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RESEARCH REPORT

Extended C termini of CPC-LIKE MYB proteins confer functional diversity in *Arabidopsis* epidermal cell differentiation

Rumi Tominaga-Wada* and Takuji Wada

ABSTRACT

The *CAPRICE* (*CPC*) gene encodes a R3-type MYB transcription factor that promotes differentiation of root hair cells in *Arabidopsis thaliana*. Here, we have compared the functions of five *CPC*-homologous genes for epidermal cell differentiation using *CPC* promoter-driven transgenic plants. Our results show that TRIPTYCHON (TRY) and ENHANCER OF TRY AND CPC2 (ETC2) were less effective in root hair cell differentiation and were unstable in root epidermal cells when compared with CPC, ETC1 or CPC LIKE MYB3 (CPL3). The deletion of the extended C-terminal domain of TRY and ETC2 enhanced protein stability and conferred the ability to induce root hair cell differentiation on them. Treatment with MG132, a proteasome inhibitor, also led to the accumulation of TRY, indicating that TRY proteolysis is mediated by the proteasome-dependent pathway. Our results indicate that the CPC family includes relatively stable (CPC, ETC1 and CPL3) and unstable (TRY and ETC2) proteins that might be degraded by the proteasome. Our findings provide new insights into the regulatory mechanism of CPC family proteins that mediate root hair cell differentiation and should be useful in understanding epidermal development.

KEY WORDS: CPC, TRY, ETC2, MYB, Root hair, *Arabidopsis*

INTRODUCTION

In *Arabidopsis* roots, epidermal cells differentiate into two types of cells: root hair cells and non-hair cells (Dolan et al., 1993, 1994). The protein complexes, including WEREWOLF (WER) (Lee and Schiefelbein, 1999), TRANSPARENT TESTA GLABRA (TTG1) (Walker et al., 1999) and GLABRA3/ENHANCER OF GL3 (GL3/EGL3) (Bernhardt et al., 2003; Payne et al., 2000) induce the transcription of *GLABRA2* (*GL2*) (Koshino-Kimura et al., 2005) and promote non-hair cell fate determination (Bernhardt et al., 2005; Tominaga-Wada et al., 2011).

CAPRICE (*CPC*), which encodes a R3-type MYB transcription factor, has been identified as a positive regulator of root hair cell development (Wada et al., 1997). After the discovery of *CPC*, six additional homologs, including *TRY*, *ETC1*, *ETC2*, *ETC3/CPL3*, *TRICHOMELESS1* (*TCL1*) and *TRICHOMELESS2* (*TCL2*), were identified (Esch et al., 2004; Gan et al., 2011; Kirik et al., 2004a,b; Schellmann et al., 2002; Tominaga et al., 2008; Tominaga-Wada and Nukumizu, 2012; Wang et al., 2007). In general, these CPC-like MYB genes are thought to act as negative regulators of trichome formation and positive regulators of root hair formation (Tominaga-Wada et al., 2011; Tominaga-Wada and Nukumizu, 2012). Wang et al. (2008) suggested that, although the CPC-like MYB genes have largely overlapping functions in controlling the epidermal development, their precise functions differ (Wang et al., 2008). In fact, the *cpc* mutant was shown to have a reduced root hair number phenotype (Wada et al., 1997). However, an obvious reduction in the root hair number was not observed in any of the other single mutants of the *CPC* family genes (Kirik et al., 2004 a,b; Tominaga et al., 2008). In contrast, the double mutants *cpc try* and *cpc etc1* lacked root hairs (Kirik et al., 2004b; Schellmann et al., 2002). This suggests that these homologous genes contribute to root hair cell differentiation more weakly than *CPC* does.

To better understand the precise functions of the CPC-like MYB gene family members in controlling the epidermal development, we conducted phenotypic analyses of plants expressing *CPC*, *TRY*, *ETC1*, *ETC2* and *CPL3* under a *CPC* promoter and performed protein localization studies.

