Control of final organ size by Mediator complex subunit 25 in Arabidopsis thaliana

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SUMMARY
Control of organ size by cell proliferation and cell expansion is a fundamental developmental process, but the mechanisms that establish the final size of organs and whole organisms remain elusive in plants and animals. We have previously demonstrated that DA1, which encodes a predicted ubiquitin receptor, controls the final size of seeds and organs by restricting cell proliferation in Arabidopsis. Through a genetic screen for mutations that enhance the floral organ size of da1-1, we have identified an enhancer of da1-1 (eod8-1). The eod8-1 mutation was identified, using a map-based cloning approach, in Mediator complex subunit 25 (MED25; also known as PFT1), which is involved in the transcriptional regulation of gene expression. Loss-of-function mutants in MED25 form small organs with fewer cells as a result of a reduced period of cell proliferation and cell expansion, whereas plants overexpressing MED25 have small organs owing to decreases in both cell number and cell size. Our genetic and physiological data suggest that MED25 acts to limit cell and organ growth independently of MED25-mediated phytochrome signaling and the jasmonate pathway. Genetic analyses show that MED25 functions redundantly with DA1 to control organ growth by restricting cell proliferation. Collectively, our findings show that MED25 plays a crucial role in setting final organ size, suggesting that it constitutes an important point of regulation in plant organ size control within the transcriptional machinery.

KEY WORDS: MED25 (PFT1), DA1, Organ size, Cell proliferation and expansion, Arabidopsis

INTRODUCTION
Although the final size of organs is influenced by environmental cues, the developing organs possess intrinsic information about their final size (Mizukami, 2001; Sugimoto-Shirasu and Roberts, 2003; Tsukaya, 2003; Ingram and Waites, 2006; Tsukaya, 2006; Anastasiou and Lenhard, 2007). In animals, the target of rapamycin (TOR) pathway and the Hippo pathway are two major pathways of organ size control (Arsham and Neufeld, 2006; Dong et al., 2007; Zeng and Hong, 2008). In plants, several plant-specific regulators of organ size control have been identified (Disch et al., 2006; White, 2006; Li et al., 2008), suggesting that plants possess novel mechanisms of organ size control. However, how the final size of organs is determined in plants is largely unknown.

Growth of plant organs up to their species-specific size is regulated by both cell number and cell size, which are the consequence of coordination of cell proliferation and cell expansion during organogenesis (Mizukami, 2001; Sugimoto-Shirasu and Roberts, 2003). Several factors that promote organ growth by increasing cell proliferation have now been isolated in plants, including AINTEGUMENTA (ANT), JAGGED (JAG), growth-regulating factors (AtGRFs), GRI-interacting factors (AtGIFs), ARGOS and KLUH (also known as CYP78A5) (Krizek, 1999; Mizukami and Fischer, 2000; Anastasiou et al., 2007). Overexpression of some of these genes (e.g. ANT and KLUH) causes enlarged organs with increased numbers of cells due to increases in the duration of proliferative growth, whereas their loss-of-function mutants form small organs with fewer cells as a result of a reduced period of cell proliferation (Mizukami and Fischer, 2000; Anastasiou et al., 2007). By contrast, several regulators have been described that restrict organ size by limiting the period of proliferative growth, such as CINCINNATA (CIN), AUXIN RESPONSE FACTOR 2 (ARF2), BIG BROTHER (BB) and DA1 (Nath et al., 2003; Okushima et al., 2005; Disch et al., 2006; Schruff et al., 2006; Li et al., 2008). In addition, PEAPOD (PPD) genes redundantly restrict dispersed meristematic cell (DMC) proliferation of leaf cells (White, 2006). These studies suggest that modulation of the time and location of cell proliferation is a key point of regulation in organ size control. Plant organ size is also regulated by cell expansion. For example, AtEXP10, ROTUNDIFOLIA 3 (ROT3), ANGUSTIFOLIA (AN) and ARGOS-LIKE (ARL) regulate organ growth by promoting cell expansion (Tsuge et al., 1996; Kim et al., 1998; Kim et al., 1999; Cho and Cosgrove, 2000; Kim et al., 2002; Hu et al., 2006), whereas BIGPETALp (BPEp) and RPT2a control organ growth by limiting cell expansion (Szecsi et al., 2006; Kurepa et al., 2009; Sonoda et al., 2009). Furthermore, increase in cell size is often associated with an increase in ploidy resulting from endoreduplication (Sugimoto-Shirasu and Roberts, 2003; Inze and De Veylder, 2006). Thus, the developmental regulation of endoreduplication has been suggested as a possible mechanism employed to control plant organ size (Mizukami, 2001; Sugimoto-Shirasu and Roberts, 2003).

