

RESEARCH REPORT

The MED30 subunit of mediator complex is essential for early plant development and promotes flowering in *Arabidopsis thaliana*

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ABSTRACT

Mediator is a large multiprotein complex that is required for the transcription of most, if not all, genes transcribed by RNA Polymerase II. A core set of subunits is essential to assemble a functional Mediator *in vitro* and, therefore, the corresponding loss-of-function mutants are expected to be lethal. The MED30 subunit is essential in animal systems, but is absent in yeast. Here, we report that MED30 is also essential for both male gametophyte and embryo development in the model plant *Arabidopsis thaliana*. Mutant *med30* pollen grains were viable and some were able to germinate and target the ovules, although the embryos aborted shortly after fertilization, suggesting that MED30 is important for the paternal control of early embryo development. When gametophyte defects were bypassed by specific pollen complementation, loss of MED30 led to early embryo development arrest. Later in plant development, MED30 promotes flowering through multiple signaling pathways; its downregulation led to a phase change delay, downregulation of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3)*, *FLOWERING LOCUS T (FTI)* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)*, and upregulation of *FLOWERING LOCUS C (FLC)*.

KEY WORDS: MED30, Mediator complex, *Arabidopsis thaliana*, Pollen, Embryo development, Flowering

INTRODUCTION

Mediator is a large protein complex that is conserved in eukaryotes and has an essential role in RNA polymerase II (RNA pol II)-mediated transcription. It was first discovered in yeast as an essential component for activation of *in vitro* transcription (Flanagan et al., 1991; Kelleher et al., 1990), and was subsequently purified from human cells (Fondell et al., 1996) and later from plant cells (Bäckström et al., 2007). Mediator components were also identified from genomic sequences of many eukaryotes, indicating that this complex has been widely conserved during evolution (Bourbon, 2008). Biochemical purification of Mediator from plants identified

21 conserved subunits and six plant-specific ones (Bäckström et al., 2007). Bioinformatics studies of 16 plant species representing diverse groups across the plant kingdom completed the identification of all known yeast and/or metazoan Mediator subunits in plants (Mathur et al., 2011).

Although Mediator is thought to act as a complex, the MED subunits appear to have specific roles (Buendia-Monreal and Gillmor, 2016), as evidenced by the specific phenotypes of viable individual subunit mutants in yeast and *Arabidopsis* (Conaway and Conaway, 2011; Malik et al., 2017). In *Arabidopsis*, for instance, subunits MED25, MED18 and MED8 are involved in flowering, defense signaling and organ development (Cerdán and Chory, 2003; Iñigo et al., 2012a, b; Kidd et al., 2009; Cevik et al., 2012; Chen et al., 2012; Xu and Li, 2012; Xu and Li, 2011; Lai et al., 2014; Kim et al., 2011; Zheng et al., 2013). Interestingly, MED8 is required for normal pollen tube growth (Lalanne et al., 2004), whereas tomato MED18 is required for anther development and pollen viability (Pérez-Martin et al., 2018).

In vitro biochemical studies suggest that functional Mediator comprises a core of 15 subunits (Cevher et al., 2014; Plaschka et al., 2015), although it is currently unknown whether this set of subunits is essential for the assembly of the complex *in vivo*. MED30 is part of this assembly and, consistent with an important role for MED30 in metazoans, mouse *med30* knockouts are not viable (Krebs et al., 2011). Here, we report the roles for MED30 in plant development. *Arabidopsis* MED30 is essential for allele transmission through the male gametophyte, is then essential for embryo and seed development and, later on, promotes phase transitions during subsequent developmental stages.

RESULTS AND DISCUSSION

Arabidopsis med30 loss-of-function alleles are not transmitted through the male gametophyte

To study the role of the MED30 subunit, we obtained two T-DNA lines from the SALK collection (Alonso et al., 2003), which we named *med30-1* (SALK_094948) and *med30-2* (SALK_117444). As confirmed by PCR genotyping and sequencing, the two T-DNAs were inserted into the first and second intron, respectively (Fig. S1). We genotyped more than 90 plants for each T-DNA segregating population but did not find any homozygous T-DNA plants, suggesting that homozygous knockouts of *MED30* are not viable. *MED30* was widely expressed in various tissues (Fig. S2), including pollen, embryos and seeds, suggesting that these reproductive stages would be affected by loss-of-function mutations in *MED30*.

