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CRYPTIC PRECOCIOUS/MED12 is a Novel Flowering Regulator with Multiple Target Steps in Arabidopsis

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Rapid Paper

The proper timing of flowering is of crucial importance for reproductive success of plants. Regulation of flowering is orchestrated by inputs from both environmental and endogenous signals such as daylength, light quality, temperature and hormones, and key flowering regulators construct several parallel and interactive genetic pathways. This integrative regulatory network has been proposed to create robustness as well as plasticity of the regulation. Although knowledge of key genes and their regulation has been accumulated, there still remains much to learn about how they are organized into an integrative regulatory network. Here, we have analyzed the *CRYPTIC PRECOCIOUS* (*CRP*) gene for the Arabidopsis counterpart of the MED12 subunit of the Mediator. A novel dominant mutant, *crp-1D*, which causes up-regulation of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *FRUITFULL* (*FUL*) and *APETALA1* (*AP1*) expression in a *FLOWERING LOCUS T* (*FT*)-dependent manner, was identified in an enhancer screen of the early-flowering phenotype of *35S::FT*. Genetic and molecular analysis of both *crp-1D* and *crp* loss-of-function alleles showed that MED12/CRP is required not only for proper regulation of *SOC1*, *FUL* and *AP1*, but also for up-regulation of *FT*, *TWIN SISTER OF FT* (*TSF*) and *FD*, and down-regulation of *FLOWERING LOCUS C* (*FLC*). These observations suggest that MED12/CRP is a novel flowering regulator with multiple regulatory target steps both upstream and downstream of the key flowering regulators including FT florigen. Our work, taken together with recent studies of other Mediator subunit genes, supports an emerging view that the Mediator plays multiple roles in the regulation of flowering.

Keywords: Arabidopsis • Flowering • *FLOWERING LOCUS C* • *FLOWERING LOCUS T* • Mediator • MED12.

Abbreviations: *AP1*, *APETALA1*; bZIP, basic region/leucine zipper; *CCT*, *CENTER CITY*; CDK, cyclin-dependent kinase; CycC, cyclin C; *CO*, *CONSTANS*; *CRP*, *CRYPTIC PRECOCIOUS*; EMS, ethylmethane sulfonate; *FLC*, *FLOWERING LOCUS C*; *FT*, *FLOWERING LOCUS T*; *FUL*, *FRUITFULL*; *GCT*, *GRAND CENTRAL*; GFP, green fluorescent protein; GUS, β-glucuronidase; *HEN*, *HUA ENHANCER*; *LFY*, *LEAFY*; *MAB*, *MACCHIBOU*; MED, Mediator complex subunit; MS, Murashige and Skoog; *NRT1.7*, *NITRATE TRANSPORTER 1.7*; *PFT*, *PHYTOCHROME AND FLOWERING TIME*; *PID*, *PINOID*; qRT–PCR, quantitative reverse transcription–PCR; RT–PCR, reverse transcription–PCR; 35S, Cauliflower mosaic virus 35S RNA promoter; SAM, shoot apical meristem; *SOC1*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*; *STM*, *SHOOTMERISTEMLESS*; *SULTR2;1*, *SULFATE TRANSPORTER2*; *1* promoter; *TSF*, *TWIN SISTER OF FT*; ZT, Zeitgeber time.

The nucleotide sequences reported in this paper have been submitted to DDBJ under accession numbers: MED12/CCT/CRP locus (AB690343) and MED12/CCT/CRP cDNA (AB690341 and AB690342).

Introduction

The plant life cycle is divided into distinct developmental phases by the morphological and functional features of the organs formed at the flank of the shoot apical meristem (SAM) (Araki 2001, Poethig 2003, Bäurle and Dean 2006). The proper timing of the transition from leaf-forming vegetative phase to flower-forming reproductive phase, called flowering, is especially important for reproductive success. Studies of Arabidopsis (*Arabidopsis thaliana*) have shown that regulation of flowering is orchestrated by inputs of multiple

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environmental and endogenous signals such as daylength, light quality, temperature and hormones, and that flowering time genes construct several parallel and interactive genetic pathways (Simpson and Dean 2002, Boss et al. 2004, Fornara et al. 2010). This integrative regulatory network has been proposed to create robustness as well as plasticity to increase fitness under genetic and environmental perturbation (Wilczek et al. 2009).

Several genes represent key nodes in the regulatory network. *FLOWERING LOCUS T* (*FT*) encodes a small 20 kDa protein, which belongs to the phosphatidylethanolamine-binding protein (PEBP)/Raf kinase inhibitor protein (RKIP) family, and plays a pivotal role in promoting flowering, integrating signals from converging pathways (Kardailsky et al. 1999, Kobayashi et al. 1999). Expression of *FT* is strictly controlled by transcription factors and chromatin-associated proteins in the leaf vasculature (Takada and Goto 2003, Kobayashi and Weigel 2007, Adrian et al. 2010, Imaizumi, 2010). A B-box zinc-finger protein CONSTANS (CO) plays a central role in *FT* regulation in the photoperiod pathway (Kardailsky et al. 1999, Kobayashi et al. 1999, Samach et al. 2000). The transcriptional and post-translational controls of *CO* are important for monitoring the seasonal daylength changes (Suárez-López et al. 2001, Yanovsky and Kay 2002, Valverde et al. 2004, Kobayashi and Weigel 2007). As the florigen, FT protein is translated in the leaf, moves via phloem to the SAM, and activates several positive floral regulators to initiate the formation of flowers (Corbesier et al. 2007, Jaeger and Wigge 2007, Mathieu et al. 2007, Notaguchi et al. 2008). FT forms a protein complex involving a bZIP (basic region/leucine zipper) transcription factor FD, and directly activates *APETALA1* (*AP1*) expression (Abe et al. 2005, Wigge et al. 2005), and either directly or indirectly activates *FRUITFULL* (*FUL*) expression (Teper-Bamnolker and Samach 2005). FT also promotes expression of *LEAFY* (*LFY*) via up-regulation of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Searle et al. 2006, Liu et al. 2008). A MADS box transcription factor, *FLOWERING LOCUS C* (*FLC*), is a potent repressor of *FT*, *TWIN SISTER OF FT* (*TSF*), *FD* and *SOC1*, and thereby negatively regulates both florigen production and response (Searle et al. 2006). *FLC* expression is regulated by histone modification in response to vernalization and autonomous pathways (Bäurle and Dean 2006, Schmitz and Amasino 2007). Although knowledge of the regulation of key genes has been accumulated, there still remains much to learn about how they are organized into an integrative regulatory network.

Mediator is an evolutionarily conserved multiprotein complex in eukaryotes, whose biochemistry and functional roles have been extensively studied in yeast and animals. Mediator plays numerous roles in controlling the function of RNA polymerase II pre-initiation complex, and the structural plasticity of the multisubunit complex enables Mediator to regulate virtually all protein-coding genes (Malik and Roeder 2005). Genetic and molecular studies collectively suggest that the Mediator complex is essential for the proper development of the organism. Mediator complex consists of a 'core module' and 'CDK8 module'. The CDK8 module contains cyclin-dependent kinase 8 (CDK8), cyclin C (CycC), Mediator complex subunit (MED) 12 (MED12) and MED13. Of the four subunits, MED12 and MED13 have been shown to be important for proper control of developmental programs in animals such as zebrafish, *Drosophila* and *Caenorhabditis elegans* (Treisman 2001, Janody et al. 2003, Hong et al. 2005, Yoda et al. 2005, Rau et al. 2006, Henteges 2011). Recently, plant Mediator subunits have been identified (Bäckström et al. 2007, Bourbon 2008), and the physiological roles of some subunits have been revealed (Autran et al. 2002, Kidd et al. 2009, Gillmor et al. 2010, Ito et al. 2011, Kidd et al. 2011, Kim et al. 2011). For example, a subunit of the core module, MED25, which was originally described as PHYTOCHROME AND FLOWERING TIME 1 (PFT1), was shown to act in jasmonate signaling and in the light quality pathway of flowering acting downstream of phytochrome B (phyB) to regulate expression of *FT* (Cerdán and Chory 2003, Wollenberg et al. 2008, Elfving et al. 2011).

In this study, we identified and investigated the *CRYPTIC PRECOCIOUS* (*CRP*) gene for the Arabidopsis counterpart of MED12. We isolated a novel dominant mutant, *crp-1D*, as a genetic modifier of the precocious-flowering phenotype in *35S::FT*. Genetic and molecular analyses of both dominant *crp-1D* and recessive *crp* loss-of-function alleles suggest that MED12/CRP is a novel flowering regulator with multiple regulatory target steps both upstream and downstream of the key flowering regulators including FT florigen. Furthermore, our present work and previous works (Gillmor et al. 2010, Ito et al. 2011) strongly suggest that MED12/CENTER CITY (CCT)/CRP and MED13/GRAND CENTRAL (GCT)/MACCHIBOU 2 (MAB2) proteins act closely together in the same pathways to regulate various aspects of plant development from embryogenesis to flowering and floral morphogenesis.

Results

cryptic precocious-1D, a dominant enhancer mutation of *35S::FT*

To understand the regulatory mechanisms acting downstream of the floral integrator *FT*, we carried out ethylmethane sulfonate (EMS) mutagenesis of a weak line of *35S::FT* and screened for mutations that affect its precocious-flowering phenotype. We isolated one dominant mutation, *cryptic precocious-1D* (*crp-1D*), which strongly enhanced the precocious-flowering phenotype to skip the vegetative phase (**Fig. 1A–C, E**, **Supplementary Table S1**). The phenotype of *crp-1D; 35S::FT* plants was very similar to that of *soc1-101D; 35S::FT* plants (**Fig. 1D**), implying that *crp-1D* mutation causes up-regulation of genes acting downstream of *FT*, such as *SOC1*.