The Arabidopsis struwelpeter (swp) mutant shows small aerial organs and various defects in development (Atur et al., 2002). SWP encodes a homolog of the yeast and metazoan Mediator subunit MED14 (Atur et al., 2002), suggesting that the Mediator complex is involved in the regulation of organ growth and development. The Mediator complex is a large multiprotein complex that coordinates the transcription of cellular activities. Plant Mediator complex is involved in the regulation of organ growth and development.
complex that is conserved in all eukaryotes and which functions to transmit various signals from activators and repressors, as well as general transcription factors, to the RNA polymerase II machinery to initiate transcription (Kim et al., 1994; Koleske and Young, 1994). The Mediator complex has only recently been identified in Arabidopsis; it contains 21 conserved and six putative plant-specific Mediator subunits (Backstrom et al., 2007). However, how the Mediator complex subunits control plant organ size remains largely unknown.

We have previously identified Arabidopsis DA1 as a negative regulator of seed and organ size, and the da1-1 mutant forms large seeds and organs due to increases in the period of proliferative growth (Li et al., 2008). Here, we describe an enhancer of da1-1 with a mutation in MEDIATOR COMPLEX SUBUNIT 25 (MED25; also known as PFFT), which has known roles in shade avoidance and stress responses in Arabidopsis (Cerdan and Chory, 2003; Backstrom et al., 2007; Wollenberg et al., 2008; Kidd et al., 2009; Elfving et al., 2011), but no previously identified function in organ size control. Loss of MED25 function causes enlarged organs with larger and slightly increased numbers of cells, whereas overexpression of MED25 results in small organs with smaller and fewer cells, indicating that MED25 regulates organ growth by restricting both cell proliferation and cell expansion. Genetic analysis suggests that MED25 acts redundantly with DA1 to control organ size by restricting cell proliferation. Our findings provide new insights into the role of plant MED25 in setting organ size.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis ecotype Col-0 was the wild-type line used. All mutants were in the Col-0 background, except for med25-3 and ft-7, which were in CS8846 and Ler backgrounds, respectively. Plants were grown at 22°C under standard conditions (a 16-hour light/8-hour dark cycle).

Identification of med25 mutants

eod8-1 (med25-1) was identified as a phenotypic enhancer of da1-1 from ethyl methanesulfonate (EMS)-treated M2 populations of da1-1. med25-2 (SALK_080230) and med25-3 (CS870982) were obtained from the Arabidopsis Stock Center NASC and ABRC collections. T-DNA insertions were confirmed by PCR (for primers, see Table S3 in the supplementary material).

Map-based cloning of MED25

The eod8-1 mutation was mapped in the F2 population of a cross between med25-1 da1-1 and da1-1Ler. To fine-map the eod8-1 mutation, new molecular markers were developed using public databases (see Table S1 in the supplementary material). We further sequenced an 18.8 kb interval between markers F2J7-6 and F2J7-10 (for primers, see Table S2 in the supplementary material).

Plasmid construction and plant transformation

MED25 coding sequence (CDS) was amplified by PCR (primers are described in Table S4 in the supplementary material) and products subcloned into T-vector (Promega). The MED25 CDS was then inserted into the EcoRI site of the 35S::pGreen vector to generate the transformation plasmid 35S::MED25-pG. The MED25 CDS was also cloned into the KpnI site of the 35S::pC1300 vector to generate the transformation plasmid 35S::MED25-pC. The MED25-KpnI primers for the 35S::MED25-pC construct are described in Table S4 in the supplementary material. Plasmids 35S::MED25-pG and 35S::MED25-pC were introduced into Col-0 and med25-2, respectively, using Agrobacterium tumefaciens GV3101. Transformants were selected on medium containing hygromycin (50 μg/ml) or kanamycin (50 μg/ml).

The MED25 promoter was amplified by PCR (using MED25PROM primers, see Table S4 in the supplementary material) and subcloned into T-vector (Promega). The MED25 promoter was then inserted into the SucI and Ncol sites of the binary vector pGreen-GUS to generate the transformation plasmid pMED25::GUS. The plasmid pMED25::GUS was introduced into Col-0 plants using Agrobacterium tumefaciens GV3101 and transformants were selected on medium containing kanamycin (50 μg/ml).

Morphological and cellular analyses

Area measurements of petals (stage 14) and leaves were made by flattening the organs, scanning to produce a digital image, and then calculating the area using ImageJ software (NIH). Each value for petal area represents measurements from more than 30 petals. Each value for leaf area represents measurements from more than ten leaves. To measure cell size and cell number, petals and leaves were mounted in clearing solution (8 g chloral hydrate, 11 ml water, 1 ml glycerol). Cleared samples were imaged using differential interference contrast (DIC) optics on a Leica DM2500 microscope, photographed with a SPOT Flex cooled CCD digital image system, and processed using Photoshop (Adobe). Each value for cell size represents measurements from more than 100 cells. Each value for cell number in terms of petal length and petal width represents measurements from more than 10 petals.