After selfing heterozygous *med30*^{+/+} plants, there was a statistically significant deviation from the 2:1 ratio (*med30*^{+/+} versus *med30*^{+/-}; *P*=0.042 and *P*=0.033 for *med30-1*^{+/+} and *med30-2*^{+/+}, respectively) expected for embryo lethality; however, there was no deviation for either allele from the 1:1 ratio, expected for gametophyte lethality (Table 1). Maturing siliques of *med30*^{+/+} heterozygous plants had

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Table 1. Genetic analysis of *med30*⁺ lines predicts a male gametophytic defect

Crosses	Parent (female×male)	Progeny observed		Progeny expected 1:1		1:1 χ^2 P value	Progeny expected 2:1		2:1 χ^2 P value
		<i>MED30</i> ^{+/-}	<i>MED30</i> ^{+/+}	<i>MED30</i> ^{+/-}	<i>MED30</i> ^{+/+}		<i>MED30</i> ^{+/-}	<i>MED30</i> ^{+/+}	
Self-crosses	<i>med30-1</i> ^{+/+}	54	41	47.5	47.5	0.182	63.33	31.67	0.042
	<i>med30-2</i> ^{+/+}	56	43	49.5	49.5	0.191	66	33	0.033
Reciprocal crosses	<i>med30-1</i> ^{+/+} ×wild type	27	23	25	25	0.5716			
	Wild type× <i>med30-1</i> ^{+/+}	0	45	22.5	22.5	P<0.001			
	<i>med30-2</i> ^{+/+} ×wild type	24	20	22	22	0.5465			
	Wild type× <i>med30-2</i> ^{+/+}	0	77	38.5	38.5	P<0.001			

The progeny of four independent self-crosses of each T-DNA line and three independent reciprocal crosses with the wild type were genotyped by PCR; the segregation data were tested for adjustment to a 1:1 distribution and to a 2:1 (*med30*⁺ versus ^{+/+}) distribution. *P*<0.05 was considered statistically significant.

aborted ovules or embryos, fewer seeds and a higher number of abnormal siliques compared with wild-type plants (Fig. S3 and Table S1). Taken together, these results suggested that *med30* alleles were not properly transmitted through at least one of the gametophytes. To distinguish between defects of either the male or female gametophytes, we conducted reciprocal crosses. Whereas a 1:1 Mendelian transmission was observed through the mutant female gametophyte, no transmission of the *med30* alleles was detected through the male, suggesting a defect at the male gametophyte level (Table 1). Thus, we then transformed *med30-1*^{+/+} and *med30-2*^{+/+} heterozygous plants with a construct harboring the cDNA of *MED30* under its own promoter. From these lines, we successfully segregated homozygous *med30/med30* plants bearing the *pMED30:MED30* construct, confirming that a loss of function of *MED30* was the cause of seed inviability.

To establish whether the *med30* mutation impaired pollen viability, we stained pollen grains with Fluorescein Diacetate (FDA). First, we obtained *med30-1*^{+/+} mutants in a *quartet* (*qrt/qrt*) background to be able to follow the four products of meiosis. When pollen grains were stained with FDA, the percentage of observed tetrad phenotypes did not differ significantly between the *qrt/qrt* and the *qrt/qrt med30-1*^{+/+} lines, suggesting that *med30* pollen is viable (Fig. 1A and Fig. S4).

Moving forward in the analysis of pollen defects, we then hypothesized that failure in allele transmission was the result of low pollen germination. We performed *in vitro* germination assays with pollen collected from wild-type and *med30*⁺ lines. After evaluating the pollen germination capacity, the *med30* allele was found to cause a decrease in pollen germination (Fig. 1B). We obtained a germination rate of 69.6% for wild type, of 52.6% for the *med30-1*^{+/+} line and of 46.3% for the *med30-2*^{+/+} line. However, although pollen germination was compromised, this defect alone could not account for the complete absence of transmission of *med30* alleles through the male gametophyte. In addition, the presence of aborted ovules or embryos in the *med30*⁺ siliques indicated that *med30* pollen could compete with wild-type pollen to some extent, but, in those cases, successful fertilization or proper embryo development might be compromised.

