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RESEARCH REPORT

Cytokinin response factors integrate auxin and cytokinin pathways for female reproductive organ development

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ABSTRACT

The developmental programme of the pistil is under the control of both auxin and cytokinin, and crosstalk between these factors converges on regulation of the auxin carrier PIN-FORMED 1 (PIN1). Here, we show that in the triple transcription factor mutant *cytokinin response factor 2* (*crf2*) *crf3 crf6* both pistil length and ovule number were reduced. *PIN1* expression was also lower in the triple mutant and the phenotypes could not be rescued by exogenous cytokinin application. *pin1* complementation studies using genomic *PIN1* constructs showed that the pistil phenotypes were only rescued when the PCRE1 domain, to which CRFs bind, was present. Without this domain, *pin* mutants resemble the *crf2 crf3 crf6* triple mutant, indicating the pivotal role of CRFs in auxin-cytokinin crosstalk.

KEY WORDS: Plant hormones, Pistil, Ovule primordia, CRFs, PIN1

INTRODUCTION

In *Arabidopsis*, ovules emerge as lateral organs from the placenta, a meristematic tissue that originates after the fusion of the carpel margin meristem (CMM) (Reyes-Olalde et al., 2013; Schneitz et al., 1995). Placenta formation and ovule growth require auxins. Reduced local auxin biosynthesis or transport causes severe defects in pistil development with a consequent loss of placental tissue and ovules (Nemhauser et al., 2000; Nole-Wilson et al., 2010). The auxin efflux carrier PIN-FORMED 1 (PIN1) is one of the main elements modulating auxin accumulation during all phases of ovule development (Benková et al., 2003; Ceccato et al., 2013). Although *pin1-201* does not develop any flowers, in the *pin1-5* mutant the gynoecium has shorter valves and contains a few ovules (Bencivenga et al., 2012; Sohlberg et al., 2006).

Cytokinins (CKs) positively regulate ovule formation and pistil development. Indeed, mutants that have a reduced capacity for CK production or perception exhibit a dramatic reduction in ovule number and pistil size, and compromised female fertility (Kinoshita-Tsujimura and Kakimoto, 2011; Riefler et al., 2006; Werner et al., 2003). By contrast, increased CK levels results in a bigger pistil with a greater number of ovules compared with wild type, confirming a positive correlation between CK levels and ovule numbers (Bartrina et al., 2011; Bencivenga et al., 2012; Galbiati et al., 2013). It has been shown that CK treatment positively influences the number of ovules per pistil via a strong increase in *PIN1* expression (Bencivenga et al., 2012; Galbiati et al., 2013; Zúñiga-Mayo et al., 2014).

Conversely, in roots, CKs modulate organogenesis by downregulating *PIN1* expression (Dello Ioio et al., 2012; Ruzicka et al., 2009) and PIN1 protein endocytic recycling (Marhavý et al., 2011).

Cytokinin response factors (CRFs) are encoded by closely related members of the *Arabidopsis* AP2 gene family and mediate a large proportion of the CK transcriptional response that functionally overlaps with the B-type ARR-mediated response (Rashotte et al., 2006). Recently, Šimášková and colleagues (2015) found that CRFs bind directly to the PIN1 cytokinin response element (PCRE1) in the *PIN1* promoter and thus modulate *PIN1* expression in response to CKs. Deletion of the PCRE1 *cis*-regulatory element uncouples *PIN1* transcription from CRF regulation and affects root sensitivity to CKs (Šimášková et al., 2015). Here, we show that CRF2, CRF3 and CRF6 redundantly induce the expression of *PIN1*, which is required for ovule development, supporting the crucial and general role of CRF factors as mediators of auxin-CK crosstalk guiding plant organogenesis.