Kinematic analysis of petal growth was performed as described (Li et al., 2008). To detect the influence of the med25-2 mutation on cell proliferation, a pCYC11::GUS reporter gene was introgressed into the med25-2 mutant. The total cell number and the number of cells with GUS activity in petals were counted and expressed as a mitotic index (percentage of cells with GUS activity/number of total cells). Each value represents measurements from at least ten petals.

GUS staining

Samples were stained in 1 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-gluc), 50 mM NaPO4 pH 7.0, 0.4 mM K4Fe(CN)6, 0.4 mM K4Fe(CN)6, 0.1% (v/v) Triton X-100 and incubated at 37°C for 6 hours. After GUS staining, chlorophyll was removed using 70% ethanol.

Jasmonate treatment

Jasmonate treatments were performed as previously described (Brioudes et al., 2009). Fully open flowers of Col-0 and med25-2 were removed and flower buds on the apical cluster were sprayed with 100 μM methyl jasmonate and 0.1% Tween 20 until the inflorescence was visibly wet. Mock treatments were performed with 0.1% Tween 20.

RNA isolation, RT-PCR and quantitative real-time RT-PCR analysis

Total RNA was extracted from Arabidopsis roots, stems, leaves and inflorescences using the RNeasy Plant Mini Kit (Tiangen, Beijing, China). Reverse transcription (RT) PCR was performed as described (Li et al., 2006). cDNA samples were standardized according to the ACTIN 7 transcript. Quantitative real-time RT-PCR analysis was performed with a LightCycler 480 Engine (Roche) using the LightCycler 480 SYBR Green I Master (Roche), with ACTIN 2 mRNA as an internal control. For RT-PCR and quantitative real-time RT-PCR primers, see Table S5 in the supplementary material.

RESULTS

Isolation and genetic analysis of an enhancer of da1-1

The da1-1 mutant forms large seeds and floral organs as a result of increased cell numbers (Fig. 1A) (Li et al., 2008). To identify other components in the DA1 pathway or additional regulators of organ size, we performed a genetic screen for phenotypic enhancers of da1-1. An EMS mutagenesis screen carried out in a da1-1 mutant background resulted in the isolation of several mutants that had larger organs than da1-1. One enhancer of da1-1, named eod8-1, significantly enhanced the floral organ size phenotypes of da1-1 (Fig. 1A,B). The eod8-1 da1-1 double mutant exhibited substantially larger flowers than da1-1 (Fig. 1A). Plots of eod8-1 da1-1 were 32% longer and 43% wider than those of da1-1, resulting in a reduction in the ratio of petal length to petal width.
At1g25540 insertions in the of organ size, we obtained mutant and two homozygous lines pft1 products without the Gln-rich region. resulting in a frameshift that produces premature translational was indistinguishable from those of MED25 restricts organ growth EOD8 suggesting that the eod8-1, petal area (PA) of Col-0, (PL), petal width (PW), the ratio of petal length to petal width (PL/PW) in respectively wild-type values. Scale bar: 1 mm.

Expression patterns of MED25 Although the roles of Arabidopsis MED25 in shade avoidance and stress responses have been reported (Cerdan and Chory, 2003; Backstrom et al., 2007; Wollenberg et al., 2008; Kidd et al., 2009; Elfving et al., 2011), its expression patterns have not been described. To investigate expression of MED25, we performed quantitative RT-PCR analysis. MED25 mRNA was detected in various tissues, including roots, stems, leaves, seedlings and inflorescences (Fig. 3A), indicating that MED25 is ubiquitously expressed. Relatively high mRNA levels were observed in inflorescences, consistent with the large flower phenotype of med25 mutants.

To evaluate the expression of MED25 in further detail, we generated transgenic Arabidopsis plants containing a MED25 promoter::GUS fusion (pMED25::GUS). In seedlings, GUS activity was detected in cotyledons, leaves and roots (Fig. 3B-E). Higher GUS activity was observed in older leaves than young ones (Fig. 3D, see Fig. S3A in the supplementary material). After bolting, MED25 was primarily expressed in floral organs, leaves and roots, but not in stems (Fig. 3F). In floral organs, GUS activity was detected in sepalas, petals, stamens and carpels (Fig. 3G-J). We compared the GUS expression patterns of pMED25::GUS and pCYCB1;1::GUS and found that MED25 was expressed in both the proliferation and expansion phases of petal development (see Fig. S3B,C in the supplementary material). However, although MED25 was strongly expressed during the early stages of petal formation, levels were substantially reduced at later stages of petal development (Fig. 3J, see Fig. S3B,C in the supplementary material). By contrast, GUS activity was stronger in old than young pedicels (Fig. 3I). These results indicate that MED25 is expressed in a temporally and spatially regulated manner.