To assess this hypothesis, we conducted a hand-pollination assay in which emasculated wild-type pistils were manually pollinated with a single tetrad (Takahashi et al., 2017) of *qrt/qrt med30-1*^{+/+} plants and stained with Aniline Blue 24 h after pollination. We analyzed only those tetrads that showed at least three germinated pollen grains and, therefore, one or two *med30* pollen grains were analyzed in each cross. All germinated pollen grains were capable of contacting the ovule through the micropyle (eight pistils and 26 pollen grains were analyzed) (Fig. S5). To assess which steps failed after the pollen tubes contacted the micropyle, we analyzed the developing embryos present in siliques of wild-type plants 48 h

after manual pollination with pollen collected from *med30*⁺ or wild-type plants (Fig. 1C-F). We identified a significant number of ovules showing embryogenesis arrested at the zygote stage in pistils pollinated with *med30*⁺ pollen compared with those pollinated with

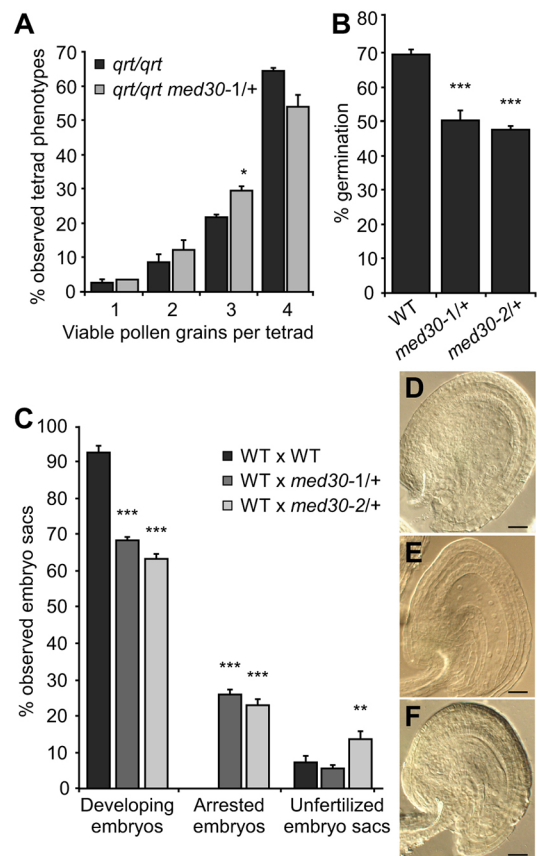


Fig. 1. *MED30* is essential for allele transmission through the male gametophyte. (A) Pollen tetrads of *qrt/qrt* (*n*=379) and *qrt/qrt med30-1*^{+/+} (*n*=425) were stained with FDA and observed under GFP filters. Bars represent the mean percentages of the observed tetrad phenotypes±s.e.m. of two independent assays. (B) *In vitro* pollen germination assay of pollen grains from wild-type and *med30*⁺ plants. Bars represent the mean percentages of each genotype±s.e.m. of two independent assays. (C) Wild-type pistils were emasculated and manually pollinated with pollen from *med30*⁺ plants. After 48 h, pistils were dissected, cleared with Hoyer's solution and observed under DIC optics. Bars represent the mean±s.e.m. of the percentages of embryo sacs observed at the indicated stages. In A-C, the significance of Dunnett's post-hoc tests conducted after one-way ANOVA is indicated with asterisks above each bar: **P*<0.05, ***P*<0.01, ****P*<0.001. (D-F) DIC images of representative developing embryos (D), embryogenesis arrested at the zygote stage (E) and unfertilized ovules (F). Scale bars: 20 μ m.

wild-type pollen, which did not contain any arrested embryos (Fig. 1C; $P < 0.001$). Collectively, our data showed that *med30* pollen grains were viable and, although their germination capacity was compromised, some of them were still capable of contacting the ovules. The presence of aborted embryos in developing siliques after fertilization with *med30* pollen suggests that MED30 is important for the paternal control of early embryo development, as has been shown previously for the *SHORT SUSPENSOR (SSP)* gene (Bayer et al., 2009).

MED30 is also essential for embryo development

To overcome the stall at the *med30* gametophyte level and to enable the study of *med30* homozygous-knockout plants, we transformed *med30-1*⁺ and *med30-2*⁺ heterozygous plants with two constructs generated by cloning *MED30* cDNA under the control of two different male gametophyte-specific promoters: *HOMOLOG OF BRASSICA CAMPESTRIS POLLEN PROTEIN 1 (BCP1, AT1G24520)*, the expression of which is restricted to the microspore and mature pollen stages, and *AT5G17340*, which we named *MALE* during the current study, and which is expressed from the uninucleate microspore to the pollination stage (Costa-Nuñez, 2013; Xu et al., 1993). However, we were unable to obtain complemented homozygous knockouts of *med30-1* or *med30-2* lines. This suggested that expression of *MED30* under these pollen-specific promoters was either insufficient to complement gametophyte defects in *med30* mutants or another defect in *med30* mutants led to unviability.

