Cell-to-cell movement of the CAPRICE protein in *Arabidopsis* root epidermal cell differentiation

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Summary

CAPRICE (CPC), a small, R3-type Myb-like protein, is a positive regulator of root hair development in *Arabidopsis*. Cell-to-cell movement of CPC is important for the differentiation of epidermal cells into trichoblasts (root hair cells). CPC is transported from atrichoblasts (hairless cells), where it is expressed, to trichoblasts, and generally accumulates in their nuclei. Using truncated versions of CPC fused to GFP, we identified a signal domain that is necessary and sufficient for CPC cell-to-cell movement. This domain includes the N-terminal region and a part of the Myb domain. Amino acid substitution experiments indicated that W76 and M78 in the Myb domain are critical for targeted transport, and that W76 is crucial for the nuclear accumulation of CPC:GFP. To evaluate the tissue-specificity of CPC movement, CPC:GFP was expressed in the stele using the *SHR* promoter and in trichoblasts using the *EGL3* promoter. CPC:GFP was able to move from trichoblasts to atrichoblasts but could not exit from the stele, suggesting the involvement of tissue-specific regulatory factors in the intercellular movement of CPC. Analyses with a secretion inhibitor, Brefeldin A, and with an *rhd3* mutant defective in the secretion process in root epidermis suggested that intercellular CPC movement is mediated through plasmodesmata. Furthermore, the fusion of CPC to tandem-GFPs defined the capability of CPC to increase the size exclusion limit of plasmodesmata.

Key words: *Arabidopsis*, Epidermis, *CAPRICE*, Myb, Protein movement

Introduction

Position dependency in developmental processes is a critical aspect for building the multicellular body of plants and requires intercellular communication. Intercellular communication is mediated by secreted signal molecules, such as small peptides (Matsubayashi et al., 2001). A mode of cellular communication in plants is the direct movement of signaling molecules through plasmodesmata, the cell wall pores that provide cytoplasmic continuity in plant cells. Plasmodesmata enable the transport of nutrients, as well as of macromolecules such as RNA and proteins (Crawford and Zambryski, 1999; Haywood et al., 2002), and have specific size exclusion limits (SELs) that depend on the developmental phase of the plant (Oparka et al., 1999). The cell-to-cell movement of macromolecules is generally considered to be crucial in plant development (Zambryski, 2004), and recent studies have underscored the significance of the cell-to-cell movement of plant transcription factors (Ruiz-Medrano et al., 2004). The intercellular movement of transcription factors in animal cells has also been described. Several animal homeodomain proteins, such as

Engrailed (En), Hoxa5, Hoxb4, Hoxc8, Emx1, Emx2, Otx2 and Pax6, move intercellularly in cultured cells (ex vivo) (Prochiantz and Joliot, 2003). Intercellular movement of En protein is driven by secretion and internalization, and this secretion process is regulated by the phosphorylation of En itself (Joliot et al., 1998; Maizel et al., 1999; Maizel et al., 2002). The intercellular movement of animal transcription factors may provide a model for analysis of the movement of transcription factors in plants.

SHORT-ROOT (SHR), a member of the GRAS family of putative transcription factors, moves from stele cells to the endodermis, where it activates endodermal cell differentiation and cell division in *Arabidopsis* (Helariutta et al., 2000; Nakajima et al., 2001). When the SHR:GFP fusion protein was expressed in several tissues under the control of tissue-specific promoters, it was unable to move from phloem companion cells and epidermal cells, suggesting a requirement for tissue-specific SHR movement factors (Sena et al., 2004). Further investigation demonstrated that SHR must be localized in the cytoplasm to move intercellularly. However, the mere presence

of SHR in the cytoplasm is not sufficient for movement (Gallagher et al., 2004).

The maize homeobox protein KNOTTED1 (KN1) controls leaf formation and has been shown to move from inner cells to epidermal cells, possibly through plasmodesmata (Lucas et al., 1995). Leaf injection experiments demonstrated that KN1 increases the SEL of plasmodesmata and induces the movement of the *kn1* RNA/KN1 protein complex (Kragler et al., 2000; Lucas et al., 1995). Cell-to-cell movement of KN1:GFP fusion proteins was investigated in *Arabidopsis* using heterologous promoters. Like the natural protein, the fusion product was able to move from inner tissue layers to the leaf epidermis in *Arabidopsis*. By contrast, KN1:GFP moved from the epidermal L1 layer towards the inner cell layers in the shoot apical meristem. Thus, the movement of KN1 is regulated in a tissue-specific manner (Kim et al., 2002; Kim et al., 2003).

The mode of movement was investigated for the transcription factor LEAFY (LFY), one of the floral identity genes in *Arabidopsis*. By using GFP-fusion proteins controlled by the L1-specific promoter *ATML1*, the movement of LFY was shown to be non-targeted and driven by diffusion. This interpretation was supported by the correlation between cytoplasmic localization and the ability of LFY to move (Wu et al., 2003). These studies suggest that there are at least two modes of protein cell-to-cell movement, including non-targeted translocation by diffusion, and targeted, regulated transport. Targeted movement is thought to be mediated by specific interactions between the transported protein and plasmodesmata components, leading to an increase in the SEL. Plant viral movement proteins (MPs) provide the best-characterized case of targeted protein movement. Cytoskeletal elements, including actin filaments and microtubules, are involved in the targeted movement of MPs (Kawakami et al., 2004; Kragler et al., 2003). However, the movement mechanisms of endogenous proteins are obscure.

To elucidate the mechanisms of cell-to-cell movement of regulatory proteins, we are studying the small Myb-like protein CAPRICE (CPC), a positive regulator of root hair formation in *Arabidopsis*. CPC is a small protein of 94 amino acids, with a single Myb-R3 domain. Loss-of-function mutants develop a reduced number of normal-shaped root hairs (Wada et al., 1997). Although this morphological defect in *cpc* mutants is obviously related to hair-cell differentiation in trichoblasts (hair cells), *CPC* mRNA expression is detected only in atrichoblasts (hairless cells). Using CPC:GFP fusion products, we established that CPC was localized to the nuclei of all epidermal cells, implying that the CPC:GFP fusion protein moved from atrichoblasts to trichoblasts in the *Arabidopsis* root epidermis (Wada et al., 2002).

In the present study, we confirmed by immunohistochemistry that the native CPC protein is localized in hair cells. By employing truncated CPC proteins fused to GFP, we further demonstrated that two motifs, one in the N-terminal region and the other in the Myb domain, are responsible for the cell-to-cell movement of CPC. Amino acid substitution experiments on CPC:GFP indicated that both W76 and M78 in the Myb domain are critical for cell-to-cell movement. Moreover, the W76A mutation reduced the nuclear accumulation of CPC:GFP. We also expressed CPC:GFP in stele cells and in root hair cells by linking the gene construct

to the *SHR* or *EGL3* promoters, respectively. CPC:GFP moved from root hair cells to hairless cells, but not from the stele to the epidermis, indicating a tissue-specific regulation of CPC movement. Analyses with a secretion inhibitor, Brefeldin A, and an *rhd3* mutant suggested that plasmodesmata are the route of CPC movement. Fusion of CPC to tandem-GFPs showed the ability of CPC to increase the SEL of plasmodesmata. Finally, we discuss the structural properties required for CPC movement in the *Arabidopsis* root epidermis.

Materials and methods

Gene constructs

Primers
Primer sequences are listed in Table S1 in the supplementary material.

CPCp::HA:CPC
To create a plasmid with a gene encoding HA-tagged CPC, a *CPC* genomic DNA fragment *pBS-gCPC* including the 1.3 kb 5′ region, the 0.9 kb coding region and the 0.45 kb 3′ regions (Wada et al., 1997) was used. HA1-F/HA1-R oligonucleotides (for HA peptide) were annealed and inserted into the 5′ portion of the coding region of a PCR-amplified linear *CPC* genome fragment with TK383 and TK384 (*pBS-CPCp-HA-CPC*). The *Xba*I and *Sal*I fragments of *pBS-CPCp-HA-CPC* were cloned into the pJHA212K binary vector (Yoo et al., 2005).