**med25 mutants form large floral organs** The effect of the single mutation in MED25 on organ size was investigated further. As med25-1, med25-2, med25-3 and pft1 exhibited similar phenotypes (Fig. 2H,J,K, see Fig. S2 in the supplementary material), we chose the med25-2 mutant for further characterization. Before analysis, the med25-2 mutant was crossed twice with Col-0. The med25-2 mutant had large sepals and petals, long stamens and carpels, and enlarged inflorescences, as well as increased biomass compared with the wild type (Fig. 4A-D).
med25-2 petals were 46% larger by area, 13% longer and 34% wider than wild-type petals, resulting in a reduction in the ratio of petal length to petal width (Fig. 4C). The size of the early developing rosette leaves was similar to that of wild type, whereas later developing rosette leaves were slightly larger than those of wild type (see Fig. S4D in the supplementary material). The med25-2 mutant had significantly more rosette and cauline branches than wild-type plants (see Fig. S4A-C in the supplementary material), suggesting that MED25 might be involved in the regulation of shoot branch patterns. In addition, med25-2 was slightly late flowering under long-day growth conditions (see Fig. S4A in the supplementary material), consistent with previous findings (Cerdan and Chory, 2003; Wollenberg et al., 2008).

**med25-2 increases the period of cell proliferation and cell expansion**

The regulation of cell proliferation and cell expansion is essential for the growth of an organ during organogenesis (Sugimoto-Shirasu and Roberts, 2003; Tsukaya, 2003; Weiss et al., 2005; Tsukaya, 2006). In Arabidopsis, petals have a simple laminar structure with a small number of cell types, facilitating the analysis of organ growth and development (Irish, 2008). As the most dramatic phenotypic alteration in med25 mutants was in flowers, and especially petals, we used the petal as a representative organ to further investigate the cellular basis of the increase in organ size. We measured the size of adaxial epidermal cells in petals. As shown in Fig. 5, the size of epidermal cells in the maximal width region of med25-2 petals (stage 14) was dramatically increased (by...
MED25 restricts organ growth

~42%) compared with wild type (Fig. 5A,B,E), indicating that MED25 restricts organ growth. To further understand how MED25 restricts organ growth, we analyzed the expression of cell proliferation in the indicated Arabidopsis tissues. Mean ± s.d. (B-J) Histochemical analysis of GUS activity of pMED25::GUS transgenic lines in a 6-day-old seedling (B), root meristem (C), 13-day-old seedling (D), leaf (E), 38-day-old soil-grown plant (F), inflorescence (G), flower (H), siliques (I) and petals (J). Scale bars: 1 mm in B,D,E,G,I; 10 μm in C; 10 mm in F; 100 μm in J.

To examine further the cellular basis of the reduced organ size in plants overexpressing MED25, we investigated cell number and cell size in petals and leaves. In petals, the size of adaxial epidermal cells was reduced by ~14% compared with wild type (Fig. 5E), and the number of adaxial epidermal cells in terms of petal length and petal width was also slightly decreased (Fig. 5E). In leaves, cells stopped expanding earlier than those in the wild type, resulting in a reduction in final cell size (Fig. 5D). This implies that an alteration in the duration of cell proliferation and/or cell expansion might lead to the altered organ size in med25-2 plants. We measured cell size over time in wild-type and med25-2 petals. As shown in Fig. 5E, cells in med25-2 petals continue growing for a longer period than those in wild-type petals. These results indicate that MED25 limits the duration of cell expansion. To further investigate how MED25 regulates cell proliferation, we measured the mitotic index using a pCYCB1;1::GUS reporter fusion in wild-type and med25-2 petals. Cells in med25-2 petals continue to proliferate for slightly longer than those in wild-type petals (Fig. 5F). Taken together, our results show that MED25 limits the period of cell expansion and cell proliferation.

