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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 large organs, with larger and slightly increased numbers of cells as a result of an increased 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), GRF-interacting factors (AtGIFs), ARGOS and KLUH (also known as CYP78A5) (Krizek, 1999; Mizukami and Fischer, 2000; Hu et al., 2003; Kim et al., 2003; Dinneny et al., 2004; Kim and Kende, 2004; Ohno et al., 2004; Horiguchi et al., 2005; 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 struwwelpeter* (*swp*) mutant shows small aerial organs and various defects in development (Autran et al., 2002). *SWP* encodes a homolog of the yeast and metazoan Mediator subunit MED14 (Autran 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

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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 *PFT1*), 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 M_2 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 F_2 population of a cross between *med25-1 da1-1* and *da1-1*^{Ler}. 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 *Eco*RI site of the *35S::pGreen* vector to generate the transformation plasmid *35S::MED25-pG*. The *MED25* CDS was also cloned into the *Kpn*I 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 (30 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 *Sac*I and *Nco*I 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 *pCYCB1;1::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 $NaPO_4$ (pH 7.0), 0.4 mM $K_3Fe(CN)_6$, 0.4 mM $K_4Fe(CN)_6$, 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, seedlings 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). Petals 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

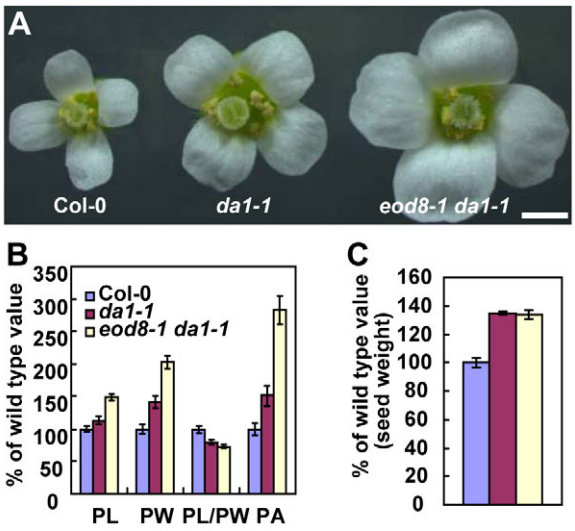

Fig. 1. *eod8-1* enhances the organ size phenotype of *da1-1*. (**A**) Col-0, *da1-1* and *eod8-1 da1-1 Arabidopsis* flowers. (**B**) Petal length (PL), petal width (PW), the ratio of petal length to petal width (PL/PW) and petal area (PA) of Col-0, *da1-1* and *eod8-1 da1-1*. (**C**) Seed weight of Col-0, *da1-1* and *eod8-1 da1-1*. Mean ± s.d. relative to the respective wild-type values. Scale bar: 1 mm.

(Fig. 1B). However, the weight of seeds from *eod8-1 da1-1* plants was indistinguishable from those of *da1-1* plants (Fig. 1C), suggesting that the *eod8-1* mutation does not affect seed growth.

EOD8 encodes Mediator complex subunit 25

The *eod8-1* mutation was identified by map-based cloning in an F_2 population of a cross between *eod8-1 da1-1* and *da1-1*^{Ler}. It was mapped into an 18.8 kb interval between markers F2J7-6 and F2J7-10 on chromosome I (Fig. 2A). DNA sequencing revealed that *eod8-1* has a G-to-A transition at the junction between the seventh exon and seventh intron of *At1g25540* (Fig. 2B). The cleaved amplified polymorphic sequence marker *At1g25540* CAPS (see Table S1 in the supplementary material) was developed based on this mutation in *eod8-1*, and it co-segregated with the *eod8-1* phenotypes (Fig. 2A,D). *At1g25540* encodes Mediator complex subunit 25 (MED25, or PFT1), which is known to regulate phytochrome signaling 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), although no function in organ size control has previously been described. MED25 contains one von Willebrand type A (vWF-A) domain, which has been shown to be crucial for the binding of MED25 to the Mediator complex, and one Gln-rich region that is predicted to act as a putative transcriptional activation domain (Fig. 2C) (Cerdan and Chory, 2003; Mittler et al., 2003; Backstrom et al., 2007). In *eod8-1*, the 5′ exon-intron boundary of intron 7 is changed from GT to AT (Fig. 2B), replacing a G that is highly conserved at plant gene splice sites. The mutation in *eod8-1* altered the splicing of *At1g25540* mRNA (Fig. 2E), resulting in a frameshift that produces premature translational products without the Gln-rich region.

