DEVELOPMENT-RELATED PcG TARGET IN THE APEX 4 controls leaf margin architecture in Arabidopsis thaliana

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SUMMARY
In a reverse genetics screen based on a group of genes enriched for development-related Polycomb group targets in the apex (DPAs), we isolated DPA4 as a novel regulator of leaf margin shape. T-DNA insertion lines in the DPA4 locus display enhanced leaf margin serrations and enlarged petals, whereas overexpression of DPA4 results in smooth margins. DPA4 encodes a putative RAV (Related to AB13VP1) transcriptional repressor and is expressed in the lateral organ boundary region and in the sinus of leaf serrations. DPA4 expression domains overlap with those of the known leaf shape regulator CUP-SHAPED COTYLEDON 2 (CUC2) and we provide evidence that DPA4 negatively regulates CUC2 expression independently of MIR164A, an established regulator of CUC2. Taken together, the data suggest DPA4 as a newly identified player in the signalling network that controls leaf serrations in Arabidopsis thaliana.

KEY WORDS: Leaf serration development, Polycomb group, Auxin, CUP-SHAPED COTYLEDON 2, Arabidopsis thaliana

INTRODUCTION
Plant leaves have a major role in the photosynthetic supply of nutrients, gas exchange, the distribution of nutrients and water transport (Tsukaya, 2006). To optimise these functions in different habitats, plants need to adapt leaf positioning, size and shape (Hasson et al., 2010; Nicotra et al., 2011).

An important trait of leaf shape variation is the outline of the leaf margin, which can be smooth, serrated (toothed) or lobed. If the gaps between lobes reach the midvein, leaves are designated as compound leaves and the lobes are referred to as leaflets, which can reiterate a dissecting pattern. In the simple Arabidopsis thaliana (Arabidopsis) leaf, the degree of serration differs between accessions and is developmentally regulated: juvenile leaves are smooth and serration increases in later-produced leaves. Increased serration can be caused by an increased number of teeth or by an increased sinus (the region between the teeth) depth (Nikovics et al., 2006; Tsukaya, 2006).

Leaf serration formation is mechanistically related to leaf primordia formation in the shoot apical meristem (SAM) (Scarpella et al., 2010). The auxin efflux carrier PIN-FORMED 1 (PIN1) establishes local auxin maxima that direct the outgrowth of the serrations (Scarpella et al., 2006). Accordingly, loss of PIN1 function results in smooth leaf margins (Hay and Tsiantis, 2006). It was shown that the transcription factor CUP-SHAPED COTYLEDON 2 (CUC2) facilitates the initiation of serrations by enabling PIN1 to reorientate within the membranes of margin cells to change the auxin flux from a leaf tip oriented flux into a segmented flux towards convergence points where the serrations develop (Bilsborough et al., 2011). Accordingly, CUC2 is essential for the formation of leaf serrations (Nikovics et al., 2006; Hasson et al., 2011). cuc2 mutants fail to initiate auxin convergence points at the leaf margin early in development and subsequently display a no longer discrete, but homogeneous, auxin distribution at their smooth leaf margins (Kawamura et al., 2010; Bilsborough et al., 2011; Hasson et al., 2011). Expression of CUC2 is observed along the leaf margin prior to the actual outgrowth of the teeth, which occurs at points of local CUC2 repression (Bilsborough et al., 2011). After the initiation of the teeth, CUC2 expression persists at the sinus region, where it functions, together with the partially redundant gene CUC3, in the maintenance of serration outgrowth (Hasson et al., 2011). CUC2 is post-transcriptionally downregulated in leaves by a microRNA (miRNA) encoded by the MIR164A locus. mir164a mutant plants display enhanced leaf serrations, whereas overexpression of MIR164A results in smooth leaf margins (Nikovics et al., 2006). In a feedback loop, auxin downregulates CUC2 both transcriptionally and post-transcriptionally by activation of MIR164A (Bilsborough et al., 2011).