RESULTS AND DISCUSSION

Of the seven CPC-like MYB transcription factors, we focused on five, *CPC*, *TRY*, *ETC1*, *ETC2* and *CPL3* (Fig. 1A), because they specifically contribute to root hair differentiation and trichome formation in *Arabidopsis* leaves. These transcription factors were ∼50% identical at the amino acid sequence level (Fig. 1A). A phylogenetic analysis performed in a previous study suggested that these five CPC-like MYBs can be divided into two groups, with CPL3, ETC1 and CPC belonging to one group, and ETC2 and TRY belonging to the other (Fig. 1B) (Simon et al., 2007; Tominaga et al., 2008; Wang et al., 2007). These five CPC-like MYBs presumably function redundantly in root hair and trichome formation because *35S:CPC*, *35S:TRY*, *35S:ETC1*, *35S:ETC2* and *35S:CPL3* transgenic plants have greater numbers of root hairs than the wild type and show a trichome-deficient phenotype (Kirik et al., 2004a,b; Schellmann et al., 2002; Tominaga et al., 2008; Wada et al., 1997). We also produced *35S:CPC*, *35S:TRY*, *35S:ETC1*, *35S:ETC2* and *35S:CPL3* transgenic lines in the Col-0 background, and precisely compared them and obtained similar results (Fig. S1). We used the promoters of the five homologs because the *35S* promoter is widely expressed (Benfey and Chua, 1990). To investigate the effect of native regulatory sequences, we introduced the genomic sequences of *CPC*, *TRY*, *ETC1*, *ETC2* and *CPL3* fused with GFP under the control of putative promoters into *Arabidopsis* (Fig. 1C,D). The extra copy of the transgene caused a gene-dose effect that was functionally equivalent to overexpression. *ETC1:ETC1-GFP* produced a significantly increased number of root hairs, as observed in *CPC:CPC-GFP* transgenic plants (Fig. 1C,D). In contrast, *TRY:TRY-GFP*, *ETC2:ETC2-GFP* and *CPL3:CPL3-GFP* transgenic plants did not show significantly higher root hair number than that in the wild-type plants (Fig. 1C,D). All the transgenic lines, except for *ETC2:ETC2-GFP*, lacked trichomes on the leaf surface (Fig. 1C,D). Strong GFP

Graduate School of Biosphere Sciences, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima 739-8528, Japan.

*Author for correspondence (rtomi@hiroshima-u.ac.jp)

