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CAPRICE family genes control flowering time through both promoting and repressing *CONSTANS* and *FLOWERING LOCUS T* expression

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ABSTRACT

CAPRICE (*CPC*) and six additional *CPC* family genes encode R3-type MYB transcription factors involved in epidermal cell fate determination, including *Arabidopsis* root hair and trichome differentiation. Previously, we reported that the *CPC* and *CPC* family genes *TRIPTYCHON* (*TRY*) and *CAPRICE LIKE MYB3* (*CPL3*) also affect flowering time. The *cpl3* mutant plants flower earlier, with fewer but larger leaves, than do wild type plants, and mutations in *CPC* or *TRY* delay flowering in the *cpl3* mutant. In this study, we examined flowering time, leaf number, and fresh weight for *CPC* family gene double and triple mutants. Mutation in *ENHANCER OF TRY AND CPC1* (*ETC1*) shortened the flowering time of the *cpl3* single mutant. Mutation in *ETC2* significantly reduced fresh weight in the *cpl3* mutant. Expression levels of the flowering-related genes *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) were higher in the *cpl3* mutant than in wild type plants. The high expression levels of *CO* and *FT* in *cpl3* were significantly reduced by mutations in *CPC, TRY, ETC1*, or *ETC2*. Our results suggest that *CPC* family genes antagonistically regulate flowering time through *CO* and *FT* expression.

1. Introduction

The *CAPRICE* (*CPC*) gene encodes an R3-type myeloblastosis (MYB) transcription factor, which has been identified as a key regulator of epidermal cell fate determination in *Arabidopsis thaliana* [1]. Six *CPC*-homologous genes exist in the *Arabidopsis* genome: *TRIPTYCHON (TRY), ENHANCER OF TRY AND CPC1 (ETC1), ENHANCER OF TRY AND CPC2 (ETC2), ENHANCER OF TRY AND CPC3 (ETC3)/CPC LIKE MYB3 (CPL3), TRICHOMELESS1 (TCL1)*, and *TRICHOMELESS2 (TCL2)/CPC LIKE MYB4 (CPL4)* [2–10]. These seven *CPC* family genes have been reported to promote root hair differentiation and reduce trichome formation.

In addition to the CPC family transcription factors, several regulatory factors have been identified as involved in epidermal cell fate determination. The R2R3-type MYB transcription factor WEREWOLF (WER) induces *GLABRA2* (*GL2*) expression, which encodes a homeodomain-leucine zipper protein and inhibits root hair differentiation [11–14]. The other R2R3-type MYB genes *GLABRA1* and *MYB23* are homologous to *WER* and are involved in trichome formation [15–17]. The basic helix-loop-helix (bHLH) transcription factors *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*) reduce root hair formation [18]. The WD40-repeat protein *TRANSPARENT TESTA GLABRA1* (*TTG1*) induces trichome formation and inhibits root hair differentiation [19,20].

The CPC family proteins, WER, GL1, and TTG1 have been demonstrated to interact with GL3 and EGL3 proteins in a yeast two-hybrid system and to act as a transcription regulatory complex in *Arabidopsis* epidermis [8,21–24]. The *GL2* gene acts as the most downstream transcription factor and is regulated by the above transcription complexes in both root hair and trichome differentiation in *Arabidopsis* [1,11,14,19,25]. The TTG1-GL3/EGL3-WER transcriptional complex activates the expression of *GL2* [26,27]. Conversely, CPC disrupts the TTG1-GL3/EGL3-WER transcriptional complex by competing with WER and its binding to TTG1-GL3/EGL3, thus negatively regulating the expression of *GL2* [28].

We have previously investigated *CPL3* gene functioning in *Arabidopsis* [8]. In addition to its regulatory functions in epidermal cell fate determination, *CPL3* is also specifically involved in flowering time [8]. Plants with a mutation in the *CPL3* gene exhibit early flowering and have fewer leaves than do wild type plants [8]. Surprisingly, mutations in the *CPC* or *TRY* genes negate the early flowering phenotype of the *cpl3* mutant [29].

Flowering time in *Arabidopsis* is mainly regulated by four genetic pathways involving the photoperiod, vernalization, autonomous, and GA-dependent pathways [30–32]. These pathways regulate downstream target genes, the supposed flowering pathway inte-

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grators *CONSTANS (CO)*, *FLOWERING LOCUS T (FT)*, *SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1)*, and *LEAFY (LFY)* [31]. The transcription levels of these genes control flowering time in *Arabidopsis* [33–39]. *FT* encodes a Raf kinase inhibitor-like protein, which integrates multiple floral pathways [33,34]. *CO*, encoding a zinc finger transcription factor, controls flowering time by promoting the expression of *FT* and *SOC1*, which encodes a MADS box domain proteins [40]. Thus, *FT* and *SOC1* are thought to function in parallel under the control of *CO* [40]. FT moves from the leaf phloem to the shoot apex, interacting with the bZIP transcription factor FD to promote flowering [41]. *FT* overexpressor and loss-of-function mutants exhibit strong changes in flowering time [33,34,42]. Therefore, FT is considered to be a florigen, a compound that controls flowering.

WER, which was initially cloned as a root epidermal–specific R2R3 type MYB, has been shown to be involved in the regulation of *FT* mRNA stability [43]. The *wer* mutant exhibits a late-flowering phenotype [43]. The transcription level of *FT* is reduced in the *wer* mutant [43]. In summary, it is very interesting that the *CPC* family genes and *WER* are involved in not only epidermal cell differentiation but also flowering time regulation.