CPCp::truncatedCPC:GFP
To create plasmids with truncated *CPC* sequences, the *2×rsGFP-NosT* fragment was amplified with TK106/TK107 from *pRTL2-2×rsGFP* (Crawford and Zambryski, 2000) as a template, and cloned into the pT7 blue T-vector (Novagen). *Eco*RV and *Xba*I fragments of *2×rsGFP-NosT* were cloned into pBluescriptII SK+ (*pBS-2×rsGFP-NosT*). The *CPC* promoter region was amplified with TK100/TK101 using Pyrobest DNA polymerase (Takara, Japan), and was cloned into pT7 (*pT-CPCp*). *pT-CPCp* was digested with *Sph*I, blunt-end filled with T4 DNA polymerase (Takara, Japan) and re-digested with *Sma*I. After pBluescriptII had been digested with *Sac*I and *Eco*RV, and treated with T4 DNA polymerase to abolish the *Sac*I site, the *CPCp* fragment was cloned into this modified pBluescriptII (*pBS-CPCp*). All truncated *CPC*-coding regions were amplified by PCR with the following primers: TK102/TK103 for NMG, TK104/TK103 for MG, TK102/TK128 for NG and TK 104/TK201 for MCG. TK102 was used as an upstream primer for the PCR-amplification of constructs of the truncated NM region. Downstream primers were TK186 for 1-79G, TK180 for 1-75G, TK181 for 1-65G, TK182 for 1-55G and TK183 for 1-45G. For additional plasmids containing the truncated NM region, the following primers were used: TK184/TK103 for 10-83G, TK185/TK103 for 21-83G and TK184/TK182 for 10-55G. Amplified, truncated *CPC*-coding regions were cloned into pT7, and digested with *Sac*I and *Eco*RV. These fragments were cloned into the *Sac*I and *Eco*RV sites of *pBS-CPCp* (*pBS-CPCp-trCPC*). A *CPCp-trCPC* fragment was created by digestion with *Sal*I and *Eco*RV, and cloned into *pBS-2×rsGFP-NosT* (*pBS-CPCp-trCPC-GFP-NosT*). Finally, the entire region was cloned into the *Sal*I and *Xba*I sites of the pJHA212K binary vector.

CPCp::substitutedCPC:GFP
For the substitution of specific amino acids in the NM region of CPC, PCR-mediated mutagenesis was carried out on *pBS-CPCp::CPC:GFP-NosT* (Wada et al., 2002) using the QuickChange Site-Directed Mutagenesis Kit (Stratagene), with the primers: TK112/TK113 for K6A:K9A, TK320/TK321 for K79A, TK340/TK341 for W76A, TK342/TK343 for L77V and TK379/TK380 for M78A. Each mutagenized region was cloned into the *Sal*I and *Xba*I sites of pJHA212K.

Ectopic expression controlled by heterologous promoters

EGL3p::CPC:GFP was created by ligating the Klenow fragment blunt-ended *Sal*I and *Bam*HI fragment of the *EGL3* promoter from *pBS-EGL3p* (R.S., K.O. and T.W., unpublished) into the 5′portion (*Cla*I digestion and fill-in by Klenow) of *pBS-CPC-GFP-NosT* (*pBS-EGL3p-CPC-GFP-NosT*). The *Sal*I and *Xba*I fragment from *pBS-EGL3p-CPC-GFP-NosT* was then ligated into pJHA212K.

The *SHORT-ROOT* promoter can be divided into two regions: a 1.8 kb upstream region (SHR-A) and a 0.7 kb downstream region (SHR-B) (Helariutta et al., 2000). These regions were amplified with TK233/TK240 and TK237/TK234, respectively. Amplified fragments were ligated into pT7 (*pT-SHR-A* or *pT-SHR-B*). The *Eco*RV/*Sma*I fragment of *pT-SHR-B* was cloned into *pT-SHR-A* (*pT-SHRp*). The *Sal*I/*Sma*I fragment of *pT-SHRp* was ligated into the *Sal*I/*Eco*RV sites of *pBS-CPC-GFP-NosT* (*pBS-SHRp-CPC-GFP-NosT*). The *Sal*I/*Eco*RV fragment of the *SCR* (*SCARECROW*) promoter from *pJH-SCRp-GFP-NosT* (T.K., K.O. and T.W., unpublished) was replaced by the *Sal*I/*Sma*I fragment of *pT-SHRp*.

CPCp::CPC:tandem GFPs

To construct *CPCp::CPC:tandem GFPs*, three plasmids containing 1×GFP were constructed. 1×GFP was amplified with GFPspacer/multiGFP-r from *pBS-CPCp::CPC:GFP* (Wada et al., 2002), and cloned into the *Eco*RV/*Bam*HI sites of pBluescriptII SK+ (*pBS-1×GFP-A*). Two other 1×GFPs were amplified from *pBS-2xrsGFP-NosT*, with multiGFP-f/multiGFP-r and multiGFP-r/M13R primer pairs, and cloned into the pT7 (*pT7-1×GFP-B*) and *Pst*I/*Bam*HI sites of pBluescriptII SK+ (*pBS-1×GFP-C*). The *Sal*I/*Bam*HI fragment of *pBS-1×GFP-A* was cloned into the *Sal*I/*Bgl*II sites of *pBS-1×GFP-C* (*pBS-2×GFP-NosT*). For 3×GFP, the *Sal*I/*Bam*HI fragment of *pT7-1×GFP-B* was cloned into the *Sal*I/*Bgl*II sites of *pBS-1×GFP-C* (*pBS-2×GFP-NosT-2*), then the *Sal*I/*Bam*HI fragment of *pBS-1×GFP-A* was inserted into the *Sal*I/*Bgl*II sites of *pBS-2×GFP-NosT-2* (*pBS-3×GFP-NosT*). The *Sal*I/*Bam*HI fragment of *pT7-1×GFP-B* was also cloned into the *Sal*I/*Bgl*II sites of *pBS-2×GFP-NosT-2* (*pBS-3×GFP-NosT-2*). The *pBS-4×GFP-NosT* and *pBS-5×GFP-NosT* plasmids were constructed by repeated insertions of the amplified fragment as above. Next, the *Sal*I/*Nco*I fragment of *pBS-CPCp::CPC:GFP* was cloned into the *Sal*I/*Nco*I sites of each *pBS-tandem GFPs-NosT* (*pBS-CPCp::CPC:tandem GFPs-NosT*). Finally, the *Sal*I/*Xba*I fragments of *pBS-CPCp::CPC:tandem GFPs-NosT* were ligated into pJHA212K.

Plant materials, growth conditions

Col-0 or Ws *Arabidopsis* ecotypes were used as wild type. The *35S::CPC* transgenic line and *cpc-1* have been described previously (Wada et al., 1997). The *cpc-2* mutant (KG12704) used in this study was isolated from Kazusa T-DNA lines. *cpc-2* contains a T-DNA insert in the second intron of *CPC*. This mutant was backcrossed to Col-0 twice. Seeds of *rhd3-1* were provided from the Arabidopsis Biological Resource Center at Ohio State University (OH, USA). Lines homozygous for mutations containing the transgene were constructed by crossing mutant and transgenic plants, the phenotype and GFP fluorescence was then examined. Transformation was performed as described previously (Kurata et al., 2003). For the observation of seedlings, plants were grown on agar plates as described by Okada and Shimura (Okada and Shimura, 1990). For the inhibitor experiment, BFA (ICN, BFA stock solution was 50 mM) was added in agar medium at the indicated concentrations.

In situ hybridization

In situ hybridization was as described by Kurata et al. (Kurata et al., 2003). A DIG-labeled antisense RNA probe for *CPC:GFP* was generated by transcribing *Nco*I-digested *pBS-1xrsGFP* (R.S., K.O. and T.W., unpublished) using a T3 polymerase.

Microscopy

Confocal laser scanning microscopy (CLSM)

Roots were stained with 5 µg/ml propidium iodide (PI) for 30 seconds and mounted in water. Confocal images were obtained with a 40× water-immersion objective on a Zeiss LSM-Pascal or a Zeiss LSM-510 Meta confocal laser-scanning microscope using 488 nm laser lines for GFP excitation. Image processing was done with Adobe Photoshop version 7.0 (Adobe Systems, CA, USA).

Light microscopy

For the observation of root hairs and trichomes, images of seedlings were recorded with a VC4500 3D digital fine scope (Omron, Kyoto, Japan). The pattern of epidermal cell types was determined according to the protocol of Lee et al. (Lee et al., 2002), with minor modifications. A light microscope equipped with differential interference contrast (Nomarski) optics was used to determine cell type and relative locations of epidermal cells. The proportions of trichoblasts and atrichoblasts in root epidermis were determined by examining a minimum of ten 5-day-old seedlings from each line. An epidermal cell was counted as a trichoblast if any protrusion was visible, regardless of its length.

RT-PCR

RNA extraction and semi-quantitative RT-PCR reaction were as described by Kurata et al. (Kurata et al., 2003). *CPC, CPC:GFP* and *HA:CPC* fragments were amplified with RT128/TK629, RT128/TK628 and HA-F/RT-129 primer pairs. *EF1α* was amplified as described by Kurata et al. (Kurata et al., 2003).

Antibody preparation

The GST-CPC protein was prepared as described previously (Wada et al., 2002). The His-CPC fusion was expressed using pET28a in *Escherichia coli* BL21 (DE3). Soluble His-CPC was purified with Ni-NTA agarose (Quiagen). Purified GST-CPC protein was injected into rabbits, and the antisera were affinity purified with coupled His-CPC (BioGate Company Limited, Gifu, Japan). In parallel, we extracted total soluble protein from *cpc-2* mutant seedlings and immobilized it in a HiTrap NHS-activated HP column (Amersham Biosciences). The affinity-purified fraction was further absorbed to the *cpc-2* protein gel for 7 hours at 4°C. The final unbound fraction was used for western blotting.