R.T.-W., 0000-0002-9939-346X

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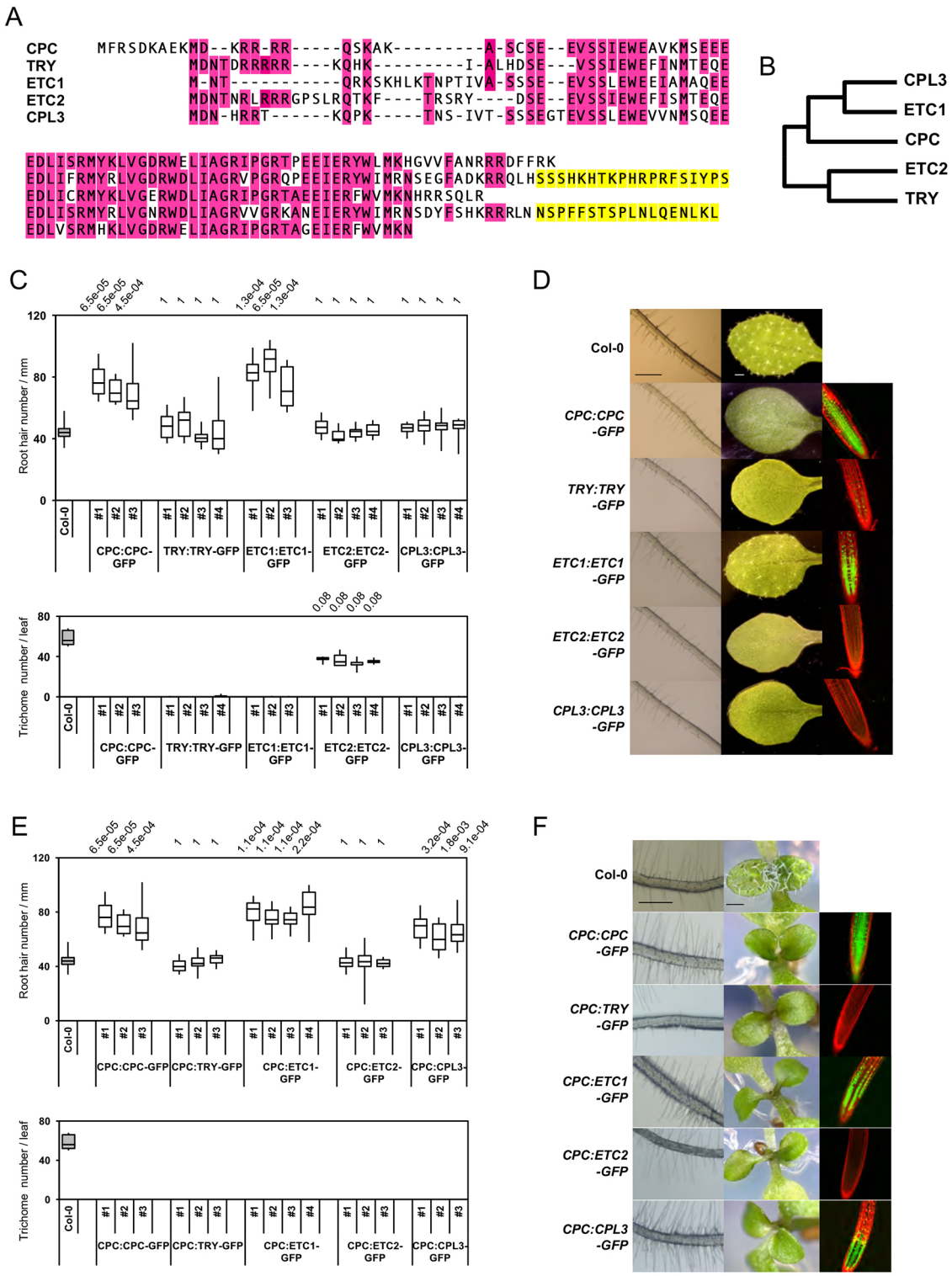

Fig. 1. Epidermal phenotypes of CPC-like MYB transgenic *Arabidopsis* plants. (A) Sequence alignment of CPC-like MYBs. Identical amino acids are shaded in pink. The extended C termini of TRY and ETC2 are highlighted in yellow. (B) Phylogenetic analysis based on the entire amino acid sequence of each protein. (C) Box-whisker plots showing root hair and trichome formation (D). (D) Epidermal phenotypes and distribution of GFP fluorescence. (E) Box-whisker plots showing root hair and trichome formation (F). (F) Epidermal phenotypes and distribution of GFP fluorescence. GFP fluorescence (green) and propidium iodide (PI) florescence (red) were observed. Scale bars: 500 µm. The *P* values between the wild-type Col-0 and the transgenic lines are provided above the boxes. Error bars indicate the whiskers that extend to the maximum and minimum value data sets. *n*=5-10.

fluorescence was observed in the root epidermal cells of *CPC:CPC-GFP* and *ETC1:ETC1-GFP* (Fig. 1D), which correlated with the increased number of root hairs (Fig. 1C). Undetectable levels of GFP fluorescence in *ETC2:ETC2-GFP* and *CPL3:CPL3-GFP* plants were expected because the *ETC2* and *CPL3* promoters are not active in the root epidermis (Tominaga et al., 2008). However,

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the weak fluorescence in TRY-GFP plants was unexpected, because the *TRY* promoter is active in the root epidermis (Tominaga et al., 2008). This suggests that the weak fluorescence could be due to rapid turnover of the TRY-GFP fusion protein. The GFP fusion did not interfere with the trafficking between the root cells (Kurata et al., 2005).