MED25 overexpression restricts organ growth

To further characterize MED25 function, we expressed MED25 under the control of the 35S promoter in the Col-0 wild type and isolated 50 transgenic plants. Integration of the transgenes into the genome was confirmed by PCR analysis using At1g25540 CAPS primers (see Table S1 and Fig. S6 in the supplementary material). Transgenic plants had significant increases in MED25 mRNA compared with wild-type plants (Fig. 6F). The majority of 35S::MED25 transgenic plants had slightly smaller flowers, narrower and shorter leaves and thinner stems than wild type (Fig. 6A,B,E). However, the bolting time of transgenic plants was similar to that of wild type (Fig. 6A,B, see Fig. S7 in the supplementary material), consistent with previous studies (Cerdan and Chory, 2003). Because the most dramatic phenotype was found in leaves, we performed a kinematic analysis of the leaves of wild-type and transgenic plants. The fifth leaf area in wild-type and transgenic plants was similar at 2 and 4 days after emergence (DAE); however, they differed remarkably at 6 DAE and thereafter (Fig. 6G). At ~10 DAE, the fifth leaves of transgenic plants reached their final size, whereas the fifth leaves of the wild type continued to grow for a longer period (Fig. 6G). These analyses show that MED25 overexpression reduces the period of leaf growth.

To examine further the cellular basis of the reduced organ size in plants overexpressing MED25, we investigated cell number and cell size in petals and leaves. In petals, the size of adaxial epidermal cells was reduced by ~14% compared with wild type (Fig. 6E), and the number of adaxial epidermal cells in terms of petal length and petal width was also slightly decreased (Fig. 6E). In leaves, cells stopped expanding earlier than those in the wild type, resulting in a reduction in final cell size (Fig. 6C,D,H,I). The number of palisade cells per leaf was also significantly decreased (Fig. 6I). These analyses suggest that overexpression of MED25 restricts both cell proliferation and cell expansion in Arabidopsis.
med25-2 does not affect endoreduplication in petal cells

An increase in ploidy caused by endocycles is often correlated with an increase in cell size (Sugimoto-Shirasu and Roberts, 2003). Previous studies have shown that petal tips do not normally endoreduplicate (Hase et al., 2005), although DNA content analysis of the entire petal showed some polyploidy (Kurepa et al., 2009). To investigate whether cell enlargement in med25 petals was caused by an increase in ploidy, we performed a flow cytometric examination with nuclei of wild-type and med25-2 petals. The distributions of ploidy classes in med25-2 were comparable to those in wild type, and the overall ploidy levels were unaltered (Fig. 7A,B), indicating that med25 does not affect nuclear DNA endoreduplication in petal cells. We also examined nuclear size in epidermal cells from fully expanded petals by measuring two-dimensional images of DAPI-stained petals. The size of petal nuclei in med25-2 was indistinguishable from that in wild type (Fig. 7C-E), supporting the conclusion that the cell and petal enlargements in med25 mutants are not associated with alterations in nuclear DNA endoreduplication.

Fig. 5. The med25-2 mutation increases the duration of cell proliferation and cell expansion. (A,B) Adaxial epidermal cells in Col-0 (A) and med25-2 (B) Arabidopsis petals. (C) The number of adaxial epidermal cells in petal length (PLD) and petal width (PWD) directions in Col-0 and med25-2. Mean ± s.d. relative to the respective wild-type values. **, P<0.01 compared with the wild type (Student’s t-test). (D) Growth of wild-type and med25-2 petals over time. The largest petals of each series are from opened flowers (stage 14). Mean ± s.e. (E) The size of adaxial epidermal cells over time in the maximal width region of wild-type and med25-2 petals. The time axis corresponds to that in D. Mean ± s.e. (F) Mitotic index versus time in wild-type and med25-2 petals. Mean ± s.e. Scale bar: 10 μm.

Fig. 6. MED25 overexpression restricts organ growth. (A,B) Soil-grown 27-day-old (A) and 35-day-old (B) Col-0 and 35S::MED25#14 Arabidopsis plants. (C,D) Palisade cells of the fifth leaf in Col-0 (C) and 35S::MED25#14 (D) plants. (E) Petal area (PA), cell area (CA) and cell number (CN) in petal length (PLD) and petal width (PWD) directions of Col-0, 35S::MED25#14 and 35S::MED25#6 petals. Mean ± s.d. relative to the respective wild-type values. **, P<0.01 and *, P<0.05 compared with the wild type (Student’s t-test). (F) Relative expression levels of MED25 in Col-0, 35S::MED25#14 and 35S::MED25#6 seedlings were measured by quantitative real-time RT-PCR. Mean ± s.d. (G) Fifth leaf area of Col-0 and 35S::MED25#14 measured over a period of 18 days after emergence (DAE). The size of the fifth leaves is ~0.45 mm² at 0 DAG. Mean ± s.e. (H) The size of abaxial epidermal cells over time in wild-type and 35S::MED25#14 fifth leaves. The time axis corresponds to that in G. Mean ± s.e. (I) The palisade cell area (CA) and the calculated palisade cell number (CN) per leaf in Col-0 and 35S::MED25#14 plants. Mean ± s.d. relative to the respective wild-type values. Scale bars: 1 cm in A,B; 100 μm in C,D.
Cell enlargement in med25 petals is independent of FT, PHYB, PHYD and PHYE

