Role of a positive regulator of root hair development, *CAPRICE*, in *Arabidopsis* root epidermal cell differentiation

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SUMMARY

In *Arabidopsis*, root hairs are formed only from a set of epidermal cells named trichoblasts or hair-forming cells. Previous studies showed *CAPRICE* (*CPC*) promotes differentiation of hair-forming cells by controlling a negative regulator, *GLABRA2* (*GL2*), which is preferentially expressed in hairless cells. Here, we show that *CPC* is also predominantly expressed in the hairless cells, but not in the neighboring hair-forming cells, and that CPC protein moves to the hair-forming cells and represses the *GL2* expression. We also show that the N terminus of bHLH protein interacts with CPC and is responsible for the *GL2* expression. We propose a model in which CPC plays a key role in the fate-determination of hair-forming cells.

Key words: *Arabidopsis*, *CAPRICE*, Myb, bHLH, Root hair, Transcriptional regulation, Epidermis, Protein movement

INTRODUCTION

Cell fate determination is a critical step in plant development. In growing roots, cells continuously proliferate and differentiate in a layer-specific manner at the root meristem, located at the tip. The root epidermal cells differentiate into two cell types, root-hair cells (trichoblasts) and hairless cells (atrichoblasts). In *Arabidopsis* roots, epidermal cells are arranged in 16 to 22 cell files. Cells in 8 symmetrically positioned files differentiate into the hair cells, and the cells of the other files, into hairless cells (Fig. 1A,B). Morphological analysis has shown that the hair cells lie over the junction of two cortical cells, whereas the hairless cells overlie one cortical cell only (Dolan et al., 1994; Galway et al., 1994). The positional relationship between cortical cells and epidermal cells was confirmed by the observation of small regions of two cell files (T-clones) that occasionally arise from a single hair cell file. One of the cell files stays in contact with the junction of the underlying cortical cells and differentiates into the hair cells, and the other cell file, in contact with only one cortical cell, does not form root hairs (Berger et al., 1998).

The molecular genetic mechanism of cell fate determination of root-hair cells is being studied by use of a set of mutants. Three *Arabidopsis* genes, *TRANSPARENT TESTA GLABRA* (*TTG*), *GLABRA2* (*GL2*), and *WERWOLF* (*WER*), are involved in the formation of the hairless cells, because all epidermal cells differentiate into hair cells in the *ttg*, *gl2* and *wer* mutants (Fig. 1D) (Galway et al., 1994; Masucci et al., 1996; Lee and Schiefelbein, 1999). *GL2* encodes a homeodomain-leucine zipper (HD-Zip) protein that is expressed preferentially in the differentiating hairless cells (Masucci et al., 1996; Rerie et al., 1994; Di Cristina et al., 1996). *TTG* has been considered to encode a bHLH protein, because the *ttg* mutation was complemented by the ectopic expression of a maize gene, *R*, encoding a protein with a bHLH domain (Lloyd et al., 1992; Galway et al., 1994). However, recent isolation of *TTG* has shown that it encodes a protein with a WD40 motif (Walker et al., 1999). *TTG* may have a role in the expression of an *Arabidopsis R* homolog. Involvement of the *Arabidopsis* R homolog in the root epidermal cell fate determination is strongly suggested. In contrast, specification of the hair cells was shown to be positively controlled by *CAPRICE* (*CPC*), a gene encoding a small protein of 94 amino acid residues with a Myb-like DNA-binding domain (Wada et al., 1997). The loss-of-function mutant of *CPC* shows only a few normal-shaped root hairs (Fig. 1C). Genetic analysis of double mutants showed that *CPC* may act together with *TTG* upstream of *GL2* in the cell fate determination process (Wada et al., 1997). Unlike other Myb proteins, CPC lacks a domain that activates transcription. Therefore, CPC may work as a negative transcriptional regulator. These previous results indicate that CPC functions as a negative regulator of *GL2*, the latter
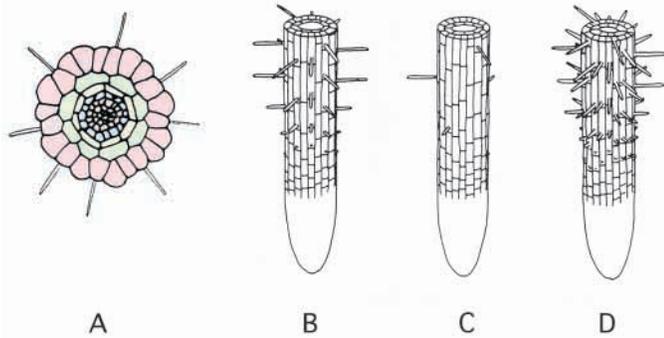
Fig. 1. Structure of *Arabidopsis* roots. Drawings of a transverse section showing the cellular organization (A), and root meristems of wild type (B), *caprice* (*cpc*) mutant (C), and *gl2*, *ttg*, *wer* and *35S::CPC* mutants (D). (A) The wild-type root is made up of five tissues, outermost is the epidermis, then inside that is cortex, endodermis, pericycle and vascular tissue. Epidermal cells are of two types: hair cells and hairless cells. The stele includes pericycle and vascular tissue. A few hairs are formed randomly in the *cpc* mutant (C), whereas hairs are formed in all of the epidermal cells in the *gl2*, *ttg* and *wer* mutants, and in the *35S::CPC* transgenic plant (D).

promoting differentiation of the hairless cells, and therefore *CPC* indirectly promotes differentiation of hair cells.

We have examined the expression pattern of *CPC* and *GL2* in the root tissue of wild-type *Arabidopsis* by in situ hybridization and by analysis of transgenic plants carrying the promoter::*GUS* fusion genes in a series of mutants, and of several transgenic plants ectopically expressing the regulatory genes. In the wild-type plant, both *CPC* and *GL2* were strongly expressed in the hairless cells. Interestingly, *GL2* was expressed in the hair cells as well as in the hairless cells in the *cpc* mutant. The expression pattern and the hair-forming phenotype of the mutants and of the transgenic lines led us to examine the interaction of the regulatory proteins at the molecular level. Analysis of *CPC* promoter::*CPC:GFP* transgenic plants confirms that the CPC protein moves from the hairless cell to the hair-cell and induce root hair formation. Combining these results, we deduced a model explaining the regulatory interaction between transcription factors controlling the fate determination of the root epidermal cells.

MATERIALS AND METHODS

Genetic and microscopic analyses

Plants were grown on agar plates under the conditions described previously (Okada and Shimura, 1990). Seeds of *Arabidopsis* Rschew ecotype were obtained from the *Arabidopsis* Biological Resources Center (The Ohio University, Columbus, OH). Seeds of *35S::R* transgenic plants, of the *rhd6-1* mutant and of the *ctr1* mutant were provided by Alan M. Lloyd, John Schiefelbein and Joseph R. Ecker, respectively. The root phenotype was observed using an Olympus Provis AX70 microscope and an Olympus SZH binocular microscope.