To compare the function and protein localization of *CPC*, *TRY*, *ETC1*, *ETC2* and *CPL3* under the same conditions, we created transgenic plants expressing *TRY*, *ETC1*, *ETC2* or *CPL3* under the *CPC* promoter. *CPC:ETC1-GFP* and *CPC:CPL3-GFP* transgenic plants produced a significantly greater number of root hairs than did the wild type, a phenotype that is similar to that of *CPC:CPC-GFP* plants (Fig. 1E,F). In contrast, the expression of *TRY* and *ETC2* did not increase the root hair number even when expressed under the control of the *CPC* promoter (Fig. 1E,F). Similarly, all the five genes, including *TRY* and *ETC2*, inhibited trichome formation (Fig. 1E,F). The GFP fluorescence was observed in the root epidermal cells of *CPC:CPC-GFP*, *CPC:ETC1-GFP* and *CPC:CPL3-GFP* transgenic plants (Fig. 1F). *CPC:TRY-GFP* and *CPC:ETC2-GFP* roots did not fluoresce at all (Fig. 1E,F). These results suggest that, unlike CPC, ETC1 or CPL3, TRY and ETC2 do not accumulate in *Arabidopsis* roots, even when expressed under the control of the *CPC* promoter (Fig. 1E,F). Based on this observation, we hypothesize that the turnover of CPC, like those of MYBs, is not same in the root epidermal cells.

To assess whether *TRY*, *ETC1*, *ETC2* and *CPL3* could substitute for the *CPC* function, the transgenic plants described in Fig. 1E were crossed into the *cpc-2* mutant. The *CPC:CPC-GFP* construct complemented the phenotype of the *cpc-2* mutant (Fig. S2) (Tominaga et al., 2007). In contrast, the expression of *TRY* or *ETC2* in *cpc-2* rescued the *cpc-2* phenotype partially (Fig. S2). These results also suggest that the effects of TRY or ETC2 were not as strong as those of CPC, ETC1 and CPL3.

A comparison of amino acid sequences revealed that TRY and ETC2 have an extended C-terminal region unlike CPC, ETC1 and CPL3 (Fig. 1A; highlighted in yellow). Therefore, we focused on the C-terminal regions of TRY and ETC2, and evaluated their possible role in the regulation of root hair and trichome formation. We generated two modified gene constructs in which the C-terminal regions of TRY and ETC2 were deleted (TRYΔC and ETC2ΔC, respectively) (Fig. 2A). The roots of *CPC:TRY*Δ*C-GFP* and *CPC:ETC2*Δ*C-GFP* transgenic plants had a significantly greater number of root hairs than that in the plants expressing the full-length TRY or ETC2, and there were no trichomes on the leaves of either of these transgenic lines (Fig. 2B,D). In addition, the *CPC:TRY*Δ*C-GFP* and *CPC:ETC2*Δ*C-GFP* constructs complemented the phenotype of *cpc-2*, whereas *CPC:TRY-GFP* and *CPC:ETC2-GFP* did not fully complement the same (Fig. 2C,D). These results strongly suggest that the deletion of the C-terminal regions of TRY and ETC2 induces their activities, which are similar to those of CPC, ETC1 or CPL3. The deletion constructs inhibited the trichome formation completely in the *cpc-2* mutant (Fig. 2C), which also indicates stronger functions in the trichomes. The roots of *CPC:TRY*Δ*C-GFP* and *CPC:ETC2*Δ*C-GFP* transgenic plants showed stronger GFP fluorescence than that in the roots of *CPC:TRY:GFP* and *CPC:ETC2-GFP* plants, respectively (Fig. 2E). In summary, our results show that the deletion of the C-terminal amino acids of TRY and ETC2 might lead to the accumulation of the proteins in the root and leaf epidermis, and induces greater number of root hairs and trichome-deficient phenotype.