In contrast to the strong enhancement of the *35S::FT* phenotype, *crp-1D* single mutation in the wild-type background had a moderate effect, leading to a slightly early-flowering phenotype

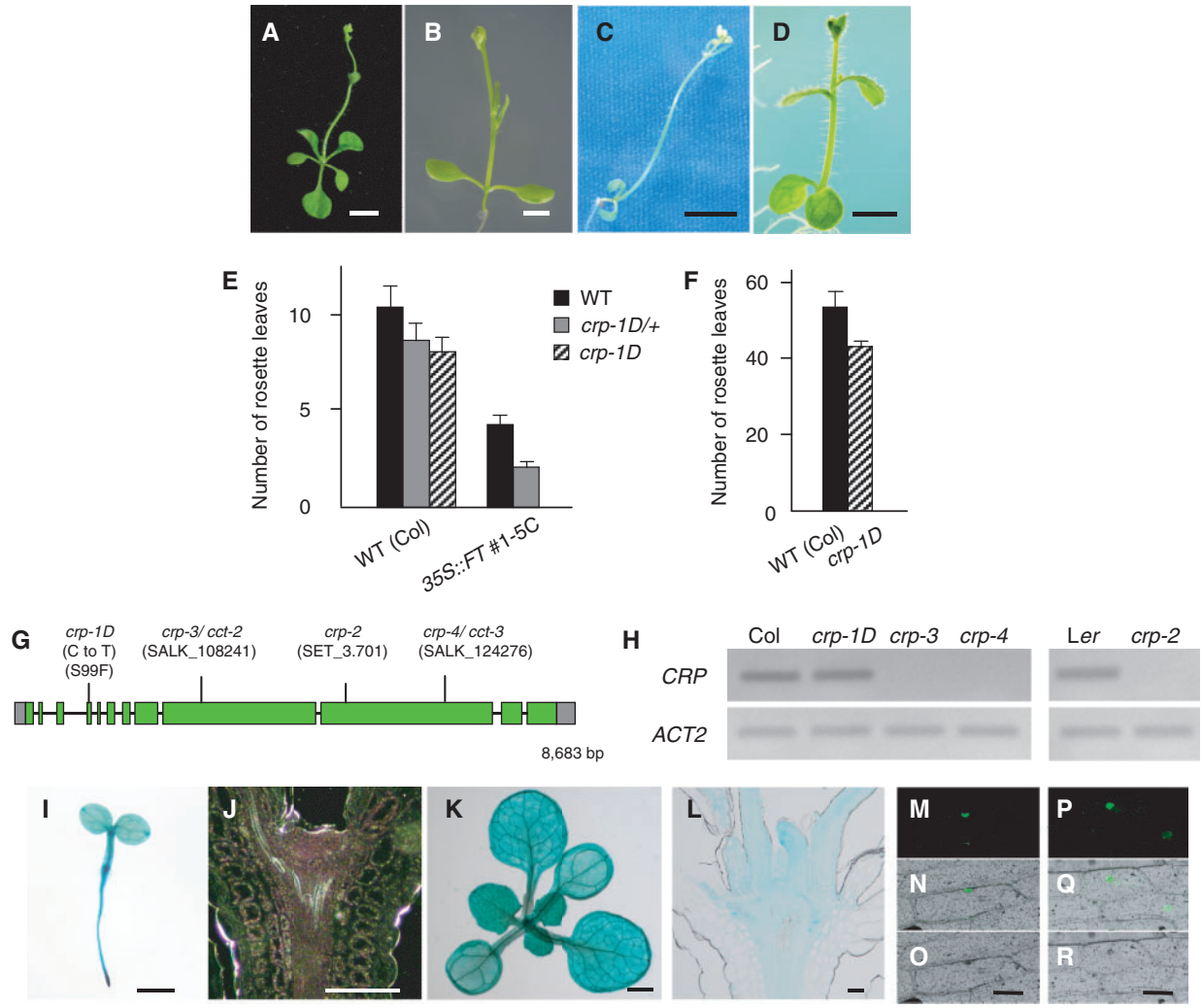

Fig. 1 Flowering phenotype of the *crp-1D* mutant and gene structure and expression patterns of *CRP*. (A–D) Effect of *crp-1D* and *soc1-101D* on the early-flowering phenotype of *35S::FT*. *35S::FT* #1-5C (A), *35S::FT* #1-5C/—; *crp-1D*/+ (B), *35S::FT* #1-5C; *crp-1D* (C) and *35S::FT* #1-5C; *soc1-101D* plants (D). 'Transgene symbol/—' and 'mutation symbol/+' indicate the hemizygote and heterozygote, respectively. (E) Flowering time of *35S::FT* #1-5C/—; *crp-1D*/+ plants under long-day conditions. There is a statistically significant difference (Student's *t*-test, $P < 0.005$) with CRP^{+} (solid bars). (F) Flowering time of the *crp-1D* mutant under short-day conditions. There is a statistically significant difference (Student's *t*-test, $P < 0.005$) between the two genotypes. (G) *CRP* gene structure and positions of mutations and T-DNA or Ds insertions in *crp* alleles. Boxes and lines indicate exons and introns of *CRP*, respectively; gray and green boxes represent untranslated regions and coding regions, respectively. (H) Semi-quantitative RT–PCR analysis of *CRP* expression in the wild type and *crp* mutants. Plants were harvested on day 10 (Col background; left panel) or 7 (L*er* background; right panel). (I–L) Spatial expression patterns of *CRP*. Whole-mount preparation and longitudinal section through the SAM of 3-day-old (I, J) and 10-day-old (K, L) *CRP::GUS* seedlings. Plants were grown under long-day conditions and subjected to GUS staining for 12–48 h. (M–R) Subcellular localizaton of CRP and crp-1D proteins. Onion epidermal cells were bombarded with GFP fusion constructs, *35S::CRP-GFP* (M–O) and *35S::crp-1D-GFP* (P–R). Dark field images (M, P), bright field images (N, Q) and merged images (O, R). Scale bars: 5 mm in A and C, 2 mm in B and D, 1 mm in I and K, and 0.1 mm in J, L, O and R. In E and F, the numbers of rosette leaves are the average of at least 11 plants. Error bars indicate the SD. Additional data and statistics of the data are summarized in **Supplementary Tables S1** and **S2**.

in both inductive long-day and non-inductive short-day conditions (**Fig. 1E, F**, **Supplementary Table S2**). *crp-1D* also showed a dominant effect in the wild-type background (**Fig. 1E**, **Supplementary Table S1**).

Identification of the *CRP* gene

To obtain knowledge of the molecular nature of the *CRYPTIC PRECOCIOUS* (*CRP*) gene, map-based cloning was performed using *crp-1D*. The *CRP* locus was mapped to a 33.5 kb interval between F5I10 and F6N23 at the top of chromosome 4 where six genes are predicted (**Supplementary Fig. S1**). The only non-synonymous substitution found in the coding region of the six genes in the *crp-1D* mutant was a C-to-T substitution in At4g00450. No obvious differences in expression levels of the other genes were observed, indicating that nucleotide substitutions in the intergenic regions causing elevated or ectopic

expression would be unlikely to be responsible for the *crp-1D* phenotype. Thus, we concluded that At4g00450 is the *CRP* gene. *CRP* was recently identified independently as the *CENTER CITY* (*CCT*) gene, which is required for proper embryogenesis (Gillmor et al. 2010). By cDNA cloning, we found that the actual coding region of *CRP* is comprised of 12 exons spanning 8.7 kb (**Fig. 1G**, DDBJ accession No. AB690343) and is different from the publicly available annotated unit adopted for *CCT* (encoding a protein of 2,144 amino acids; Gillmor et al. 2010). The *CRP* gene encodes a protein of 2,235 amino acids, which corresponds to the Arabidopsis counterpart of MED12 protein (Gillmor et al. 2010), a subunit of the transcriptional Mediator complex (Bäckström et al. 2007) (**Supplementary Fig. S2**). *crp-1D* mutation caused an amino acid substitution from serine (S) to phenylalanine (F) at position 99 (**Fig. 1G**, **Supplementary Fig. S3**). *crp-1D* mutation had no effect on the mRNA level (**Fig. 1H**).

Spatial expression patterns of *CRP* were examined using transgenic plants expressing the *β-glucuronidase* (*GUS*) reporter gene under the control of a 2.0 kb genomic sequence upstream of the initiation codon (*CRP::GUS*). Ubiquitous expression was observed in seedlings and young plants, with higher levels in vascular tissue and the shoot apex (**Fig. 1I–L**). In mature plants, strong expression was observed in young floral buds and developing floral organs as described below. To examine the subcellular localization of CRP protein, green fluorescent proteins (GFPs) fused to full-length wild-type CRP (CRP–GFP) and S99F mutant CRP (crp-1D–GFP) were expressed in onion epidermal cells. Both CRP–GFP and crp-1D–GFP fusion proteins were localized in the nuclear region in a similar manner (**Fig. 1M–R**).

Effects of *crp* loss-of-function mutations on flowering time

To investigate whether the *CRP* gene has any role in the flowering process, we obtained a Ds-transposon insertion line in the Landsberg *erecta* (L*er*) background (*crp-2*) and two T-DNA insertion lines in the Columbia (Col) background (*crp-3* and *crp-4*) (**Fig. 1G**). Reverse transcription–PCR (RT–PCR) analysis revealed no accumulation of the CRP full-length transcript in *crp-2*, *crp-3* or *crp-4* plants (**Fig. 1H**). As homozygous *crp* loss-of-function plants were sterile, we analyzed the phenotypes in homozygous mutants segregating from self-fertilized heterozygous *crp* plants.

crp loss-of-function mutants flowered much later than the wild type in long-day conditions (**Fig. 2A**, **Supplementary Table S2**) (see also Gillmor et al. 2010), whereas no significant difference from the wild type was observed in short-day conditions (**Fig. 2B**, **Supplementary Table S2**). This phenotype is comparable with that of loss-of-function mutants of genes in the photoperiod pathway, such as *ft*, *co* and *pft1/med25* (Koornneef et al. 1991, Cerdán and Chory 2003), suggesting that *CRP* is likely to be a promoter of flowering acting in the photoperiod pathway.