To further investigate potential roles of *MED25* in the regulation of organ size, we obtained *pft1* mutant and two homozygous lines (SALK_080230 and CS870982) harboring independent T-DNA insertions in the *At1g25540* gene. *med25-2* (SALK_080230) is in the Col-0 background, whereas *med25-3* (CS870982) is in the CS8846 background. *med25-2* and *med25-3* were identified with T-DNA insertions in the second intron and the ninth exon of *At1g25540*, respectively (Fig. 2B, see Fig. S1 in the supplementary material). RT-PCR analysis revealed that *med25-2* and *med25-3* mutants had no detectable full-length transcripts of *At1g25540* (Fig. 2F,G), suggesting that they are null mutant alleles. Like *eod8-1*, the *pft1*, *med25-2* and *med25-3* mutants exhibited larger flowers than their parental lines (Fig. 2H,J,K, see Fig. S2 in the supplementary material). F_1 progeny of crosses of these four lines (*eod8-1*, *med25-2*, *med25-3* and *pft1*) all exhibited the phenotypes of the *eod8-1* mutant (data not shown), indicating that these lines were allelic and also suggesting that mutations in *At1g25540* cause large organs. Transformation of *med25-2* with wild-type *At1g25540* CDS expressed from an *35S* promoter rescued the large organ phenotype of the *med25-2* mutant (Fig. 2I,L), further supporting the conclusion that mutations in *At1g25540* are responsible for the large organ phenotype of *med25* mutants. Thus, we renamed the *eod8-1* mutant *med25-1*, referring to the order of discovery of the *med25* alleles.

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 younger 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 sepals, 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 pedicles (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).