To investigate the effect of C-terminal regions of TRY and ETC2 on their transcriptional or post-transcriptional levels, real-time PCR was performed (Fig. 2F,G). The expression levels of the genes for these proteins varied among different lines; however, there were no substantial differences in the mRNA levels between the full-length (Fig. 2F,G; blue bars) and truncated versions of the respective genes (Fig. 2F,G; green bars). We also checked the expression levels using GFP-specific primers because these constructs were fused to *GFP* (Fig. S3). These results suggest that the C-terminal regions of TRY and ETC2 do not influence their expression levels, suggesting that the regulation of these genes is post-transcriptional.

The apparent disparity in the accumulation levels of TRY-GFP and TRYΔC-GFP fusion proteins in the root epidermal cells was addressed by immunoblot analysis of the proteins extracted from the root tissue of wild-type (Col-0), and *CPC:TRY-GFP* and *CPC:TRY*Δ*C-GFP* transgenic plants using an anti-GFP antibody. In the transgenic plants expressing *CPC:TRY*Δ*C-GFP*, strong bands of the predicted molecular mass (65 kDa) corresponding to TRYΔC-GFP were detected, but only faint bands corresponding to TRY-GFP were detected in the *CPC:TRY-GFP* transgenic plants (Fig. 3A). Deletion of the C terminus of TRY apparently contributed to the stability of TRY. In addition to the results shown in Fig. 2F, the C-terminal region of TRY was observed to be involved in its turnover.

To investigate whether the low levels of TRY-GFP protein in transgenic roots were due to the degradation of TRY mediated by the 26S proteasome, we tested the effect of a proteasome inhibitor, MG132, on the accumulation of TRY. The *CPC:TRY-GFP* transgenic seedlings were treated with or without MG132. As shown in Fig. 3B, the degradation of TRY-GFP was greatly reduced in the presence of MG132, suggesting that TRY may be degraded through the 26S proteasome. We also tested the effect of MG115, another proteasome inhibitor, on the accumulation of TRY. Significant accumulation of TRY-GFP was also observed in the MG115-treated *CPC:TRY-GFP* roots (Fig. S4), which again indicates that TRY might be degraded through the 26S proteasome.

Plants selectively degrade proteins via the ubiquitin (Ub)/26S proteasome proteolytic pathway to achieve an additional layer of regulatory control (Hershko and Ciechanover, 1998; Pickart, 2001). Our results suggest that the Ub/26S proteasomal pathway controlled the TRY activity. In addition, MG132 did not change the promoter activity and protein stability of CPC (Fig. 3C; Fig. S5), indicating that MG132 specifically targeted the stability of TRY thorough the C-terminal region.

We demonstrated that TRY was degraded through the Ub/26S proteasome-mediated pathway because of the properties of the C-terminal domain. We did not detect any possible PEST sequences in TRY or in ETC2; these sequences target themselves for proteolytic degradation (Rechsteiner and Rogers, 1996). However, there is a possibility that S89 and T94 in the extended C terminus of TRY, and S96 and S102 in the extended C terminus of ETC2 might be phosphorylated, and consequently become the target of ubiquitylation. We show that CPC-like MYBs are regulated not only at the transcriptional level but also by protein degradation.