Construction of chimeric genes and transgenic plants

To make *CPC* promoter::*GL2*, we subcloned a 3.6 kb *Hin*dIII fragment obtained from pgl2gen into pBluescript SK+ (Stratagene). After digestion of this plasmid with *Nhe*I and *Apa*I, the larger fragment was purified and ligated into the *Xba*I and *Apa*I sites of CPC cDNA prepared from SK+CPC (SK+GL2::CPC). The GL2::CPC

region was removed with *Hin*dIII and *Apa*I from SK+GL2::CPC and ligated into the *Hin*dIII and *Sac*I sites of a binary vector, pARK5. For 35S::RN construction, a *Xba*I-*Sse*8387I fragment including the 5′ UTR region and the N-terminal region of R was ligated to a *Pst*I and *Eco*RV-digested fragment including the 3′ UTR region of R, and inserted into pBluescript SK+. This plasmid DNA was digested with *Xba*I and *Hin*cII, and subcloned into the *Xba*I and *Hpa*I sites of pMAT137Hm (Matsuoka and Nakamura, 1991).

The *GL2* promoter::GUS chimeric gene was constructed by ligation of a *Xho*I-*Sal*I fragment of 4 kb at the 5′ upstream region of *GL2* into a *Sal*I site of pBI101 (Masucci et al., 1996; Szymanski et al., 1998). For construction of the *CPC* promoter::GUS, a *Pst*I-*Bbs*I fragment of 1.2 kb was blunted by T4 DNA polymerase and subcloned into a *Sma*I site of pBI101-Hm3 (provided by H. Hirano and K. Nakamura, Nagoya University, Nagoya, Japan) (Mita et al., 1995).

Binary plasmids were introduced into an *Agrobacterium tumefaciens* strain C58::pGV2260 by electroporation using a Gene Pulser (Bio-Rad). Plant transformation of *Arabidopsis* wild type (ecotype WS) was performed by a vacuum infiltration procedure (Bechtold et al., 1993). Selection of transformants was performed on B5 agar medium containing 20 mg/l hygromycin (Wako Junyaku, Osaka, Japan) or 50 mg/l kanamycin.

The *GL2::GUS* and *CPC::GUS* constructs were introduced onto the various mutant backgrounds by crossing plants harboring the markers and analyzing F$_2$ seedlings for homozygous mutants.

GUS staining

Samples of the transgenic plants were stained under vacuum in X-Gluc solution containing 5.7 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-glucronide), 1.5 mM K$_3$Fe(CN)$_6$, 1.5 mM K$_4$Fe(CN)$_6$, 50 mM NaPi (pH 7.0), and 0.9% Triton X-100 (Jefferson et al., 1987). Stained roots were embedded in 5% low-melting point agarose (BRL) and sectioned with a microslicer DTK-3000 (Dohann EM, Kyoto, Japan).

In situ hybridization

RNA probes used for detecting CPC transcripts in situ were prepared by PCR using the following primers:
   CPCF2, 5′ TTAAGCTTTCTCACTCTTTTCTTTT 3′;
   CPCB2, 5′ GGAATTCTTTCCTAAAAAAGTCTC 3′.
   A PCR fragment (400 bp) was digested with *Hin*dIII and *Eco*RI, and cloned into Bluescript SK+ (for use as a sense probe) or into KS+ (for use as an antisense probe) (Stratagene). To prepare the antisense and the sense probe, we linearized the plasmids with *Hin*dIII or *Eco*RI, respectively, prior to adding them to the in vitro transcription mixture (Trans Probe kit, Pharmacia) containing T3 RNA polymerase and ^{35}S-UTP. RNA probes for detection of GL2 transcripts in situ were prepared as previously reported (Masucci et al., 1996; Rerie et al., 1994).

Tissue fixation in paraffin, hybridization and washing were carried out as described elsewhere (Di Laurenzio et al., 1996; Drews et al., 1991). 10 μm thick transverse sections of roots and 8 μm longitudinal sections were prepared. Slides were emulsion coated and exposed for 5 weeks before development. The sections were observed under a Zeiss Axiophot microscope.

Two-hybrid analysis

Vectors and yeast strains were obtained from Clonetech (MATCHMAKER Two-Hybrid System). The plasmid carrying the various forms of truncated R were prepared as follows:

R deleted of residues 1-525 (pGBTR525). pSPUTK-R (provided by Alan M. Lloyd) was digested with *Nco*I, and the obtained fragment was cloned into the *Sma*I site of pGBT9 after blunting with a Klenow Fragment.

R deleted of residues 1-371. pSPUTK-R was digested with *Nco*I and *Ehe*I, and cloning into the *Sma*I site of pGBT9 or of pGAD424.

R deleted of residues 1-298 or 1-206 combined to the GAL-AD.

pGADR1-371 was digested with *Bam*HI and *Sse*8387 I or *Sac*II, respectively, blunted by T4 DNA polymerase, and self-ligated.

R deleted of residues 1-206. pGADR1-371 was digested with *Sac*II and *Bam*HI, blunted, and self-ligated.

R deleted of residues 1-312, 1-291, 1-244, or 1-239. pGADR371 was digested with *Mlu*I, *Alw*NI, *Msl*I or *Tth*HB8I, respectively, blunted, digested with *Eco*RI, and ligated to pGAD424, which was then digested with *Eco*RI and *Sma*I.

R deleted of residues 23-298 and 30-298. The *Pst*I fragment or the *Bgl*II-*Pst*I fragment, respectively, obtained from pSPUTK-R, was cloned into pGAD424 and digested with *Pst*I or *Bam*HI, respectively.

A series of the CPC deletion constructs was prepared by PCR using primers below:
CPCN, 5′ CGGAATTCATGTTTCGTTCAGAC 3′
CPCC, 5′ ACGCGTCGACTTCCTAAAAAAGTC 3′
MybF, 5′ CGGAATTCTGGGAAGCTGTGAAG 3′
MybB, 5′ CATAGTCGACGACGCCGTGTTTC 3′.

PCR was performed using Pfu DNA polymerase (Stratagene) and a combination of primers as follows: CPCN and CPCC for full-length CPC protein, CPCN and MybB for CPC of residues 1-83, MybF and CPCC for CPC of residues 33-94, and MybF and MybB for CPC of residues 33-88. The PCR-amplified fragments were digested with *Eco*RI and *Sal*I and inserted into the *Eco*RI and *Sal*I sites in pGAD424 or pGBT9. For preparation of CPC of residues 1-65, pGAD- full-length CPC was linearized by *Sma*I and *Sal*I and self-ligated. To prepare CPC of residues 44-94, we digested pGAD- full-length CPC with *Bgl*II and *Sal*I, and cloned the obtained fragment into the *Bam*HI and *Xho*I sites of pGAD GL. To prepare CPC of residues 1-75, we subcloned into pGAD424 a PCR-amplified fragment from the *cpc* mutant using CPCN and a oligonucleotide corresponding to the T-DNA (5′ CATAGTCGACTATCTCTCTATCTCC 3′) as primers.