Chromatin-mediated gene repression through Polycomb group (PcG) proteins has an impact on leaf development (Katz et al., 2004). PcG proteins are structurally unrelated repressive proteins that assemble in several Polycomb repressive complexes (PRCs), and the components of PRCs are highly conserved in higher eukaryotes (Schwartz and Pirrotta, 2008). Genes to be repressed are methylated at lysine 27 of histone H3 (H3K27me3) by PRC2, and this mark is subsequently recognised by a chromodomomain component of PRC1 (Kuzmichev et al., 2005; Schwartz and Pirrotta, 2008). PRC1 stays associated with the repressed locus and catalyses mono-ubiquitylation of lysine 119 in histone H2A (H2AK119ub1), leading to a compaction of chromatin that represses transcription (Morey and Helin, 2010). In plants, PRC2 is conserved and was shown to be involved in deposition of the H3K27me3 mark (Pien and Grossniklaus, 2007). The catalytic core of the sporophytic PRC2 comprises one of the partially redundant

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DPA4 controls leaf shape

MATERIALS AND METHODS

Plant material

Seeds of mir164a-4 (GABI_867E03) (Nikonov et al., 2006) and T-DNA insertion lines for DPA4 (At5g06250) (SALK_150707 and Salk_088181C) (Alonso et al., 2003) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Lines clf-28 (SALK_139371) (Doyle and Amasino, 2009), swn-7 (SALK_109121) and clf-28/swn-7 (Lafos et al., 2011), cuc2-1 (Aida et al., 1997), DR5:GFP (Benková et al., 2003), CUC2::CUC2:VENUS (Heisler et al., 2005) and 35S:LHP1:HA in Landsberg erecta (Ler) ecotype (Turck et al., 2007) were described previously. Sequencing of SALK_150707 revealed an insertion in the intron of At5g06250 for SALK_150707 (line was annotated in first exon). All further experiments were conducted using homozygous plants of SALK_088181C (dpa4-1) and SALK_150707 (dpa4-2). Genotyping primers are listed in supplementary material Table S4. cuc2-1 was genotyped as described (Gómez-Mena and Sablowski, 2008).

Growth conditions

PeG mutants and plants for chromatin immunoprecipitation (ChIP) experiments were grown on germination medium (GM; half-strength Murashige and Skoog medium supplemented with 1% sucrose) in growth cabinets (Percival) in a controlled environment at 20°C in long days (LD) of 16 hours light and 8 hours darkness. All other plants were grown on soil in LD in a greenhouse at 20°C or for short days (SD) of 8 hours light and 16 hours darkness in growth cabinets at 20°C, 70% humidity. Prior to sowing, all seeds were stratified at 4°C for 4 days. Plant age is indicated as days after sowing. Unless indicated otherwise, plants were harvested at Zeitgeber (ZT) 4 hours. Plants grown on soil were cut above the soil for sampling; for seedlings/calli grown on plates, whole plants were harvested.

Generation of transgenic plants

The coding region of DPA4 was amplified from Arabidopsis (Col-0) cDNA and cloned into the binary pAlligator2 vector (Bensmihen et al., 2004) using the Gateway cloning system according to the manufacturer’s instructions (Invitrogen). With this vector, transgenic plants overexpressing DPA4 in Col-0 wild type, DR5::GFP and CUC2::CUC2:VENUS plants were generated as described (Bensmihen et al., 2004). Only single T-DNA insertion lines were used for phenotypic characterisation. For DR5::GFP and CUC2::CUC2:VENUS analysis, transgenic lines with phenotypes resembling that of the 35S::DPA4-1 line were chosen.

Expression analysis

Samples named ‘flowers’ contained flowers from stage 14, and ‘apex/inflorescence’ (apex/infl.) samples contained main inflorescences including closed flowers. For ‘apex’ samples, all leaves larger than 5 mm and the roots were removed. About 2-5 mm of hypocotyls remained in the sample. For ‘leaf’ samples, the leaves removed for ‘apex’ samples were collected. This results in apex- or leaf-enriched samples, but samples do not contain exclusively the respective tissue. Total RNA was isolated and transcribed into cDNA as described (Adrian et al., 2010). Quantitative real-time PCR (qRT-PCR) was performed using a BioRad iCycler iQ5 Real-Time PCR Detection System with EvaGreen dye (Biotium) to detect the product. Primer sequences are listed in supplementary material Table S4. PP2A3 (At1g13320) was used as reference gene (Martin-Tryon et al., 2007).

Genome-wide expression data were obtained using the AGRONOMICs Tiling Array (Affymetrix) (Rehrauer et al., 2010). Three biological replicates were processed for each genotype. cRNA synthesis and array hybridisation were performed as described (Rehrauer et al., 2010).

The aroma.affymetrix package (Bengtsson et al., 2008) together with the scripts agronomicsTools01.r were used to perform RNA normalisation and calculation of mean values over all probes per gene in R. To assign probes to genes, a CDF file was created according to aroma.affymetrix instructions using the AGRONOMICs_AT_TAIRG R package (Version 13.0.0, TAIR9 annotation) obtained from the Brainarray project webpage (Dai et al., 2005). Differentially expressed genes were determined using the Bioconductor (www.bioconductor.org) package RankProd (Hong et al., 2006). Genes with false discovery rate-corrected P-values below 0.05 were considered as differentially expressed. Data of this study are available at ArrayExpress (www.ebi.ac.uk/arrayexpress/) under accession number E-TABM-1195. To confirm the array results, we performed qRT-PCR and semi-quantitative RT-PCR on selected differentially expressed genes using biological replicates of the samples used for the array (supplementary material Fig. S7A,B).