MATERIALS AND METHODS

Plant materials and growth conditions

The *Arabidopsis thaliana cpc-2* mutant, *CPC:CPC-GFP*, *CPL3:CPL3-GFP*, *CPC:CPL3-GFP*, *35S:CPC*, *35S:ETC3* and *CPC:CPC* in *cpc-2* transgenic plants have all been described previously (Tominaga et al., 2008; Tominaga-Wada and Wada, 2016; Wada et al., 2002, 1997). The seeds were sown on 1.5% agar plates as described previously (Okada and Shimura, 1990). The number of root hairs per millimeter was determined using ten 5-day-old seedlings from each line. The number of trichomes per leaf was determined using the third leaves from five 2-week-old seedlings of each line. The data were compared between the wild-type Col-0 and mutant or

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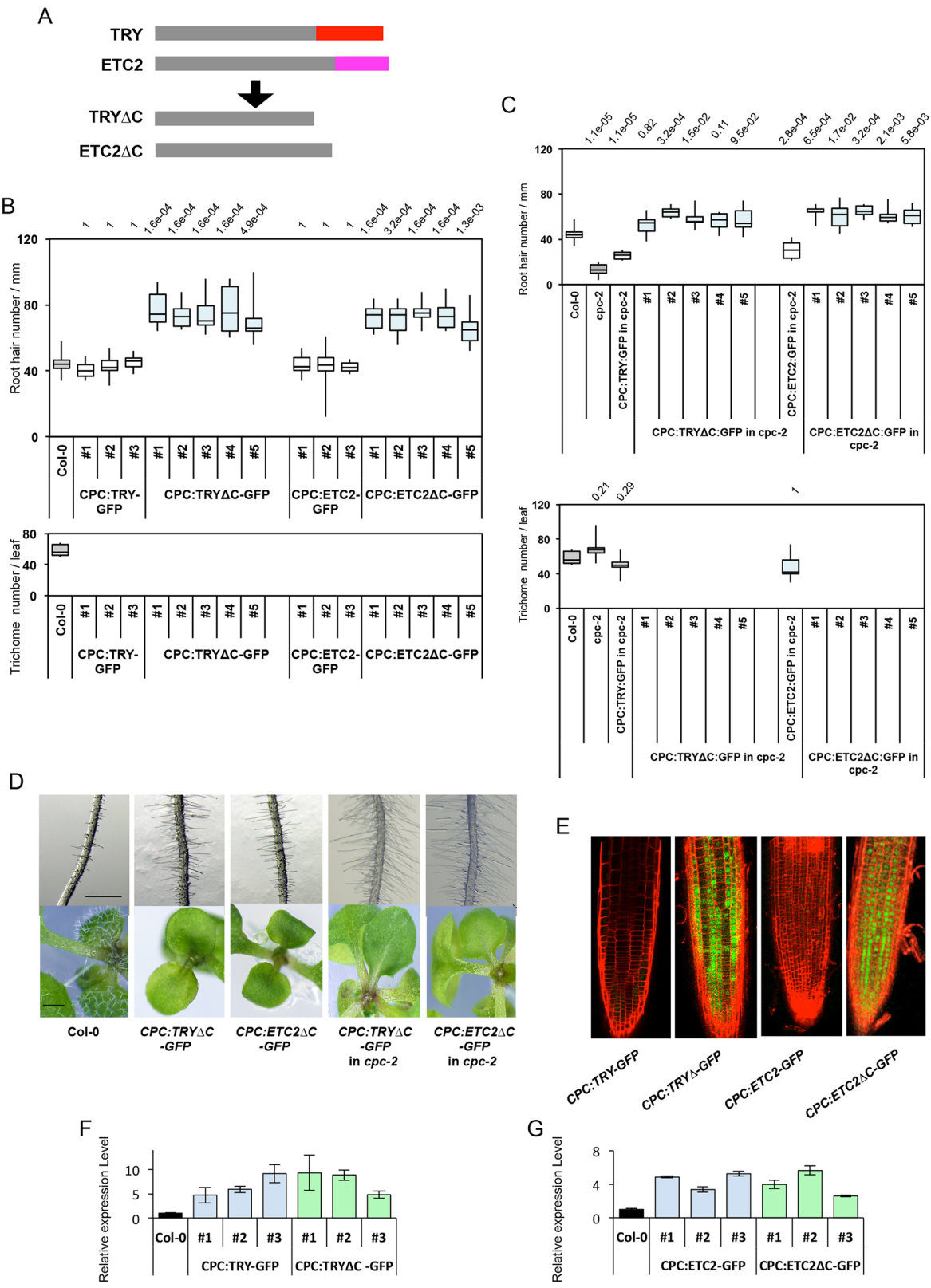