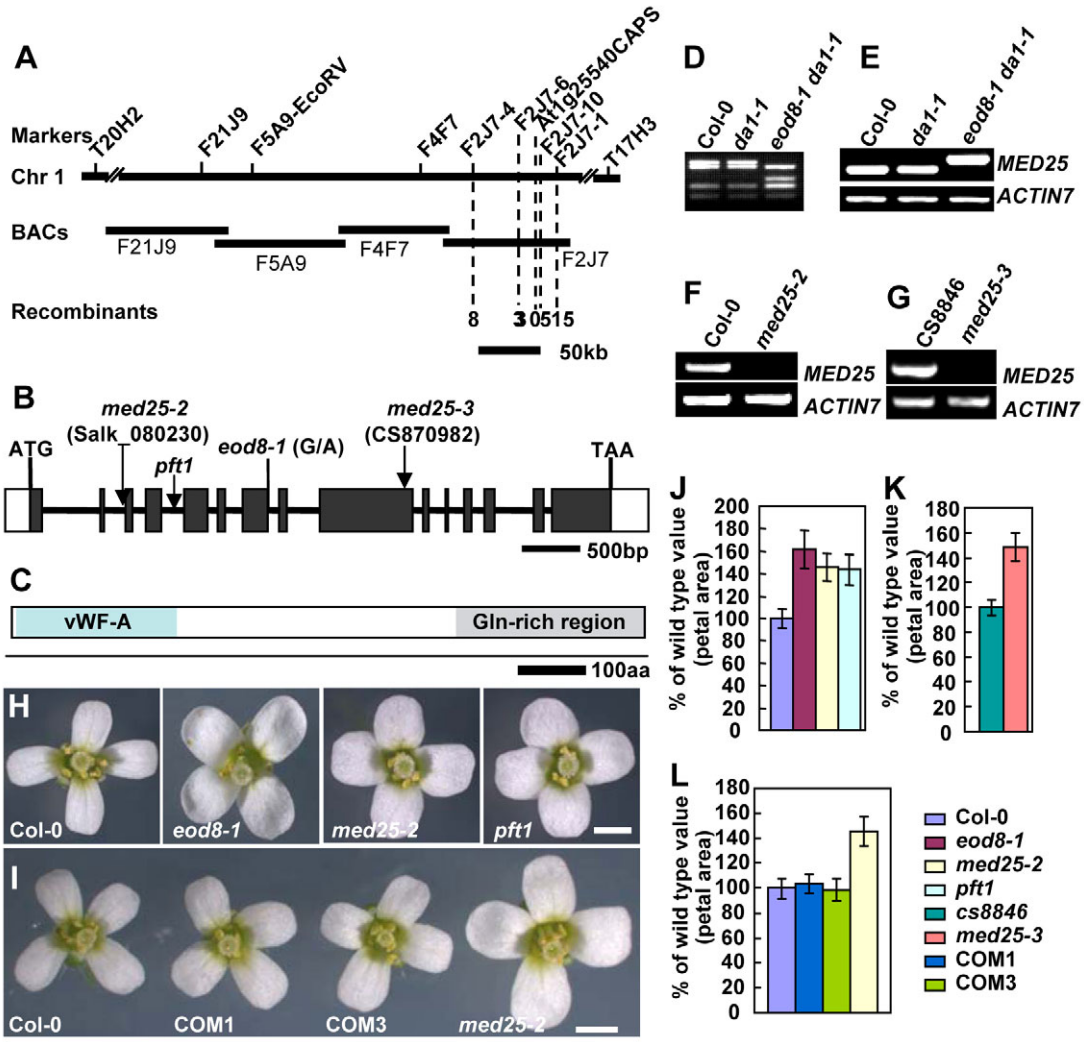

Fig. 2. Identification of the *MED25* gene. (**A**) Fine mapping of the *eod8-1* mutation to an 18.8 kb region of the *Arabidopsis* genome between markers F2J7-6 and F2J7-10, co-segregated with marker *At1g25540* CAPS. The numbers of recombinants identified from an F_2 population of a cross between *eod8-1 da1-1* and *da1-1*^{Ler} are indicated beneath the dashed lines. (**B**) *MED25* gene structure, showing the mutation site of *eod8-1* and the T-DNA insertion sites in *med25-2*, *med25-3* and *pft1*. The start codon (ATG) and the stop codon (TAA) are indicated. Black boxes indicate exons and lines between boxes indicate introns. (**C**) The predicted MED25 protein contains one von Willebrand factor type A (vWF-A) domain and one glutamine (Gln)-rich region. (**D**) The mutation in the *eod8-1* allele produces a *Hin*fI site that is used for the *At1g25540* CAPS marker. (**E-G**) RT-PCR analysis of *MED25* expression in *eod8-1*, *med25-2* and *med25-3* alleles. RT-PCR was performed on first-strand cDNA prepared from 2-week-old seedlings. (**H**) Flowers of Col-0, *eod8-1*, *med25-2* and *pft1*. (**I**) Flowers of Col-0, COM1, COM3 and *med25-2*. COM is *med25-2* transformed with *MED25* coding sequence (CDS) driven by the *35S* promoter. (**J**) Petal area of Col-0, *eod8-1*, *med25-2* and *pft1*. (**K**) Petal area of *CS8846* and *med25-3*. (**L**) Petal area of Col-0, COM1, COM3 and *med25-2*. Mean ± s.d. relative to the respective wild-type values. Scale bars: 1 mm.