RESULTS AND DISCUSSION

CRFs are required for placenta elongation and ovule development

In the placenta, CKs promote *PIN1* expression , which is needed for the establishment of the auxin gradient that leads to ovule primordia development (Bencivenga et al., 2012; Benková et al., 2003; Ceccato et al., 2013; Galbiati et al., 2013). Recently, it has been shown that three members of the CRF family, CRF2, CRF3 and CRF6, directly regulate *PIN1* expression upon CK signalling in roots (Šimášková et al., 2015). *CRF2* and *CRF6* promoters were able to drive reporter gene expression in stage 9 and 10 of pistil development, whereas the *CRF3* promoter did not show any activity (Fig. S1). These results are consistent with recently published transcriptomic data of the gynoecial medial domain, which show high expression of *CRF2* and *CRF6* and low expression of *CRF3* (Villarino et al., 2016).

To investigate whether these three CRFs control *PIN1* expression during early stages of pistil development, we have analysed *crf2*, *crf3* and *crf6* single, double and triple mutants. Ovule counts were performed on ovules from stage 1-II (primordia) to stage 2-I (finger-like), which corresponds to stages 9 and 10 of pistil development [according to Schneitz et al. (1995) and Roeder and Yanofsky (2006)]. Analysis of single *crf3* and *crf6* mutants, as well as the *crf3 crf6* double mutant, did not reveal any significant difference in ovule number compared with wild type, whereas the single *crf2* and the double *crf2 crf3* mutant showed a small but significant decrease in ovule number (Fig. S2). Instead, the *crf2 crf6* double mutant presented ovule numbers comparable to wild type, suggesting a compensatory mechanism between *crf2* and *crf6*

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(Fig. S2). Finally, in the *crf2 crf3 crf6* (*crf2/3/6*) triple mutant, a reduction of 31.68% in ovule number was observed with respect to the wild type (Fig. 1A). Wild-type *Col-0* plants grown under long-day conditions developed on average (mean±s.e.m.) 46.36±1.24 ovules per pistil whereas 31.67±2.01 ovules were formed in the *crf2/3/6* triple mutant pistils (Fig. 1A).

Placenta length was measured at the same developmental stages. In the wild type, the average length of the placenta was found to be 351±12 µm at stage 9 and 517±12 µm at stage 10, whereas in the *crf2/3/6* mutant it was significantly shorter (269±20 µm at stage 9 and 436±19 µm at stage 10) (Fig. 1B). Ovule density (number of ovules per µm placenta) was also reduced in the *crf2/3/6* mutant (Fig. 1C).

CK treatment results in an increase in pistil size and ovule number (Bencivenga et al., 2012; Galbiati et al., 2013). Because CRFs regulate the transcriptional response to cytokinins (Rashotte et al., 2006), we tested the CK response in wild type and the *crf2/3/6* triple mutant. Wild-type plants treated with the synthetic cytokinin 6-benzylaminopurine (BAP) yielded 60% more ovules and a 58% longer placenta than untreated plants (Fig. 1D,E). The *crf2/3/6* triple mutant treated with BAP produced 19% more ovules and an increase in placenta length of 32% (Fig. 1D,E), indicating that the capacity to respond to CKs is strongly reduced in the absence of *CRF2*, *CRF3* and *CRF6* activities.

CRFs regulate *PIN1* transcription during pistil growth

To investigate whether the pistil phenotypes observed in the *crf2/3/6* mutant were due to changes in *PIN1* expression, we performed real-time qPCR experiments. In the *crf2/3/6* triple mutant, *PIN1* expression was significantly lower than in the wild type (Fig. 2A). As previously reported (Bencivenga et al., 2012; Galbiati et al., 2013), *PIN1* expression was at least twofold higher in BAP-treated wild-type inflorescences. Interestingly, the level of *PIN1* mRNA in the *crf2/3/6* mutant did not increase upon CK application, suggesting that CRFs are required for CK-dependent *PIN1* expression (Fig. 2A).