Fig. 2. Epidermal phenotypes of CPC-like MYB-modified transgenic *Arabidopsis* plants. (A) Schematic diagrams of the C-terminal modified constructs. The C termini of TRY and ETC2 (red and pink) were truncated. (B) Box-whisker plots showing root hair and trichome formation in *CPC:TRYΔC-GFP* and *CPC:ETC2ΔC-GFP* transgenic plants (D). (C) Box-whisker plots showing root hair and trichome formation in *CPC:TRYΔC-GFP* transgenic plants in the *cpc-2* background and in *CPC:ETC2ΔC-GFP* transgenic plants in the *cpc-2* background (D). The *P* values between the wild-type Col-0 and the mutant or individual transgenic lines are mentioned above the boxes. Error bars indicate the whiskers that extend to the maximum and minimum value data sets. *n*=5-10. (D) Root hair and trichome formation. Scale bars: 500 µm. (E) Distribution of GFP fluorescence. (F) Expression analyses of *TRY*. (G) Expression analyses of *ETC2*. The expression level in each line relative to that in the wild type is indicated. Data are mean±s.d.

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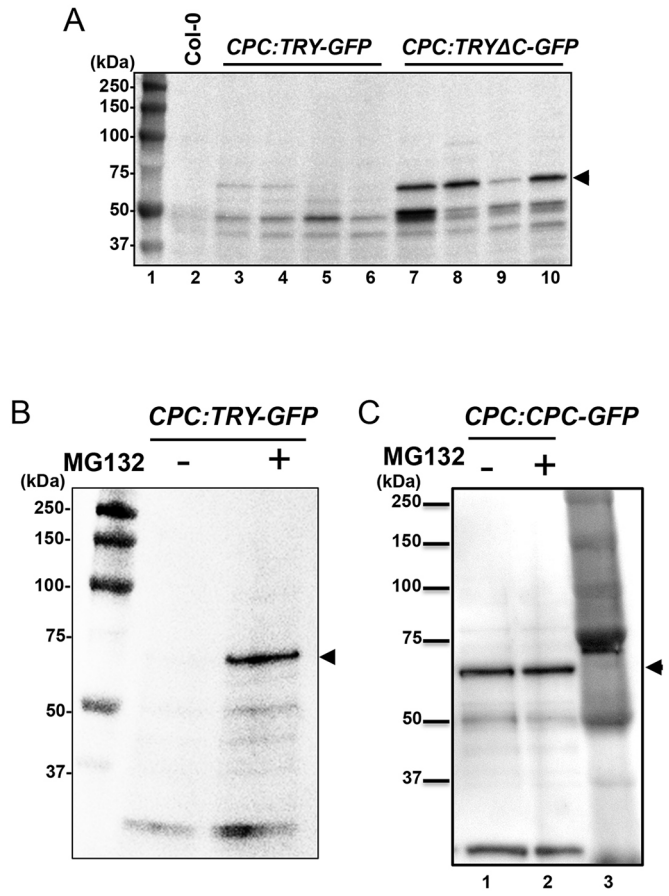

Fig. 3. Immunoblot analyses of GFP fusion proteins. (A) TRY-GFP (lanes 3-6) and TRYΔC-GFP (lanes 7-10) fusion proteins (indicated by the arrowhead) were detected using anti-GFP antibodies. Molecular weights are shown on the left (lane 1). (B) Effect of MG132 treatment on TRY-GFP protein. The CPC:TRY-GFP seedlings were incubated for 24 h with (+) or without (–) 50 µM MG132. The TRY-GFP fusion protein (indicated by the arrowhead) was detected. Molecular weights are shown on the left. (C) Effect of MG132 treatment on CPC-GFP protein. CPC:CPC-GFP seedlings were incubated for 24 h with (+) or without (–) 50 µM MG132. The CPC-GFP fusion protein (indicated by the arrowhead) was detected. Molecular weights are shown on the left.

individual transgenic lines using the Mann–Whitney *U*-test combined with Bonferroni correction. Statistical analyses were performed using Microsoft Excel. A value of $P<0.05$ was considered to be significant.