To explore further the molecular basis of the late-flowering phenotype in *crp*, we first examined expression levels of genes which are regulated by *FT* as well as genes with important roles in the *FT*-dependent flowering pathway, in *crp* loss-of-function mutants and in wild-type plants. While the mRNA level of *CO*, an upstream promoter of *FT*, remained unchanged, the mRNA level of *FLC*, a strong repressor of *FT* and *TSF*, was increased in *crp-3* and *crp-4* plants at every time point examined (**Fig. 2C**). Consistent with this, *FT* and *TSF* mRNA levels were much lower in *crp* loss-of-function mutants than in wild-type plants (**Fig. 2C**). The robust expression of the *NRT1.7* gene in *crp* mutants, which has been reported as a marker gene of phloem companion cells of mature leaf minor veins (Fan et al. 2009), confirmed that the reduction of *FT* and *TSF* expression is likely to be due to the direct effect of *crp* mutation rather than the secondary effect of disturbed vascular patterning in *crp* (**Supplementary Fig. S4A**; details are described later). In the shoot apical region, expression of *FD*, encoding an FT partner bZIP transcription factor, and *SOC1* and *FUL*, downstream targets of *FT*, were significantly reduced in *crp* mutants (**Fig. 2D**). This is again consistent with the increased level of *FLC* which is a negative regulator of *FD* and *SOC1* expression in the shoot apex (Searle et al. 2006). That the reduced expression of *FD*, *SOC1* and *FUL* was not the consequence of possible abnormal shoot apical morphology in the *crp* mutant or unequal collections of shoot apical samples among genotypes was confirmed by monitoring the expression level of the *SHOOTMERISTEMLESS* (*STM*) gene (Long et al. 1996) (**Supplementary Fig. S4B**). In accordance with the reduced expression of flowering promoters, *LFY* and *AP1* expression was not induced in *crp* mutants even at day 15 when these floral meristem identity genes are up-regulated in wild-type plants (**Fig. 2D**).

Because *CRP* seems to act both upstream and downstream of the *FT*-dependent flowering pathway, we next analyzed the genetic interaction between *CRP* and *FT*. Since the *35S::FT* transgene was silenced in F_2 progeny from the cross between *35S::FT* and *crp* T-DNA insertion alleles (data not shown), we instead used a *SULTR2;1::FT* transgenic line in which the *FT* gene is specifically expressed in the phloem (Abe et al. 2005) to examine whether the artificial expression of *FT* can suppress the late-flowering phenotype of *crp* loss-of-function mutants. Introduction of *SULTR2;1::FT* partially, but not completely, suppressed the late-flowering phenotype of *crp-3* and *crp-4* (**Fig. 2E**, **Supplementary Table S3**). This result is consistent with *CRP* controlling not only the expression of *FT*, but also the expression of downstream genes of *FT* in an, in part, *FT*-dependent manner. This was further supported by the observation that *crp*; *ft-2* double mutants flowered later than the *ft-2* single mutant (**Fig. 2A**, **Supplementary Table S4**). A *tsf* loss-of-function mutant (*tsf-1*) had no effect on the flowering time of *crp*, whereas both *fd* and *soc1* mutations showed an additive effect on the late-flowering phenotype of the *crp* single mutant (**Fig. 2A**, **Supplementary Table S4**).

To investigate whether the elevated expression level of *FLC* is responsible for the late-flowering phenotype of *crp* loss-of-

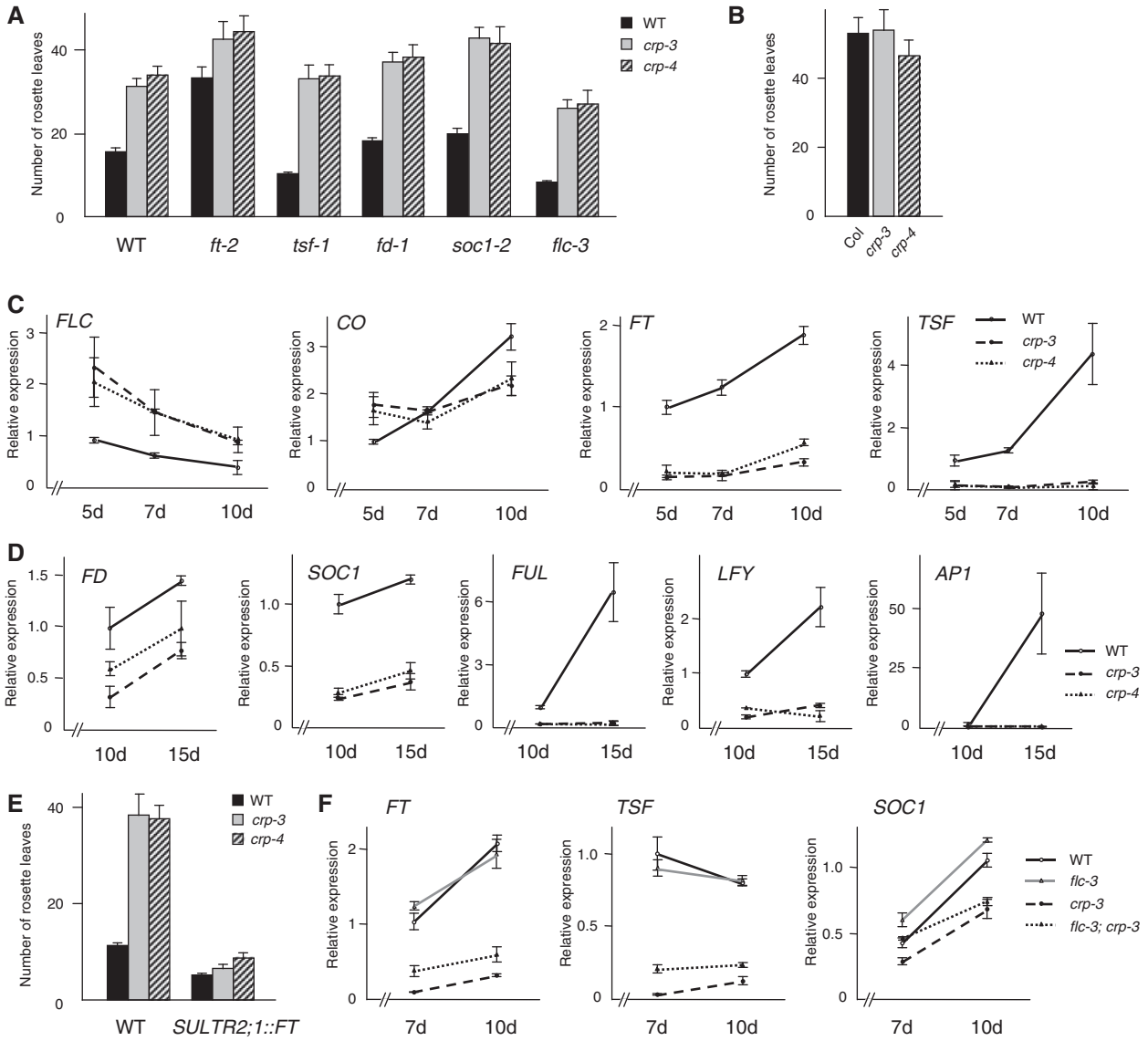

Fig. 2 Effect of *crp* loss-of-function mutations on the flowering phenotype. (A) Flowering time of *crp* loss-of-function mutants and flowering-time mutants with wild-type CRP^+ or *crp* loss-of-function alleles under long-day conditions. (B) Flowering time of *crp* loss-of-function mutants under short-day conditions. (C) *CO*, *FLC*, *FT* and *TSF* expression in wild-type Col, *crp-3* and *crp-4* plants. Aerial parts of seedlings grown under long-day conditions were harvested on days 5, 7 and 10 for qRT–PCR analysis. (D) *FD*, *SOC1*, *FUL*, *LFY* and *AP1* expression in wild-type Col, *crp-3* and *crp-4* plants. Shoot apical regions of seedlings grown under long-day conditions were harvested on days 10 and 15 for qRT–PCR analysis. (E) Flowering time of *crp* loss-of-function mutants with or without the *SULTR2;1::FT* transgene. There is a statistically significant difference (Student's *t*-test, $P < 0.005$) between CRP^+ and *crp* in the *SULTR2;1::FT* background. (F) *FT*, *TSF* and *SOC1* expression in wild-type Col, *flc-3*, *crp-3* and *flc-3; crp-3* plants. Plants were grown under long-day conditions and aerial parts were harvested on days 7 and 10 for qRT–PCR analysis. In A, B and E, the numbers of rosette leaves are the average of at least seven plants. Error bars indicate the SD. Additional data and statistics of the data are summarized in **Supplementary Tables S2–S4**. Error bars in C, D and F indicate the SEM ($n = 9$).

function mutants, *crp; flc-3* double mutants were compared with *crp* single mutants. The *crp; flc-3* plants flowered earlier than *crp* single mutants under long-day conditions (**Fig. 2A**, **Supplementary Table S4**), suggesting that the increased expression of *FLC* does partially explain the flowering delay of *crp* mutants. This was further confirmed by the observation that the reduction of *FT* and *TSF* in the *crp* single mutant was partially restored in *crp; flc-3* double mutant plants (**Fig. 2F**, **Supplementary Fig. S5**).

Effect of overexpression of *CRP* on flowering time

To understand further the function of *CRP*, we generated transgenic plants overexpressing *CRP* under the control of the 35S promoter (*35S::CRP*), and obtained 15 independent lines.

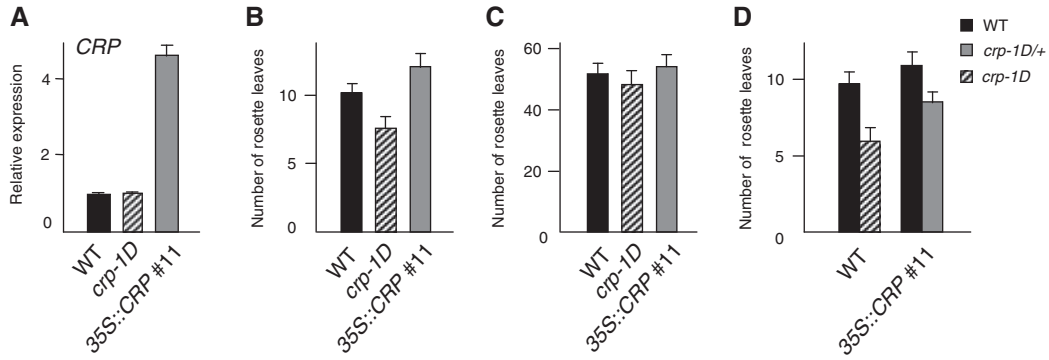

Fig. 3 *CRP* expression and flowering phenotype of *CRP*-overexpressing plants. (A) *CRP* expression levels in wild-type Col and *CRP*-overexpressing plants. Aerial parts of seedlings grown under long-day conditions were harvested on day 12 for qRT–PCR analysis. (B and C) Flowering time of wild-type Col and *CRP*-overexpressing plants under long-day (B) and short-day (C) conditions. There is a statistically significant difference (Student's *t*-test, $P < 0.005$) between the wild type and *35S::CRP* in B. (D) Flowering time of *crp-1D* plants with the *35S::CRP* transgene under long-day conditions. There is a statistically significant difference (Student's *t*-test, $P < 0.005$) between CRP^{+} (solid bar) and *crp-1D* in the *35S::CRP* background. Error bars in A indicate the SE. In B–D, the numbers of rosette leaves are the average of at least 10 plants. Error bars indicate the SD. Additional data and statistics of the data are summarized in **Supplementary Tables S5** and **S6**.