In situ hybridisation

In situ hybridisation was performed as described (Bradley et al., 1993) with the modifications described (Jang et al., 2009). Templates for T7 RNA polymerase for probe labelling were created by PCR; for CUC2 detection, a template created as described (Chandler et al., 2011) was used; the template for DPA4 was amplified using the primers listed in supplementary material Table S4. The specificity of the DPA4 probe (IS DPA4) was confirmed by the absence of signal in the dpa4-2 mutant (supplementary material Fig. S9).

Identification of hexameric motifs in promoter regions

To find hexameric motifs in promoters the Motif Analysis tool from The Arabidopsis Information Resource (TAIR) was used (www.arabidopsis.org). The length of the upstream region considered for the analysis was 3000 bp.

Determination of PcG target genes

PeG target genes were determined by ChIP followed by hybridisation to whole genome tiling arrays (ChIP-chip) as described (Reimer and Turck, 2010; Göbel et al., 2010) in 10-day-old seedlings of 35S:LHP1:HA (polyclonal anti-HA antibodies from rabbit; Sigma, H6908). Data analysis was performed using simple loess normalisation; LHP1 target genes were determined by ChIP followed by hybridisation to whole genome tiling arrays (ChIP-chip) as described (Reimer and Turck, 2010). Three biological replicates were processed for each genotype. cRNA synthesis and array hybridisation were performed as described (Rehrauer et al., 2010).

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Clustering and Gene Ontology (GO) analysis

PeG target genes were clustered according to expression in different developmental stages and organs using the Developmental Series from the AgGenExpress project (Schmid et al., 2005). After normalising expression values per gene by root mean square, hierarchical and k-means clustering were performed using Genesis software (Sturm et al., 2002) as described (Engelhorn and Turck, 2010). A hierarchical clustering analysis of a subset...
of 500 randomly chosen H3K27me3 target genes revealed nine major branches, indicating nine groups of genes with similar expression patterns. Therefore, k=9 was used for the following k-means clustering analysis (supplementary material Fig. S10). One cluster (cluster 4) contained the group of mostly apex- and flower/floral organ-expressed genes. We used both gcRMA and MAS 5.0 normalised expression data and repeated the k-means clustering analysis ten times to obtain a final list of genes stably assigned to the apex cluster in all repetitions (105 genes). For functional enrichment analysis we used the web tool FatigO (Al-Shahrouk et al., 2007).

Scanning electron microscopy (SEM)
Fresh plant material was frozen in liquid nitrogen and remaining surface water was sublimed. Frozen samples were then spattered with platinum and transferred to the microscope under constant vacuum. Images were obtained using a Zeiss SUPRA 40VP scanning electron microscope including cryopreparation and transfer system (EMITECH K1250X).

Confocal microscopy
Images were taken using a TCS SP2 AOB5 confocal laser-scanning microscope (Leica, Mannheim, Germany). Excitation was with an argon laser at 488 nm (GFP) or 514 nm (VENUS). Emission of GFP was detected from 492 to 550 nm and of VENUS from 518 to 575 nm. As a reference, the autofluorescence of chlorophyll was simultaneously detected between 650 and 730 nm. Images presented are average projections of 8-20 optical sections.

Quantification of leaf dissection index
To obtain silhouettes of leaves for figures and quantification, leaves were adhered to white paper and digitised using a scanner at 600 dpi resolution. Outlines of leaves were coloured in black and leaf surface was determined by counting of black pixels. Leaf perimeter was determined as the length of a closed path along all margin pixels. Leaf dissection index was calculated as (perimeter squared)/(4\pi \times area) as described (Bilsborough et al., 2011). All calculations and measurements were performed using a customised C++ program.