Western blot analysis

Total plant protein was extracted from 7-day-old seedlings with a protein extraction buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1% (w/v) Triton X-100, 1 mM PMSF, 50 µM Leupeptin and 11 mM 2-mercaptoethanol. Crude protein was separated by SDS-PAGE with SuperSep gradient gel (Wako Pure Chemical Industries, Osaka, Japan) and Prestained Protein Marker (New England BioLabs). Samples were transferred to nitrocellulose membranes by wet-electroblotting, and checked by Ponceau S staining. The blotted membrane was incubated with rabbit anti-CPC primary antibody (1:2000). Incubation with secondary HRP-conjugated anti-rabbit antibody and detection were according to the manufacturer's protocol (ECL Plus, Amersham Biosciences).

Immunohistochemistry

Immunohistochemistry was performed according to Nakajima et al. (Nakajima et al., 2001), with minor modifications. Five-day-old seedlings were fixed in 4% (w/v) paraformaldehyde in PBS [10 mM sodium phosphate (pH 7.5), 130 mM NaCl] overnight at 4°C, washed with PBS and passed through an ethanol series. Samples were cleaned with Histo-clear II (National Diagnostics, GA, USA), and embedded in Histologie (MERCK, Darmstadt, Germany). Sections (9 µm) were cut with a microtome (Leica RM2165, Nubloh, Germany) and placed on MAS-coated slides (MATSUNAMI GLASS IND, Osaka, Japan).

After deparaffinization and rehydration, the sections were treated with 2N HCl for 5 minutes and 300 μg/ml pronase (Roche) for 10 minutes at 37°C. Treatment with pronase was stopped by incubation in glycine-TBS [TBS: 25 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2.68 mM KCl] and TBS wash. After blocking with TBST [TBS plus 0.05% (w/v) Tween 20] containing 1% (w/v) bovine serum albumin and 2% (v/v) goat serum for 5 hours at room temperature, the sections were incubated with anti-HA antibodies (Clone 3F10, Roche) at a 1:200 dilution in the blocking solution overnight at room temperature. Slides were washed six times with TBST for 10 minutes and incubated with alkaline phosphatase-conjugated anti-rat IgG (1:500, Zymed, CA, USA) for 2 hours at room temperature. After washing four times in TBST and twice in TBS, the signal was developed with NBT/BCIP solution (Western Blue, Promega) with 1 mM levamisole for 4 hours at room temperature.

Results

The CPC:GFP fusion protein moves from atrichoblasts to trichoblasts

In wild-type roots, root hair cells and hairless cells are arranged in alternating files in the root epidermis (Wada et al., 2002). Transgenic plants in which the expression of 2×rsGFP was driven by the *CPC* promoter (*CPCp::GFP*) showed GFP fluorescence exclusively in hairless cell files, and had normal root hairs, as well as trichomes on leaf surfaces (Fig. 1A-C). The 2×rsGFP construct was chosen because 1×GFP could move freely from hairless cells to hair cells in a non-targeted manner (Y.K. and K.O., unpublished) (Crawford and

Fig. 1. Phenotypes and distribution of GFP fluorescence in *CPCp::GFP* (A-C) and *CPCp::CPC:GFP* (D-F) transgenic plants. (A,D) Confocal laser scanning microscopy images of GFP fluorescence (green) and propidium iodide (PI) fluorescence (red) in the root epidermis of 5-day-old seedlings. (A) GFP is restricted to hairless cell files and is absent from the file of root hair cells (asterisk). (D) CPC:GFP has moved from hairless cell files to a hair cell file (asterisk; note CPC:GFP accumulation in the nuclei). The inset in D shows that *CPC:GFP* RNA was localized in hairless cells, as detected by in situ hybridization with an antisense *GFP* probe. (B,E) Distribution of root hairs in the root epidermis of 5-day-old seedlings. *CPCp::CPC:GFP* transgenic plants grow ectopic root hairs in positions normally occupied by hairless cells. (C,F) Phenotype of 8-day-old seedlings. No trichome formation was observed in *CPCp::CPC:GFP* transgenic plants (F). Scale bars: 50 μm in A; 200 μm in B; 1 mm in C.

Zanbryski, 2000). By contrast, the roots of transgenic plants which expressed the CPC:2×rsGFP (CPC:GFP) fusion protein under the control of the *CPC* promoter (*CPCp::CPC:GFP*) exhibited GFP fluorescence in all epidermal cell nuclei, and developed ectopic hair cells in positions where hairless cells would usually develop (Fig. 1D,E). Moreover, *CPCp::CPC:GFP* plants lacked trichomes on the surface of rosette leaves (Fig. 1F). These phenotypes, observed in *CPCp::CPC:GFP* plants, resembled *35S::CPC* overexpression lines (Wada et al., 1997). To exclude the possibility of the movement of *CPC:GFP* mRNA from hairless cells to hair cells, we checked the expression pattern of *CPC:GFP* mRNA by in situ hybridization in *CPCp::CPC:GFP* transgenic plants. *CPC:GFP* mRNA was detected in hairless cells with an antisense *GFP* probe (inset in Fig. 1D); this observation was similar to the wild-type transcripts detected with an antisense *CPC* probe (Wada et al., 2002). We also examined *CPCp::CPC:GFP* transgenes in the *cpc-1* mutant background and observed GFP fluorescence in all epidermal cell nuclei, which was similar to the fluorescence distribution in the wild-type background (see Fig. S1 in the supplementary material). The *cpc-1* plants containing *CPCp::CPC:GFP* showed normal hair formation in the hair-cell files and ectopic root hairs in the hairless-cell files, suggesting that CPC:GFP is functional in the *cpc-1* mutant background (see Fig. S1 in the supplementary material).

To confirm the localization of CPC using immunohistochemistry, we used haemagglutinin A (HA)-tagged CPC. HA-tagged CPC was expressed under the control of the *CPC* promoter in the *cpc-1* mutant (*CPCp::HA:CPC* in *cpc-1*). These transgenic plants showed a complemented phenotype without ectopic root hair formation (Fig. 2B), suggesting that the HA:CPC fusion protein behaved like the endogenous CPC protein. Immunostaining with anti-HA antibody of root transverse sections from *CPCp::HA:CPC* transgenic *cpc-1* plants led to strong signals in the nuclei of
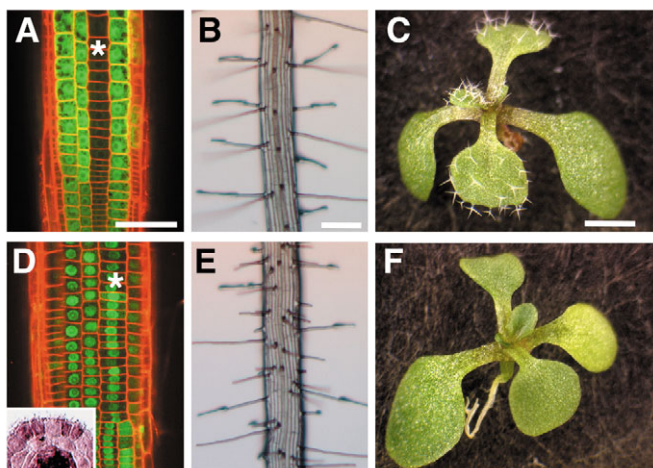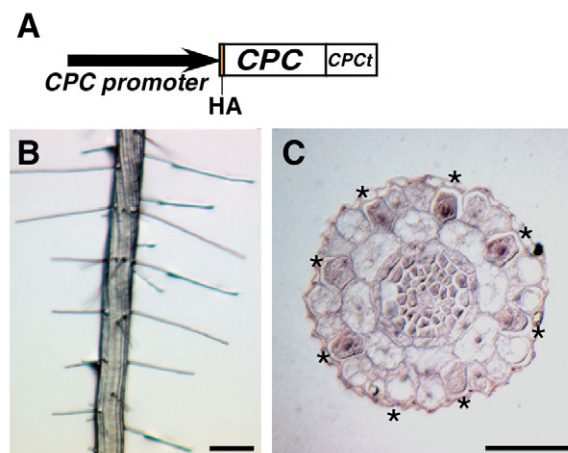

Fig. 2. Immunolocalization of HA-tagged CPC in roots of the *cpc-1* mutant. (A) Schematic representation of the gene construct with the HA-tag at the N terminus of the CPC-coding region (*CPCp::HA:CPC*). (B) Root phenotype. (C) Immunostaining of HA:CPC in a root transverse section. Signals in nuclei of hair cells are stronger than those of non-hair cells. Root hair cells are marked by asterisks. Scale bars: 200 μm in B; 50 μm in C.

root hair cells, but not in the nuclei of hairless cells (Fig. 2C). Detection of CPC protein in the nuclei of root hair cells confirms the results obtained in CPC:GFP fusion plants (Fig. 1D), and supports our model that CPC moves from atrichoblasts to trichoblasts in the root epidermis. However, Fig. 2C shows that the amount of CPC protein in the nuclei of hairless cells falls below detectable levels. This is not consistent with the previous result shown in Fig. 1D.