In roots, CRFs regulate *PIN1* expression by binding the PCRE1 sequence in the *PIN1* promoter (Šimášková et al., 2015); therefore, we analysed plants carrying a *ΔPIN1::PIN1-GFP* construct in which a *PIN1* promoter lacking the PCRE1 element drives the expression of a fully functional PIN1-GFP fusion protein. Real-time qPCR experiments were performed on *GFP* instead of *PIN1* in order to avoid the detection of endogenous *PIN1*. The level of *PIN1-GFP* transcripts in *ΔPIN1::PIN1-GFP* inflorescences was lower than that in plants carrying the same fusion protein construct under the control of a wild-type version of the *PIN1* promoter (Fig. 2B). The reduction in *PIN1-GFP* expression under control of the *ΔPIN1* promoter was also evident by confocal microscopy in placenta cells and ovule primordia at stages 1-I and 1-II (compare Fig. 2C,D with Fig. 2E,F). Although *PIN1* expression was dramatically reduced (Fig. 2B), PIN1-GFP protein in *ΔPIN1::PIN1-GFP* plants was correctly localized at the membrane of placenta cells (Fig. 2E,F).

To understand whether PCRE1 is the only element in the *PIN1* promoter required for CK-mediated *PIN1* expression in inflorescences, we also analysed *GFP* expression after treatment with CKs in *PIN1::PIN1-GFP* and *ΔPIN1::PIN1-GFP* plants. Interestingly, *GFP* expression increased in both *PIN1::PIN1-GFP* and *ΔPIN1::PIN1-GFP* inflorescences compared with the control (mock treatment) (Fig. 2B), suggesting that CRFs might bind to other regions of the *PIN1* promoter besides PCRE1. The possibility that other CK-induced transcription factors regulate *PIN1* expression is unlikely as *PIN1* expression remains unchanged in CK-treated *crf2/3/6* inflorescences (Fig. 2A). The same reduction of GFP expression in *ΔPIN1::PIN1-GFP* compared with *PIN1::PIN1-GFP* was observed in a second independent *ΔPIN1::PIN1-GFP* line (Fig. S3). Also, in this second line (*ΔPIN1::PIN1-GFP_2*) *GFP* expression increased after BAP treatment, reconfirming the results obtained with line 1 (Fig. S3). These results confirm that CRFs are