Plasmid DNAs used as positive controls, pVA3 and pTD1, which encode murine p53/GAL4 and SV40 large T-antigen, respectively, were supplied from Clonetec. Cultures of yeast strains SFY526 and HF7c were transformed with appropriate plasmids using carrier DNA and the lithium acetate method. Then, the cells were pelleted, resuspended in TE (Tris-EDTA) buffer, and spread on plates containing SD synthetic medium (2% dextrose, 1× yeast nitrogen base) lacking Trp and Leu. For the His requirement test, the yeast cells were streaked on plates with SD synthetic medium lacking Trp, Leu and His and containing the appropriate concentration of 3-amino-1, 2, 4-triazole (3-AT; Sigma #A-8056). To assay β-galactosidase activity, cells were grown in 2 ml of liquid SD medium lacking Leu and Trp until an $OD_{600}=0.6\sim0.9$ was obtained. The cells were collected and resuspended in the reaction buffer containing ONPG (o-nitrophenyl β-D-galactopyranoside; Sigma #N-1127) as a substrate. After incubation, the samples were centrifuged, and absorbance of the supernatant at 420 nm was then measured.

In vitro binding analysis

R proteins were prepared by in vitro transcription/translation using a system of TNT SP6-coupled rabbit reticulocyte lysate (Promega). R-coding sequence that was cloned in an in vitro translation vector into pSPUTK (Stratagene) (pSPUTK-R) (Symanski, 1998) was used as a template for synthesis of intact R protein. The DNA template for the N-terminal region of R was constructed by digesting pSPUTKR with *Mlu*I and *Cla*I and purifying the larger fragment. This fragment was blunted by a Klenow fragment and self-ligated. The self-ligation created a stop codon at the junction between the R-coding sequence and the multiple cloning sites. The DNA template for the C-terminal region of R was made by digesting pSPUTKR with *Kas*I and *Nco*I. After the ends had been blunted by a Klenow fragment, the fragment was inserted into the *Nco*I and *Cla*I sites of pSPUTK. The ligated DNA also created a stop codon, at the C terminus of the inserted DNA.

The GST-CPC DNA was made by digesting pGAD424-CPC with *Eco*RI and *Sal*I. The fragment was cloned into pGEX4T-1 (Pharmacia). The plasmid DNA was used to transform *E. coli* strain BL21 (DE3). After incubation of the transformed bacteria at 37°C for 3 hours, IPTG was added to a final concentration of 1 mM, and the incubation continued for 3 hours. The culture was harvested, and the GST-CPC protein was purified by passage through a glutathione-Sepharose (Pharmacia) column.

For in vitro association assay, appropriate aliquots of [^{35}S]methionine-labeled R proteins were mixed with 2 μg of the purified GST-CPC protein or GST protein in 50 μl PBS and incubated for 1 hour at room temperature. Then, 30 μl of a 50% suspension of glutathione-Sepharose was added to the reaction mixture and gently agitated for 15 minutes. The protein complex bound to the resin was eluted with a solution of 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) after the resin had been washed three times with PBS. The eluted proteins were analyzed on a SDS-polyacrylamide gel.

GFP imaging of gene expression

To prepare the *CPC* promoter::*GFP* and *CPC* promoter::*CPC*::*GFP*, the *CPC* promoter that was used in *CPC* promoter::*GUS* construct, was combined with 2XrsGFP (Crawford and Zambryski, 2000) (gift from Katrina Crawford). To prepare the *CPC* promoter::SV40 NLS:2X *GFP*, synthesized SV40 NLS sequence (ATGCCTAAGAA-GAAGCGTAAGGTCGAT) was inserted between the CPC promoter and 2XGFP (Kalderon et al., 1984).

Seedlings were incubated for 5 minutes in 5 μg/ml propidium iodide to stain the cell walls. GFP fluorescence was visualized in whole mount using a confocal laser scanning microscope (Zeiss LSM 5 Pascal) with the FITC channel (green, GFP) and the rhodamine channel (red, propidium iodide).

RESULTS

CPC is a positive regulator of root hair development

Root hairs are linear structures about 1 mm long formed by tip-growth from the epidermal cells (Fig. 1A,B, Fig. 2A,H). Of about 20 epidermal cell files of a wild-type root, cells of eight files form root hairs (hair-cells, asterisks in Fig. 2H), whereas cells of the other files do not, i.e., they are hairless cells (Dolan et al., 1994; Galway et al., 1994). The *cpc* mutant formed a few root hairs at random positions (Fig. 1B, Fig. 2B, Table 1), but failed to form files of hair cells. However, unlike other *Arabidopsis* mutants with no root hairs, the *cpc* mutant retains the function of the genes required for root hair growth, because the shape of the occasionally formed hairs was normal. The data suggest, therefore, that *CPC* is a positive factor controlling the differentiation of the hair-forming cells. This hypothesis was confirmed by the observation that root hairs were formed from cells of all epidermal cell files in the roots of transgenic

Table 1. Phenotype of root epidermal cells

Genotype	Number of root hairs per mm (a)	Length of epidermal cell (μm) (b)	Relative hair number*
WT (WS)	43.2±1.0	257.1±10.9	11.1
WT (Rschew)	53.1±2.1	261.5±5.2	13.9
cpc	10.6±0.6	289.0±12.0	3.1
GL2::CPC in cpc	39.6±1.4	269.3±8.5	10.6
35S::R†	0.6±0.4	161.1±6.2	0.1
35S::N terminal region of R (1-298)	1.3±0.5	261.5±5.2	0.3