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

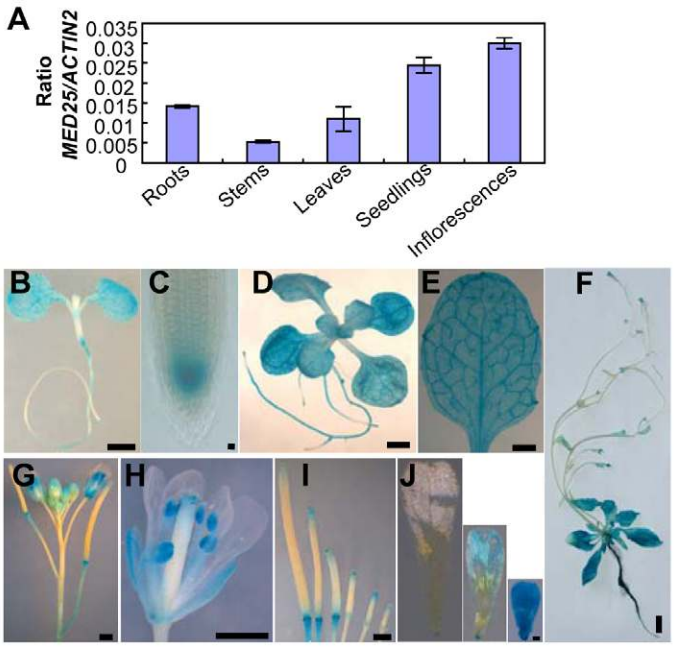

Fig. 3. Expression of the *MED25* gene. (**A**) Quantitative real-time RT-PCR analysis of *MED25* expression in the indicated *Arabidopsis* tissues. Mean ± s.d. (**B-J**) Histochemical analysis of GUS activity of *pMED25::GUS* transgenic lines in a 6-day-old seedling (B), root meristem (C), 13-day-old 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.

~42%) compared with wild type (Fig. 5A,B,E), indicating that *MED25* restricts cell expansion. In addition, *med25* mutants formed more lobes in adaxial and abaxial epidermal cells compared with the wild type (Fig. 5A,B, see Fig. S5 in the supplementary material), suggesting that *MED25* might be involved in the regulation of cell shape. The number of adaxial epidermal cells in the petal-length direction was comparable in *med25-2* and wild-type petals, whereas the number of adaxial epidermal cells in the petal-width direction was increased (Fig. 5C), suggesting that *MED25* also limits cell proliferation. We also counted the total number of adaxial epidermal cells in petals. As shown in Fig. 10G, *med25-2* had more cells than wild type. These data imply that mutations in *MED25* predominantly increase cell size but also increase cell number slightly.

To understand how *MED25* restricts organ growth, we analyzed the growth dynamics of petals in wild-type and *med25-2* plants. Compared with wild type, *med25-2* mutant petals stopped growing later, resulting in a larger final 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*.

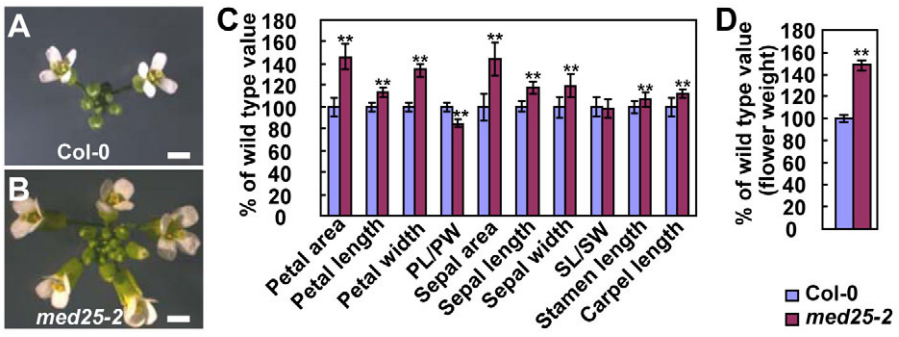

Fig. 4. Organ size in *med25-2*. (**A**,**B**) Inflorescences of Col-0 (A) and *med25-2* (B) *Arabidopsis*. (**C**) Petal area, petal length, petal width, the ratio of petal length to petal width (PL/PW), sepal length, sepal width, the ratio of sepal length to sepal width (SL/SW), stamen length and carpel length of Col-0 and *med25-2*. (**D**) Mass of Col-0 and *med25-2* flowers. Mean ± s.d. relative to the respective wild-type values. **, $P<0.01$ compared with the wild type (Student's *t*-test). Scale bars: 1 mm.