To address these issues, we first obtained homozygous *BCP1: MED30* and *MALE: MED30* transgenic lines in the *med30-1*⁺ and *med30-2*⁺ heterozygous backgrounds. If these constructs complemented the gametophyte defects, we expected to obtain a deviation from the 1:1 ratio for selfed *med30*⁺ plants. Indeed, we found a ratio close to 2:1 for most of the independent transgenic lines (Table S2), suggesting a lack of viability at the embryo level. To further confirm that *BCP1: MED30* and *MALE: MED30* complemented the gametophyte defects, we used these lines as pollen donors, crossing them with wild-type plants. The transgenic *BCP1: MED30/BCP1: MED30 med30*⁺ and *MALE: MED30/MALE:*

MED30 med30⁺ lines transferred the *med30* alleles through the male gametophyte, confirming that complementation had occurred (Table S2). Interestingly, the *MALE* promoter led to full complementation, whereas the *BCP1* promoter led to partial complementation, which is consistent with the lower expression of *BCP1* early during pollen development (Xu et al., 1993).

To test the hypothesis that, after complementing the male gametophyte defects, *med30* loss of function led to embryo development defects, we studied maturing siliques from *BCP1: MED30/BCP1: MED30 med30*⁺ and *MALE: MED30/MALE: MED30 med30*⁺ plants. White aborted seeds were observed together with wild-type green seeds in the siliques from plants of both lines, in contrast to the siliques from wild-type plants, which contained only green seeds in a well-ordered array (Fig. 2A-C). Consistent with the partial complementation observed in *BCP1: MED30/BCP1: MED30 med30*⁺ lines, these plants also had unfertilized ovules along with white aborted seeds.

To further address the role of MED30 in embryo development, we analyzed embryos in developing siliques of complemented lines 4 days after fertilization. For *MALE: MED30/MALE: MED30 med30*⁺ lines, a high percentage of embryos were arrested at the one-cell stage or delayed in their development at the four-cell stage (Fig. 2D,E; 28–33%). In addition, for the *BCP1: MED30/BCP1: MED30 med30*⁺ lines, the abortions occurred at both the zygote and the one-cell stages (Fig. 2F-H). Consistent with a partial complementation, unfertilized ovules were also found in the *BCP1: MED30/BCP1: MED30 med30*⁺ line. Additionally, some of the developing seeds of the C.8 line of the *MALE: MED30/MALE: MED30 med30*⁺ and of the A.17 line of the *BCP1: MED30/BCP1: MED30 med30*⁺ line contained embryos without endosperm development (Fig. S6), suggesting that MED30 is necessary not only for early embryo development, but also, to some extent, for endosperm development.

Knockdown lines of MED30 display a delayed flowering phenotype

Given that MED30 is essential during early development, we used artificial micro RNAs (amiRNAs) to generate knockdown lines to