*Relative hair number indicates the number of root hairs formed on a segment of root with an average length of epidermal cells (a×b/100).
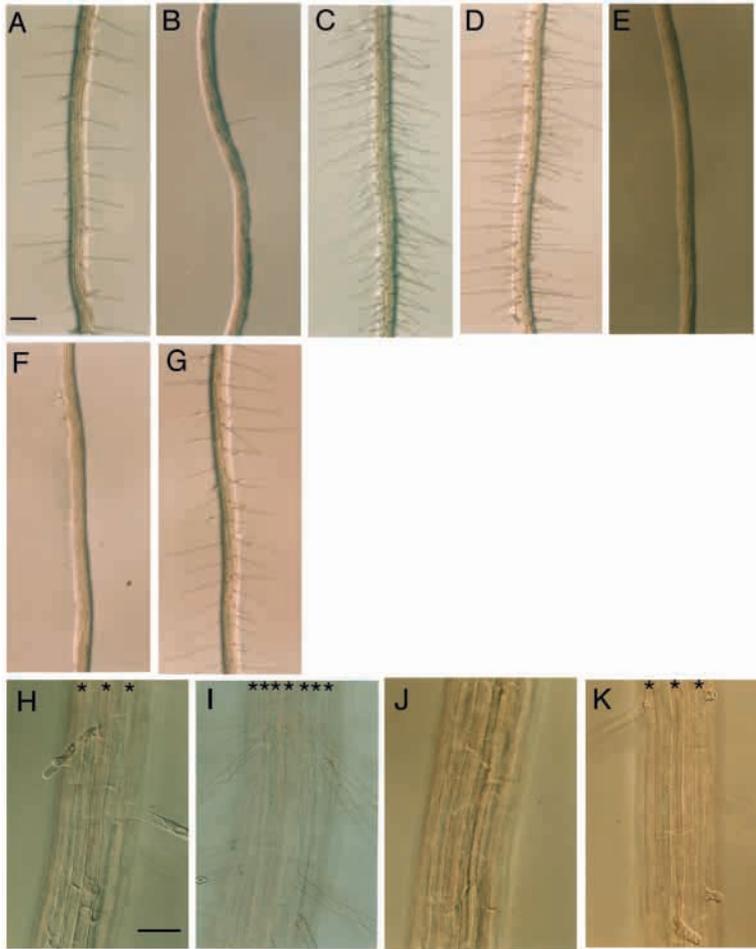†The ecotype of 35S::R is Rschew. The ecotype of other lines is WS.

Fig. 2. Patterns of root hair formation in the primary roots of 5-day-old seedlings. (A,H) Wild type, (B) *cpc*, (C) *35S::CPC*, (D,I) *gl2-1*, (E) *35S::R*, (F,J) *35S::RN* and (G,K) *GL2* promoter::*CPC* in *cpc*. Asterisk indicates cell files forming root hairs. (A to G and H to K are of the same magnification. Scale bars in A (for A-G) 200 µm; in H (for H-K) 50 µm.

plants ectopically expressing *CPC* under the control of the 35S promoter (Fig. 1D, Fig. 2C) (Wada et al., 1997).

GL2, TTG and R are negative regulators of root hair development

In contrast to the *cpc* mutant, the *gl2* and *ttg* mutants formed root hairs in all epidermal cells, indicating that *GL2* and *TTG* are required for generating the hairless cells (Fig. 1D, Fig. 2D,I) (Galway et al., 1994; Masucci et al., 1996). Formation of root hairs was reduced when the maize *R* gene was introduced into wild-type *Arabidopsis* under the control of the 35S promoter (Lloyd et al., 1992; Galway et al., 1994) (Fig. 2E, Table 1). Formation of the hairs was abolished when R was introduced into the *ttg* mutant, but not when it was introduced into the *gl2* mutant (Galway et al., 1994; Hung et al., 1998). These results indicate that maize *R* is a strong negative regulator of root hair development. The *R* gene encodes a protein of 610 amino acid residues with a bHLH domain at the C terminus and an acidic region at the N terminus (Fig. 5A) (Ludwig et al., 1989). bHLH proteins are known to work as transcription factors. There are many genes encoding bHLH

proteins in plants, but, so far, only R has been reported to complement the *ttg* mutation in formation of root hairs (Lloyd et al., 1992; Galway et al., 1994). Therefore, some region outside the HLH domain of R is likely to be responsible for the regulation of root hair development. In order to examine the function of the N-terminal region of the R protein, we constructed transgenic plants carrying a chimeric gene covering residues 1-298 driven by the 35S promoter. Like the transgenic plants ectopically expressing the intact R protein, the transgenic plants carrying the N-terminal region of R failed to form root hairs (Fig. 2F,J, Table 1). This result indicates that the N-terminal region including an acidic domain is required for the negative control of root hair development.

Expression pattern of *CPC*

Because the expression of *CPC* is required for the development of the root hair cells, *CPC* was postulated to be expressed in the root hair cells. The gene was also expected to be expressed in the cells at the root tip, because root epidermal cells elongate and begin to differentiate into the hair cells after dividing from the epidermal initial cells located at the root meristem (Dolan et al., 1994; Galway et al., 1994).

In order to examine the type and position of cells expressing CPC, we transformed wild-type plants with the *GUS* reporter gene driven by the *CPC* promoter including an approximately 1.2 kb region upstream of the initiation codon, the region sufficient to complement the *cpc* mutation (Wada et al., 1997). Histochemical staining of the primary roots of the 5-day-old transgenic plants showed vertical stripes of stained cells (Fig. 3K). Epidermal cells in the elongation zone were strongly stained, and cells in the division zone below the elongation zone and cells that had shifted into the differentiation zone were weakly stained. The blue stain was not observed in the root cap or in cells in the fully differentiated regions. The longitudinal pattern of the *CPC* expression is consistent with the model that *CPC* is involved in the development of hair cells. In transverse sections, strong staining was observed in the hairless cells, and weak staining was seen in the hair cells and in the stele cells (Fig. 3A). Cells of other tissues also were stained weakly. This result was contrary to our expectation that CPC would be expressed in hair cells, but not in the hairless cells. It is not clear whether the weak staining reflects low-level expression, or diffusion from the neighboring strongly stained cells.

The expression pattern of *CPC* was further confirmed by in situ hybridization with a probe of the CPC antisense RNA corresponding to the 5′ UTR and the coding region. Although the probe included the Myb region, genomic Southern blots with this probe showed no extra bands in addition to the fragments encoding CPC, even after washing under moderately stringent conditions, showing that the signal obtained in the in situ hybridization may not have included any 'noise' originating from transcripts of other Myb genes. The results of the in situ hybridization were clear: a strong signal was detected in hairless cells, but not in hair-forming cells and other cells (Fig. 4D), confirming the results of the *CPC*

promoter::*GUS* staining. In situ hybridization experiments using longitudinal sections also confirmed that *CPC* mRNA had accumulated in the epidermal cells (data not shown). Weak signals were observed in root hair cells and other types of cells. In situ hybridization experiments using the sense strand of *CPC* as a control showed no significant signals (Fig. 4N).