Gene constructs and transgenic plants

The sequences of all the primers used in this study are listed in Table S1. To generate the *TRY:TRY-GFP*, *ETC1:ETC1-GFP* and *ETC2:ETC2-GFP* transgenic plants, PCR-amplified 4.0 (primers RT89/RT90), 2.3 (primers RT67/RT68) and 4.0 kb (primers RT69/RT70) fragments were ligated into *pBS-GFP* (Kurata et al., 2005). To generate the *CPC:TRY-GFP*, *CPC:ETC1-GFP* and *CPC:ETC2-GFP* transgenic plants, 2.6 (primers RT299/CF2_NOSterSma), 1.9 (primers RT296/CF2_NOSterSma) and 2.4 kb (primers RT297/CF2_NOSterSma) fragments were ligated into the *pBS-CPC* promoter (Kurata et al., 2005). To generate the *CPC:TRYΔC-GFP* and *CPC:ETC2ΔC-GFP* transgenic plants, 7.0 (primers RT322/RT323) and 7.0 kb (primers RT320/RT321) fragments were self-ligated. The sequenced fragments were recloned into a binary vector, *pJHA212K* (Yoo et al., 2005), using appropriate restriction sites. To generate the *35S:TRY*, *35S:ETC1* and *35S:ETC2* transgenic plants, PCR-amplified 1.0 (primers RT91/RT92), 0.5 (primers TW1169/TW1170) and 1.0 kb (primers TW1165/TW1166) fragments were subcloned into pBluescript SK+ (Stratagene). The sequenced fragments were recloned into a binary vector, *pCHF3* (Jarvis et al., 1998), using appropriate restriction sites. Plant transformation was performed using the floral dip method (Clough and Bent, 1998). Several constructs were introduced into the *cpc-2* mutant by conventional crosses. The confocal images for the GFP-fusion lines were obtained with a Zeiss LSM-510 Meta confocal laser scanning microscope.

Real-time RT-PCR

Total RNA was extracted from the roots and used for real-time RT-PCR analysis, as described previously (Wada and Tominaga-Wada, 2015), using primer pairs for *CPC*, *TRY*, *ETC1*, *ETC2*, *GFP* and *ACT2* (Table S1).

Western blotting

The proteins were extracted from the whole-cell-extracts of root tissue using the P-PER Plant Protein Extraction Kit (Thermo Scientific), according to the manufacturer's instructions. The extracted proteins (20 µg) were separated by SDS-PAGE on a 10% Mini-PROTEAN EGX Precast Gel (Bio-Rad), and were transferred onto a PVDF membrane (Bio-Rad). We used mouse anti-GFP antibody (1:10,000; Living Colors A.v. Monoclonal Antibody, Clontech) and sheep anti-GFP antibody (1:10,000; Amersham ECL anti-mouse IgG HRP-linked species-specific whole antibody from sheep, GE Healthcare). The immunoblotted proteins were detected with the ImmunoSter LD system (Wako) and the Ez-Capture MG imaging system (ATTO, Tokyo, Japan).

Inhibitors

For the proteasome inhibition experiment, MG132 and MG115 (Peptide Institute, Osaka, Japan) were dissolved in DMSO to final concentrations of 50 and 10 µM, respectively. MG132 is not specific for the proteasome (Gu et al., 2010).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.T.-W., T.W.; Investigation: R.T.-W.; Writing - original draft: R.T.-W., T.W.; Writing - review & editing: T.W.; Funding acquisition: R.T.-W.

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Supplementary information

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