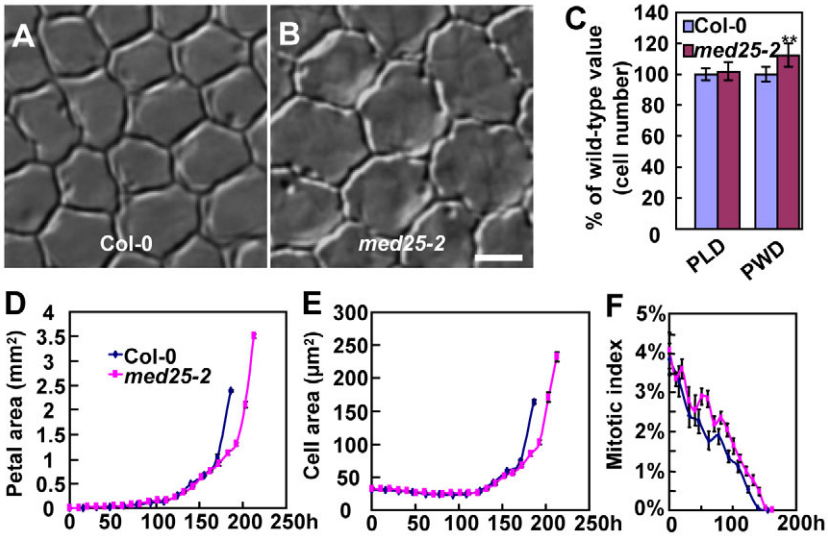

Fig. 5. The *med25-2* mutation increases the duration of cell proliferation and cell expansion. (**A**,**B**) Adaxial epidermal cells in Col-0 (A) and *med25-2* (B) *Arabidopsis* petals. (**C**) The number of adaxial epidermal cells in petal length (PLD) and petal width (PWD) directions in Col-0 and *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.

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.

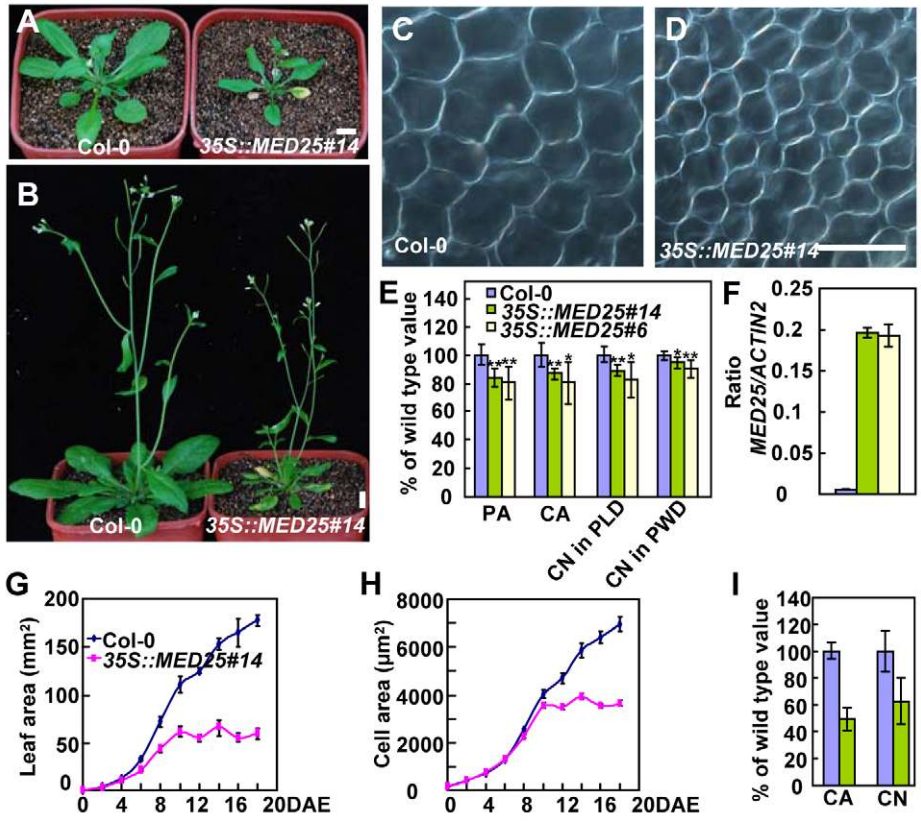

Fig. 6. *MED25* overexpression restricts organ growth. (**A**,**B**) Soil-grown 27-day-old (A) and 35-day-old (B) Col-0 and *35S::MED25#14 Arabidopsis* plants. (**C**,**D**) Palisade cells of the fifth leaf in Col-0 (C) and *35S::MED25#14* (D) plants. (**E**) Petal area (PA), cell area (CA) and cell 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 mm^2 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.