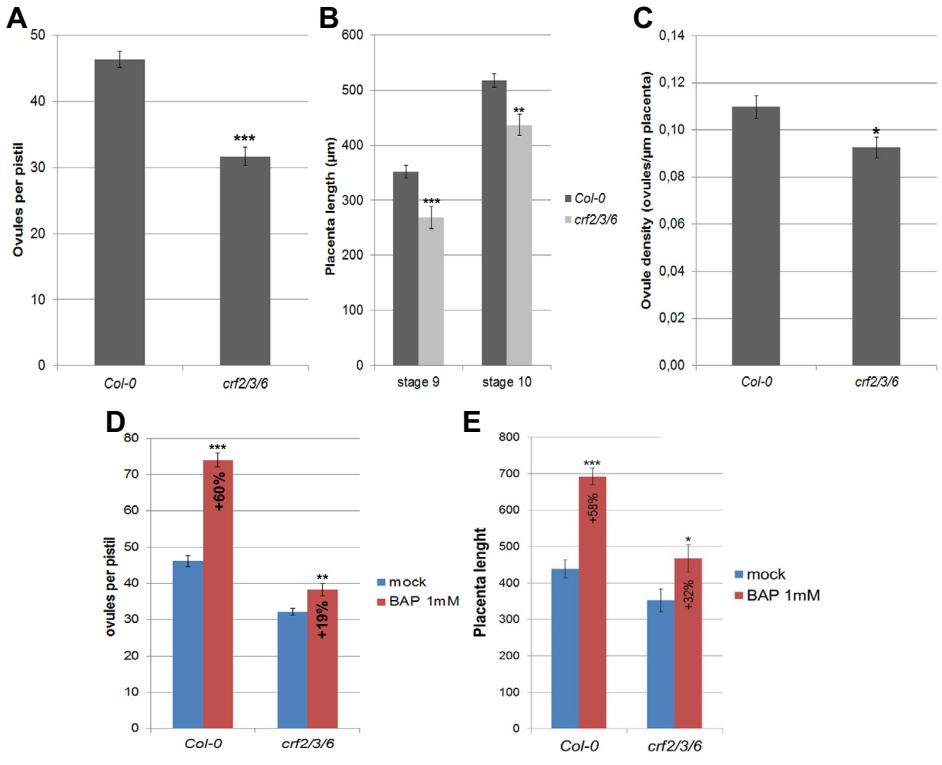

Fig. 1. CRFs influence pistil length and ovule numbers. (A-C) Number of ovules (A), placenta length (B) and ovule density (C) of wild-type (*Col-0*) and *crf2/3/6* pistils. (D,E) Ovule number (D) and placenta length measurements (E) in mock- and 1 mM BAP-treated wild-type and *crf2/3/6* inflorescences 48 h after treatment. Mean±s.e.m. is shown. **P*<0.05; ***P*<0.01; ****P*<0.001 (Student's *t*-test; *n*=20). Percentage increment is also reported in D,E.

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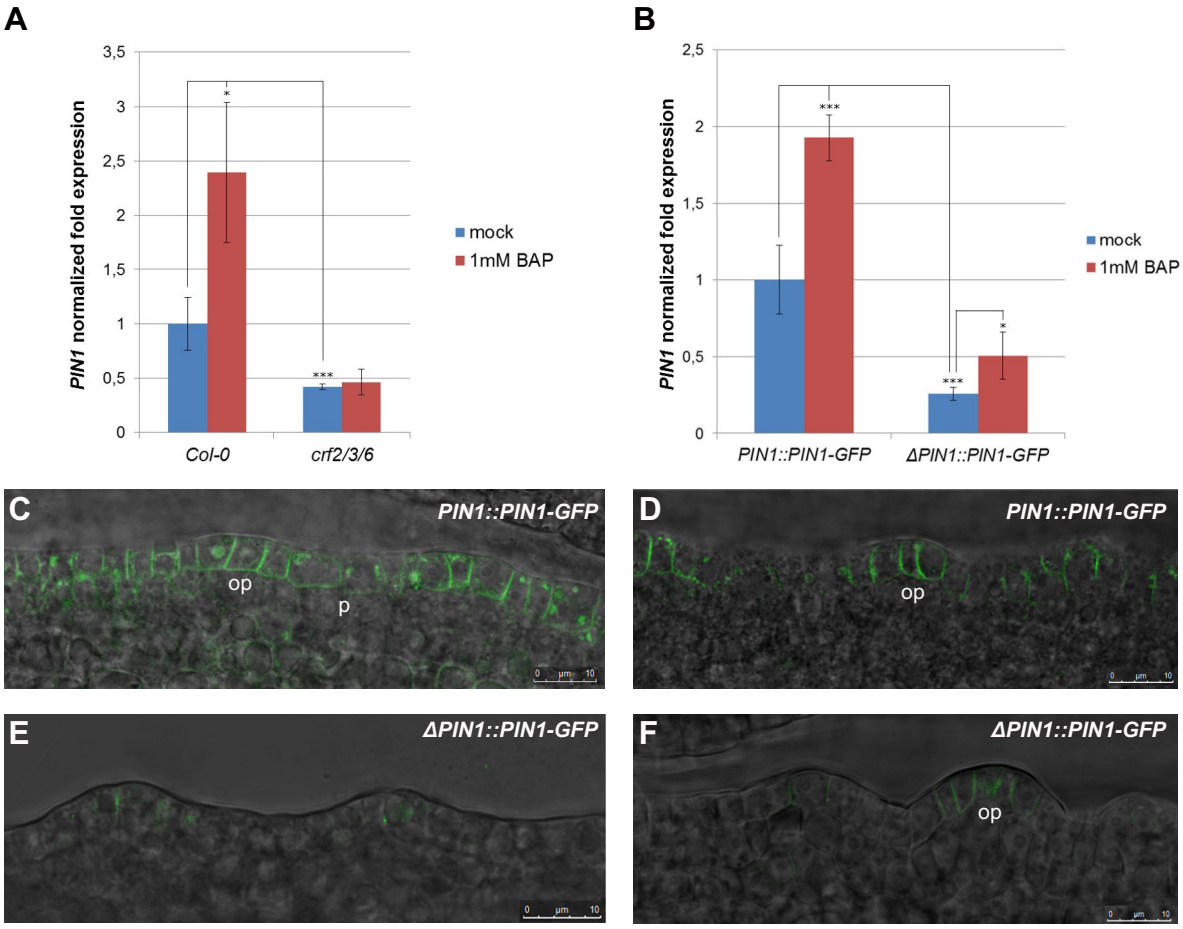