For the purpose of analyzing the expression pattern of *CPC* on a series of mutant backgrounds, we crossed the *CPC* promoter::*GUS* plants with various root-hair mutants. In the *ttg* mutant, GUS staining was not detected in the epidermal cells, but weak GUS expression was observed in the stele (Fig. 3C). Also in situ hybridization experiment did not reveal any significant signals in the transverse sections of primary root of the *ttg* mutant (Fig. 4F). The results indicate that *TTG* positively controls the expression of *CPC* in the epidermal cells, but is possibly not involved in expression in the stele cells. In plants overexpressing *R*, strong GUS expression was observed in almost all cells (Fig. 3D). Staining was not found to be stronger in any particular files. In addition, GUS expression was observed in cells of columella and lateral root cap, except the two columella cell layers at the top (Fig. 3M). The in situ hybridization experiments also showed uniform *CPC* RNA accumulation in longitudinal sections of the primary root overexpressing R (data not shown). These results indicate that R functions as a positive regulator of *CPC*. In the other mutant with ectopic root hairs, *gl2*, the expression pattern of *CPC* was essentially the same as that in the wild type, as shown by in situ hybridization (Fig. 4E). The results confirmed our previous conclusion obtained from double mutant analysis (Wada et al., 1997), that *GL2* acts downstream of *CPC*. Interestingly, the level of *CPC* promoter::*GUS* expression was dramatically enhanced in the *cpc* mutant background in all epidermal cells (Fig. 3B). In contrast, expression of the *CPC* promoter::*GUS* gene was repressed in epidermal cells when *CPC* was overexpressed under the *35S* promoter (Fig. 3L). These results indicate the presence of a self-regulation system for *CPC* expression; namely, its expression is promoted in the absence of *CPC*, but it is repressed by overexpression.

ctr1 and *rhd6* are known to control the hormone-dependent formation of root hairs (Dolan et al., 1994; Kieber et al., 1993; Masucci and Schiefelbein, 1994), and in plants mutant for these genes the pattern of *CPC* expression was found to be the same as that of wild type (Fig. 3E,F), indicating that *CTR1* and *RHD6* work downstream of *CPC*.

Expression pattern of *GL2*

Previous studies using the promoter-GUS analysis indicated that *GL2* is expressed in hairless cells but not in hair-forming cells of wild-type roots (Masucci et al., 1996). Our observations using *GL2::GUS* gene expression and in situ hybridization with the *GL2* probe confirmed this. In transverse sections, *GL2* was expressed preferentially in the hairless cells
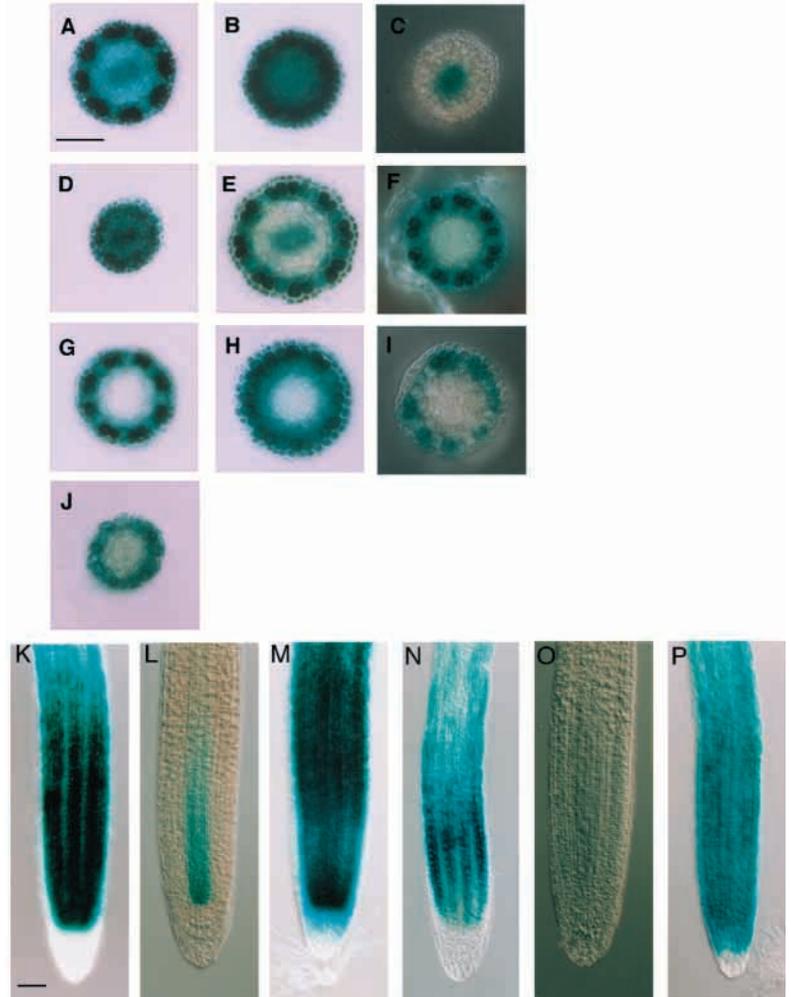

Fig. 3. Spatial expression pattern of *CPC promoter::GUS* and *GL2 promoter::GUS* in different mutant backgrounds. Transverse (A-J) and longitudinal (K-P) sections of 5-day-old seedlings were stained with X-Gluc. (A-F) Expression of *CPC::GUS* in wild type (A), *cpc* (B), *ttg-1* (C), *35S::R* (D), *rhd6-1* (E) and *ctr-1* (F). (G-J) Expression of *GL2::GUS* in wild type (G), *cpc* (H), *ttg-1* (I), and *35S::R* (J). (K-M) Expression of *CPC::GUS* in wild type (K), *35S::CPC* (L), *35S::R* (M). (N-P) Expression of *GL2::GUS* in wild type (N), *35S::CPC* (O), *35S::R* (P). Photos and are of the same magnification. Scale bars in A (for A-J) and K (for K-P), 50 μm.

(Fig. 3G), a pattern consistent with the model that *GL2* is a negative regulator of root hair development. *GL2* was expressed in the cells of the elongation zone, but the expression level was gradually decreased as cells entered the differentiation zone, and the expression was hardly detected in cells that had initiated root hair formation (Fig. 3N). The pattern of GL2 expression was confirmed by in situ hybridization. As shown in Fig. 4J, the *GL2* mRNA was localized in the hairless epidermal cells. Expression of *GL2* overlapped that of *CPC*, but the amount of the *GL2* message appeared to be higher than that of *CPC*. In addition, unlike *CPC*, expression of *GL2* was not detected in the stele cells.