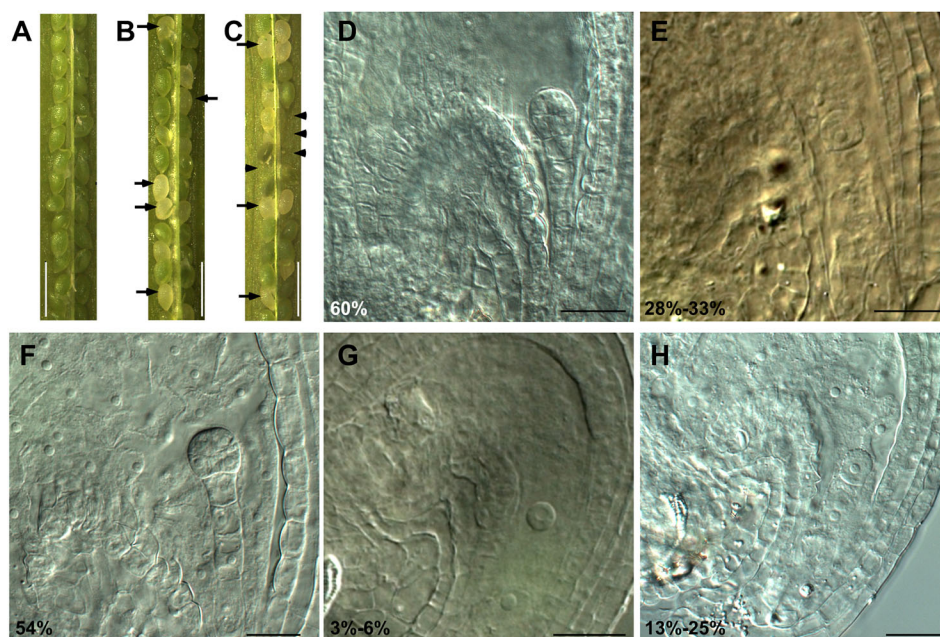


Fig. 2. Once the male gametophyte defect is bypassed, MED30 is essential for embryo development. (A-C) Dissected siliques of wild-type (A) and *med30*⁺ lines complemented with *MALE: MED30* (B) and *BCP1: MED30* (C) constructs. Arrows indicate aborted seeds and arrowheads indicate early abortions. (D-H) DIC images of developing embryos of the complemented lines. Dermatogen embryos of *MALE: MED30/MALE: MED30 med30*⁺ lines (D) and *BCP1: MED30/BCP1: MED30 med30*⁺ lines (F). *MALE: MED30/MALE: MED30 med30*⁺ line embryo arrested at the one-cell stage (E), and embryos from the *BCP1: MED30/BCP1: MED30 med30*⁺ arrested at the zygote (G) and one-cell stages (H). The percentages observed are indicated on each panel. Scale bars: 1 mm (A-C) and 20 μ m (D-H).

study the role of MED30 later in plant development. We designed two independent amiRNAs that target *MED30*, which we named *amiMed30-1* and *amiMed30-2* (Fig. S1B), and obtained transgenic lines bearing the *p35S:amiMed30-1* or the *p35S:amiMed30-2* constructs. Five independent transgenic lines, one for *p35S:amiMed30-1* and four for *p35S:amiMed30-2* constructs, were analyzed by RT-quantitative PCR (RT-qPCR). Four out of five amiRNA lines displayed a significant reduction in the expression of *MED30* in seedlings (Fig. S7). We then analyzed the flowering time of these lines when grown under three different photoperiods: long days (LD) (16 h light/8 h dark), intermediate days (ID) (12 h light/12 h dark) and short days (SD) (8 h light/16 h dark). Under all three conditions, all amiRNA lines with reduced *MED30* expression flowered late compared with the wild type (Fig. 3A). As expected, the *amiMed30-2 X3.7* line, which displayed *MED30* mRNA levels similar to wild type, also flowered similarly to wild-type plants. Despite the late flowering phenotype displayed by *MED30*-knockdown lines under the three photoperiods tested, they still flowered earlier under LD compared with SD (Fig. 3A), suggesting that the photoperiod pathway was functional. To determine whether other flowering pathways were affected in *MED30*-knockdown lines, we analyzed the flowering time under vernalization, low temperature, and gibberellin (GA) treatments. Additionally, we measured phase transition, controlled by the aging pathway, as the appearance of trichomes in the abaxial surface of the leaves and the change in the leaf length:width ratio (Kerstetter and Poethig, 1998). *amiMed30* lines responded to vernalization, low temperature, and GA treatments, despite continuing to flower later than the wild type (Fig. S8), albeit with a delay in phase transition (Fig. S9A-C).

To determine which flowering factors were affected by *MED30* knockdown, we measured the expression in 7-day-old seedlings of four flowering time genes: *FLOWERING LOCUS T (FT)*, *SUPPRESSOR OF CONSTANS OVEREXPRESSION 1 (SOC1)*, *CONSTANS (CO)* and *FLOWERING LOCUS C (FLC)*; and two targets of the miR156 that control the aging pathway: *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3)* and *SPL9*. There was a statistically significant reduction in the expression of *FT* and *SPL3*, but not of *SOC1*, *CO*, *FLC* and *SPL9* in 7-day-old seedlings (Fig. 3B; *FT*: $P=0.0013$ for *amiMed30-1 T6.1* and $P<0.0001$ for *amiMed30-2 Z3.2*; *SPL3*: $P=0.0168$ for *amiMed30-1 T6.1* and

$P=0.004$ for *amiMed30-2 Z3.2*). *SPL3* is a direct regulator of the expression of *FT* (Kim et al., 2012) and, together with *SPL4* and *SPL5*, forms a structural and functional subfamily among the *SPL* factors. We analyzed the expression levels of these two additional *SPLs* but found no statistically significant difference between the expression levels in the *amiMed30* lines and in the wild type (Fig. S9D). Remarkably, *spl3* mutants have no evident delay in flowering time (Wu and Poethig, 2006) and, therefore, we searched by RNA sequencing (RNAseq) for additional factors responsible for the late flowering phenotype of the *amiMed30* lines. We compared the transcriptomes of wild-type with *amiMed30-2 Z3.2* 10-day-old seedlings grown in LD. We found a reduction not only in *FT*, but also in *SOC1*. Among the upregulated genes, we identified *FLC*, *MAF4* and *MAF5* (Table S3). We confirmed these transcriptional changes in independent lines by RT-qPCR (Fig. 3B and Fig. S10). Interestingly *CO* levels remained unchanged (Fig. 3B), which is consistent with the normal photoperiodic response of *amiMed30* lines (Fig. 3A).