Fig. 2. CRFs regulate *PIN1* expression. (A) *PIN1* expression levels in mock- and BAP-treated pre-fertilization inflorescences of wild-type and *crf2/3/6* triple mutant. (B) *GFP* expression levels in mock- and BAP-treated *PIN1::PIN1-GFP* and *ΔPIN1::PIN1-GFP* pre-fertilization inflorescences. Error bars indicate the s.e.m. based on three biological replicates. **P*<0.05; ****P*<0.001 (Student's *t*-test; *n*=3). Data were normalized with respect to *ACT8-2* and *UBI10* mRNA levels. (C-F) Confocal microscope images of *PIN1::PIN1-GFP* (C,D) and *ΔPIN1::PIN1-GFP* (E,F) placenta cells and ovule primordia at stages 1-I (C,E) and 1-II (D,F). Scale bars: 10 µm. op, ovule primordia; p, placenta.

required to regulate the expression of *PIN1* in the pistil. The possibility of other CRF regulatory regions needs to be investigated as the lack of PCRE1 does not cause complete CK insensitivity. It is important to recall that in roots *PIN1::PIN1-GFP* expression is reduced by CKs and that *ΔPIN1::PIN1-GFP* is completely CK insensitive (Šimášková et al., 2015), indicating that there might be a specific regulation of *PIN1* expression depending on the developmental context.

PCRE1 is required for pistil development and ovule primordia formation

Introducing *ΔPIN1::PIN1-GFP* in a wild-type *A. thaliana* does not lead to any abnormalities in pistil and ovule development (Fig. 3A-C). To examine the functional significance of the CRF regulatory regions in the *PIN1* promoter (PCRE1), we introgressed *ΔPIN1::PIN1-GFP* into the *pin1-5* mutant. *pin1-5* is a hypomorphic mutant that has shorter pistils and develops an average of nine ovules per pistil (Bencivenga et al., 2012; Sohlberg et al., 2006). Confirmation of the presence of *ΔPIN1::PIN1-GFP* construct in the *pin1* mutant is shown in Fig. S4.

PIN1::PIN1-GFP completely rescued the *pin1-5* mutant phenotype whereas *ΔPIN1::PIN1-GFP* was unable to rescue the pistil growth phenotype of *pin1-5* (Fig. 3A-C). The placenta length of *pin1-5 ΔPIN1::PIN1-GFP* remained the same as in *pin1-5* (Fig. 3A,B). Placenta length in *pin1-5 ΔPIN1::PIN1-GFP* was similar to that of the *crf2/3/6* mutant (Fig. 3A,B). This suggests that PCRE1-mediated transcriptional regulation of *PIN1* is necessary for correct elongation of the pistil. Furthermore, ovule density in *pin1-5 ΔPIN1::PIN1-GFP* (0.0902±0.008 ovules/µm placenta) was similar to that of *crf2/3/6* (0.0926±0.004 ovules/µm placenta). By contrast, *ΔPIN1::PIN1-GFP* did rescue the ovule number phenotype of *pin1-5*, raising the ovule count of *pin1-5* from an average of 8.5±1.7 to 28.67±1.84 (Fig. 3C).