Interestingly, the majority of *35S::CRP* transgenic plants showed a flowering time phenotype either indistinguishable from that of wild-type plants or similar to that of *crp* loss-of-function mutants in both long-day and short-day conditions. In the latter case, the extent of delay in long days was not as severe as that of *crp* loss-of-function mutants (**Fig. 3A–C, Supplementary Table S5**). A similar observation has been reported for *Drosophila kohtalo* (*kto*), which is the *Drosophila* counterpart of *CRP* encoding MED12 (Janody et al. 2003). It is likely that the excessive amount of MED12 protein (CRP in Arabidopsis and kto in *Drosophila*) interferes with the proper formation of protein complex(es) that contain MED12. Consistently, expression levels of *FT* and *TSF* were reduced in the *35S::CRP* plants as in the case of *crp* loss-of-function mutants (**Supplementary Fig. S6**).

Based on the phenotypes of *crp* loss-of-function mutants and *35S::CRP* transgenic plants, it is suggested that *crp-1D* acts as neither a dominant-negative mutation nor a gain-of-function mutation which increases protein stability as reported for other genes (El-Assal et al. 2001, Reed 2001, Dieterle et al. 2005). By taking advantage of *35S::CRP* transgenic plants, we further addressed the question of whether the mutant crp-1D protein functions in the same molecular context as that of the wild-type CRP protein. If this is indeed the case, then increased amounts of wild-type CRP protein should attenuate the *crp-1D* phenotype. In contrast, there should be little effect if crp-1D protein functions in a different molecular context. As shown in **Fig. 3D**, the *crp-1D* phenotype was significantly attenuated by the introduction of the *35S::CRP* transgene (see also **Supplementary Table S6**). Therefore, it is likely that *crp-1D* acts in the same molecular context as that which involves wild-type CRP, rather than acting as a 'neomorphic' allele whose activity is unrelated to normal *CRP* function. As described below, this was further supported by the observation that expression of the *crp-1D* phenotype required functional *MED13/MAB2*, another subunit functioning together with MED12/CRP (see Genetic interaction of *MED12/CRP* and *MED13/MAB2* in the flowering process and auxin response).

Genetic interaction between the dominant *crp-1D* mutation and flowering-related genes

Since *crp-1D* is likely to act in the same molecular context as the wild-type *CRP*, it will shed some light on the normal *CRP* function. With this in mind, genetic and regulatory interactions between *crp-1D* and flowering-related genes were investigated. We first examined the effect of *crp-1D* mutation on the precocious-flowering phenotype of *35S::TSF*. As expected from redundancy between *FT* and *TSF*, F_1 progeny of crosses between *crp-1D* and *35S::TSF* showed a much earlier flowering phenotype than *35S::TSF* (**Fig. 4A**, **Supplementary Table S1**). Previously, it was reported that *FT*, *TSF*, *SOC1* and *LFY* have both overlapping and independent functions such that *soc1-101D* and *35S::LFY*, respectively, had additive effects on early-flowering phenotypes of *35S::FT* or *35S::TSF* (Kardailsky et al. 1999, Kobayashi et al. 1999, Moon et al. 2005, Yamaguchi et al. 2005). This raises the possibility that *crp-1D* may enhance the precocious-flowering phenotype of *FT*- or *TSF*-overexpressing plants via up-regulation of *SOC1* or *LFY*. To test the interaction between *crp-1D* and these genes, we generated plants which have a *crp-1D* mutation with *soc1-101D* or *35S::LFY*. F_1 progeny of a cross between *crp-1D* and *35S::LFY* flowered slightly earlier than *35S::LFY* (**Fig. 4A**, **Supplementary Table S1**). In contrast, *crp-1D* did not enhance the early-flowering phenotype of *soc1-101D* (**Fig. 4A**, **Supplementary Table S1**). In addition, no enhancement by *crp-1D* was observed for the early-flowering phenotype of *35S::AP1* (**Fig. 4A**, **Supplementary Table S1**). These results suggest that *crp-1D* may affect step(s) upstream of *SOC1* and *AP1*.

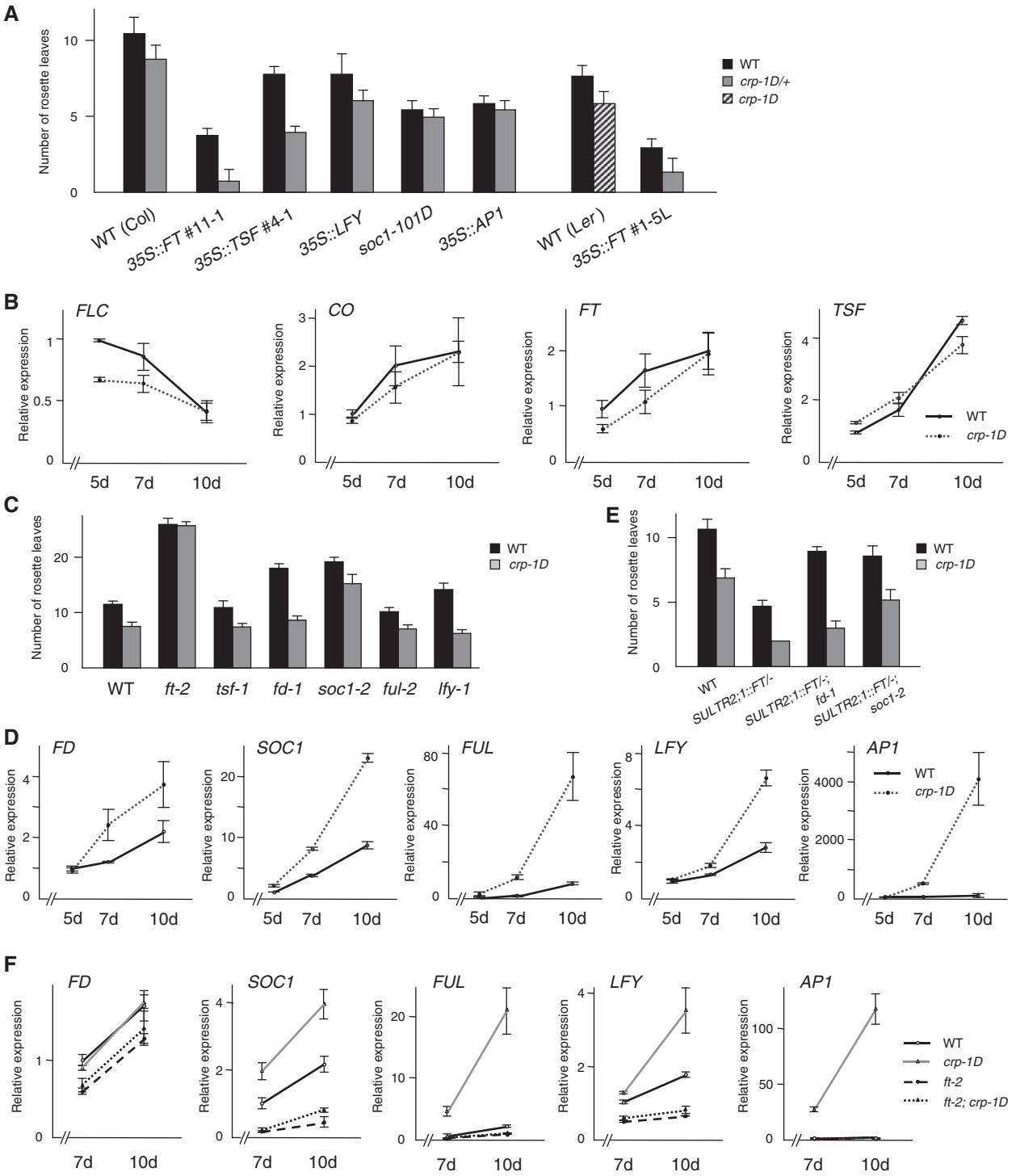

Fig. 4 Effect of *crp-1D* mutation on the flowering phenotype. (A) Flowering time of plants overexpressing flowering regulator genes with or without the dominant *crp-1D* mutation. There is a statistically significant difference (Student's *t*-test, $P < 0.005$) with CRP^{+} (solid bars). (B) *CO*, *FLC*, *FT* and *TSF* expression levels in wild-type Col and *crp-1D* plants. Aerial parts of seedlings grown under long-day conditions were harvested on days 5, 7 and 10 for qRT–PCR analysis. (C) Flowering time of double mutant plants with the *crp-1D* mutation and loss-of-function mutation of flowering regulator genes. (D) *FD*, *SOC1*, *FUL*, *LFY* and *AP1* expression levels in wild-type Col and *crp-1D* plants. Shoot apical regions of seedlings grown under long-day conditions were harvested on days 5, 7 and 10 for qRT–PCR analysis. (E) Flowering time of *SULTR2;1::FT/—; crp-1D/+* plants with an *fd-1* or *soc1-2* mutation. (F) *FD*, *SOC*1, *FUL*, *LFY* and *AP1* expression levels in wild-type Col, *crp-1D*, *ft-2* and *ft-2; crp-1D* plants. Shoot apical region of seedlings grown under long-day conditions were harvested on days 7 and 10 for qRT–PCR analysis. In A, C and E, the numbers of rosette leaves are the average of at least eight plants. Error bars indicate the SD. Additional data and statistics of the data are summarized in **Supplementary Tables S1**, **S7** and **S8**. Error bars in B, D and F indicate the SEM ($n = 9$).