MED25 has been reported to act downstream of PHOT1 and PHOT2 (PHYB) to regulate the expression of FLOWERING LOCUS T (FT) in Arabidopsis (Cerdan and Chory, 2003). As med25 mutations predominantly increase cell expansion, we investigated whether cell enlargement in med25 petals is mediated by FT and PHYB. The size of adaxial epidermal cells in fl-7 petals was indistinguishable from that in wild type (Fig. 8A), suggesting that

\[ \text{Area of wild-type and} \]
\[ \text{med25-2 petals.} \]
\[ \text{Mean} \pm \text{s.d.} \]
\[ \text{(n=80). Scale bars: 10 \( \mu \text{m}. \)} \]

\[ \text{Fig. 8. Cell enlargement in med25-2 is independent of MED25-mediated phytochrome signaling and the jasmonate pathway.} \]
\[ \text{(A) Adaxial epidermis cell area of Ler and fl-7 Arabidopsis.} \]
\[ \text{(B) Adaxial epidermis cell area of Col-0, pft1, phyB;D;E and pft1 phyB;D;E.} \]
\[ \text{(C) Flowers of wild type, col1 and aos.} \]
\[ \text{(D) Inflorescences of col1 med25-2 and aos med25-2.} \]
\[ \text{col1 med25-2 and aos med25-2 exhibit defects in} \]
\[ \text{flower development and opening.} \]
\[ \text{(E) Area of wild-type and med25-2 petals treated with 100 \mu M methyl jasmonate (MeJA) or 0.1 % Tween 20 (mock).} \]
\[ \text{Mean} \pm \text{s.d. relative to the respective wild-type values.} \]
\[ \text{**, P<0.01 compared with the respective mock-treated petals (Student's t-test).} \]
\[ \text{Scale bars: 1 mm in C, 1 cm in D.} \]

\[ \text{FT is not required for cell size control. Similarly, the size of adaxial} \]
\[ \text{epidermal cells in phyB;D;E petals was similar to that in wild type,} \]
\[ \text{and the size of cells in pft1 phyB;D;E petals was comparable with} \]
\[ \text{that in pft1 (Fig. 8B). This suggests that cell enlargement in med25} \]
\[ \text{petals is independent of FT, PHYB, PHYD and PHYE.} \]

Genetic interactions of med25-2 with jasmonate mutants

MED25 is a key regulator of jasmonate-dependent defense, and expression of MED25 is reduced in response to methyl jasmonate (MeJA) in Arabidopsis (Kidd et al., 2009). The jasmonate biosynthesis mutant opr3 forms large petals as a result of increased cell size (Brioudes et al., 2009). We examined whether MED25 restricts cell and organ growth through the jasmonate pathway by investigating the petal size of the jasmonate signaling mutant col1 and the jasmonate biosynthesis mutant aos (Xie et al., 1998; Gfeller et al., 2010). col1 and aos mutants were sterile, but produced larger flowers than wild type (Fig. 8C), suggesting that both jasmonate signaling and biosynthesis pathways influence petal growth. To understand the genetic interaction between MED25 and the jasmonate pathway, we generated col1 med25-2 and aos med25-2 double mutants. Surprisingly, flowers produced by col1 med25-2 and aos med25-2 double mutants did not open normally and exhibited defects in growth and development (Fig. 8D, see Fig. S8 in the supplementary material), suggesting that MED25 and the jasmonate pathway are required for normal flower growth and opening. To further understand the effect of the jasmonate pathway on med25-2 petals, we treated wild-type and med25-2 young flower buds with MeJA and measured their petal sizes at flower stage 14. Exogenous application of jasmonate slightly reduced the petal size of wild type and med25-2 (Fig. 8E); however, med25-2 petals exhibited a similar jasmonate response to wild-type petals (Fig. 8E), suggesting that MED25 might function independently of the jasmonate pathway to limit petal growth.