These results suggest that PCRE1-mediated control of *PIN1* expression is required for determining the correct size of the pistils, whereas it seems to be less relevant for ovule formation. However, it should be taken into account that transcription of *pin1-5* (which encodes a partially functional protein) could be induced by CKs. For this reason, we also analysed the phenotype of *ΔPIN1::PIN1-GFP* in *pin1-201* mutant. This mutant fails to develop any lateral organs due to a loss-of-function mutation (Fig. S5). Pistil length in *ΔPIN1::PIN1-GFP pin1-201* is similar to that in *ΔPIN1::PIN1-GFP pin1-5* and *crf2/3/6* (Fig. 2A,B). Regarding the ovule number, *ΔPIN1::PIN1-GFP pin1-201* showed a reduction in comparison with *ΔPIN1::PIN1-GFP pin1-5 and crf2/3/6* (Fig. 3C). The reduction in ovule number highlighted in *ΔPIN1::PIN1-GFP pin1-201* compared with *ΔPIN1::PIN1-GFP pin1-5* might be explained by residual function of the PIN1-5 mutant protein. The

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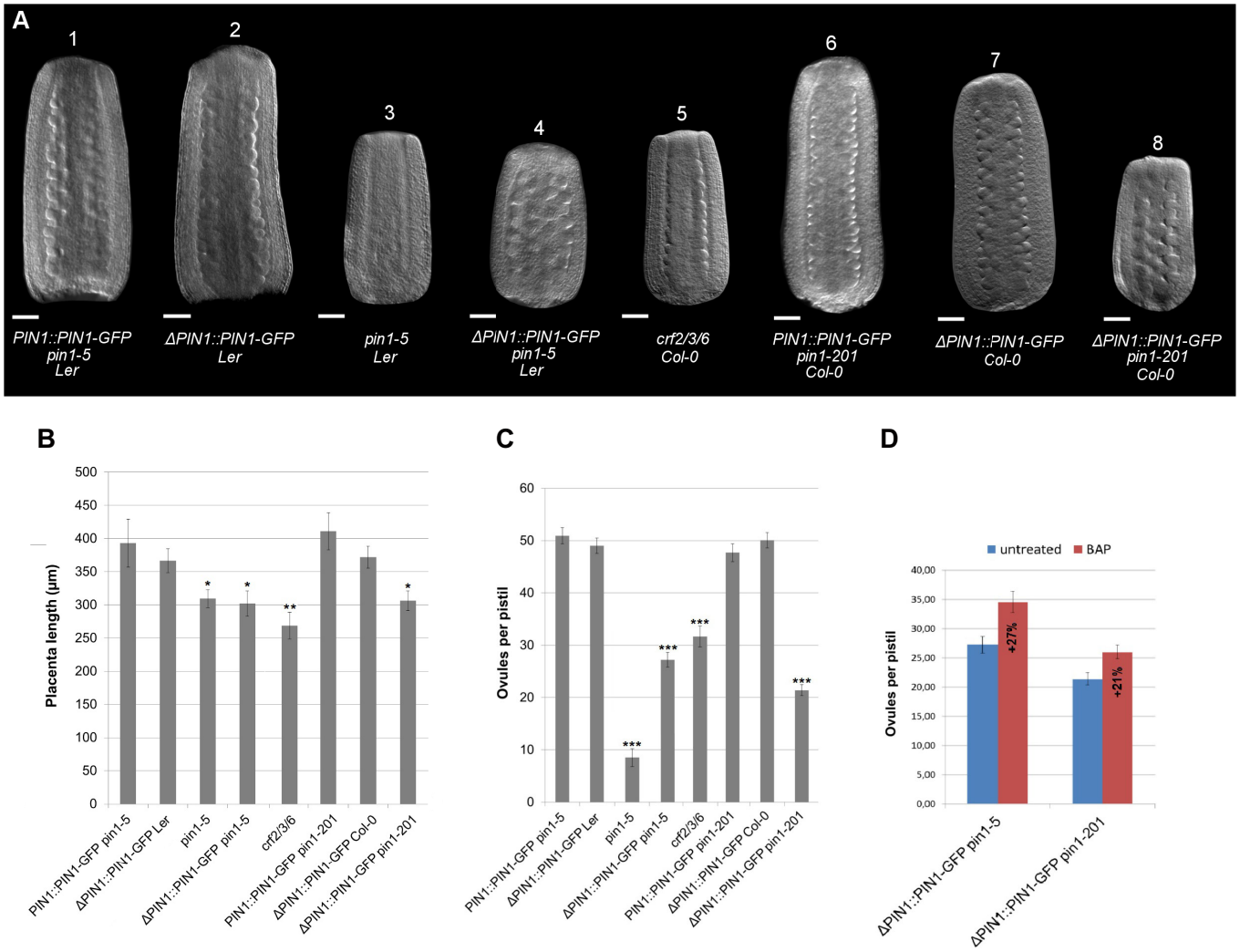