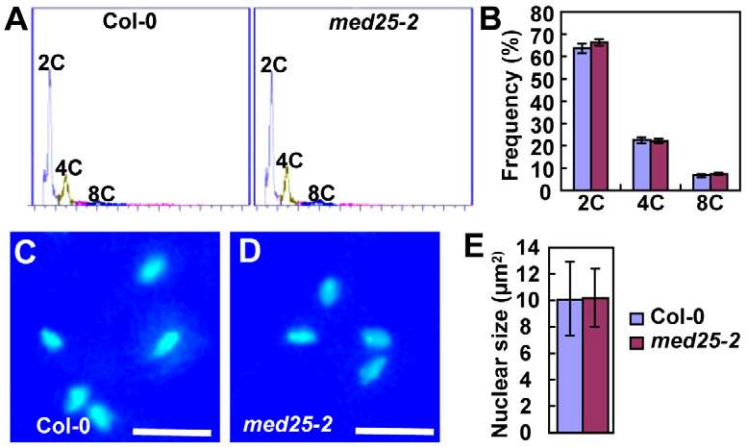

Fig. 7. Ploidy levels and nuclear size of Col-0 and *med25-2* petals. (**A**) DNA ploidy level distribution of Col-0 and *med25-2 Arabidopsis* petals (stage 14). (**B**) Percentage of cells with different classes of nuclear ploidy. Mean ± s.d. (*n*=3). (**C**,**D**) DAPI staining of the nuclei of Col-0 and *med25-2* petals. (**E**) Nuclear area of Col-0 and *med25-2* petals. Mean ± s.d. (*n*=80). Scale bars: 10 μm.

Cell enlargement in *med25* petals is independent of *FT*, *PHYB*, *PHYD* and *PHYE*

MED25 has been reported to act downstream of *PHYTOCHROME B* (*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 *ft-7* petals was indistinguishable from that in wild type (Fig. 8A), suggesting that *FT* is not required for cell size control. Similarly, the size of adaxial epidermal cells in *phyB;D;E* petals was similar to that in wild type, and the size of cells in *pft1 phyB;D;E* petals was comparable with that in *pft1* (Fig. 8B). This suggests that cell enlargement in *med25* petals is independent of *FT*, *PHYB*, *PHYD* and *PHYE*.

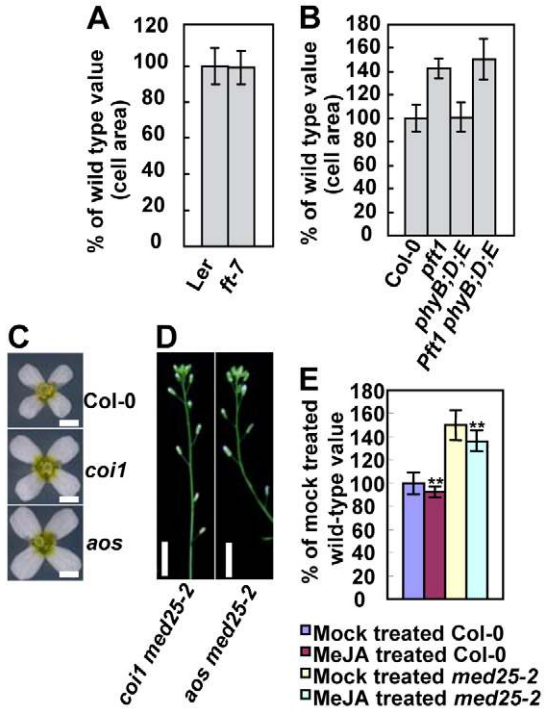

Fig. 8. Cell enlargement in *med25-2* is independent of *MED25*-mediated phytochrome signaling and the jasmonate pathway. (**A**) Adaxial epidermis cell area of Ler and *ft-7 Arabidopsis*. (**B**) Adaxial epidermis cell area of Col-0, *pft1*, *phyB;D;E* and *pft1 phyB;D;E*. (**C**) Flowers of wild type,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

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 *coi1* and the jasmonate biosynthesis mutant *aos* (Xie et al., 1998; Gfeller et al., 2010). *coi1* 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 *coi1 med25-2* and *aos med25-2* double mutants. Surprisingly, flowers produced by *coi1 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* inflorescences, 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).