Increased expression of expansin genes in med25-2

To further understand how med25 mutants affect cell expansion, we examined the expression levels of other characterized Arabidopsis genes involved in cell expansion, including ROT3 (Kim et al., 1998), AN (Kim et al., 2002), AtAF2 (Delessert et al., 2005), BPEp (Szecsi et al., 2006) and ARL (Hu et al., 2006). Their expression was not significantly changed in med25-2 flowers than wild type (Fig. 8F), suggesting that the role of MED25 in regulating cell expansion does not involve ROT3, AN, AtAF2, BPEp and ARL at the mRNA level (see Fig. S9 in the supplementary material).
Expansins are now generally accepted as key regulators of cell wall extension during cell enlargement (Lee et al., 2001; Cosgrove et al., 2002). Plants overexpressing AtEXP10 have large organs with enlarged cells (Cho and Cosgrove, 2000), supporting the role of expansins in cell enlargement. The Arabidopsis genome contains 26 α-expansin genes and at least five β-expansin genes (Lee et al., 2001; Cosgrove et al., 2002), which might act redundantly to influence plant growth and development because most loss-of-function mutations in individual family members do not cause growth defects (Cho and Cosgrove, 2000). The expression levels of AtEXP10 in wild-type and med25-2 inflorescences were essentially similar (see Fig. S9 in the supplementary material). By contrast, the expression levels of other expansin genes examined (AtEXP1, AtEXP3, AtEXP5, AtEXP9, AtEXP11 and AtEXP13) were significantly higher in med25-2 than in wild type (Fig. 9, see Fig. S9 in the supplementary material). AtEXP3 has been proposed to promote cell expansion (Kwon et al., 2008) and the Atexp5-1 mutant has smaller leaves than wild type (Park et al., 2010). These results suggest that cell enlargement in med25 mutants might, in part, result from increased expression of particular expansin genes.

**MED25 functions redundantly with DA1 to control the final size of organs**

As the med25-1 mutation enhanced the floral organ size phenotype of da1-1, we sought to determine the genetic relationship between MED25 and DA1 in organ size control. We crossed med25-2 with da1-1 to generate the med25-2 da1-1 double mutant and determined its petal size. Surprisingly, the med25-2 mutation synergistically enhanced the petal size phenotype of da1-1 (Fig. 10A-E). Interestingly, mutations in MED25 increased cell size but increased cell number slightly (Fig. 5A-C,E), whereas the da1-1 mutant had more cells than wild type (Li et al., 2008). To determine which parameter is affected in the double mutant, we measured cell number and cell size in med25-2 da1-1 petals. The size of adaxial epidermal cells in med25-2 da1-1 petals was similar to that in med25-2 petals (Fig. 10F), suggesting that there is no synergistic interaction between DA1 and MED25 in the context of cell size. We then counted the number of epidermal cells in Col-0, da1-1, med25-2 and med25-2 da1-1 petals. As shown in Fig. 10G, the med25-2 mutation enhanced the cell number phenotype of da1-1, revealing a synergistic interaction between MED25 and DA1 in cell proliferation and also indicating that da1-1 is required for the dramatic effects of the med25 mutations on cell proliferation. These analyses suggest that MED25 acts redundantly with DA1 to restrict cell proliferation.

**DISCUSSION**

In this study, we identified MED25 as a regulator of organ size, with loss and gain of function producing opposite effects on organ size. Organ growth is a well-coordinated process that is regulated by cell proliferation and cell expansion in plants and animals (Conlon and Raff, 1999; Sugimoto-Shirasu and Roberts, 2003; Tsukaya, 2006). Failure to maintain correct cell proliferation and/or expansion will lead to an alteration in organ growth. MED25 restricts organ growth by limiting the period of cell proliferation and cell expansion in Arabidopsis, suggesting that modulation of this period is a crucial point in the regulation of organ size. Expression of MED25 was detected in the proliferation and expansion phases of petal development (see Fig. S3B,C in the supplementary material), supporting the proposed roles of MED25 in cell proliferation and cell expansion. To the best of our knowledge, MED25 provides one of the few examples in Arabidopsis of a loss-of-function mutant that is associated with increases in both cell number and cell size.

MED25 was first described as a positive regulator of shade avoidance and later as a regulator of the basal defense and abiotic stress responses (Cerden and Chory, 2003; Backstrom et al., 2007; Wollenberg et al., 2008; Kidd et al., 2009; Elfving et al., 2011), suggesting that MED25 might be involved in the regulation of various biological processes. Our findings show that MED25 also controls the final size of organs by limiting both cell proliferation and cell expansion, suggesting that MED25 might provide a link in the transcriptional machinery

**Fig. 9. Increased expression of expansin genes in med25-2.**

Expression levels of AtEXP1, AtEXP3, AtEXP5, AtEXP9, AtEXP11 and AtEXP13 are higher in med25-2 inflorescences than in wild-type Arabidopsis inflorescences. Mean ± s.d.