RESULTS
Identification of developmental regulators among PcG target genes
PcG target genes with tissue-specific expression were previously shown to play a major role in development according to Gene Ontology (GO) analysis (Zhang et al., 2007a). We generated genome-wide distribution data of the PcG protein LHP1 and classified target genes according to their expression pattern using publicly available expression data for different developmental stages and tissues and k-means clustering (supplementary material Fig. S10) (Schmid et al., 2005). A GO analysis for the 105 shoot apex-expressed LHP1 target genes revealed a significant over-representation of several developmental functions. This over-representation was only found for apex-expressed targets, not for all LHP1 targets or all apex-expressed genes (supplementary material Table S1). Since 50% of the genes in the apex cluster had not yet been characterised, we performed a screen for developmental abnormalities in T-DNA insertion lines that were likely loss-of-function alleles of these uncharacterised genes. We identified fourteen putative development-related PcG targets in the apex (DPAs). Here we report our characterisation of DPA4 (AT5G06250). Based on sequence similarity, DPA4 is a member of the B3 superfamily and a RAV [Related to ABRICISIC ACID-INSSENSITIVE 3 (AB13)/VIVIPAROUS1 (VP1)] transcriptional repressor containing the repressive motif described for B3 transcription factors (LVRLFVG N/D M/L/V) in the variety VRLFVGVLN (Romanel et al., 2009; Ikeda and Ohme-Takagi, 2009) (Fig. 1A,B).

DPA4 controls leaf margin outline and floral organ size
We analysed two independent T-DNA insertion lines in DPA4 (dpa4-1 and dpa4-2); both T-DNAs were inserted in the only intron of the gene but in opposite orientation (Fig. 1A). Expression of the full-length transcript was undetectable in dpa4-2 and strongly reduced in dpa4-1 (Fig. 1C).

Both dpa4-1 and dpa4-2 displayed enhanced leaf margin serration and elongated petals (Fig. 1D and supplementary material Fig. S1). No statistically significant difference in size was observed for other floral organs, although a tendency for bigger sepal length in dpa4-2 plants was observed. In accordance with the presence of residual full-length transcript in dpa4-1, serrations were stronger in dpa4-2 than in dpa4-1 (supplementary material Fig. S1A).

Serrations in dpa4 plants were increased by a deepening of the sinuses and not by an increased number of serrations (supplementary material Fig. S1B,C). As in wild type, serrations were stronger in later-produced rosette leaves of dpa4 plants, at which point the differences with wild type became more obvious (Fig. 1D). Rosette diameter and leaf surface were on average the same in dpa4 and Col-0 plants (data not shown) and the shape of leaf epidermal cells was unchanged in dpa4-2 plants (supplementary material Fig. S2). The leaf margin of Arabidopsis thaliana is characterised by highly elongated epidermal cells (Kawamura et al., 2010). Margin cells in dpa4-1 plants appeared to elongate and develop normally, except for the increase in sinus depth and the associated more pronounced bending of the leaf margin cells (supplementary material Fig. S2D). Owing to the stronger deepening of serrations and absence of the full-length transcript, dpa4-2 plants were used in all subsequent experiments.

Four independent 35S::DPA4 overexpressor lines [35S::DPA4-1, 35S::DPA4-2, 35S::DPA4-4 (in CUC2::CUC2:VENUS) and 35S::DPA4-9 (in DR5::GFP)] developed narrow leaves without serrations (Fig. 1D and supplementary material Fig. S3A) and further lines displayed reduced serrations in several nuances, correlated with the amount of DPA4 transcript (supplementary material Fig. S3B,C). The strongest lines, which did not show leaf serrations, could not be propagated as homozygous. In each sowing, up to 20% of the plants were very small (~1.5 cm in diameter), produced progressively smaller leaves and died after several weeks, prior to flowering. These individuals expressed DPA4 at approximately twice the level of their hemizygous siblings and were thus likely to be homozygous (supplementary material Fig. S3).

Hemizygous 35S::DPA4-1 and 35S::DPA4-2 plants produced more leaves prior to flowering than wild type and flowered at least 10 days later (Fig. 1D). In several transgenic lines, leaves of individual plants turned in a left-handed spiral (Fig. 1E). Additionally, these lines displayed several other abnormal phenotypes, such as a pronounced reduction in height, fascination of inflorescences, reduced number of petals in some flowers and a broader replum resulting in abnormally shaped siliques (Fig. 1F-1).