The apparent disparity in accumulation levels between CPC:GFP and HA:CPC fusion proteins in hairless cells was addressed by western blot with proteins extracted from wild type (Col-0), *CPCp::CPC:GFP* transgenic plants, *CPCp::HA:CPC* transgenic *cpc-1* plants, *35S::CPC* and *cpc-2*. In transgenic plants expressing *CPCp::CPC:GFP*, a predicted band (65 kDa) corresponding to CPC:GFP was detected but there was no corresponding signal for HA:CPC in *CPCp::HA:CPC* transgenic *cpc-1* plants (Fig. 3A). Transgenic plants expressing *35S::CPC* yielded an 11 kDa band, which correlates with the predicted size of CPC. However, we did not detect a band corresponding to CPC in either the wild type (Col-0) or the *cpc-2* mutant (Fig. 3A). Quantitation by RT-PCR with gene-specific primer pairs suggested that there was no significant difference in the amounts of mRNA between *CPCp::CPC:GFP* and *CPCp::HA:CPC* plants, but these transgenic plants had a higher concentration of *CPC:GFP* and *HA:CPC* fusion transcripts than wild type (Col-0) did (Fig. 3B). *35S:CPC* had the highest concentration of *CPC* transcripts, whereas no *CPC* transcripts were detected in the *cpc-2* mutant, suggesting that *cpc-2* was a null mutant (Fig. 3B). These data indicate that the CPC:GFP fusion protein is more stable than the HA:CPC protein is and that it accumulated in hairless cells. Therefore, a small amount of HA:CPC, and presumably also endogenous CPC, could be effectively transported into root hair cells from hairless cells. However, we used the CPC:GFP fusion for qualitative analyses of the cell-to-cell movement of CPC, as GFP fusion proteins can be conveniently and non-invasively monitored, and our focus is on the ability of CPC to move into hair cells. Furthermore, both

CPC:GFP and HA:CPC accumulated in the nuclei of hair cells, implying that the movement activity of CPC:GFP from hairless cells to hair cells is equivalent to that of HA:CPC (and presumably endogenous CPC).

In a series of experiments, we used the wild-type background for the construction of transgenic plants because intact endogenous CPC is required for monitoring the intercellular movement of CPC fusions. If the mutated CPCs fused to GFP, which do not move intercellularly, were to be transformed into the *cpc* mutant background, GFP fluorescence might be observed in all epidermal cell files. Because CPC itself is indispensable for the transcriptional self-repression of CPC in hair cells (Wada et al., 2002), it would be unlikely that the defects of intercellular movement of mutated CPC could be detected.

A region spanning the N-terminal and Myb domains is required and sufficient for the cell-to-cell movement of CPC

To define which regions are required or sufficient for the cell-to-cell movement of CPC, we generated a series of truncated CPC proteins fused to the N terminus of 2×rsGFP and expressed them in transgenic plants. The CPC protein was divided into three non-overlapping domains: the N-terminal (residues 1 to 32), Myb (residues 33 to 83) and C-terminal (residues 84 to 94) domains (Fig. 4A). We made four constructs (Fig. 4A) and analyzed the ability of their expression products to move between cells. Transformant plant lines with NMG, containing the N-terminal and Myb (residues 1 to 83) domains, had GFP fluorescence in all epidermal cell nuclei, similar to the full-length CPC:GFP (Fig. 1D versus Fig. 4B). Lines containing MG, consisting of the Myb region only, had GFP signal mostly restricted to the nuclei of hairless cells (Fig. 4C). Transformants with the third product, NG, with only the N-terminal domain, showed neither the capability for cell-to-cell movement nor nuclear accumulation (Fig. 4D). MCG, containing the Myb and C-terminal (residues 33 to 94) domains, resembled MG: most of the GFP signal was observed in the nuclei of hairless cells (Fig. 4E). These results suggested that the region spanning from the N terminus to the Myb domain functions as a trafficking signal domain of CPC. In addition, the Myb domain appeared likely to contain the nuclear localization signal. To further delimit the CPC regions required for cell-to-cell movement, we constructed a series of NMG plasmids with deletions extending from both the N-terminal and C-terminal ends (Fig. 5A). 1-79G, with only four amino acids deleted from the C terminus of the Myb domain, gave results similar to those of NMG
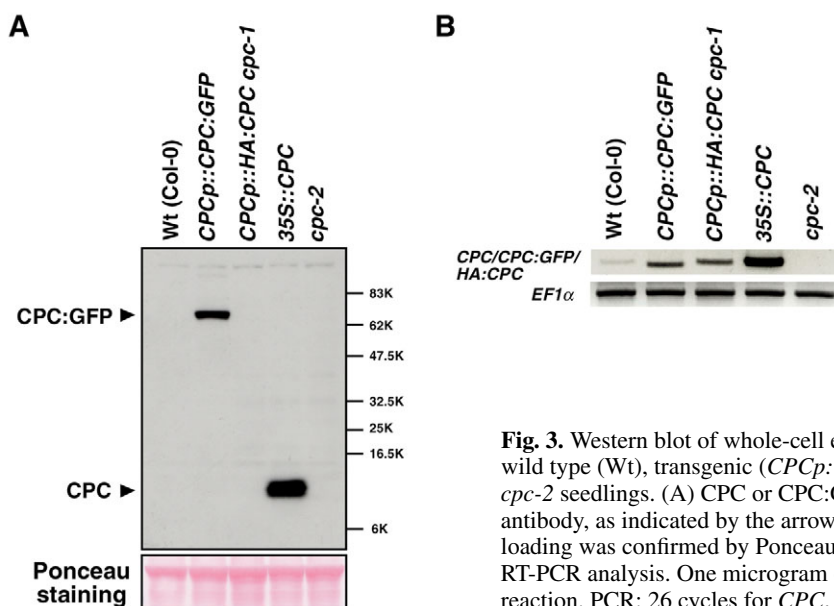

Fig. 3. Western blot of whole-cell extracts and semi-quantitative RT-PCR analysis in Col-0 wild type (Wt), transgenic (*CPCp::CPC:GFP*, *CPCp::HA:CPC cpc-1* and *35S::CPC*) and *cpc-2* seedlings. (A) CPC or CPC:GFP fusion proteins were detected with anti-CPC antibody, as indicated by the arrowheads. Molecular weights are shown on the right. Equal loading was confirmed by Ponceau staining of the blotted membrane. (B) Semi-quantitative RT-PCR analysis. One microgram of total RNA was used for each reverse transcription reaction. PCR: 26 cycles for *CPC*, *CPC:GFP* or *HA:CPC* ; 22 cycles for the *EF1α* control.

Fig. 4. Localization of truncated CPC in cells of the root epidermis. (A) Schematic representation of CPC, consisting of three non-overlapping domains (N, N-terminal domain; Myb, Myb domain; C, C-terminal domain), and the four truncated forms of the CPC:GFP fusion protein. At the bottom, the construct consisting of the CPC promoter, a truncated CPC and 2×rsGFP is shown. (B-E) CLSM images showing GFP fluorescence (green) and PI fluorescence (red) in the root epidermis of 5-day-old seedlings. (B,D) NMG moved from hairless cells to hair cells (hair cell file is highlighted by an asterisk), whereas NG did not. (C,E) Reduced movement of MG and MCG was indicated by the patchy occurrence of GFP fluorescence in hair cells. NMG, MG and MCG accumulated in the nuclei (B,C,E), while NG was dispersed throughout the hairless cells (D). Scale bar: 50 μm.
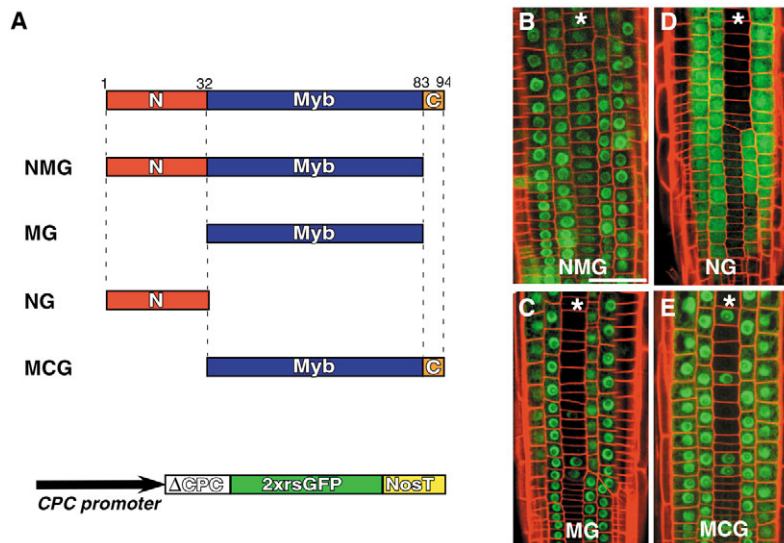
(Fig. 5B). By contrast, 1-75G transgenic plants had GFP fluorescence only in hairless cell files and lacked nuclear fluorescence (Fig. 5C). Thus, the four amino acids (WLMK) deleted in 1-75G are crucial for cell-to-cell movement and may also be required for a nuclear localization of CPC. Transgenic lines harboring *CPC* constructs with more extensive C-terminal deletions than 1-75G (i.e. 1-65G, 1-55G and 1-45G;

Fig. 5A) had GFP signals similar to the 1-75G transgenic plants (data not shown).