We next compared the temporal regulation of *FLC*, *CO*, *FT* and *TSF* in *crp-1D* and wild-type plants. With the possible exception of *FLC* in young seedlings (5 d), no significant differences were observed (**Fig. 4B**). This suggests that *crp-1D* may have an effect on step(s) downstream of *FT* and/or *TSF*. To test for genetic interaction between *crp-1D* and *FT* and *TSF*, we generated *crp-1D; ft-2* and *crp-1D; tsf-1* double mutants. The early-flowering phenotype of *crp-1D* was suppressed by *ft-2*, but was not affected by *tsf-1* in long-day conditions (**Fig. 4C**, **Supplementary Table S7**), suggesting that *crp-1D* affects flowering mainly in the *FT*-dependent pathway.

The results so far described prompted us to examine the relationship between *crp-1D* and genes acting at the shoot apex in steps downstream of *FT*, such as *FD*, *SOC1* and *FUL*. The expression level of *FD* was not significantly affected in *crp-1D* (**Fig. 4D**), and *fd-1* had little effect on the early-flowering phenotype of *crp-1D* (**Fig. 4C**, **Supplementary Table S7**). In addition, *fd-1* only very weakly suppressed the early-flowering phenotype of *crp-1D/+; SULTR2;1::FT/−* (**Fig. 4E**, **Supplementary Table S8**). These results suggest that *crp-1D* is likely to regulate flowering independently of FD. In contrast, the expression level of *SOC1* and *FUL*, both of which are regulatory targets of *FT*, was elevated in *crp-1D* at every time point tested (**Fig. 4D**). Consistently, the precocious-flowering phenotype of *crp-1D* plants was strongly suppressed by *soc1-2* (**Fig. 4C**, **Supplementary Table S7**). In addition, *soc1-2* suppressed the early-flowering phenotype of *SULTR2;1::FT/−; crp-1D/+* plants (**Fig. 4E**, **Supplementary Table S8**). *ful-2*, which has little effect on flowering time in the wild-type background, did not delay flowering in *crp-1D* (**Fig. 4C**, **Supplementary Table S7**). These results suggest that *crp-1D* acts in the same pathway in which endogenous and overexpressed *FT* regulates *SOC1* and *FUL* expression. This was further supported by the observation that up-regulation of *SOC1* and *FUL* in *crp-1D* was strongly suppressed by introduction of *ft-2*, while the expression level of *FD* was little affected by genotypes (**Fig. 4F**). As expected from elevated *SOC1* expression, levels of *LFY* and *AP1* expression were increased in *crp-1D* compared with the wild type in an *FT*-dependent manner (**Fig. 4D, F**). Since *lfy* loss-of-function mutants (*lfy-1*) had no effect on the precocious-flowering phenotype of *crp-1D* (**Fig. 4C**, **Supplementary Table S7**), *crp-1D* may affect *LFY* (and *AP1*, as well) expression indirectly.

Genetic interaction between *MED12/CRP* and *MED13/MAB2* in the flowering process and auxin response

It was reported that MED12 and MED13 act as a pair in the CDK8 Mediator kinase module in yeast and *Drosophila* (Samuelsen et al. 2003, Kornberg 2005, Bäckström et al. 2007, Loncle et al. 2007). Moreover, mutations in *Drosophila MED12* and *MED13* cause identical phenotypes across multiple traits, again supporting the idea that these two components act closely together in the same module of the Mediator (Treisman 2001, Janody et al. 2003). To test whether this is also true for Arabidopsis *MED12/CRP* and *MED13/MACCHI-BOU2* (*MAB2*), we compared the flowering phenotype of *mab2* loss-of-function mutant (*mab2-1*) with that of *crp-2* in the L*er* background. Similar to the *crp-2* mutant, *mab2-1* flowered later than the wild type in long-day conditions and no difference in flowering time was observed in short-day conditions (**Fig. 5A, B**, **Supplementary Table S2**). We further examined the genetic interaction between *CRP* and *MAB2*. In *Drosophila*, the phenotype of *med12/kohtalo; med13/skuld* double mutants was indistinguishable from that of either single mutant (Janody et al. 2003, Loncle et al. 2007). Similarly, the *crp-2; mab2-1* double mutant and each parental single mutant showed the same phenotype in seedling morphology and flowering time in long-day conditions (**Fig. 5C**, **Supplementary Table S9**) (Ito et al. 2011). These observations suggest that *MED12/CRP* and *MED13/MAB2* act closely together in the same pathway in Arabidopsis development. We further examined genetic interaction between *crp-1D* and *mab2*. *mab2-1* completely suppressed the early-flowering phenotype of *crp-1D* and the precocious-flowering phenotype of *35S::FT/-; crp-1D/+* (**Fig. 5D, E, F–H**, **Supplementary Table S10**), indicating that *crp-1D* requires functional *MED13/MAB2* for its effect on flowering. These results confirmed that *MED13/MAB2* is required for *crp-1D*-mediated floral induction as well.

In addition to a late-flowering phenotype, *crp* loss-of-function mutants showed pleiotropic phenotypes. These include occasional embryo lethality, such that a certain fraction of seeds on the selfed *crp/+* heterozygote were aborted (**Fig. 6A**), abnormal shape of cotyledons (**Fig. 6B**), delay in early growth (**Fig. 6C**), dwarfism (**Fig. 6D**), abnormal leaf shape and vascular patterning (**Fig. 6E**), ectopic bract-like organs subtending the first few flowers as reported for *mab2* (Ito et al. 2011) (**Fig. 6F**), abnormal development of floral organs including chimerism (**Fig. 6G, H**), and sterility due to poor production of pollen grains and anther dehiscence (**Fig. 6I**). In accordance with the floral phenotype, strong expression was observed in young floral buds and developing floral organs (**Fig. 6K, L**). Interestingly, *crp-1D* showed the opposite effect in some of the seedling phenotypes (**Fig. 6A, B**). These phenotypes were in agreement with a recent report that *center city-1* (*cct-1*), another loss-of-function allele of MED12, affected the timing of a variety of morphogenetic events, including embryogenesis, specification of peripheral–abaxial identity and vascular patterning (Gillmor et al. 2010), supporting the generally accepted concept that *MED12* is involved in diverse developmental aspects through gene regulation.

Interestingly, gynoecium morphology of *crp* loss-of-function mutants (**Fig. 6J**) resembles that of auxin-related mutants such as *ettin* (*ett*), namely shorter valves, longer style and elongated basal internode (Liu and Meyerowitz 1995, Nemhauser et al. 2000, Franks et al. 2002). A similar gynoecium phenotype was also observed in *mab2* mutants. It was recently reported that double mutant combinations of *mab2* and *pinoid* (*pid*), which

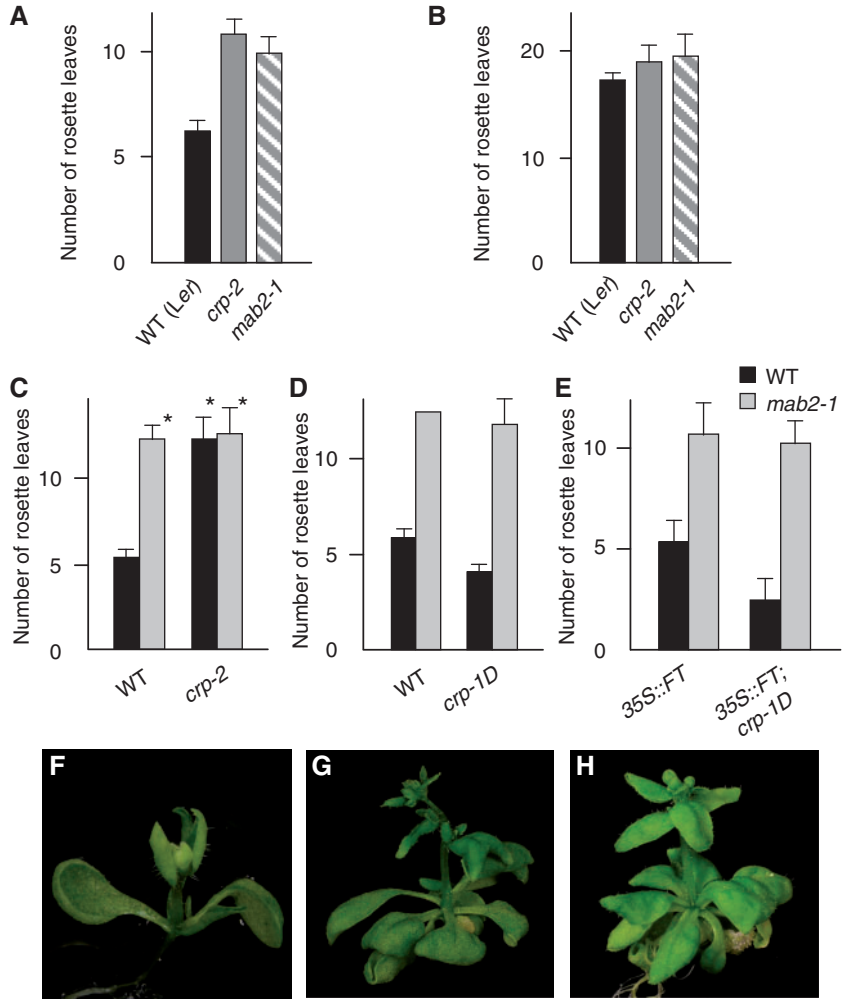

Fig. 5 Effect of the *mab2* loss-of-function mutation on the flowering time of *crp* mutants. (A and B) Flowering time of *crp-2* and *mab2-1* mutants under long-day (A) and short-day (B) conditions. (C) Flowering time of the *crp-2* mutant with a wild-type $MAB2^{+}$ or *mab2* loss-of-function allele. There is no statistically significant difference (Student's *t*-test, $P > 0.5$) among genotypes marked with an asterisk. (D) Flowering time of the *crp-1D* mutant with a wild-type $MAB2^{+}$ or *mab2* loss-of-function allele. (E) Flowering time of *35S::FT* #1-5L/—; *crp-1D* L/+ plants with a wild-type $MAB2^{+}$ or *mab2* loss-of-function allele. (F–H) Effect of *mab2-1* on the flowering phenotype of *crp-1D* plants. *35S::FT* #1-5L/—; *crp-1D* L/+ (15 d old) (F), *35S::FT*/—; *crp-1D*/+; *mab2-1* (20 d old) (G) and *crp-1D* L; *mab2-1* (30 d old) (H). Plants were grown under long-day conditions. In A, C and E, the numbers of rosette leaves are the average of at least 10, six and five plants, respectively. B and D include genotypes with two or three plants. Error bars in A–E indicate the SD, except for *mab2-1* ($n = 2$) in D (left gray bar). Additional data and statistics of the data are summarized in **Supplementary Tables S2, S9** and **S10**.

abolishes directional flow of auxin in developing embryo (Robert and Offringa 2008), caused severe defects in organogenesis such that cotyledons and bilateral symmetry are completely missing (Ito et al. 2011). These observations prompted us to generate *pid; crp* double mutants. Similar to the *pid-2; mab2-1* double mutant, seedlings of *pid-2; crp-2*, *pid-3; crp-3* and *pid-3; crp-4* double mutants completely lacked cotyledons (**Fig. 7**). These results suggest that *MED12/CRP* has a similar role in auxin-dependent organogenesis to that of *MED13/MAB2*, supporting the notion that these two act closely together possibly within the same module of the Mediator complex.