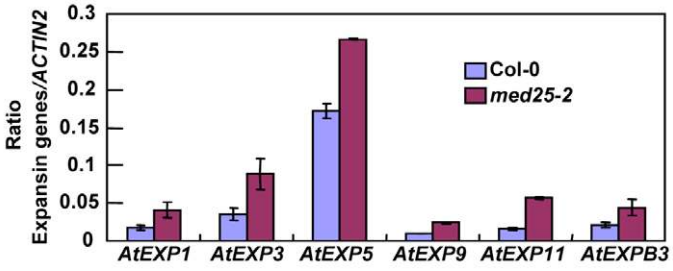

Fig. 9. Increased expression of expansin genes in *med25-2*. Expression levels of *AtEXP1*, *AtEXP3*, *AtEXP5*, *AtEXP9*, *AtEXP11* and *AtEXPB3* are higher in *med25-2* inflorescences than in wild-type *Arabidopsis* inflorescences. Mean ± s.d.

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 *AtEXPB3*) 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 (Cerdan 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

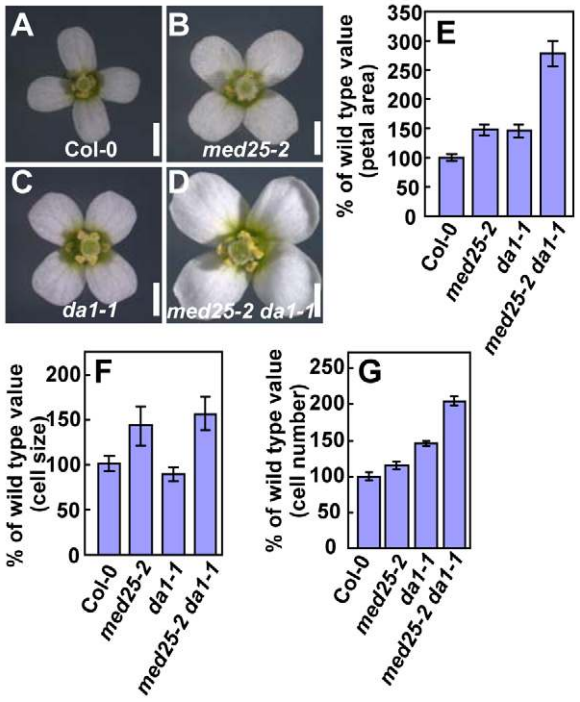

Fig. 10. Genetic interaction between *med25-2* and *da1-1*. (**A-D**) Flowers of Col-0, *med25-2*, *da1-1* and *med25-2 da1-1* double-mutant *Arabidopsis*. (**E**) Petal area of Col-0, *med25-2*, *da1-1* and *med25-2 da1-1* double mutant. (**F**) The epidermal cell area on the adaxial side of Col-0, *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.

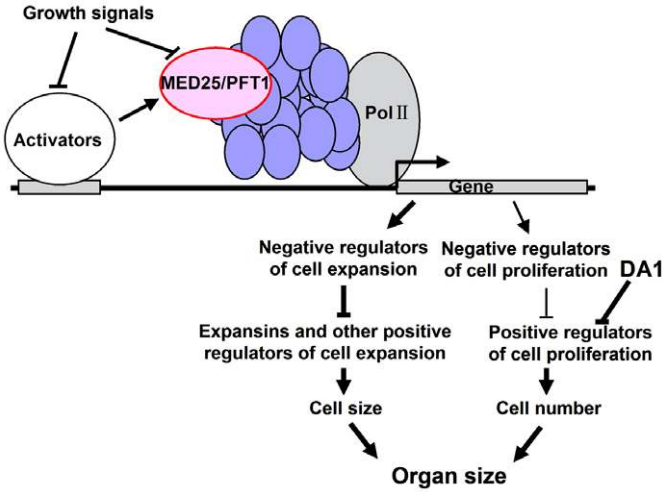

Fig. 11. Model of *MED25* control of organ size. Growth signals are transmitted to the Mediator complex by direct action on MED25 (PFT1) or via activators to regulate the transcription of target genes, which include negative regulators of both cell proliferation and cell expansion. These negative regulators of cell expansion may regulate cell size by repressing the expression of expansin genes and other positive regulators of cell expansion. DA1 and MED25 might share a common downstream target to restrict cell proliferation.

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,I). 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.

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Competing interests statement
The authors declare no competing financial interests.

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