Fig. 3. CRF regulate *PIN1* expression required for pistil growth. (A) DIC images of pistils with visible lines of ovules in *Ler* and *Col-0* genetic backgrounds. Scale bars: 50 µm. (B,C) Placenta length (B) and ovule number (C) in xxxxxxxxxxxxx. (D) Ovule number measurements in untreated and 1 mM BAP-treated *ΔPIN1::PIN1-GFP pin1-5* and *ΔPIN1::PIN1-GFP pin1-201* inflorescences 48 h after treatment. Percentage increment is also reported in the graphs. Mean±s.e.m is shown. **P*<0.05; ***P*<0.01; ****P*<0.001 (Student's *t*-test; *n*=20).

analysis of *ΔPIN1::PIN1-GFP* in both *pin1-5* and *pin1-201* allelic backgrounds confirmed that pistil elongation is affected when *PIN1* expression is uncoupled from regulation of CRFs. Finally, we also tested the capacity of *ΔPIN1::PIN1-GFP pin1-5* and *ΔPIN1::PIN1-GFP pin1-201* to respond to CK by checking the number of ovules after BAP treatment. Interestingly, both lines are still able to respond to CK showing an increase in the number of ovules per pistil of 27% and 21%, respectively (Fig. 3D). This result is in agreement with the fact that *PIN1-GFP* expression level increases in *ΔPIN1::PIN1-GFP* after BAP treatment (Fig. 2B; Fig. S3). confirming the importance of CRF-mediated *PIN1* expression for pistil elongation.

The reduction in pistil size observed in *crf* mutants could be due to defective cell division or cell expansion processes or a combination of both. Auxin plays a prominent role in controlling cell expansion. For example, elongation of the primary root and the hypocotyl require specific auxin transport to determine their expansive growth rates (Rayle and Cleland, 1992; Spartz et al., 2012). Interestingly, a reduction in pistil and anther elongation has been reported for *tir1 afb1 afb2 afb3*, a quadruple mutant with compromised auxin signalling (Cecchetti et al., 2008). Our understanding of the influence of auxin on the cell cycle is still fragmentary, but primary evidence indicates that auxin acts on several targets involved in the control of cell cycle (Perrot-Rechenmann, 2010). On the other side, the ability of CKs to promote cell division, in particular through their action on D-type cyclins, was described several years ago (Dewitte et al., 2007; Riou-Khamlichi et al., 1999), and it has been recently been shown that the transcript levels of several cell cycle-related genes were decreased in roots of the *crf1,3,5,6* quadruple mutant (Raines et al., 2016).