Plasmid 10-83G, from which nine amino acids had been deleted at the N terminus of CPC (Fig. 5A), gave GFP fluorescence in most nuclei of hairless cell files (Fig. 5D), suggesting that the truncated fusion protein had lost intercellular movement ability. Similar results were obtained with plasmid 21-83G, in which the N terminus had been further shortened (data not shown).

From these deletion analyses, we concluded that CPC carries a signal domain (residues 1 to 79), which is necessary and sufficient for cell-to-cell movement, and which is made up of the whole N terminus and a part of the Myb domain (Fig. 5E). We tentatively named the two regions required for CPC movement S1 (sequence 1) and S2.

Identification of active sites for cell-to-cell movement of CPC:GFP

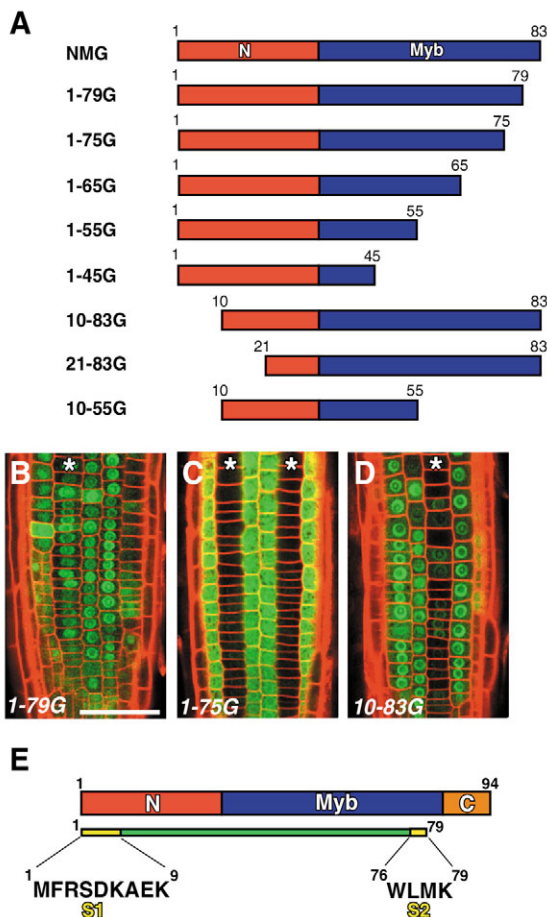To identify crucial amino acid(s) in the S1 and S2 regions, we

Fig. 5. Identification of a signal domain that is necessary and sufficient for inter- and/or intracellular movement of the protein. (A) Diagram of the deletion mutants of CPC. The N-terminal domain is shown in red, the Myb domain in blue. (B-D) CLSM images showing GFP fluorescence (green) and PI fluorescence (red) in the root epidermis of 5-day-old seedlings. (B) Like full-length CPC fused to GFP, 1-79G moved from hairless cells to root hair cells (file of hair cells is indicated by an asterisk) and accumulated in the nuclei of both cell types. (C) In contrast to 1-79G, 1-75G remained distributed throughout the cytoplasm of the hairless cells. (D) A partial defect in CPC movement in 10-83G was indicated by the occurrence of the GFP signal only in a fraction of hair cells, with 1-79G and 10-83G accumulated in nuclei. (E) Diagrammatic representation of a signal domain (residues 1 to 79, as indicated by the yellow and green bar) that is necessary and sufficient for CPC cell-to-cell movement. The domain, amino acid residues 1 to 79, contains the N terminus and a part of the Myb domain. The regions whose deletions conferred defects in the intercellular movement of CPC:GFP were tentatively designated S1 and S2 (as indicated by the yellow bars). As the behavior of 1-75G shows (C), S2 also is required for the nuclear accumulation of CPC. Scale bar: 50 μm.
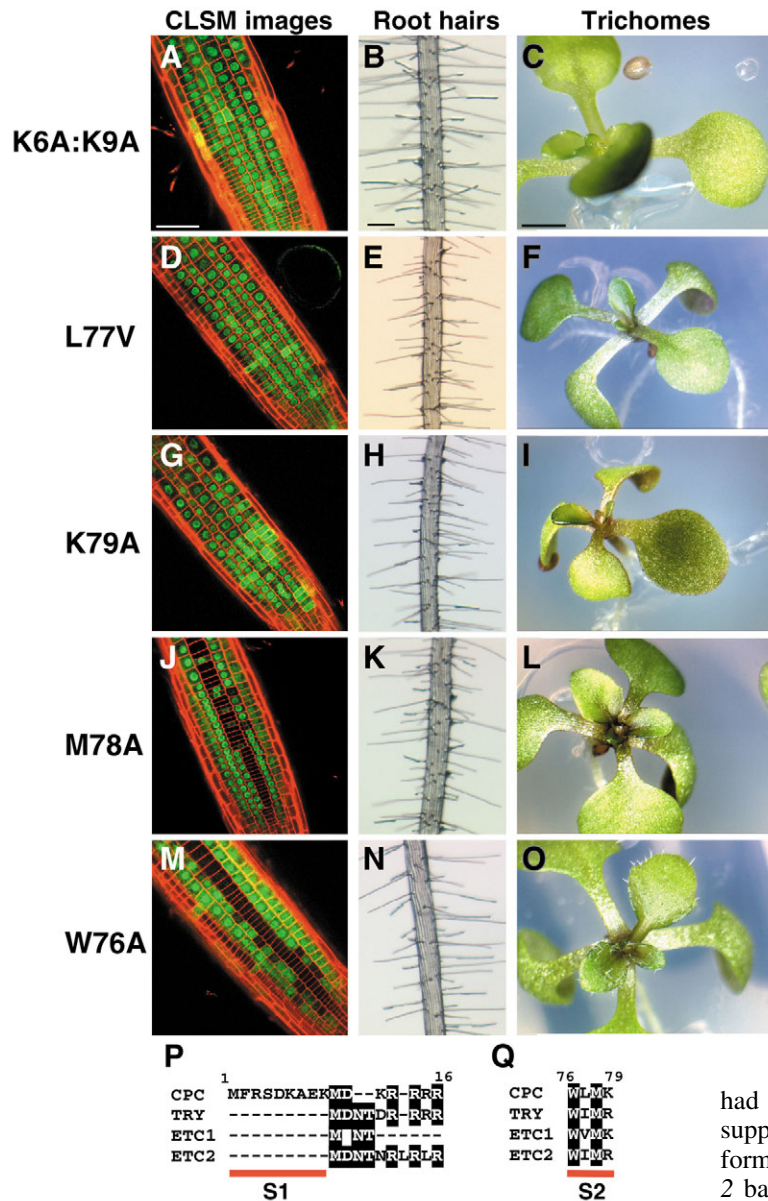
Development

Fig. 6. Localization of amino acid substitution mutants of CPC in roots, and phenotypes of the mutant plants. (A,D,G,J,M) CLSM images of GFP fluorescence (green) and PI fluorescence (red) in the root epidermis of 5-day-old seedlings. (B,E,H,K,N) Root hair phenotypes of 5-day-old seedlings. (C,F,I,L,O) Trichome phenotypes of 9-day-old seedlings. K6A:K9A, L77V and K79A moved from hairless cells to hair cells, and accumulated in the nuclei of both cell types (A,D,G). The plants developed ectopic root hairs (B,E,H) but no trichomes, thus resembling lines expressing CPC:GFP (C,F,I). The capability for cell-to-cell movement was reduced in M78A, but it accumulated in the nuclei (J). M78A formed ectopic root hairs but no trichomes (K,L). The W76A form of the protein did not move from hairless cells to root hair cells, and GFP-derived fluorescence was dispersed throughout the cells (M). Normal root hair formation and trichome formation were observed in this mutant (N,O). (P,Q) Alignment of the N termini, and of the C-terminal regions of the Myb domains, respectively, of CPC and homologous small Myb proteins. Small Myb protein sequences were compiled from Schellmann et al. (Schellmann et al., 2002) (TRY) and Kirik et al. (Kirik et al., 2004a; Kirik et al., 2004b) (ETC1 and ETC2). The S1 region is unique to CPC, whereas W76 and M78 in S2 are conserved in all small Myb proteins. Scale bars: 50 μm in A; 200 μm in B; 1 mm in C.