DPA4 is expressed in organ boundaries and in the sinus of leaf serrations
We selected DPA4 as a candidate for our screen because of its exclusive expression in the shoot apex and floral organs. Visualisation of the Developmental Series data with the Arabidopsis eFP browser (www.bar.utoronto.ca) showed strongest expression of DPA4 in the inflorescence shoot apex and in siliques containing stage 3 seeds. Strong expression was also observed in transition/vegetative apices and flowers at stage 9. We confirmed
that DPA4 expression is higher in apex-enriched samples than in the corresponding leaf samples collected at different stages (supplementary material Fig. S4). In situ hybridisation experiments showed that expression of DPA4 within the shoot apex is tightly restricted to the areas of primordia formation. In vegetative and transition apices, expression was observed at the emerging boundary between the shoot apex and the leaf primordium. DPA4 expression persisted in this area as the leaf developed (Fig. 2A,B). In inflorescence apices, DPA4 was expressed in the boundary between the inflorescence meristem and the emerging flowers; in floral meristems, DPA4 expression marked the boundaries between emerging floral organs (Fig. 2C,D). In flowers, DPA4 expression persisted between organs, and also between the two fused carpels (Fig. 2E). Thus, DPA4 is expressed wherever organs separate from the SAM or from each other. In addition, DPA4 expression was observed in the leaf sinus, suggesting a role of DPA4 during the early stages of organ initiation and during early development in young organs (Fig. 2F,G).

The DPA4 expression level but not its tissue specificity is regulated via PcG proteins

The very restricted spatial expression pattern of DPA4 in organ boundaries suggests a tight control of DPA4 expression that might be conferred through chromatin-mediated gene repression by PcG proteins. We probed the expression of DPA4 in plants mutated in components of the PcG pathway. In the severe clf/swn double mutants, PcG-mediated repression is abolished in the developing seedling. DPA4 was ~6-fold upregulated at 22 days in clf/swn, whereas upregulation at 11 days was much weaker (Fig. 3A). In the single clf mutant, DPA4 expression was slightly lower than in wild type. This observation is in accordance with results from our previous studies, in which DPA4 expression was, among ~500 other PcG target genes, slightly upregulated in clf/swn (1.23-fold) and slightly downregulated in clf (Farrona et al., 2011).

To further elucidate the possible regulation of DPA4 by PcG proteins, we performed ChIP with an anti-H3K27me3 antibody followed by semi-quantitative PCR on the DPA4 locus (supplementary material Fig. S5). Strong H3K27me3 signal was detected in wild-type and clf plants, whereas the chromatin mark was absent from clf/swn. These results are in accordance with the DPA4 expression data in the mutants. Whereas clf plants develop relatively normally apart from an early flowering phenotype and an upward curling of leaves, clf/swn double mutants develop into callus-like structures after germination (Fig. 3B) (Schubert et al., 2005). At 22 days, the formation of ectopic tissue is already visible, whereas the 11-day-old seedlings contain mainly differentiated tissue. Thus, DPA4 expression is elevated with the loss of differentiation, suggesting that the strict tissue-specific regulation of DPA4 depends on organ differentiation and might be lost in clf/swn. However, in situ hybridisation revealed that expression of DPA4 was still restricted to discrete clusters of few cells in clf/swn callus tissue, indicating that additional regulation, apart from PcG-mediated repression, is involved in the tight regulation of DPA4 (Fig. 3B).

DPA4 influences later stages of leaf serration development

The expression of DPA4 in the boundaries of initiating and developing organs does not answer the question of whether the gene controls development at early stages in the shoot apex or later.
in the developing organ. Defects in serration formation can be caused by a failure in serration initiation (as in cuc2 mutants) or by defects in serration outgrowth (as in cuc3 mutants) (Hasson et al., 2011). The deepened serrations observed in the mutants could be caused by an earlier onset of serration formation in developing leaves. However, no alterations during early serration initiation were observed in dpa4 plants: serrations occurred at approximately the same leaf length in dpa4 and Col-0 leaves (Fig. 4A,B). Therefore, the defects in dpa4 plants originate later during the outgrowth of serrations. Differences to wild type only became apparent when the leaves reached a length of ~600 µm (supplementary material Fig. S6). We never observed serrations in homozygous plants of strong 35S::DPA4 lines, including at early stages of leaf serration development (Fig. 4C,D). Taken together, these observations suggest that DPA4 can also prevent serration initiation if expressed ectopically, although its function in the accession Col-0 seems restricted to serration outgrowth.

**Global expression profiling links DPA4 to auxin signalling**

As a putative transcriptional repressor, DPA4 is expected to participate in leaf development by downregulating the transcription of target genes. Transcriptional profiling of dpa4 and wild-type 'apex' tissue of 28-day-old plants grown in SD conditions identified 16 significantly upregulated and 61 downregulated genes in dpa4 (supplementary material Table S2).

Functional characterisation of the differentially expressed genes provided no obvious candidates that might directly explain the leaf serration phenotype (supplementary material Fig. S7 and Table S3).

