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 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 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 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 CPC genomic fragment with PK383 and PK384 (pB8-CPCp-HA-CPC). The XbaI and SalI fragments of pBS-CPCp-HA-CPC were cloned into the pJHA212K binary vector (Yoo et al., 2005).

To create a plasmid with a gene encoding HA-tagged CPC, a CPC genomic fragment pBS-gCPC included 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 CPC genomic fragment with PK383 and PK384 (pB8-CPCp-HA-CPC). The XbaI and SalI 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 PK106/PK107 from pRTL2-2×rsGFP (Crawford and Zambryski, 2000) as a template, and cloned into the pT7 blue T-vector (Novagen). EcoRV and XbaI fragments of 2×rsGFP-NosT were cloned into pBluescriptII SK+ (pBS-2×rsGFP-NosT). The CPC promoter region was amplified with PK100/PK101 using Pyrobest DNA polymerase (Takara, Japan), and was cloned into pT7 (pT-CPCp). pT-CPCp was digested with SpeI, blunt-end filled with T4 DNA polymerase (Takara, Japan) and re-digested with SmaI. After pBluescriptII had been digested with SacI and EcoRV, and treated with T4 DNA polymerase to abolish the SacI site, the CPC fragment was cloned into this modified pBluescriptII (pBS-CPCp). All truncated CPC-coding regions were amplified by PCR with the following primers: PK102/PK103 for NM, PK104/PK103 for MG, PK102/PK128 for NG and PK104/PK201 for MCG. PK102 was used as an upstream primer for the PCR-amplication of constructs of the truncated NM region. Downstream primers were PK186 for 1-79G, PK180 for 1-75G, PK181 for 1-55G and PK182 for 1-45G. For additional plasmids containing the truncated NM region, the following primers were used: PK184/PK103 for 10-83G, PK185/PK103 for 21-83G and PK184/PK182 for 10-55G. Amplified, truncated CPC-coding regions were cloned into pT7, and digested with SacI and EcoRV. These fragments were cloned into the SacI and EcoRV sites of pBS-CPCp (pBS-CPCp-trCPC). A CPCp-trCPC fragment was created by digestion with SacI and EcoRV, and cloned into pBS-2×rsGFP-NosT (pBS-CPCp-trCPC-GFP-NosT). Finally, the entire region was cloned into the SacI and XbaI 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: PK112/ PK113 for K6A/K9A, PK320/PK321 for K79A, PK340/PK341 for W76A, PK342/PK343 for L77V and PK379/PK380 for M78A. Each mutagenized region was cloned into the SacI and XbaI sites of the pJHA212K.
Ectopic expression controlled by heterologous promoters

**EGL3p::CPC-GFP** was created by ligating the Klenow fragment blunt-ended **SalI** and **BamHI** fragment of the **EGL3** promoter from **pBS-EGL3p** (R.S., K.O. and T.W., unpublished) into the **5'** portion (CiaI digestion and fill-in by Klenow) of **pBS-CPC-GFP-NosT** (**pBS-EGL3p::CPC-GFP-NosT**). The **SalI** and **XbaI** fragment from **pBS-EGL3p::CPC-GFP-NosT** was then ligated into pHA212K.

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**) (Wada et al., 2002). These regions were amplified with **pBS-CPC::CPC-tandem GFPs** (**pBS-CPC::CPC-tandem GFPs**). Amplified fragments were ligated into **pT7** (**pT7-SHR-A** or **pT7-SHR-B**). The EcoRV/Smal fragment of **pT7-SHR-B** was cloned into **pT7-SHR-A** (**pT7-SHRp**). The **SalI/Smal** fragment of **pT7-SHRp** was ligated into the **SalI/EcoRV** sites of **pBS-CPC::CPC-GFP-NosT** (**pBS-SHRp::CPC-GFP-NosT**). The **SalI/EcoRV** fragment of the **SCR** (**SCARECROW** promoter from **pHS-CRPC-GFP-NosT** (**pT7::SCRp-GFP-NosT**)), then the **SalI/Smal** fragment of **pT7-SHRp** was replaced by the **SalI/Smal** fragment of **pT7-SHRp**.