Discussion

In this study, we identified a dominant mutation, *crp-1D*, as a genetic modifier of the precocious-flowering phenotype of *35S::FT*. Map-based cloning revealed that *crp-1D* is a missense mutation of the Arabidopsis gene for the MED12 subunit of the Mediator. Genetic and molecular analysis of both dominant *crp-1D* and recessive *crp* loss-of-function alleles showed that MED12/CRP is a novel flowering regulator which suppresses *FLC* expression, promotes *FT* and *TSF* expression and up-regulates *SOC1* and *FUL* mainly in an *FT*-dependent manner under long-day conditions (**Supplementary Fig. S7**).

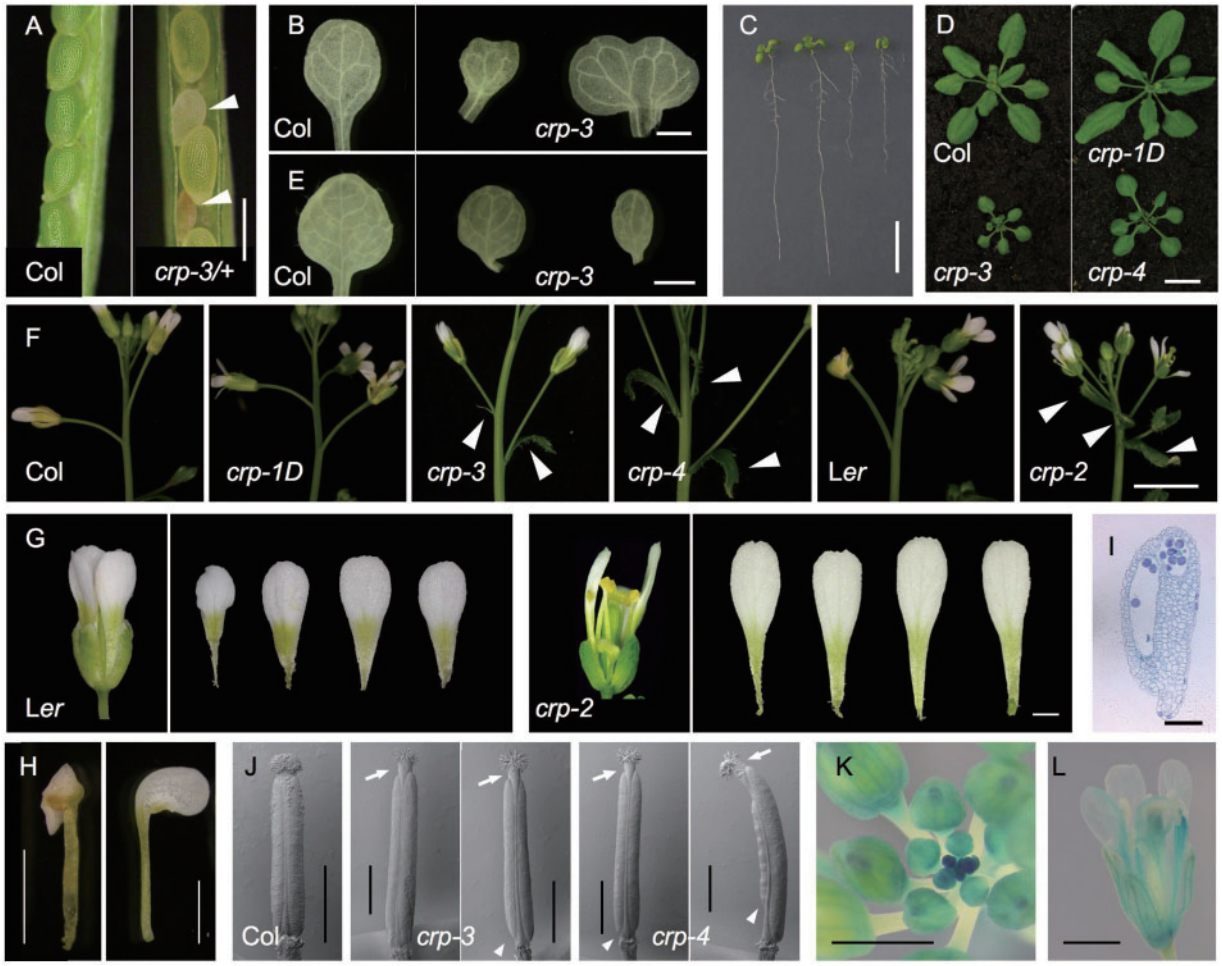

Fig. 6 Pleiotropic phenotype of *crp* mutants. (A) Opened siliques of wild-type Col (left) and *crp-3*/+ (right) plants. Arrowheads indicate aborted seeds. (B) Cotyledons from 10-day-old wild-type Col (left) and *crp-3* (right) seedlings. (C) Eight-day-old seedlings of wild-type Col, *crp-1D*, *crp-3* and *crp-4* (from left to right). (D) Twenty-two-day-old wild-type Col (top left), *crp-1D* (top right), *crp-3* (bottom left) and *crp-4* (bottom right) plants. (E) First leaves from 10-day-old wild-type (left) and two *crp-3* (right) seedlings. (F) First and second flowers with bract-like organs on the primary inflorescence shoot of wild-type Col, *crp-1D*, *crp-3*, *crp-4*, wild-type L*er* and *crp-2* (from left to right). Arrowheads indicate bract-like organs. (G) Flower and petals of wild-type L*er* (left) and *crp-2* (right). (H) Partial stamen-to-petal transformation observed in *crp-3*. (I) Longitudinal section through the anther of *crp-2*. (J) Scanning electron micrographs of pistils from wild-type Col (left), two *crp-3* (middle) and two *crp-4* (right) flowers. Arrows and arrowheads indicate elongated styles and basal internodes, respectively, of *crp* mutants. (K and L) Expression of *CRP::GUS* in inflorescence (K) and flower (L). Scale bars: 0.5 mm in A, 1 mm in B, E, G, H, J, K and L, 1 cm in C and D, 5 mm in F, 0.1 mm in I.

Although protein interaction has not yet been successfully demonstrated for MED12/CRP and MED13/MAB2, our present work and previous work (Gillmor et al. 2010, Ito et al. 2011) strongly suggest that these two proteins act closely together in the same pathways to regulate various aspects of plant development from embryogenesis to floral morphogenesis, some of which are mediated through modulation of the auxin response (Ito et al. 2011). Among them, floral transition represents a developmental process largely independent of auxin signaling and a unique common target process shared by MED12/CRP and MED13/MAB2 and core subunits of the Mediator, such as MED8 and MED25/PFT1.

CRP encodes the MED12 subunit of the Mediator

We have shown that the *CRP* gene encodes a protein of 2,235 amino acids, which corresponds to the Arabidopsis counterpart of the MED12 subunit of the Mediator (**Supplementary Fig. S2**). The gene for MED12 was recently identified independently as the *CCT* gene required for proper embryogenesis (Gillmor et al. 2010). By cDNA analysis, we found that the actual coding region of *MED12/CCT/CRP* differs from the publicly available annotated unit (**Fig. 1G**). *MED12/CCT/CRP* is ubiquitously expressed in seedlings and young plants, with higher levels of expression in vascular tissues and the shoot apex.