W76A and M78A (Fig. 6J,M; for a quantitative assessment see Table 1). In the W76A mutant, GFP fluorescence appeared to be dispersed in the cytoplasm (Fig. 6M). These results suggested that W76 was crucial for both cell-to-cell movement and nuclear accumulation. The fact that W76A had an almost normal root hair formation and a partial inhibition of trichome development (Fig. 6N,O; Table 2) is consistent with this interpretation. Furthermore, W76A in the *cpc-2* mutant background had a reduced number of hair cells (see Fig. S2 in the supplementary material), suggesting that the W76A mutant form was not functional. The GFP signal of W76A in the *cpc-2* background was observed to be dispersed in all epidermal cells, suggesting that the movement-defective W76A mutant form could not repress its own expression in hair cells in a *cpc-2* background, as there was no endogenous CPC present, and repression of CPC in hair cells did not occur. In M78A mutants, the cell-to-cell movement of CPC was defective but nuclear accumulation remained unaffected (Fig. 6J,L). The proportion

conducted site-directed mutagenesis. Alanine substitutions in both K6 and K9 (K6A:K9A) showed no difference in the distribution of GFP fluorescence when compared to the *CPCp::CPC:GFP* transgenic line (Fig. 6A). The K6A:K9A mutant transgenic line developed ectopic root hairs but no trichomes on leaves (Fig. 6B,C), suggesting that K6 and K9 are not required for cell-to-cell movement of CPC. We also structured mutants with alanine substitutions beyond the C-terminal border of S1 in the N-terminal domain of CPC (K12A:R13A, R14A:R15A, R15A:R16A and K19A:K21A), but no effects on CPC:GFP movement were observed (data not shown).

Three alanine substitutions (W76A, M78A, and K79A) and one valine substitution (L77V) were introduced into the S2 region. K79A and L77V mutants showed distributions of GFP fluorescence and morphological phenotypes like those of the *CPCp::CPC:GFP* line (Fig. 6D-I). Conversely, the GFP signal was mostly restricted to files of hairless cells in the mutants

Table 1. CPC:GFP localization in developing root epidermis

Genotype	GFP-expressing cells in a trichoblast position (%)	GFP-expressing cells in an atrichoblast position (%)
CPCp::CPC:GFP	100±0	100±0
CPCp::GFP	0±0	100±0
W76A-1	1.3±1.5	95.1±4.0
W76A-2	0±0	91.3±5.5
W76A-3	0±0	88.0±7.2
M78A-1	14.4±15.7	99.1±1.1
M78A-9	14.3±12.4	98.7±2.3

5-day-old seedlings; *n*=3 or 4. Values represent means±s.d.

Table 2. Specification of cell types in root epidermis

Genotype	Hair cells in epidermis (%)	Root hair-cell position		Hairless-cell position	
		Hair cells (%)	Hairless cells (%)	Hair cells (%)	Hairless cells (%)
Wild type (Col-0)	50.0±6.7	93.4±9.5	6.7±9.5	0±0	100±0
CPCp::CPC:GFP	90.6±12	98.7±4.2	1.3±4.2	84.8±16.6	15.2±16.6
CPCp::GFP	48.8±9.1	94.7±8.2	5.3±8.2	2.1±4.5	99.0±2.6
W76A-3	51.2±4.7	100±0	0±0	7.1±7.5	92.9±7.4
M78A-1	78.7±12.7	97.8±4.9	2.2±4.9	61.9±24.2	38.1±24.1

A minimum of 10 5-day-old seedlings were examined for each strain. Values represent means±s.d.

of GFP-expressing cells in root hair-cell positions was higher in M78A than in W76A (Table1). For M78A, we examined the effects on phenotype in the *cpc-2* mutant background. M78A
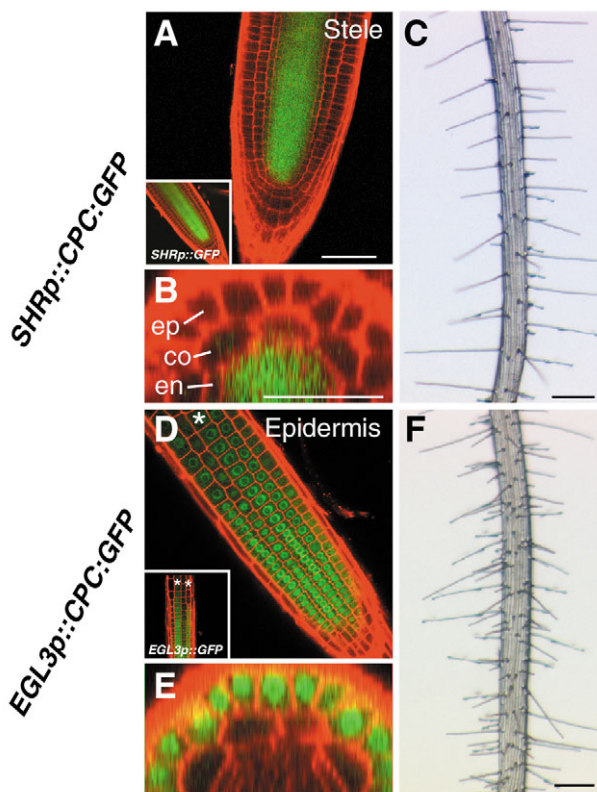

Fig. 7. Tissue distribution of the CPC:GFP fusion protein expressed under the control of heterologous promoters. The figure shows longitudinal (A,D) and transverse (B,E) CLSM images with GFP (green) and PI fluorescence (red) in *SHRp::CPC:GFP* (A,B) and *EGL3p::CPC:GFP* (D,E) transgenic plants at 5 days post germination, and the corresponding root hair phenotypes (C,F). (A) Images are focused on the stele. When *CPC:GFP* is controlled by the stele-specific *SHR* promoter, the GFP signal is restricted to stele cells, where it is distributed throughout the cytoplasm (A). No signal was detected in the root cortex or the epidermis (B). No ectopic root hairs were observed in these plants (C). By contrast, GFP fluorescence was restricted to the epidermis when *CPC:GFP* expression was controlled by the hair cell-specific *EGL3* promoter (D,E). The signal occurred in all epidermal cells and accumulated in nuclei. Root hairs were formed ectopically in these plants (F). Insets in A,D show the distribution of GFP fluorescence in *SHRp::GFP* and *EGL3p::GFP* control plants, respectively. The asterisk in D highlights a hair-cell file. ep, epidermis; co, cortex; en, endodermis. Scale bars: 50 μm in A,B; 200 μm in C,F.

in the *cpc-2* background produced hair formation in the hair-cell and hairless-cell files, and ectopic root hairs, as observed with wild-type CPC (see the Fig. S2 in the supplementary material). M78A in the *cpc-2* background also produced GFP fluorescence in all epidermal cell nuclei for the same reason as W76A; that is, the movement-defective M78A form could not repress CPC expression in hair cells.

Mode of cell-to-cell movement of CPC:GFP in root stele and epidermis

To examine the possibility of cell-to-cell movement of CPC in tissues other than the epidermis, we expressed CPC:GFP using the promoter of the *SHORT-ROOT* (*SHR*) gene. Control plants (*SHRp::GFP*) expressed GFP fluorescence preferentially in the stele (inset in Fig. 7A). This result is consistent with a previous study (Helariutta et al., 2000). Practically the same results were obtained from plants expressing *SHRp::CPC:GFP* (Fig. 7A,B), suggesting that the CPC:GFP fusion protein did not selectively move out of the stele. In contrast to epidermal cells, CPC:GFP did not specifically accumulate in the nuclei of stele cells (Fig. 7A,B). We confirmed the expression of the CPC:GFP fusion protein by western blot analysis in *SHRp::CPC:GFP* lines (data not shown). CPC:GFP expressed in stele cells had no effect on root hair formation. These results suggest that nuclear accumulation of CPC may be required for its action on root hair development, or that CPC functions with unidentified auxiliary factors that are not present in the stele because CPC has to be in the epidermis during root hair formation.