The delay in phase transition in the *amiMed30* lines could be explained by the downregulation of *SPL3*, a factor that positively regulates the expression of *FT* through the aging pathway (Wang et al., 2009; Kim et al., 2012). Both *SPL3* and *FT* appeared to be downregulated early, in 7-day-old seedlings (Fig. 3B). Lower levels of *SOC1* and higher levels of *FLC*, *MAF4* and *MAF5* were found in 10-day-old seedlings, which might contribute to the delay of flowering (Fig. 3B). However, the *amiMed30* lines responded to vernalization (Fig. S8A). Therefore, although our data suggest that *MED30* affects the autonomous and age pathways, we cannot rule out that a more substantial decrease in *MED30* mRNA levels leads to defects in other flowering pathways. These results are consistent with previous reports showing that other MED subunits also affect flowering through multiple pathways (Iñigo et al., 2012a; Kidd et al., 2009).

Individual Mediator subunits have been linked to several responses to environmental clues, abiotic and biotic stresses and hormonal inputs. However, the roles of essential subunits have remained unknown for the most part. Here, we have shown that the *MED30* subunit is essential for male gametophyte and embryo development, and that it also promotes flowering. More interestingly, our data suggest that *MED30* is necessary for paternal control of early embryo development. Further research is

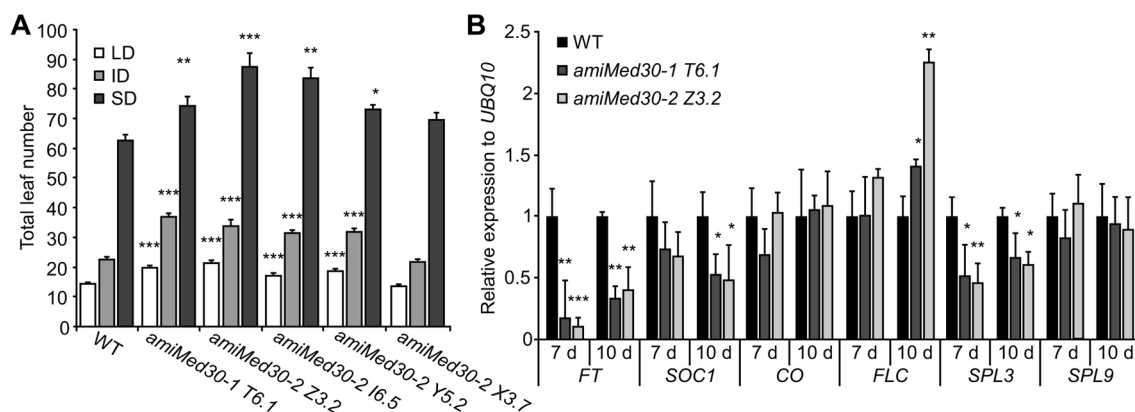


Fig. 3. *MED30* downregulation leads to late flowering and misexpression of flowering genes. (A) Flowering time of independent lines for two amiRNAs designed to target *MED30* mRNA. All genotypes were grown under LD, ID or SD at 23°C. The total leaf number (rosette plus cauline) was recorded at the time of flowering. Bars represent the mean \pm s.e.m. of two independent experiments, with 18 plants for each genotype. (B) mRNA expression levels relative to *UBQ10*. For RNA extraction, wild-type and *amiMed30* seedlings were grown for 7 or 10 days under LD at 23°C, and samples were extracted 2 h before the start of the night. Bars represent mean \pm s.e.m. of two independent experiments, each with three biological replicates that were analyzed in triplicate. In each panel, the significance of Dunnett's post-hoc test conducted after one-way blocked ANOVA is indicated with asterisks above each bar: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

needed to determine whether MED30 is also essential during subsequent developmental stages. We hope that the study of essential Mediator subunits will aid our understanding of which of those subunits form the core set of subunits required *in vivo*.