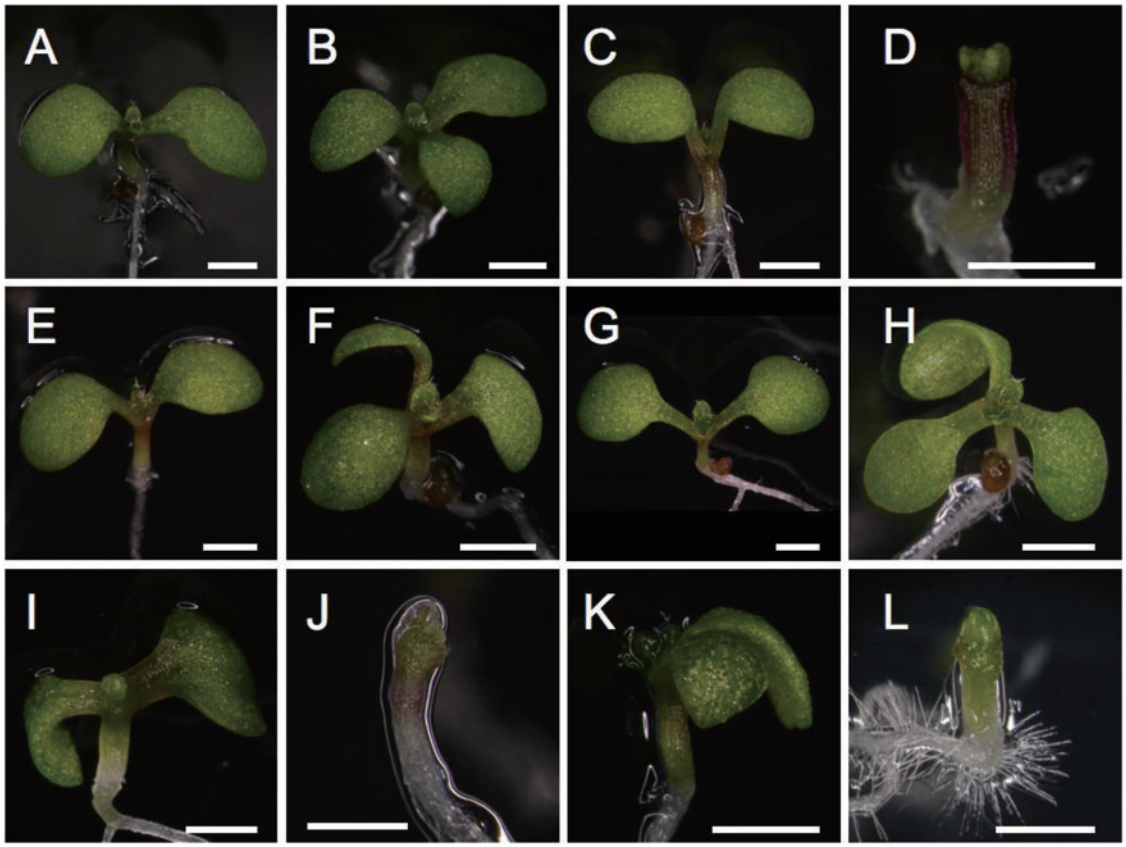

Fig. 7 Phenotype of *crp; pid* seedlings. Five-day-old seedlings of wild-type L*er* (A), *pid-2* (B), *crp-2* (C), *pid-2; crp-2* (D), wild-type Col (E), *pid-3* (F), *crp-1D* (G), *pid-3; crp-1D* (H), *crp-3* (I), *pid-3; crp-3* (J), *crp-4* (K) and *pid-3; crp-4* (L). Plants were grown under long-day conditions. Scale bars: 1 mm.

A dominant *crp-1D* mutation caused an S-to-F amino acid substitution at position 99. This serine residue is not located in either the conserved MED12 domain or signature sequence motifs (SSMs) of MED12 defined by Bourbon (2008), and is not conserved even among plant MED12 proteins (**Supplementary Fig. S3**). This makes interpretation of the *crp-1D* mutation difficult. However, that *crp-1D* is likely to act in the same molecular context as that involving wild-type MED12/CRP is suggested by its requirement for MED13/MAB2 to exert its phenotypic effect and attenuation of the *crp-1D* phenotype by an increased level of wild-type *CRP* (**Figs. 3**, **5**). One interesting feature of *crp-1D* is its limited effect on development (mainly restricted to the floral transition) as opposed to pleiotropic effects throughout the entire life cycle observed in *cct* and *crp* loss-of-function mutants **(Figs. 5–7**) (Gillmor et al. 2010). Even during flowering, *crp-1D* affects only a subset of genes whose expression is affected by *crp* loss-of-function mutations (**Figs. 2**, **4**). These findings are reminiscent of a recently reported dominant allele of *CURLY LEAF* (*CLF*), the Arabidopsis ortholog of *Enhancer of Zeste* (Doyle and Amasino 2009). *clf* loss-of-function mutations cause a severe pleiotropic phenotype and ectopic expression of various genes including *AGAMOUS* (*AG*). In contrast, the effect of a dominant *clf-59* mutation (P704S) is limited to flowering, and ectopic *AG* expression was not observed. *crp-1D* and *clf-59* may be useful tools to understand further the biochemical function of the respective proteins.

MED12/CRP is a novel flowering regulator with multiple target steps

Genetic and molecular analysis of both dominant *crp-1D* and loss-of-function *crp* alleles suggested that MED12/CRP acts as a flowering regulator with multiple target steps. That the Mediator plays multiple roles in the regulation of flowering is an emerging scheme, as clearly demonstrated by recent studies of MED25/PFT1 (Kidd et al. 2009, Elfving et al. 2011, Iñigo et al. 2011). Our present work with MED12/CRP gives further supports to this idea (**Supplementary Fig. S7**).

First, MED12/CRP is a negative regulator of *FLC* expression (T-bar #1 in **Supplementary Fig. S7**). An increased *FLC* mRNA level in *crp* loss-of-function mutants (**Fig. 2C**) is in part responsible for the late-flowering phenotype (**Fig. 2A**). It also, at least in part, explains reduced expression levels of downstream target genes of FLC, namely *FT* and *TSF* in the vasculature, and *FD* and *SOC1* in the shoot apex (Searle et al. 2006) (**Fig. 2C, D**). Genetic analysis suggests a similar role for MED13/MAB2 as well (**Fig. 5**). It is interesting to note that genome-wide association analysis of recombinant inbred lines revealed a promising association at the *CRP* locus with the *FLC* mRNA level,

although its close linkage (<100 kb) with *FRIGIDA*, whose allelic variation is a major determinant of the *FLC* expression level among natural accessions, made it difficult to be conclusive (Atwell et al. 2010). Involvement of the Mediator in regulation of *FLC* expression has recently been shown for several core subunits, such as MED8 and MED25/PFT1 (Kidd et al. 2009, Elfving et al. 2011). Similar to *crp* loss-of-function mutants, *med8* and *med25/pft1* mutants have increased *FLC* and reduced *FT* expression levels and showed the late-flowering phenotype in long-day conditions. Taken together, these and our results suggest that the Mediator plays an important role in suppression of *FLC*. In zebrafish and humans, it was shown that the SOX9 transcription factor physically or functionally interacts with MED12 and MED25 (Zhou et al. 2002, Rau et al. 2006, Nakamura et al. 2011). Therefore, it is likely that MED12/CRP, and possibly MED13/MAB2 as well, act with MED25/PFT1 in the same Mediator complex in regulation of *FLC*.

Secondly, MED12/CRP also positively regulates *FT* and *TSF* independently of *FLC* (arrow #2 in **Supplementary Fig. S7**). Reduced expression levels of *FT* and *TSF* were observed in *crp* loss-of-function mutants. The increased *FLC* level mentioned above is not the sole cause, since only partial recovery of expression of these two genes was observed in *crp; flc* double mutants (**Fig. 2F**). There should be another pathway independent of *FLC*. This is not through the transcriptional regulation of *CO*, because neither dominant *crp-1D* nor loss-of-function *crp* mutants showed a significant change in *CO* expression (**Figs. 2C**, **4B**). MED12/CRP, and possibly also MED13/MAB2, may act either downstream of *CO* expression or independently of the CO function. Interestingly, it was shown that MED25/PFT1 regulates *FT* (but not *TSF*) in both CO-dependent (through up-regulation of *CO* expression) and CO-independent pathways (Iñigo et al. 2011). MED12/CRP may participate in the latter pathway. Alternatively, since MED12/CRP regulates both *FT* and *TSF*, it may represent a unique pathway.

Thirdly, MED12/CRP acts downstream of *FT* in regulation of *SOC1* and *FUL* (arrow #3 in **Supplementary Fig. S7**). *SOC1* and *FUL* expression in the shoot apical region was severely reduced in *crp-3* and *crp-4* mutants and increased in the *crp-1D* mutant (**Figs. 2D**, **4D**). Reduction of the *SOC1* level in *crp* loss-of-function mutants is, again, only partly explained by increased *FLC*, which is a negative regulator of *SOC1* (**Fig. 2F**). In contrast, increased *SOC1* and *FUL* levels in *crp-1D* clearly required functional *FT* (**Fig. 4F**), suggesting that MED12/CRP acts with or downstream of FT. That MED12/CRP acts in part downstream of *FT* is further supported by partial suppression of the precocious-flowering phenotype of *FT*-overexpressing (*SULTR2;1::FT*) plants by *crp* loss-of-function mutations (**Fig. 2E**). It has been shown that *FT* is an upstream activator of *SOC1* (Yamaguchi et al. 2005, Yoo et al. 2005, Searle et al. 2006) and *FUL* (Abe et al. 2005, Teper-Bamnolker and Samach 2005, Wigge et al. 2005) expression. MED12/CRP, and possibly also MED13/MAB2, may act with FT and transcription factors such as FD in transcriptional regulation of *FUL* and *SOC1*. Again, it is interesting to note that it was recently suggested that MED25/PFT1 is also involved in regulation of *SOC1* expression (Iñigo et al. 2011). MED12/CRP, and possibly also MED13/MAB2, and MED25/PFT1 may act in the same pathway for *SOC1* activation.

Recent studies in animals showed that the CDK8 module (comprising CDK8, CycC, MED12 and MED13) is stable and biochemically active apart from the Mediator (Knuesel et al. 2009). CDK8 phosphorylates histone H3S10, and the kinase activity of the CDK8 module is dependent on MED12 in humans (Knuesel et al. 2009). MED12 can repress transcription through interaction with histone H3K9 methyltransferase in HeLa cells (Ding et al. 2008). MED12 acts as a chromatin regulator and a 'hub' protein that modifies expression of a large number of genes from multiple signaling pathways in *C. elegans* (Moghal and Sternberg 2003, Lehner et al. 2006). These observations may support the possibility that MED12/CCT/CRP could be involved in the epigenetic control of genes such as *FLC* or *FT* in the context of the CDK8 module outside of the Mediator. However, our observations discussed above are slightly in favor of the alternative view that it functions in the context of the Mediator with core subunits.

MED12/CCT/CRP and MED13/GCT/MAB2 in plant development

In addition to the control of flowering, MED12/CRP and MED13/MAB2 are involved in various developmental processes. It was shown that MED12/CCT and MED13/GCT are absolutely required for *KANADI* expression during early embryogenesis and are important for the proper temporal regulation of genes involved in radial pattern formation during embryogenesis (Gillmor et al. 2010). This is likely to be achieved in part through modulation of the auxin response, as clearly demonstrated for MED13/MAB2 (Ito et al. 2010). That MED12/CRP closely shares the role in the auxin response with MED13/MAB2 was supported by a similar genetic interaction of *crp* and *mab2* mutations with *pid* (**Fig. 7**). The fact that the gynoecium phenotype of the *crp* loss-of-function mutants is reminiscent of that of auxin-related mutants such as *ett* (**Fig. 6J**) lends additional support for the role of MED12/CRP in the auxin response. Taken together, MED12/CCT/CRP and MED13/GCT/MAB2 function closely together to regulate the auxin response. Dwarfism (**Fig. 6D**), inflorescence defects including ectopic bract formation (**Fig. 6F**), and various floral defects (**Fig. 6G–I**) observed in *crp* loss-of-function mutants, some of which are shared by *mab2* (Ito et al. 2010), imply that MED12/CCT/CRP, and possibly also MED13/GCT/MAB2, are involved in other aspects of development. Some are probably through modulation of the auxin response. In contrast to the role in regulation of flowering, which is shared by core subunits of the Mediator, such as MED8 and MED25/PFT1, the roles of MED12/CCT/CRP and MED13/GCT/MAB2 in other aspects of development, such as embryogenesis, seem to be unique to these two subunits of the Mediator.