In summary, we propose that *PIN1* expression mediated by CRFs is required for the determination of pistil size. The greater number of ovule primordia in CK-treated pistils correlates with the increased pistil size. Therefore, it is likely that when enough space occurs between two ovules, CRFs and/or other CKs-dependent factors induce *PIN1* expression to create a new auxin maximum.

MATERIALS AND METHODS

Plant materials and treatments

Arabidopsis wild-type and mutant plants were grown at 22°C under long-day conditions (16 h light, 8 h dark) in a greenhouse. *crf2-2* seeds (Schlereth et al., 2010) were provided by Dolf Weijers (xxx location/affiliation? xxx).

PIN1::PIN1-GFP (Benková et al., 2003), *pin1-5* mutant (Bencivenga et al., 2012; Sohlberg et al., 2006), *pin1-201* (Furutani et al., 2004), *crf3-1*, *crf6-S2*, *crf3-1 crf6-S2*, *crf2-2 crf3-1 crf6-S2*, *ΔPIN1::PIN1-GFP*, *ΔPIN1-GFP pin1-201* and *PIN1::PIN1-GFP pin1-201* (Šimášková et al., 2015) lines have been described previously. BAP treatment was performed on inflorescences as detailed by Bencivenga et al. (2012).

Quantitative real-time qPCR analysis

Total RNA was extracted from inflorescences at pre-fertilization stages using the Macherey-Nagel Nucleospin RNA Plant Kit and then reverse transcribed using the Promega ImProm-II RT System. Gene expression analysis was performed using the Bio-Rad iQ5 Multicolor RT-PCR Detection System with the GeneSpin SYBR Green PCR Master Mix. *ACTIN 2-8* and *UBIQUITIN 10* were used as reference genes for normalization of transcript levels. RT-PCR primers used in this work were: RT2017fw 5′-TGTTCCATGGCCAACACTTG-3′ and RT2018rev 5′-AAGTCGTGCCGCTTCATATG-3′ for GFP, RT509fw 5′-TGGTCCC-TCATTTCCTTCAA-3′ and RT510rev 5′-GGCAAAGCTGCCTGGATA-AT-3′ for PIN1, RT147fw 5′-CTGTTCACGGAACCCAATTC-3′ and RT148rev 5′-GGAAAAAGGTCTGACCGACA-3 for UBI10, and RT861fw 5′-CTCAGGTATTGCAGA CCGTATGAG-3′ and RT862rev 5′-CTGGACCTGCTTCATCATACTCTG-3′ rev for ACT2-8.

Counting ovule number by differential interference contrast (DIC) microscopy

Inflorescences were fixed with ethanol/acetic acid (9:1) overnight, rehydrated with 90% and 70% ethanol and cleared in a chloral hydrate/glycerol/water solution (8 g: 1 ml: 3 ml) for at least 2 h before dissection under a stereomicroscope. Pistils were observed using a Zeiss Axiophot D1 microscope equipped with DIC optics. Images were recorded using a Zeiss Axiocam MRc5 camera with Axiovision software version 4.1. Only ovules of pistils in which both carpels remained intact after slide preparation and where all four rows of ovules were visible and distinguishable were counted.

Confocal microscopy

For confocal laser scanning microscopy (CLSM), fresh material was collected, mounted in water and analysed immediately. CLSM analysis was performed using a Leica TCS SPE microscope with a 488 nm argon laser line for excitation of GFP fluorescence. Emissions were detected between 505 and 580 nm. Images were collected in multi-channel mode and overlay images were generated using Leica analysis software LAS AF 2.2.0.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Designed the experiments: M.C., E.B., L.C.; Performed the experiments: M.C., S.M., A.G., N.E.Q., M.A.M.; Analysed the data: M.C., E.B., L.C.; Contributed reagents/materials/analysis tools: E.B., L.C.; Wrote the paper: M.C., L.C.

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Data availability

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.143545.supplemental

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