The RAV B3 domain has been shown to recognise a CACCTG motif (Kagaya et al., 1999). Eight of the upregulated and 24 of the downregulated genes contain the CACCTG motif in their promoter region, which is not significantly different from the background distribution. Interestingly, the auxin-response element (AuxRE) TGTCCT, which is the binding motif for AUXIN RESPONSE FACTOR (ARF) family members (Ulmasov et al., 1999), was found in 57 of the 61 downregulated genes, which is a significant over-representation (P=0.00198 assuming binomial distribution). As the putative repressive function of DPA4 suggests that genes downregulated in dpa4 plants are indirect targets of DPA4, an indirect regulation through ARF-mediated auxin signalling is more likely. The AuxRE was also found in 11 of the upregulated genes, but this does not constitute an over-representation of the motif.

**Auxin distribution in 35S::DPA4 plants is similar to that in cuc2-3 mutants**

To test whether DPA4 affects auxin distribution, we analysed expression of the DR5::GFP auxin reporter in 35S::DPA4 plants (Benková et al., 2003). 35S::DPA4-9 plants showed GFP signal at the tip of initiating leaves, comparable to wild type, but failed to establish discrete auxin maxima at other positions, even at growth stages when wild-type leaves had already initiated serrations (Fig. 5A-D). At later stages, when serrations grew out in wild type, a diffuse expression of DR5::GFP was visible throughout the leaf margin of 35S::DPA4-9 plants (Fig. 5E,F). This auxin distribution pattern during leaf development resembles that observed in cuc2 mutants (Bilsborough et al., 2011). DPA4 and CUC2 expression domains overlap in almost all post-embryonic parts of the plant. CUC2, as with DPA4, is expressed in the boundary region of the SAM, in leaf sinuses, at the base of emerging floral organs and the septum region of carpels (Fig. 2) (Ishida et al., 2000; Nikovics et al., 2006).

Alterations of CUC2 transcript levels result in phenotypic changes that are similar to those observed in dpa4 mutant plants, but the effects are opposite: expression of a miR164-resistant version of CUC2 (cuc2-1D) results in enhanced leaf serration and enlarged leaf and petal size (Larue et al., 2009), similar to dpa4 (Fig. 1C,D and supplementary material Fig. S1). By contrast,
overexpression of MIR164A reduces CUC2 levels and causes smooth leaf margins (Nikovics et al., 2006), as does overexpression of DPA4. Taken together, DPA4 appears to act in the same process as CUC2 but plays an antagonistic role.

Leaf shape regulation by DPA4 depends on CUC2 but not MIR164A

To place DPA4 within the genetic network for leaf serration development, we analysed the genetic interaction of DPA4, CUC2 and MIR164A.

The alleles of dpa4 and cuc2-1 are in different genetic backgrounds, which show differences in leaf serration and leaf shape (cuc2-1 in Ler; dpa4 in Col-0). We therefore scored several double-homozygous cuc2-1/dpa4 F3 families for their degree of serration to account for phenotypic alterations caused by the genetic background. None of the cuc2-1/dpa4 plants displayed any serrations, whereas serrations occurred in all single-homozygous dpa4 families. In conclusion, serrations are abolished in cuc2-1/dpa4 double mutants (Fig. 6A and supplementary material Fig. S8A,B). These results confirm the essential role of CUC2 in serration formation, which was reported previously for the genetic interactions of CUC2 and other leaf margin-controlling genes (Hasson et al., 2011).

Based on the epistatic relationship of their loss-of-function alleles and their antagonistic effect on leaf serration development, we expected CUC2 to act downstream of DPA4. CUC2 is directly repressed by miR164, and DPA4 could antagonise CUC2 by affecting this miRNA. We therefore tested whether the absence of MIR164A compromised the inhibitory effect of DPA4 overexpression on leaf serration. All plants homozygous for mir164a-4 carrying at least a hemizygous allele of 35S::DPA4 displayed a 35S::DPA4 phenotype (Fig. 6B), suggesting that the repression of leaf serrations by DPA4 does not depend on miR164. Analysis of double mir164a-4/dpa4 mutants confirmed that DPA4 and miR164 act as independent antagonists of leaf serrations. The double mir164a-4/dpa4 mutants displayed enhanced serrations.
that \textit{MIR164A} and \textit{DPA4} have an additive effect on \textit{CUC2} repression. Compared with Col-0, \textit{CUC2} expression was increased to a similar degree in either single mutant, whereas an additive increase was observed in \textit{mir164a-4/dpa4} double mutants (Fig. 7A). In addition, \textit{CUC2} expression was strongly downregulated in \textit{35S::DPA4-1} (Fig. 7B). This downregulation occurred in both the shoot apex and young developing leaves, where no \textit{CUC2} transcript was detectable in \textit{35S::DPA4} plants (Fig. 7C-G). \textit{CUC2} is expressed in organ boundaries at the SAM and in sinuses of leaf serrations; therefore, the respective reduction and enhancement of these structures in \textit{35S::DPA4} and \textit{dpa4} plants could be the indirect cause of changes in \textit{CUC2} expression levels. We used transgenic plants expressing a \textit{CUC2::CUC2:VENUS} reporter construct (Heisler et al., 2005) to detect the impact of \textit{DPA4} on \textit{CUC2} expression prior to serration formation. As previously shown, \textit{CUC2} is expressed in a slightly expanded region at the leaf base before leaf serration initiation and this pattern is gradually restricted to the sinuses of expanding serrations (Fig. 7D,F) (Bilsborough et al., 2011). In strong \textit{DPA4}-overexpressing plants, no \textit{CUC2:VENUS} signal was detected, suggesting that \textit{DPA4} mediates \textit{CUC2} repression (Fig. 7E,G).