Previous analysis of double mutants showed that the *gl2* mutation is epistatic to the *cpc* mutation in the developmental pathway of epidermal cell differentiation (Wada et al., 1997). In order to study the regulatory network, we examined the
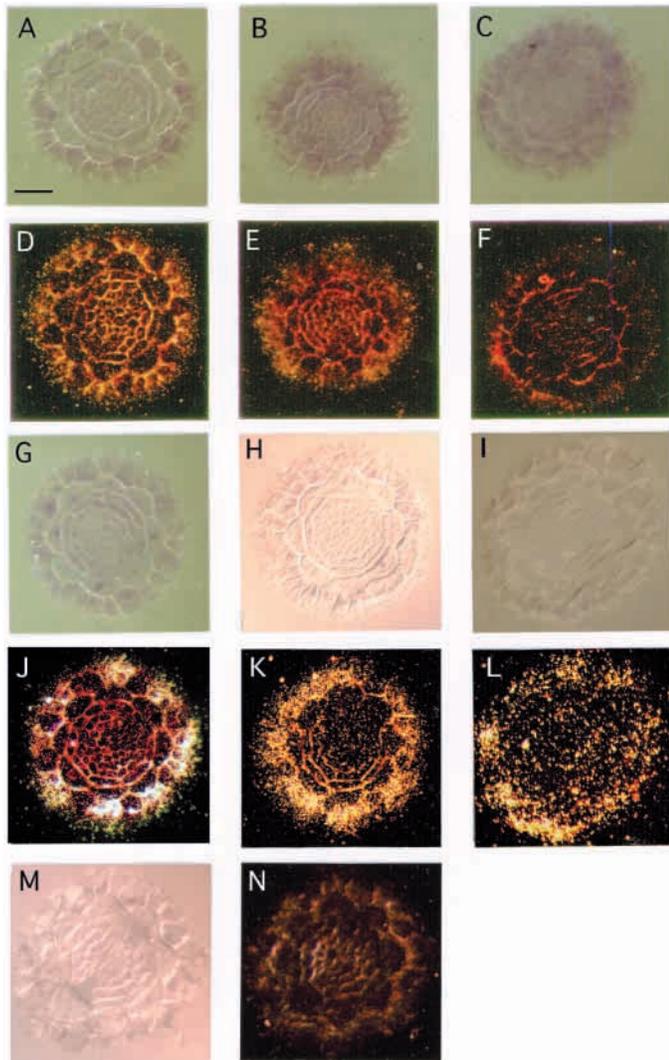
Fig. 4. In situ hybridization pattern of CPC and GL2 in roots sections. (A-N) Hybridization of transverse sections using the following probes: antisense of CPC (A-F), antisense of GL2 (G-L), and sense of CPC (M,N). Photos were taken at the same magnification in (A-C,G-I,M) Bright-field, (D-F,J-L,N) dark-field illumination. (A,D,G,J,M,N) Wild type, (B,E) *gl2-1*, (C,F,I,L) *ttg-1*, (H,K) *cpc*. Scale bar: 25 μm.

expression pattern of *GL2* on various mutant backgrounds. The *GL2* promoter::*GUS* gene was shown to be expressed in almost all epidermal cells in the *cpc* mutant (Fig. 3H). In situ hybridization also clearly demonstrated that the *GL2* expression was permitted in the hair cells at the same level as that in the hairless cell (Fig. 4K). This result strongly suggests that *CPC* represses *GL2* expression. This interpretation was also confirmed by the drastic reduction of the *GL2::GUS* expression observed in roots of the *35S::CPC* transgenic plants overexpressing *CPC* (Fig. 3O).

In the *ttg* mutant, a reduction in *GL2* expression was observed by *GL2* promoter::*GUS* and in situ hybridization experiments (Fig. 3I, Fig. 4L). The results are consistent with the reported model that *GL2* is positively controlled by *TTG* (Hung et al., 1998). In contrast, *GL2* expression was enhanced in transgenic plants overexpressing *R* (Fig. 3J,P), indicating

that *R* is a positive regulator of *GL2*. The in situ hybridization experiment also showed that *GL2* RNA was observed throughout the epidermis in the *35S::R* transgenic plant (data not shown).

The *CPC* promoter can be replaced by the *GL2* promoter

Because the expression patterns of *GL2* and *CPC* are similar in wild type, several mutants and in a transgenic background, the two genes are likely to be regulated by some common mechanism. In order to confirm that the regulatory system works similarly, we tested whether the *GL2* promoter could replace the CPC promoter. When a chimeric gene, *GL2* promoter::*CPC,* was introduced into the *cpc* mutant, the transgenic plants showed the normal pattern of root hairs (Fig. 2G,K, Table 1). This confirms that the *CPC* and *GL2* genes are controlled by similar regulatory circuits and that the *GL2* promoter has sufficient activity to support the spatial and temporal expression of *CPC*. In addition, it is likely that the expression of *CPC* in the stele cells does not contribute to the normal pattern of root hair development, because the *GL2* promoter did not support the expression in the stele cells.

CPC protein binds to the N-terminal region of R protein

The promoter::GUS expression as well as the in situ hybridization experiments revealed that expression of *GL2* is negatively controlled by *CPC*, and positively regulated by *TTG* and *R*. Root hair formation was abolished in transgenic plants ectopically expressing the N-terminal region of *R* lacking the HLH region (Fig. 2F,J, Table 1). This result suggests that the CPC protein might interact with the N-terminal region of R.

As a first approach to show the interaction between R and CPC, we employed the yeast two-hybrid analysis using the GAL4 protein-fusion system. CPC was conjugated to the GAL4 transcriptional activation domain (GAL4-AD), and assayed for its ability to bind various constructs of R fused to the GAL4 DNA binding domain (GAL4-BD). As shown in Fig. 5B, CPC interacted with residues 1-525 and residues 1-371 of R to a similar degree. However, CPC interacted with residues 372-525 of R at a very low level, similar to that with the negative controls. A domain-swapping experiment showed that residues 1-371 of R conjugated to GAL4-AD strongly interacted with CPC fused to GAL4-BD. The strength of the interaction between CPC and R was strong, about twice that of the positive control between the large T-antigen and p53. These results indicate that CPC bound to the N-terminal half of R (residues 1-371), but not to the C-terminal half (residues 372-525), which includes the HLH region.