MED12 and MED13 belong to the CDK8 module of Mediator which contains CDK8 and CycC subunits. There has been no report of a mutant phenotype and functional analysis of *CycC* orthologs in plants (Wang et al. 2004). In Arabidopsis, *HUA ENHANCER3* (*HEN3*) encodes the CDK8 subunit, and is expressed in proliferating tissues, such as the SAM, young leaves and floral buds (Wang and Chen 2004, Wang et al. 2004). However, *hen3* mutants do not show pleiotropic developmental defects such as those observed in *cct/crp* and *gct/mab2* mutants. HEN3 protein interacts with LEUNIG (LUG) and SEUSS (SEU), which repress *AG* and are involved in auxin signaling during floral organ development (Navarro et al. 2004, Pfluger and Zambryski 2004, Gonzalez et al. 2007). Interestingly, *crp* and *gct/mab2* mutants showed floral defects reminiscent of *lug* and *seu* mutants (Liu and Meyerowitz 1995, Franks et al. 2002, Gillmor et al. 2010) (**Fig. 6G–J**). It was reported that *gct; kan* double and *hen3; hua1; hua2* triple mutants showed enhanced loss of floral organ identity, although *gct/mab2* or *hen3* single mutants did not show major defects in floral organ specification (Wang and Chen 2004, Gillmor et al. 2010). These observations suggest that floral organ development may represent a shared developmental program regulated by the CDK8 module proteins of the Mediator possibly through modulation of the auxin response.

Does Mediator contribute to feed-forward loops in flowering regulation?

Flowering of monocarpic plants such as *A. thaliana* is an irreversible phase transition. It has been suggested that feed-forward loops constructed from key regulators are important to minimize external noises to achieve robustness of developmental systems (Mangan and Alon 2003, Dekel et al. 2005, Pastore et al. 2011). As summarized in a simplified model (**Supplementary Fig. S7**), MED12/CRP, and possibly also MED13/MAB2, are involved in multiple steps in the regulation of production of florigen (FT and TSF proteins) and in multiple downstream steps of florigen action. Similarly, it has been shown that a core subunit of the Mediator, MED25/PFT1, regulates multiple steps both upstream and downstream of FT florigen. These observations and the possible involvement of genetic variation at the *CRP* locus in natural variation of the *FLC* expression level among various accessions (Atwell et al. 2010) prompt us to propose a hypothesis that the Mediator may make an important contribution to flowering as a key factor to construct a cohesive feed-forward regulatory motif. Further studies to examine the role of other core subunits of the Mediator in flowering and identification of transcription factors involved will be important steps to test this hypothesis.

Materials and Methods

Plant materials and growth conditions

Col-0 and L*er* were used as wild types. T-DNA insertion alleles of *CRP* in the Col background (SALK_108241, *crp-3*; and SALK_124276; *crp-4*) were obtained from the Arabidopsis Biological Resource Center (ABRC). *crp-3* and *crp-4* were backcrossed with wild-type Col-0 three times prior to analysis. *crp-3* and *crp-4* correspond to *cct-2* and *cct-3*, respectively (Gillmor et al. 2010). A Ds-transposon insertion allele in the L*er* background (SET_3.701; *crp-2*) was kindly provided by Dr. V. Sundaresan (Parinov et al. 1999), and was backcrossed with wild-type L*er* three times prior to analysis. *crp-1D* was isolated as an enhancer mutation of the *35S::FT* #1-5C in the Col background (Kobayashi et al. 1999). Mutagenesis was carried out by soaking seeds in 0.1% EMS for 16 h. The resulting M_1 population (5,000 seeds) was sown and self-fertilized, and the M_2 population was screened for enhancers of the early-flowering phenotype in constant light conditions (24°C). *crp-1D* L was obtained by five backcrosses with wild-type L*er*. *CRP::GUS* and *35S::CRP* (see below for the transgene construction) were generated in this work. The *ft-2* allele from the L*er* accession was introgressed into Col by five backcrosses to generate *ft-2* (Col). Previously published plant materials used in this work are as follows: *35S::FT* #11-1 (a strong line), *35S::FT* #1-5C (a weak line), *SULTR2;1::FT* #1-a, *35S::TSF* #4-1 (a weak line), *35S::LFY* (DW151.2.5C), *35S::AP1*, *soc1-101D*, *flc-3*, *tsf-1*, *fd-1* (Col). *soc1-2*, *ful-2*, *lfy-1*, *pid-3* are in the Col background; *mab2-1* and *pid-2* are in the L*er* background. Further information is given in **Supplementary Table S11**.

For analysis of the flowering time phenotype, plants were grown on soil or half-strength Murashige and Skoog (MS; Wako) medium supplemented with 0.5% sucrose and 0.4% Gellan Gum (Wako) at 22°C under long-day (16 h light/8 h dark) conditions with white fluorescent light (~80 μmol m^{-2} s^{-1}) or short-day (8 h light/16 h dark) conditions with white fluorescent light (~100 μmol m^{-2} s^{-1}). Flowering time was measured by counting rosette and cauline leaves. Bars in the figures show the number of rosette leaves. For expression analysis, plants were grown on half-strength MS medium supplemented with 0.5% sucrose and 0.4% Gellan Gum. Seeds were stratified by keeping them at 4°C for 2–4 d and then transferred to 22°C long-day conditions, which was defined as day 0. Plants were harvested at Zeitgeber time (ZT) 15 on each day.

RT–PCR analysis

RNA was extracted using TRIzol reagent (Invitrogen) and was treated with RNase-free DNase I (Invitrogen) according to the manufacturer's instructions. Total RNA (0.5 μg) was reverse-transcribed in a 20 μl reaction mixture using SuperScript III (Invitrogen). After the reaction, 10 μl of the mixture was diluted with 240 μl of water, and 5 μl aliquots were analyzed. Quantitative RT–PCRs (qRT–PCRs) were performed using SYBR Premix Ex Taq II (TAKARA). Primers used in this study are listed in **Supplementary Table S12**. qRT–PCR results normalized to *ACT2*, *NRT1.7* or *STM* show the average of nine different reactions (biological×technical triplicate), except for the data shown in **Fig. 3A** (only technical triplicate).

Relative expression was obtained as the ratio to the level in wild-type Col harvested on the first day of the experiment. Semi-quantitative RT–PCR was performed using the primers listed in **Supplementary Table S13**. PCR products were electrophoresed on an agarose gel and visualized by ethidium bromide staining.

Plasmid construction and transgenic plants

To construct *CRP::GUS*, the *GUS* coding sequence from pBI101 was inserted downstream of the 2.0 kb *CRP* promoter fragment (2.0 kb sequence upstream of the presumptive initiation ATG codon) amplified by PCR from Col using the primers listed in **Supplementary Table S14**. To clone *CRP* and *crp-1D* cDNA, the coding region of *CRP* from Col and *crp-1D*, respectively, was amplified by PCR using the primers listed in **Supplementary Table S14** and recombined into the *Sal*I and *Xho*I sites of the pENTR1A vector (Invitrogen). To construct *CRP-GFP* and *crp-1D-GFP*, the coding region of sGFP was fused, in-frame, to the C-terminus of CRP or crp-1D in the pENTR vector as described above and, after sequencing, this translational fusion construct was cloned into the binary vector pGWB2 (Nakagawa et al. 2007) using LR Recombination Reactions (Invitrogen). To construct *35S::CRP*, the *CRP* coding sequence in the pENTR1A vector was transferred to the binary vector pGWB2 (Nakagawa et al. 2007) using LR Recombination Reactions (Invitrogen). To generate stable transgenic plants, the constructs were introduced into *Agrobacterium tumefaciens* strain pMP90, and Col plants were transformed by the floral dip procedure (Clough and Bent 1998).

GUS staining, histological analysis and microscopy

CRP::GUS line #13.4 was chosen for analysis. Samples were collected at ZT15 from plants grown in long-day conditions. For GUS staining, tissues were incubated at 4°C for 15 min in 90% acetone, rinsed with phosphate-buffered saline (PBS), infiltrated with staining solution (0.5 mg ml^{-1} X-Gluc, 100 mM sodium phosphate buffer, pH 7.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1% Triton X-100) under vacuum for 5 min and incubated at 37°C for about 12–48 h in the dark. After staining, for whole-mount observation, samples were cleared as described previously (Aida et al. 1997). For sectioning, samples were dehydrated through an ethanol series, embedded in Technovit 7100 (Heraeus Kulzer) and sectioned at a thickness of 4 µm with a microtome.

For visualization of seedling vasculature, plants were fixed and rehydrated as described previously (Aida et al. 1997). Gynoecium morphology was observed using a scanning electron microscope (TM3000 Miniscope, Hitachi).

Particle bombardment and GFP fluorescence detection

35S::CRP-GFP and *35S::crp-1D-GFP* were used as bioluminescent reporter constructs. These plasmid vectors were introduced into onion cells using the particle bombardment PDS-1000/He Biolistic Particle Delivery System (Bio-Rad). A 1 µg aliquot of plasmids was mixed with 8 µl of a pre-washed 1 µm diameter gold particle suspension (60 mg ml^{-1}), 3.3 µl of $CaCl_2$ (2.5 M) and 3.3 µl of spermidine (0.1 M). After incubation, the particles were washed with 80% ethanol and resuspended in 10 µl of 100% ethanol. The DNA-coated particles were fired into the onion cells using a 1,100 p.s.i. rupture disk. Onion cells were incubated in the dark at 23°C for 24–40 h after particle delivery and the epidermal cells were observed with a confocal laser scanning microscope (FV1000, Olympus), excitation wavelength 488 nm by argon laser, emission wavelength 500–600 nm.

Supplementary data

Supplementary data are available at PCP online.

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