MATERIALS AND METHODS

Plant material

The *MED30* T-DNA alleles, *med30-1* (SALK_094948) and *med30-2* (SALK_117444), were both in the *A. thaliana* Columbia background and were obtained from the Arabidopsis Biological Resource Center (ABRC; www.abrc.osu.edu). Heterozygous plants were identified by a PCR reaction with three primers: gene-specific left (LP) and right primers (RP) designed to anneal surrounding each T-DNA insertion, plus a third primer (LB) that annealed on the left border of the T-DNA. To confirm the location of the T-DNA insertions in each line, an additional primer that annealed on the right border (RB) was used. PCR products using LB+RP and LP+RB for *med30-1* and LP+LB and RB+RP for *med30-2* were sequenced. The sequences of the primers used are shown in Table S4.

Growth conditions

Seeds were sterilized with chlorine in the vapor phase, plated in MS salts medium (DUCHEFA) and stratified in the dark at 4°C for 3 days. Plates were subsequently incubated at 23°C under LD, ID or SD for 5-7 days and plants were later grown on Grow Mix MultiPro soil (Agroquímica Larrocca); every 2 weeks, the plants were fertilized with a 0.1% solution of Hakaphos (Compo Agricultura).

Segregation analysis

To evaluate transmission of *med30* alleles through male gametophytes, lines bearing the *med30* alleles were used as males and a glabra line *phot1-5 phot2-1* was used as the pollen acceptor (female); the successful crosses were selected by the presence of trichomes over the surface of the rosette leaves on F1 plants. To evaluate transmission of *med30* alleles through female gametophytes, lines bearing the *med30* alleles were used as females and plants harboring the empty vector CHF5 were used as pollen donors; F1 plants were selected in MS plates supplemented with 17 µg ml⁻¹ glufosinate ammonium (Duchefa). F1 individuals were genotyped by PCR to determine whether they had inherited a *med30* allele.

Plant assays

The fertility of wild-type, *med30-1*^{+/+} and *med30-2*^{+/+} plants was evaluated by measuring the length of mature siliques on the main stem of plants grown at 23°C and under ID for 7 weeks. All siliques were harvested and imaged using a Leica EZ4D stereomicroscope and measurements were made using ImageJ software.

For the vernalization assay, seeds were stratified at 4°C for 3 days and germinated directly on the soil at 23°C under ID for 7 days. They were transferred to vernalizing conditions at 10°C for 4 weeks and were then transferred back to 23°C until the end of the flowering process.

For the GA treatment, plants were cultivated under SD at 23°C and twice a week following the appearance of the first pair of true leaves, they were sprayed with either a solution of Silwet-77 (Rizobacter) 1% supplemented with GA₄₊₇ (Duchefa) 10 µM, or with ethanol 0.1% until the end of the flowering process.

Abaxial trichomes were scored with a stereomicroscope 3-4 weeks after planting. For leaf shape analysis, fully expanded leaves were removed and attached to a piece of white paper with transparent tape, and then scanned in a digital scanner. Leaf length and width were measured using ImageJ software.

Pollen assays

Pollen grain viability was assessed after incubation with 20 mM FDA for 10 min at room temperature in the dark (Li, 2011) using a Carl Zeiss Axioplan microscope with Green Fluorescent Protein (GFP) filters. Images were captured on an Olympus DP72 camera.

In vitro *Arabidopsis* pollen germination experiments were conducted as previously described (Boavida and McCormick, 2007). Briefly,

pollen from three independent plants of each genotype was germinated on solid pollen germination medium (PGM: 0.01% boric acid, 5 mM CaCl₂, 5 mM KCl, 1 mM MgSO₄ and 10% sucrose and 0.5% low-melting agarose, pH 7.6) at 22°C for 3 h. A pollen grain was classified as germinated if the pollen tube length was equal to or greater than the pollen grain diameter.

In vivo pollination and Aniline Blue staining of pollen tubes

For Aniline Blue staining of pollen tubes, pistils of *qrt/qrt* emasculated flowers were hand pollinated with pollen from *qrt/qrt med30-1*^{+/+} plants. Pistils were dissected 24 h after pollination and incubated in NaOH 5 N overnight at room temperature. They were then washed with ddH₂O and incubated for 3 h in the dark with 0.1% decolorized Aniline Blue in K₃PO₄ 100 mM pH 8.3 buffer. Pistils were washed again with ddH₂O and mounted on a microscope slide with a drop of glycerol and carefully pressed with a cover slip to open the pistil longitudinally. Aniline Blue fluorescence was observed under a Carl Zeiss Axioplan microscope with ultraviolet (UV) light. Images were captured with an Olympus DP72 camera.