In order to identify further the interacting domain of R, we conjugated a series of R deletions to the GAL-AD, and examined their ability to bind to CPC fused to the GAL4-BD (Fig. 5C). The results indicated that the truncated R deleted of residues 1-371, 1-312 or 1-298 interacted with CPC to about the same degree as the intact R. The strength of interaction between CPC and R truncated of residues 1-291, 1-244 or 1-239 was low; about one-tenth that of R deleted of residues 1-371. R deleted of residues 1-206 did not show any detectable interaction with CPC. Although residues 23-298 of R interacted with CPC, residues 30-298 did not. The results indicate that CPC binds to the N-terminal region of R covering
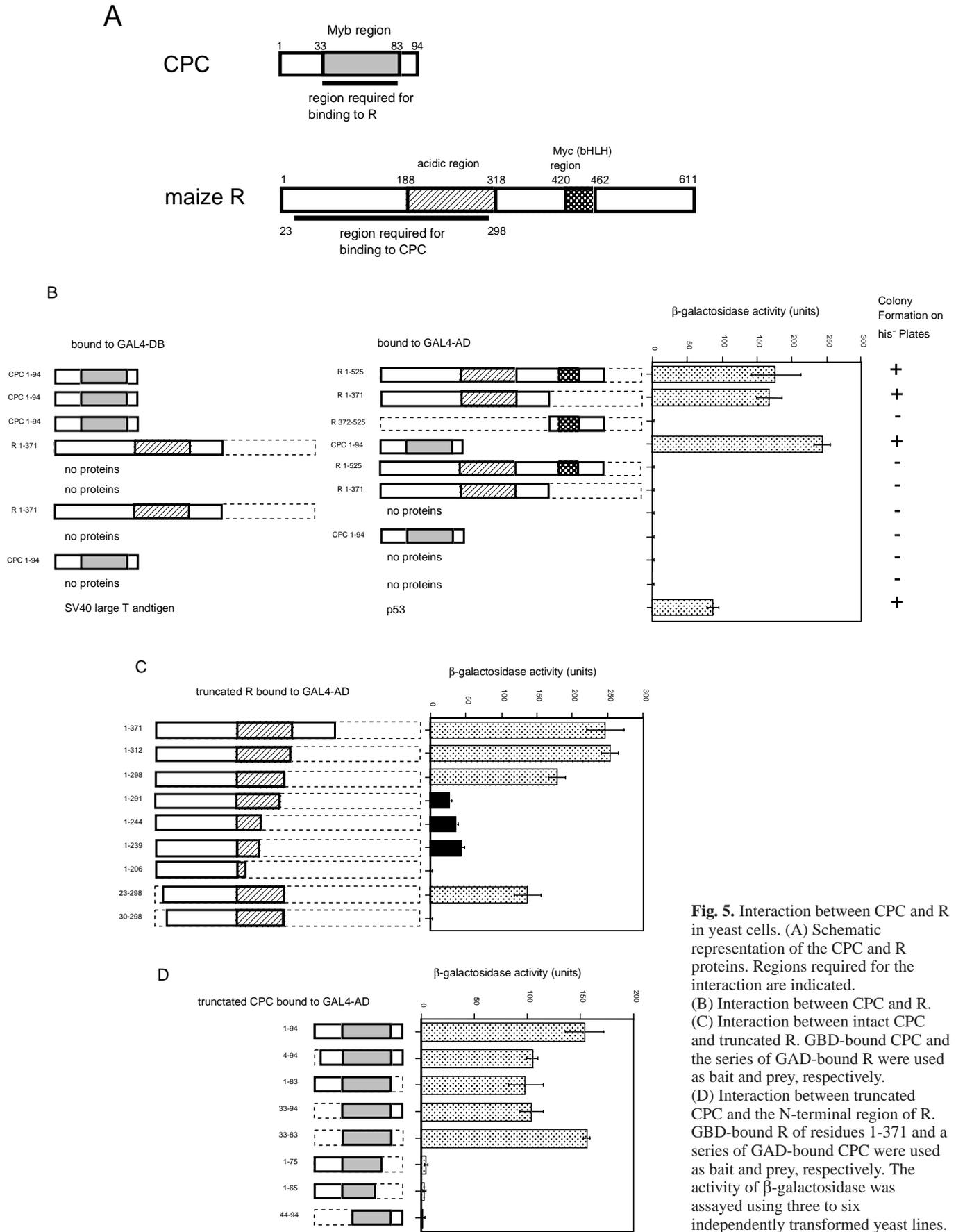
Fig. 5. Interaction between CPC and R in yeast cells. (A) Schematic representation of the CPC and R proteins. Regions required for the interaction are indicated. (B) Interaction between CPC and R. (C) Interaction between intact CPC and truncated R. GBD-bound CPC and the series of GAD-bound R were used as bait and prey, respectively. (D) Interaction between truncated CPC and the N-terminal region of R. GBD-bound R of residues 1-371 and a series of GAD-bound CPC were used as bait and prey, respectively. The activity of β-galactosidase was assayed using three to six independently transformed yeast lines.

residues 23-298 and that the HLH domain is not required for the binding.

A series of truncated CPC proteins were combined with GAL4-AD, and tested for their ability to bind to the N-terminal region of R (residues 1-371). CPC was separated into the N-terminal region, residues 1-32, the Myb-homologous region, residues 33-83, and the C-terminal region, residues 84-94 (Fig. 5A). As shown in Fig. 5D, residues 1-83, 33-94 and 33-83 of CPC, interacted with R to about the same degree as intact CPC (residues 1-94). However, residues 1-75, 1-65, and 44-94 of CPC of showed no interaction with R. It is worth noting that the *cpc* mutant is thought to express residues 1-75 of the CPC protein (Wada et al., 1997). These results show that the Myb-homologous domain of CPC, residues 33-83, is sufficient for binding to R.

As a second approach to show the interaction between CPC and R, we used an in vitro binding assay. The *CPC* construct fused to glutathione S-transferase (GST) was expressed in *E. coli* cells. Three R proteins, intact R and R truncated of residues 1-312 or 372-525, were labeled with [35S]methionine by use of an in vitro transcription/translation system, which revealed 64 kDa, 34 kDa and 17 kDa products, respectively (Fig. 6, lanes 1-3). The R proteins were incubated with the GST-CPC fusion protein, and the complex was coprecipitated with glutathione-Sepharose. As a negative control, the R proteins were incubated with GST (Fig. 6, lanes 7-9). Intact R and R deleted of residues 1-312 were coprecipitated with CPC (Fig. 6, lanes 4,5). However, residues 372-525 of R showed no significant association with CPC (Fig. 6, lane 6). These results confirm the conclusion drawn from the two-hybrid analysis showing that CPC binds to the N-terminal region of R.

Localization of the CPC protein

CPC was predominately expressed in the hairless cell (Fig. 3A,K, Fig. 4D). In the *cpc* mutant, root-hair cells were converted into hairless cells (Wada et al., 1997). To examine the localization of the CPC protein, we made a DNA construct of a CPC-GFP fusion protein (Crawford and Zambryski, 2000).
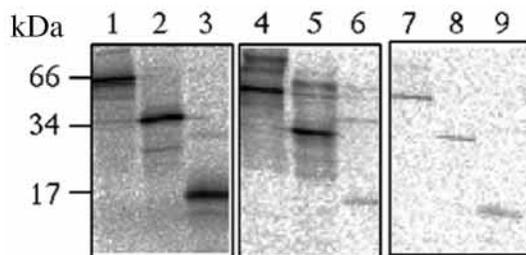

Fig. 6. In vitro binding of CPC to R. The intact and truncated R proteins were translated in vitro, labeled with [35S]methionine, incubated with or without the purified GST-CPC protein or with the GST protein, and adsorbed on glutathione-Sepharose. The Sepharose-bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Lane1: intact R protein before incubation; lane 2: N-terminal region of R (residues 1-312) before incubation; lane 3: C-terminal region of R (residues 372-525) before incubation; lane 4: intact R incubated with GST-CPC; lane 5: N-terminal region of R incubated with GST-CPC; lane 6: C-terminal region of R incubated with GST-CPC; lane 7: intact R incubated with GST; lane 8: N-terminal region of R incubated with GST; lane 9: C-terminal region of R incubated with GST.