ENHANCER OF GL3 (*EGL3*), a bHLH-type transcriptional factor gene, has been characterized in several laboratories, including our own (Zhang et al., 2003; Bernhardt et al., 2003) (R.S., K.O. and T.W., unpublished). The *EGL3* promoter is active in the root epidermis, preferentially in hair cells (R.S. et al., unpublished). In plants carrying an *EGL3p::CPC:GFP* transgene, the fusion protein was localized in the nuclei of all epidermal cells (Fig. 7D,E). Control plants expressing *EGL3p:GFP* showed GFP fluorescence predominantly in hair cells (insets in Fig. 7D), suggesting that CPC:GFP expressed under the control of *EGL3* promoter moved from the root hair cells to hairless cells. The protein is also functional in controlling root hair development, as *EGL3p::CPC:GFP* transgenic plants grew ectopic root hairs (Fig. 7F). The differences in the behavior of CPC:GFP expressed either in the stele or the epidermis suggested that epidermis-specific components were required for cell-to-cell movement of CPC and also for its nuclear accumulation.

We also expressed the CPC:GFP in another ground tissue, the endodermis, with the endodermis-specific *SCARECROW* (*SCR*) promoter (Di Laurenzio et al., 1996). When 2×GFP

were expressed under the *SCR* promoter as a control, it moved from the endodermis to the epidermis via the cortex, suggesting that 2×GFP may be passively transported in these layers (data not shown).

Inhibition of a secretion pathway does not affect CPC:GFP movement

To examine whether the classical secretion process is involved in the movement of CPC, we performed two types of experiment. First, brefeldin A (BFA) blocks the secretory pathway from the endoplasmic reticulum (ER) to the plasma membrane by inhibiting vesicle formation at the Golgi apparatus (Ritzenthaler et al., 2002; Geldner et al., 2003). Seedlings treated with BFA had phenotypes similar to plants treated with an auxin-transport inhibitor, such as a reduction in root and hypocotyl gravitropism and elongation, as well as lateral root initiation (data not shown) (Geldner et al., 2001). CPC:GFP localization was not affected in *CPCp::CPC:GFP* transgenic plants treated with 10 μM BFA (Fig. 8A,B), whereas the aberrant phenotypes described above were observed (data not shown). This result strongly suggests that the BFA-sensitive secretion pathway is not involved in the movement of CPC:GFP.

Second, the *rhd3-1* mutant allele was originally recognized and isolated by its phenotype of short wavy root hairs (Schiefelbein and Somerville, 1990), and Zheng et al. (Zheng et al., 2004) reported that this mutant has a defect in the secretion of proteins to the extracellular space. We tested the movement of CPC:GFP in the *rhd3-1* mutant. As shown in Fig. 8E, CPC:GFP was found in the nuclei of both hairless cells and root hair cells, indicating that the mutation does not affect the

Fig. 8. Effects of BFA and the *rhd3* mutation on CPC:GFP movement. (A,B,E) CLSM images of GFP (A,B,E) and PI (E) fluorescence in root epidermis of 5-day-old seedlings grown on media supplemented with (A) 0 μM BFA or (B)10 μM BFA. (C,D) Root hair phenotypes of (C) *rhd3-1* and (D) *rhd3-1 CPCp::CPC:GFP* 5-day-old seedlings. (E) CLSM image of the root epidermis of D. Asterisks in A, B and E indicate hair-cell files. Scale bars: 50 μm in A; 200 μm in C; 50 μm in E.

movement of CPC. Root hairs of the *CPCp::CPC:GFP* transgenic plant had short, wavy root-hairs on both root hair cell and hairless cell files (Fig. 8D), whereas the *rhd3-1* mutant had short, wavy root hairs only on root hair cell files (Fig. 8C). This result is consistent with the model proposing that CPC is moved from hairless cells to neighboring hair cells in the *rhd3-1* mutant. These two lines of evidence strongly suggest that Golgi-mediated secretion is not a mechanism of controlling the intercellular movement of CPC.

Increase of SEL due to CPC

If CPC protein moves via plasmodesmata, its movement, like other proteins or complex structures, is likely to have a size exclusion limit (SEL) (Zambryski and Crawford, 2000). To investigate the effect of the SEL on CPC movement, the movement abilities of CPC fused to tandem GFPs, 2×, 3×, 4× and 5×GFP, were compared. CPC:tandem-GFPs were expressed under the control of the CPC promoter. As a control, we examined the movement ability of free 1×GFP and 2×GFP under the expression control of the CPC promoter; *CPCp::1×GFP* and *CPCp::2×GFP*. Free 2×GFP could not move out of hairless cells but 1×GFP moved freely between epidermal cells (see, for example Fig. 1A; data not shown). This result suggests that the SEL of plasmodesmata under these conditions is between 27 kDa (M_r of 1×GFP) and 54 kDa (M_r of 2×GFP) in the meristematic zone of root epidermis. In contrast to free GFP multimers, CPC increased the SEL of plasmodesmata for CPC:GFP multimer fusions. Transgenic plants expressing CPC:2×GFP had GFP fluorescence in all epidermal cell nuclei (Fig. 9A). For CPC:3×GFP, the nuclei of all epidermal cells showed GFP fluorescence and partially reduced nuclear localization, as some GFP fluorescence was dispersed in the cell (Fig. 9B). Larger multimers of GFP fused to CPC reduced the GFP signal in hair cells and nuclear-specific localization (Fig. 9C,D). These data demonstrated that the SEL of plasmodesmata specifically increases to around 119 kDa (M_r of CPC:4×GFP) when CPC fused to GFPs. Furthermore, intercellular movement of CPC:GFP is apparently coupled with intracellular movement, resulting in nuclear localization.

Discussion

A domain that is necessary and sufficient for the cell-to-cell movement of CPC

Although several transcription factors such as CPC have the ability to move from cell-to-cell in plants (Ruiz-Medrano et al., 2004), little is known about the mechanisms controlling these movements. To identify sequences required or sufficient for the cell-to-cell movement of CPC, we created a series of truncated proteins. Similar experiments have been conducted to elucidate the systemic movement of plant viruses. The movement protein (MP) of the tobacco mosaic virus (TMV) provides the best-characterized mechanism by which plant viruses move between cells. Using MP and fluorescent dextrans, the viral RNA-binding domain of TMV-MP was identified as a necessary component for cell-to-cell movement (Waigmann et al., 1994). Using a different approach, a series of deletions in the MP gene was tested for the ability to mediate the intercellular movement of TMV. Three N-terminal amino acids (residues 3 to 5) were found to be essential for transport in
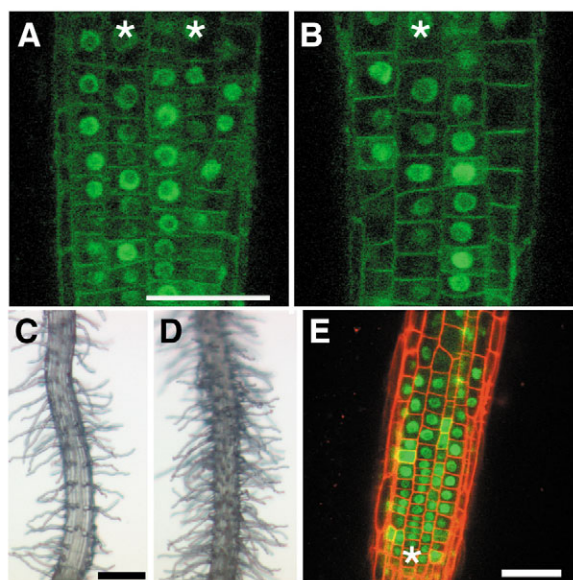
Fig. 9. Inter- and intracellular movement of CPC fused to tandem GFPmers. (A-D) CLSM images of GFP fluorescence (green) and propidium iodide (PI) fluorescence (red) in the root epidermis of 5-day-old seedlings. The *CPC* promoter-driven CPC was fused to 2×GFP (A), 3×GFP (B), 4×GFP (C) and 5×GFP (D). GFP fluorescence was observed in epidermal cell nuclei with 2×GFP or 3×GFP fusions (A,B), but patchy GFP fluorescence in hair cells was observed in CPC:4×GFP and CPC:5×GFP lines (C,D). CPC fused to greater than 3×GFP reduced the nuclear-specific localization (C,D).
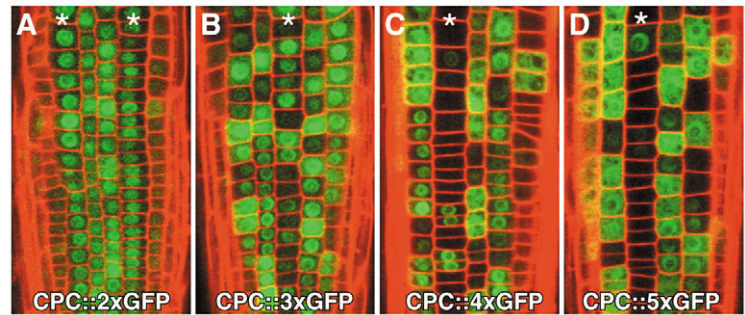
tobacco cells (Kotlizky et al., 2001). However, it remained unclear whether these three amino acids were the only ones necessary for the cell-to-cell movement of TMV. In contrast to the intracellular transport of proteins in and out of organelles, which is usually mediated by simple, short signal sequences, large sequence regions of MP may be necessary for the movement of TMV between cells. Another class of plant protein, including heat-shock cognate 70 chaperones and thioredoxin h, also moves from cell-to-cell, and motifs required for this movement were investigated, but no common conserved/similar amino-acid sequences have been identified, indicating that non-cell autonomous proteins have no consensus sequence among them for moving intercellularly (Aoki et al., 2002; Ishiwatari et al., 1998).