Microscopic embryo observations

For embryo observations, pistils of wild-type emasculated plants flowers hand pollinated with pollen from *med30*^{+/+} plants or 3- to 4-day-old developing siliques from *BCP1:MED30/BCP1:MED30 med30*^{+/+} and *MALE:MED30/MALE:MED30 med30*^{+/+} plants were dissected and then cleared overnight in Hoyer's solution. The material was observed under a Zeiss Axioplan Imaging 2 microscope equipped with differential interference contrast (DIC) optics.

Constructs

The complementation constructs were generated by fusing a PCR-amplified 717-bp fragment of the *BCP1* promoter or a PCR-amplified 1549-bp fragment of the AT5G17340 (*MALE*) promoter to a retrotranscribed *MED30* cDNA. Both combinations were cloned by restriction enzymes in a pCHF5 derivative binary plasmid (Sánchez-Lamas et al., 2016). These constructs were used to transform plants with *Agrobacterium tumefaciens* GV3101 (Clough and Bent, 1998). Lines were selected on MS medium supplemented with 17 µg ml⁻¹ Basta. Only lines showing a 3:1 segregation ratio in the T2, indicating single-locus insertions, were used for subsequent experiments.

Two amiRNA constructs directed against *MED30* were designed using WMD2-Web Micro RNA designer (Ossowski et al., 2008). Overlapping PCR was used to replace the MIR319a precursor with each amiRNA and finally subcloned into a CHF3 binary vector for plant transformation. Transgenic lines were selected on MS medium supplemented with 50 µg ml⁻¹ kanamycin. The primer sequences are shown in Table S4.

Quantitative RT-PCR

Seedlings were frozen in liquid nitrogen. Total RNA was prepared using a Plant Total RNA Mini Kit (YRP50; Real Biotech Corporation), and 1 µg was used to synthesize cDNA with M-MuLV reverse transcriptase (NEB) to quantify *UBQ10*, *MED30*, *CO*, *SOC1*, *FT*, *FLC*, *SPL3*, *SPL4*, *SPL5*, *SPL9*, *MAF4* and *MAF5* expression with the Light Cycler 480 real-time PCR system (Roche) in conjunction with SyBR Green I (Invitrogen). *UBQ10* was used as a housekeeping gene to normalize gene expression (Iñigo et al., 2012a). The average ratio value was used to determine the fold change in transcript level. Relative expression levels were determined using the comparative cycle threshold (Ct) method (Larionov et al., 2005). The primers used are described in Table S4.

Data analysis

RT-qPCR data was analyzed with one-way blocked ANOVA, followed by post-hoc Dunnett's *t*-test. GraphPad 7 software was used. *P*<0.05 was considered statistically significant.

RNAseq experiments

For the RNAseq experiments, seeds of the wild-type and *amiMed30-2 Z3.2* lines were cultivated at 23°C under LD under 100 µmoles m⁻² seg⁻¹ of

white fluorescent light for 10 days. Samples were extracted 2 h before the start of the night period and the seedlings were then frozen in liquid nitrogen. Total RNA was prepared using a Plant Total RNA Mini Kit (YRP50).

Illumina 150-bp paired-end reads were mapped to the *A. thaliana* reference genome assembly (assembly version TAIR10) with tophat2 (PMID: 23618408) and raw read counts per gene were then estimated with htseq-count (PMID:25260700).

Genes with more than five reads per million in only two or fewer samples were eliminated from the analysis. Differential expression analysis of the remaining genes was carried out with the R package EdgeR (PMID:19910308) using a quasi-likelihood negative binomial generalized log-linear model (EdgeR function glmQLFit) (PMID: 27008025).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.J., S.I., S.M.A., A.R.S., J.P.M., G.C.P., P.D.C.; Methodology: A.J., S.I., S.M.A., L.A.A., D.F.F., A.R.S., M.B.O., G.C.P., P.D.C.; Software: H.L.; Formal analysis: A.J., S.I., S.M.A., H.L., P.D.C.; Investigation: A.J., S.I., S.M.A., L.A.A., D.F.F., A.R.S., M.B.O., G.C.P.; Writing - original draft: A.J., P.D.C.; Writing - review & editing: A.J., J.P.M., G.C.P., P.D.C.; Supervision: J.P.M., G.C.P.; Project administration: P.D.C.; Funding acquisition: J.P.M., G.C.P., P.D.C.

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Data availability

Raw RNAseq data have been deposited in the BioProject archive of the SRA database under the project number PRJNA508510.

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.175224.supplemental>

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