Taken together, our results establish \textit{DPA4} as a new player in the genetic network of leaf margin regulation that represses leaf serration formation by negatively regulating the expression of \textit{CUC2} in parallel to \textit{miR164} (Fig. 8).

**DISCUSSION**

**DPA4 as repressor of leaf serrations**

We identified \textit{DPA4} as a gene with a role in development by a reverse genetics screen based on a rationally selected subset of genome-wide PcG target genes. The reduced number of candidate genes allowed screening for developmental abnormalities in several growth conditions. The relatively mild leaf serration phenotype in \textit{dpa4} T-DNA insertion lines was best observed in SD growth conditions and could easily have been overlooked in a higher throughput approach. However, the role of \textit{DPA4} in the regulation of leaf margin shape is clearly confirmed by the strong reciprocal enhancement of the single \textit{mir164a} and \textit{dpa4} phenotypes in combined double mutants (Fig. 6C,D).

Other lines of evidence indicate that \textit{DPA4} influences leaf serration by modulating \textit{CUC2} transcript levels. First, leaf serrations are absent in \textit{cuc2/dpa4} double mutants, indicating that \textit{DPA4} requires a functional \textit{CUC2} to increase leaf serrations (Fig. 6A). Furthermore, \textit{CUC2} levels are upregulated in \textit{dpa4} seedlings to levels similar to those observed in \textit{mir164a} mutants (Fig. 7A). In addition, \textit{CUC2} levels are strongly downregulated in \textit{35S::DPA4} plants even before leaf serrations have initiated, which confirms that the reduced expression of \textit{CUC2} is not only a consequence of the reduced number of serration sinuses, but is also more directly dependent on \textit{DPA4} (Fig. 7B-G). Nevertheless, it cannot be ruled out that \textit{DPA4} also plays a role downstream of \textit{CUC2}, and this will be subject to further studies.

The negative regulation of \textit{CUC2} by \textit{DPA4} is independent of \textit{miR164}. First, the phenotype of \textit{35S::DPA4} plants is not altered in the absence of \textit{MIR164A}. In addition, the enhancement of leaf serrations in double \textit{mir164a-4/dpa4} mutants is at least additive, as is the increase in \textit{CUC2} expression. Thus, \textit{DPA4} and \textit{MIR164A} represent two parallel pathways that converge to fine-tune \textit{CUC2} levels. Slight alterations in the expression of either gene might contribute to the plasticity of leaf serration development, such as the variation observed in serration depth in different culture conditions and variation dependent on

**DPA4 regulates \textit{CUC2} expression**

As a predicted transcriptional repressor, \textit{DPA4} could control leaf serration by modulating \textit{CUC2} expression. Expression analysis in the single and double mutants of \textit{MIR164A} and \textit{DPA4} confirmed compared with either single mutant, indicating a parallel action of \textit{miR164} and \textit{DPA4} (Fig. 6C,D and supplementary material Fig. S8C,D). The substantial increase in depth in the most basal serrations and juvenile leaves suggests an at least additive phenotype in the double \textit{mir164a-4/dpa4} mutant.
developmental age (Royer et al., 2009). In addition, the relative contribution of DPA4 and MIR164A might be subject to natural variation. So far, a limited number of studies have reported quantitative trait loci (QTLs) for leaf serration depth in adult leaves, but apparently not just one but several major QTLs cause the variation between Ler and Col and Ler and Cape Verde Islands (Cvi) (Pérez-Pérez et al., 2002; Juenger et al., 2005; Pérez-Pérez et al., 2010).