**Fig. 10. Genetic interaction between med25-2 and da1-1.**

(A-D) Flowers of Col-0, med25-2, da1-1 and med25-2 da1-1 double-mutant Arabidopsis. (E) Petal area of Col-0, med25-2, da1-1 and med25-2 da1-1 double mutant. (F) The epidermal cell area on the adaxial side of Col-0, med25-2, da1-1 and med25-2 da1-1 petals. (G) The epidermal cell number on the adaxial side of Col-0, med25-2, da1-1 and med25-2 da1-1 petals. Mean ± s.d. relative to the respective wild-type values. Scale bars: 1 mm.
between the cell proliferation and cell expansion pathways. Our genetic and physiological analyses show that MED25 may regulate organ growth independently of MED25-mediated phytochrome signaling and the jasmonate pathway, suggesting the importance of MED25 in the integration of key signaling pathways in plants. Another Mediator subunit, SWP (MED14), promotes cell proliferation in Arabidopsis (Autran et al., 2002). swp mutants form small, finger-shaped leaves owing to a reduction in the number of cells. This reduction is partially compensated by an increase in final cell size (Autran et al., 2002). Unexpectedly, plants overexpressing SWP exhibit small organs with an increased number of cells and smaller cells (Autran et al., 2002). By contrast, loss and gain of function in MED25 produced opposite effects on organ size. med25 mutants predominantly promote cell expansion and increase cell proliferation slightly (Fig. 5). Cell proliferation and cell expansion in med25-2 or 35S::MED25 do not compensate for each other to affect organ size (Fig. 5A-C,E, Fig. 6E,J). These studies suggest that MED25 and SWP might, at least in part, use different mechanisms to regulate organ size. It is plausible that distinct modular Mediator complexes might coexist in the nucleus, coordinately transmitting various signals from different classes of activators to RNA polymerase II to initiate transcription of the genes involved in organ size control.

Several factors have recently been identified as regulators of cell proliferation and cell expansion in plants, but little is known about the molecular or genetic regulatory mechanisms that control organ size. Based on our genetic data and the role of MED25 in transcriptional activation (Cerdan and Chory, 2003; Backstrom et al., 2007), we suggest a simple hypothesis of how MED25 controls organ size in Arabidopsis (Fig. 11). It is plausible that growth signals are transmitted to the Mediator complex by direct action on MED25 or via activators to regulate the transcription of target genes, which include negative regulators of both cell proliferation and cell expansion. Our results show that the expression of six expansin genes was significantly increased in the med25-2 mutant (Fig. 9), suggesting that these negative regulators of cell expansion might regulate cell size by repressing the expression of expansin genes and other positive regulators of cell expansion (Fig. 11). In med25 single mutants, cell size was increased dramatically, whereas cell number was increased only slightly, suggesting that the MED25-regulated genes involved in organ growth mainly function to restrict cell expansion. A synergistic genetic interaction between med25-2 and da1-1 in cell proliferation was observed, suggesting that MED25 and DA1 function redundantly to restrict cell proliferation and that they might share a common downstream target in the cell proliferation pathway. However, which activators are involved in transmitting organ growth signals to the Mediator complex through MED25 is not known. In addition, the Mediator complex subunits have been proposed to be involved in microRNA (miRNA) biogenesis by recruiting RNA polymerase II to the promoters of miRNA genes (Kim et al., 2011). It is possible that MED25 promotes the transcription of miRNA that targets positive regulators of cell proliferation and cell expansion. It will therefore be a difficult but worthwhile challenge to identify the activators and downstream targets of MED25 in organ size control.

Currently, there is a great deal of interest in using plant biomass and seeds as sustainable fuel and energy sources. In this study, MED25 was identified as an important player in regulating organ size. Our current understanding of MED25 function and of the synergistic interaction between MED25 and DA1 suggest that the combination of the MED25 and DA1 genes (and their orthologs in plants) could make a significant contribution to future biomass and biofuel production.

**Acknowledgements**

We thank anonymous reviewers and the co-editor for critical comments on the manuscript; Pablo Cerdan for pft1, phyB;D;E and pft1 phyB;D;E seeds; Michael Lenhard for pCYCB1;1:::GUS seeds; Xiangyang Hu for ft-7 seeds; Jun Fan for coi1 and aoc seeds; Michael W. Bevan, Fiona Corke, Zhen Li and Cheng Chang for their suggestions and help with the mutant screen and mapping; and the Arabidopsis Stock Center ABRC and NASC for med25 and other organ size mutants.

**Funding**

This work was supported by the National Basic Research Program of China (2009CB941503) and National Natural Science Foundation of China (30870215; 91017014; 30921003).

**Competing interests statement**

The authors declare no competing financial interests.

**Supplemental material**

Supplemental material for this article is available at http://dev.biologists.orglookup suppl doi:10.1242dev.071423R-DC1

**References**