In this study, we examined the effect of *CPC* family mutants on the flowering time, leaf number, and fresh weight of *Arabidopsis*. We precisely measured the flowering time of double and triple *CPC* family mutants and confirmed the effects of mutations in *CPC* and *TRY* on the early flowering phenotype of the *cpl3* mutant. Additionally, we revealed that *ETC1* also played a role in flowering regulation. Mutation in *ETC1* significantly shortened flowering time and reduced leaf number in the *cpl3* mutant. On the other hand, mutation in *ETC2* significantly reduced fresh weight in the *cpl3* mutant. The high expression levels of *CO* and *FT* in *cpl3* were significantly reduced by mutations in *CPC, TRY, ETC1* or *ETC2*. Our results suggest that the *CPC* family genes both positively and negatively regulate flowering time through *CO* and *FT* expression.

2. Materials and methods

2.1. Plant materials and growth conditions

The *A. thaliana* (L.) Heynh. Col-0 ecotype was used as the wild type. The *cpc-2* mutant used in this study has been described previously [44]. The *cpl3-1* mutant was isolated from a Wisconsin T-DNA population as described previously [8]. The *try-29760, etc1-1*, and *etc2-2* mutants were taken from a SALK T-DNA population as described previously [29]. All mutants were carried on the Col-0 background. Double and triple mutants of *cpc, try, etc1, etc2*, and *cpl3* were selected as described previously [8]. Seeds were surface-sterilized, sown on the surface of 1.5% agar plates as described previously [45], and grown for the observation of seedling phenotypes and flowering-related gene expression analyses (*CO, FT*, and *SOC1*). Seeded plates were kept at 4 °C for 2 d and then incubated at 22 °C under long-day conditions (16 h light/8 h dark) under white light (50–100 μmol m^{-2} s^{-1}). 7-day-old seedlings were transplanted to soil and kept under long-day conditions for the observation of phenotypes and flowering-related gene expression analyses.

2.2. Flowering time

Developmental uniformity was obtained by selecting the twelve most uniform plants on approximately 14 days after sowing, bringing the plant density to four plants per pot, and rotating the trays three times a week. Flowering time and leaf number at flowering time were determined for Col-0, *cpc, cpl3, cpc cpl3, try cpl3, etc1 cpl3, etc2 cpl3, cpc try cpl3*, and *try etc1 cpl3* plants. Flowering time was measured as the number of days from sowing to the first elongation of the floral stem at 0.1 cm in height. The number of true leaves produced by the apical meristem was recorded for bolted plants. Data represent the means (±s.d.) of at least 10 plants per mutant line. No major variation was observed in two independent repeats of the experiment.

2.3. Fresh weight

The fresh weights of 7-, 14-, 21-, and 28-day-old wild-type (Col-0) and *cpc, cpl3, cpc cpl3, try cpl3, etc1 cpl3, etc2 cpl3, cpc try cpl3*, and *try etc1 cpl3* mutant plants were measured. The fresh weight of rosette leaves for each plant was calculated from the means (±s.d.) of a minimum of three plants from each line.

2.4. Real-time RT-PCR

Total RNA was extracted from rosette leaves from 7-, 14-, 21-, and 28-day-old plants grown under long-day conditions (16 h light/8 h dark) using the RNeasy Plant Mini Kit (Qiagen). We harvested rosette leaves just before the lights were turned off, because *CO* expression was highest at this point [46]. On-column DNase I digestion was performed during RNA purification following the protocol described in the RNeasy Mini Kit handbook. First-strand cDNA was synthesized from 1 μg total RNA in a 20 μl reaction mixture using the Prime Script RT Regent Kit (Takara). Real-time RT-PCR was performed in a Chromo4 Real-time RT-PCR Detection System (Bio-Rad, Hercules, CA) using SYBR Premix Ex Taq (Takara). PCR amplification employed a 30 s denaturing step at 95 °C, followed by 5 s at 95 °C and 30 s at 60 °C for 40 cycles, for *CO, FT, SOC1*, and *ACT2*. Relative mRNA levels were calculated using iQ5 software (Bio-Rad) and normalized to the concentration of *ACT2* mRNA. The following primers were used in this experiment: *CO-F* and *CO-R* for *CO*; *FT-F* and *FT-R* for *FT*; *SOC1-F* and *SOC1-R* for *SOC1*; and *ACT2-F* and *ACT2-R* for *ACT2*, as described previously [8,47,48]. For accurate flowering-related gene expression analyses, we extracted RNA from the leaves just before the end of the photoperiod.

2.5. Light microscopy

Phenotypes of 7- and 14-day old seedlings of *cpc, cpl3, cpc cpl3, try cpl3, etc1 cpl3, etc2 cpl3, cpc try cpl3*, and *try etc1 cpl3* mutant plants were observed using a Leica MZ16FA stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were recorded using a high-sensitivity CCD color camera system (Keyence VB 7010, Osaka, Japan).

3. Results

3.1. CPC *family genes antagonistically regulate plant growth and flowering time*

Previously, we have reported the differences in flowering times among *CPC* family mutants under continuous light conditions [29]. Eighteen-day-old *cpl3, etc1 cpl3*, and *etc2 cpl3* mutant seedlings bolted earlier than did wild type plants or other mutant lines [29]. In addition to altered bolting, *cpl3* loss-of-function mutants show larger rosette leaves than do wild type plants [8]. In this study, we precisely determined the flowering times of *CPC* family double and triple mutants (Table 1). The *cpl3* mutant showed significantly earlier flowering compared to the wild type, even in the long-day condition (Table 1). The *cpc cpl3*, and *try cpl3* double mutants showed significantly prolonged flowering times compared with that of the *cpl3* mutant, comparable to the flowering time of the wild type (Table 1). These results suggest that the *cpc* or *try* mutations negate the early flowering phenotype of the *cpl3* mutant even