**CPCp::CPC::tandem GFPs**

To construct **CPCp::CPC::tandem GFPs**, three plasmids containing 1×GFP were constructed. 1×GFP was amplified with GFPspacermultigfp-r from **pBS-CPC::CPC::GFP** (Wada et al., 2002), and cloned into the **EcoRV/BamHI** sites of plasmids (**pBS2xrsGFP-NosT**). 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** (**pT7-1GFP** and **pT7-2GFP**). The **SalI/Smal** fragment of **pT7-1GFP** was cloned into the **SalI/VsbI** sites of **pBS-1GFP-NosT** (**pBS-SHRp::CPC-GFP-NosT**). For 3×GFP, the **SalI/BamHI** fragment of **pT7-1GFP-B** was cloned into the **SalI/VsbI** sites of **pBS-1GFP-C** (**pBS-2GFP-NosT**), then the **SalI/BamHI** fragment of **pT7-1GFP-A** was inserted into the **SalI/VsbI** sites of **pBS-2GFP-NosT** (**pBS-3GFP-NosT**). The **SalI/BamHI** fragment of **pT7-1GFP-B** was also cloned into the **SalI/VsbI** sites of **pBS-2GFP-NosT** (**pBS-3GFP-NosT**). The **pBS-4GFP-NosT** and **pBS-5GFP-NosT** plasmids were constructed by repeated insertions of the amplified fragment as above. Next, the **SalI/NcoI** fragment of **pBS-CPC::CPC::GFP** was cloned into the **SalI/NcoI** sites of each **pBS-CPC::GFP** (**pBS-CPC::CPC::GFP**). Finally, the **SalI/XbaI** fragments of **pBS-CPC::CPC::tandem GP** were ligated into pHA212K.

**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**) was studied in this study. The **cpc-2** mutant T-DNA line was backcrossed to Col-0 twice. Seeds of **rdh3-1** 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 examined. The expression 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). 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 *N*coI-digested **pBS-1GFP** (R.S., K.O. and T.W., unpublished) using a T3 polymerase.

**Microscopy**

Confocal laser scanning microscopy (CLSM) was performed with a 10× 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 2400× 3D digital line scope (Optron, Kyoto, Japan). The pattern of epidermal cell types was determined according to the protocol of Kurata et al. (Kurata et al., 2003). Immunofluorescence was performed with 4′,6-diamidino-2-phenylindol (DAPI) (Sigma, D-9542) at 4°C.

**Immunohistochemistry**

Immunohistochemistry was performed according to Nakajima et al. (Nakajima et al., 2001), with minor modifications. The 5-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 (USA) and embedded in Histogel (MERCK, Darmstadt, Germany). Sections (5 μm) were cut with a microtome (Leica RM2165, Nukohl, 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 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 looked 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. 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 mRNA 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.

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.
Development

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 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.
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
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

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

5-day-old seedlings; n=3 or 4. Values represent means±s.d.
of GFP-expressing cells in root hair-cell positions was higher in M78A than in W76A (Table 1). For M78A, we examined the effects on phenotype in the cpc-2 mutant background. M78A 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 (Jelariutta 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). The *EGL3* promoter 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

**Table 2. Specification of cell types in root epidermis**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hair cells in epidermis (%)</th>
<th>Hair cells (%)</th>
<th>Hairless cells (%)</th>
<th>Hairless-cell position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Col-0)</td>
<td>50.0±6.7</td>
<td>93.4±9.5</td>
<td>6.7±9.5</td>
<td>0±0</td>
</tr>
<tr>
<td>CPCp::CPC:GFP</td>
<td>90.6±12</td>
<td>98.7±4.2</td>
<td>1.3±4.2</td>
<td>84.8±16.6</td>
</tr>
<tr>
<td>CPCp::GFP</td>
<td>48.8±9.1</td>
<td>94.7±8.2</td>
<td>5.3±8.2</td>
<td>2.1±4.5</td>
</tr>
<tr>
<td>W76A-3</td>
<td>51.2±4.7</td>
<td>100±0</td>
<td>0±0</td>
<td>7.1±7.5</td>
</tr>
<tr>
<td>M78A-1</td>
<td>78.7±12.7</td>
<td>97.8±4.9</td>
<td>2.2±4.9</td>
<td>61.9±24.2</td>
</tr>
</tbody>
</table>

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

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.
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 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 (Mf of 1×GFP) and 54 kDa (Mf of 2×GFP) in the meristematic zone of root epidermis. In contrast to free GFP multimers, CPC increased the SEL of plasmodesmata for CPC:tandem 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 (Mf 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...
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 intracellular 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 (Zambrzycki and Crawford, 2000). Free GFP and LFY move by this mechanism (Crawford and Zambrzycki, 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.

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Supplementary material

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

References