Interestingly, the dpa4-1 allele, which shows residual levels of DPA4 transcript, has a milder effect on leaf serrations than the dpa4-2 loss-of-function allele, suggesting a dosage dependency for DPA4. In this context it should be mentioned that DPA4 might show redundancies with other closely related RAV transcription factors. The closest relative of DPA4, At3g11580, shows only 64% amino acid similarity and is predominantly expressed in mature seeds (Schmid et al., 2005). The T-DNA insertion lines available for At3g11580 did not generate loss-of-function alleles; therefore, the influence of redundancy has not yet been fully excluded in this study.

Severe developmental defects are observed in 35S::DPA4 plants that cannot be explained by a loss of CUC2 function alone, suggesting that high-level ectopic expression of DPA4 also affects other target genes. Indeed, we observed a strong downregulation of CUC2-related genes in DPA4-overexpressing plants (data not shown), but the direct involvement of DPA4 is difficult to evaluate. CUC3 expression is dependent on CUC2, which means that the loss of CUC2 in 35S::DPA4 plants could be the primary cause of CUC3 downregulation (Hasson et al., 2011). CUC1 is expressed in boundary regions of the SAM, which is gradually consumed in DPA4-overexpressing plants (Fig. 7C). On the other hand, repression of additional CUC genes might lead to a gradual consumption of the meristematic cells and thus explain the smaller meristem size of 35S::DPA4 plants (Aida et al., 1997; Ishida et al., 2000). Future studies will have to clarify whether any of the CUC genes, including CUC2, are indeed direct targets of the supposed transcriptional repressor DPA4 or if other factors act as intermediates in the regulatory network.

PcG-mediated repression at several levels of leaf margin development

In addition to DPA4, the leaf shape-controlling factors MIR164A, CUC2 and PIN1 are PcG target genes. Given their network topology, it is not possible to conclude whether an upregulation of
these genes in clf/swn is immediately dependent on the loss of repressive chromatin or is indirectly mediated by alterations in expression within the network.

Expression of DPA4 is restricted to defined areas in a few tissues. Interestingly, the putative target gene CUC2 displays the same expression pattern, although DPA4 is a negative regulator of CUC2. This indicates an as yet unknown regulatory mechanism that results in the co-expression of these genes, which then fine-tune their expression. PcG targets are on average more tissue specific than the remainder of the genome and several target genes display tissue-specific H3K27me3 labelling that is inversely correlated with their expression pattern (Zhang et al., 2007a; Lafos et al., 2011). This suggests that PcG-mediated regulation is responsible for the restricted expression pattern of DPA4, CUC2 and MIR164A (this work) (Nikovics et al., 2006), although we found DPA4 expression still restricted to discrete clusters of a few cells in the clf/swn callus tissue, suggesting that additional factors are responsible for the tissue-specific expression of DPA4. The preservation of tissue specificity in severe PcG mutants has also been observed for other H3K27me3 targets. In particular, FT expression is strictly dependent on the presence of a differentiated vasculature even in the absence of PcG-mediated repression (Farrona et al., 2011).

Little is known about CUC2 upstream activators, which, given the similar expression pattern, could also contribute positively to the expression of DPA4. A recent report shows that SHOOT MERISTEMLESS (STM) acts as a direct activator of CUC1 through CTGTCGA elements found in the CUC1 promoter (Spinelli et al., 2011). Prolonged expression of STM also leads to activation of CUC2 and MIR164A, but this effect is indirect (Spinelli et al., 2011). All CUC genes and DPA4 are expressed in a similar pattern in the SAM, but CUC1 is not expressed in developing leaves. Chromatin remodelling ATPases are positive regulators of CUC genes, which are expressed at lower levels in plants that carry mutations in BRAHMA (AIBRM). BRM may participate in tissue-specific expression regulation, but its ubiquitous expression pattern suggests that at least one additional tissue-specific partner should be involved in the process (Kwon et al., 2006).

Complexity of the leaf serration gene network

DPA4 and MIR164A appear to play redundant roles in fine-tuning the expression of CUC2 and show largely overlapping expression domains. The question arises as to why such redundant regulation has evolved or been maintained. MIR164 and DPA4 are both conserved in eudicots and the role of the MIR164 and CUC2 pathway in the evolution of leaf shape has been confirmed (Blein et al., 2008). The relative contribution of DPA4 orthologues to leaf development in plant species that show more complicated leaf serration patterns than Arabidopsis remains to be seen.

It will be a challenge to integrate information on how developmental timing and the perception of environmental signals influence the expression of the single components in the gene network that controls leaf serration.

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