In the present study, we identified a domain that is necessary and sufficient for CPC cell-to-cell movement. The S1 region in the N-terminal domain whose truncation resulted in the inability of CPC to move consists of nine residues and is unique in the family of R3-type small Myb-like proteins. The other region, S2, in the Myb domain, which is also required for CPC movement, consists of four amino acids (residues 76 to 79). Both the S1 and S2 region required for CPC movement possess no significant homology to the amino acid sequences of known non-cell-autonomous proteins (NCAPs). Deletion of the S2 region abolished the nuclear accumulation of CPC. This is an interesting point, because the same region is thus apparently required in inter- and intracellular movement. This S2 region is neither rich in basic amino acids nor does it resemble a typical bipartite nuclear localization signal sequence (NLS) (Kaffman and O'Shea, 1999), suggesting that it functions in an unknown type of nuclear import system. S2 overlaps with a region that is required for protein-protein interactions between CPC and R (residues 33 to 83) (Wada et al., 2002), a bHLH-type transcription factor from maize that affects root epidermal cell differentiation in *Arabidopsis* (Lloyd et al., 1992). Thus, the S2 region in the Myb domain functions as a part of multifunctional motif with roles in intercellular movement, nuclear localization (i.e. intracellular movement) and protein-protein interactions. A similar situation in which functional motifs overlap has been reported in the tobacco mitogen-activated protein kinase kinase kinase, NPK1, in which regions that function as a NLS and in protein-protein interactions are partly identical (Ishikawa et al., 2002). The crucial amino acids in the S2 region, W76 and M78, were identified by site-directed mutagenesis. Both residues are conserved in R3-type small Myb-like proteins (Fig. 6P). Thus, these conserved amino acids may be important for CPC movement. To clarify whether the two truncated sequences, S1

and S2, in the N-terminal and Myb domains are sufficient for cell-to-cell movement of CPC, we created a chimeric construct with a *CPC* promoter-driven gene in which *2×rsGFP* was sandwiched between S1 and S2 (*CPCp::S1:2×rsGFP:S2*). However, in the *CPCp::S1:2×rsGFP:S2* transgenic plants, GFP fluorescence did not move from the hairless cells to hair cells (data not shown). These results indicate that the specific conformation of the native CPC protein is important for its cell-to-cell transport. An analysis of the three-dimensional structure of CPC will help to understand its intercellular movement structural requirements. Kim et al. (Kim et al., 2005) recently reported that the relatively large KNOX homeodomain (residues 256 to 326) is required and sufficient for KN1 movement. There is no detectable sequence homology/similarity between the domains of the two proteins (CPC versus KN1). Therefore, comparative analyses of the three dimensional structures of CPC and KN1 will help to understand the role of KNOX or other domains in cell-to-cell movement.

W76 is crucial not only for cell-to-cell movement but also for intracellular movement to nuclei. In addition, increasing the number of GFP in CPC:GFP fusions also affects both inter- and intracellular movement. Nuclear accumulation and intercellular movement of proteins depend on membrane pores, nuclear pores or plasmodesmata (Lee et al., 2000), and it is tempting to speculate that some structural elements of CPC may play similar roles in its transport through the nuclear membrane and plasmodesmata. Some other factor(s) may also have a functional similarity for transporting proteins through nuclear membranes and plasmodesmata. In the case of KN1 movement, a movement-defective mutation (M6) overlapped with a potential nuclear localization signal (Lucas et al., 1995). Coupling nuclear accumulation with the intercellular movement of protein has been also observed in the case of SHR. The *shr-5* mutant allele, which converts threonine 289 into isoleucine (T289I), showed defects in both nuclear accumulation and intercellular movement (Gallagher et al., 2004).

Plasmodesmata-mediated cell-to-cell movement of proteins may proceed by either of two modes: non-targeted movement by simple diffusion, or targeted and regulated transport. In the first case, soluble proteins can cross plasmodesmata if the SEL is greater than the actual size of the protein molecule (Zambryski and Crawford, 2000). Free GFP and LFY move by this mechanism (Crawford and Zambryski, 2000; Wu et al., 2003), but CPC:GFP does not. Rather, it has to be transported by a targeted movement that requires dilation of the plasmodesmata. This process is probably mediated by specific interactions

between the transported protein and components of the plasmodesmata (Haywood et al., 2002). Thus, some specific factor must interact with CPC during targeted movement in the root epidermis. W76A transgenic plants in the *cpc-2* background showed reduced numbers of root hairs; the same phenotype as *cpc-2*. The reason for the failure of complementation with W76A mutants may be the defective nuclear accumulation or reduced repressor activity of CPC in transcriptional regulation. By contrast, the substitution M78A only reduced the cell-to-cell movement of CPC, suggesting that M78 is specifically involved in the intercellular movement of CPC.

Tissue-specific regulation of cell-to-cell movement of CPC

The SHR:GFP fusion protein expressed under control of the stele-specific *SHR* promoter moved from the stele to the adjacent endodermis (Nakajima et al., 2001). When the expression of the CPC:GFP fusion protein was driven by the *SHR* promoter, CPC:GFP was not able to leave the stele. Apparently, factors required for CPC movement are absent from stele cells. As CPC:GFP expressed in the stele also did not accumulate in the nuclei, CPC apparently requires tissue-specific factors for nuclear import. Although the CPC:GFP fusion protein expressed under the control of the hairless-cell-specific *CPC* promoter moved from hairless cells to root hair cells, the direction of movement was reversed when expression was limited to hair cells by the hair cell-specific *EGL3* promoter. Thus, CPC can move through the epidermal layer, implying that the specific factors required for CPC movement are present in the epidermis as a whole. Similar tissue-specificity in the regulation of protein movement has been observed with SHR and KN1. When SHR:GFP is expressed in phloem companion cells by the tissue-specific *SUC2* (sucrose-H$^+$ symporter gene) promoter, it is unable to exit from the companion cells. SHR:GFP also remains trapped in root epidermal cells when expressed there through specific promoters (Sena et al., 2004). In the case of KN1, movement is directional depending on the developmental state of the tissue. In leaves, the GFP:KN1 fusion protein was able to move from mesophyll cells to epidermal cells, but not in the opposite direction. In the shoot apical meristem, however, GFP:KN1 can move from epidermal (L1) to inner layers (L2, L3) (Kim et al., 2003). Such developmentally regulated competency for protein movement probably depends on the quantity or quality of tissue-specific factors required to support movement.

Plasmodesmata-mediated movement of CPC

Based on what is currently known about the transport of transcriptional regulatory proteins, there are two possible modes of intercellular movement of CPC; one is via plasmodesmata-mediated trafficking, the other is secretion-internalization-dependent apoplastic transport, such as the transport of homeodomain proteins in animal cell-cultured systems (ex vivo) (Prochiantz and Joliot, 2003).

Localized accumulations of GFP:KN1 have been observed in the periphery of epidermal cells expressing the protein (Kim et al., 2003). These foci appeared to co-localize with plasmodesmata. We could not detect similar focal accumulations in cells expressing CPC:GFP. However, it still appears likely that CPC travels through plasmodesmata, as its movement was not inhibited by BFA nor by the *rhd3* mutation.

Furthermore, CPC increased the SEL between root epidermal cells, and does not have the putative signal sequences commonly observed in secretory proteins, supporting the conclusion that CPC moves from cell to cell through plasmodesmata or possibly by some as yet unknown mechanism. Recently, nonclassical protein secretion systems that are not inhibited by BFA have been reported (Nickel, 2003), and Haupt et al. (Haupt et al., 2005) have shown that the membrane trafficking (endocytic) pathway is employed in the intercellular movement of viral movement protein. Thus, further investigation is needed to clarify the involvement of unknown mechanism(s), including the endocytic pathway, on CPC movement.

In conclusion, this study has revealed the complex structural requirements for CPC cell-to-cell movement. A domain containing both the N-terminal domain and a part of Myb domain is required and sufficient to move the CPC:GFP fusion protein. Amino acid substitution experiments defined the critical residues, W76 and M78, for CPC movement, and suggested that CPC moves from atrichoblasts to trichoblasts in a regulated, targeted manner. Furthermore, substitution experiments indicated a coupling of intercellular and intracellular movement to nuclei, as W76 was required for both. We have also demonstrated that tissue-specific factors are involved in the cell-to-cell movement of CPC. These findings open the way for further investigations into the mechanisms and biological functions of CPC movement.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/24/5387/DC1

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