.8 merely plays an auxiliary role to MP in vascular formation in leaf primordia. Thus, the molecular basis of this is currently unclear. More detailed analysis of dof5.8 mutants in combination with mutations within other Dof genes that are expressed in provascular cells or other genes downstream of MP, as well as the identification of target genes of Dof5.8 would be necessary to reveal the role of Dof5.8 in vascular formation in leaves.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Genotyping of segregating populations of the arf5-2 mutant harbouring the heterozygous arf5-2 allele and the mutants that are homozygous for the arf5-2 allele and homozygous for the dof5.8 allele.

Table S1. Primer list.

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