In *CPC* promoter::*GFP* transgenic plants, GFP fluorescence was observed mainly in the cytoplasm of hairless cells. This pattern is the same as that of *CPC* promoter::*GUS* (Fig. 7A). However, GFP fluorescence was observed in the nuclei of all root epidermal cells in *CPC* promoter::*CPC:GFP* protein::2XrsGFPs transgenic plants (Fig. 7B). To avoid the possibility that targeting the GFP protein into the nucleus simply increase the sensitivity of the assay, we produced transgenic plants harboring *CPC* promoter::NLS:*GFP*. We observed GFP fluorescence in the nucleus of hairless cells, but not of hair cells (Fig. 7C).

In situ hybridization showed that *CPC* RNA also localizes in hairless cells (Fig. 4D). These results indicate that CPC protein is translated in hairless cells, and it then moves into the hair cells, where it represses *GL2* transcription.

DISCUSSION

### GL2 is the key to the formation of hairless cells

Microscopic analysis of the pattern of root hairs in a series of mutants and transgenic plants clearly distinguished positive and negative regulators of the hair-forming process. Mutant phenotypes indicated that *GL2* and *TTG* are negative regulators of root hair development (Fig. 1D, Fig. 2D,I) (Galway et al., 1994; Masucci et al., 1996). In addition to *GL2* and *TTG*, the maize *R* gene was also found to work as a negative regulator of root hair development, because ectopic expression of the gene resulted in a defect in hair formation (Fig. 2E, Table 1). Involvement of an *R* gene homolog in root hair development in *Arabidopsis* is strongly suggested. However, *CPC* was confirmed to be a positive regulator of root hair development, because the *cpc* mutant failed to form root hairs, and transgenic plants overexpressing *CPC* converted all the root epidermal cells into hair-forming cells (Fig. 1C,D, Fig. 2B,C) (Wada et al., 1997).

A series of genetic analyses showed that *GL2* functions downstream of the other regulators. Phenotype analysis of double mutants suggested that *CPC* acts upstream of *GL2* in the regulatory process of root hair development because the double mutant showed ectopic root hairs similar to the *gl2* single mutant (Wada et al., 1997). *TTG* and the *R* homolog are also considered to act upstream of *GL2*, because ectopic expression of maize *R* complemented the *ttg* mutation, but not the *gl2* mutation (Galway et al., 1994; Hung et al., 1998). This result suggests that the *R* homolog mediates a regulatory process between *TTG* and *GL2*. In addition, the pattern of root hair development in the *cpc ttg* double mutant suggested that *CPC* and *TTG* may act together (Wada et al., 1997). These results suggest a genetic model in which *GL2* is a key regulator controlling the development of hairless cells: i.e. *TTG* and the *R* homolog work as negative regulators of root hair development by promoting *GL2*, and *CPC* serves as a positive regulator by repressing *GL2*.

Expression pattern of GL2 supports the genetic model

Staining of the *GL2::GUS* construct and in situ hybridization using the *GL2* gene as a probe clearly demonstrated that the expression of *GL2* was promoted by *TTG* and *R*, but repressed by *CPC*. In wild-type roots, *GL2* was preferentially expressed

Fig. 7. Analysis of *CPC* promoter::*CPC*:*GFP* transgenic roots. Confocal images of (A) *CPC* promoter:*GFP*, (B) *CPC* promoter::*CPC*::*GFP* and (C) *CPC* promoter::NLS:*GFP*. Asterisks in A and C indicate hairless cell file. Scale bar, 30 µm.
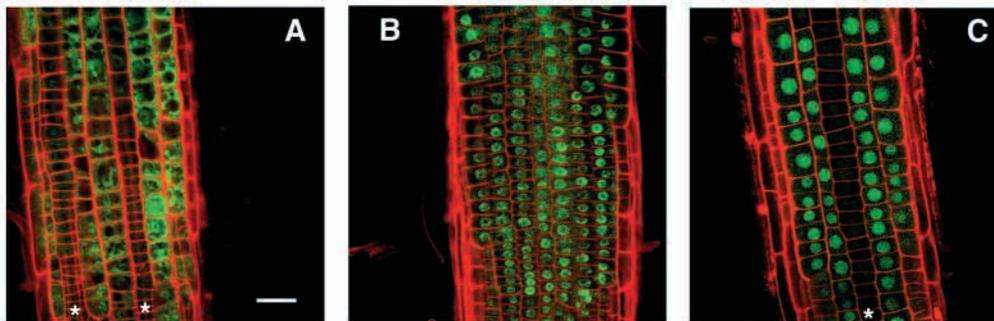
in the hairless cells (Fig. 3G,N, Fig. 4J) (Masucci et al., 1996), consistent with the model that *GL2* is a negative regulator of hair development. The *ttg* mutation repressed *GL2* expression in both the hairless and hair cells (Fig. 3I, Fig. 4L), whereas ectopic expression of *R* induced *GL2* expression in all the epidermal cells (Fig. 3J,P). These results confirm that both *TTG* and *R* promote *GL2* expression (Hung et al., 1998). The *cpc* mutation also induced the expression of *GL2* in the hair-forming cells as well as in the hairless cells, as clearly shown by both in situ hybridization and *GL2::GUS* analysis (Fig. 3H, Fig. 4K). In contrast, ectopic expression of *CPC* repressed *GL2* expression in epidermal cells (Fig. 3O). Another Myb gene, *WER*, is expressed in hairless cells (Lee and Schiefelbein, 1999). *GL2::GUS* expression has been shown to be reduced in *wer-1* mutants (Lee and Schiefelbein, 1999). These results support the genetic model that *CPC* represses *GL2* expression and *WER* activates *GL2* expression.

Expression of *CPC* is under several regulatory controls

Unlike *GL2*, the expression pattern of *CPC* did not correlate with its site of action. First, *CPC* promoter::*GUS* staining and in situ hybridization showed that *CPC* was strongly expressed in the hairless cells (Fig. 3A,K, Fig. 4D). Weak GUS staining was observed in the hair cells, but this was not clear from the in situ hybridization using the *CPC* gene as a probe. The results of *CPC* promoter::NLS:*GFP* expression indicate that CPC is not expressed in the hair cell. The weak GUS staining in the hair cell may represent diffusion from the strongly stained hairless cells. Second, *CPC* expression was observed in stele cells as well as in epidermal cells. *CPC* expression in the stele cells was shown not to be involved in the normal root hair patterning, because the *cpc* mutation was complemented by transforming the *GL2::CPC* gene (Fig. 2G,K, Table 1). Third, the data suggested that a self-repression system is at work in the expression of *CPC*. When the *CPC::GUS* gene was introduced into the *cpc* mutant, a high level of GUS staining was observed (Fig. 3B), but GUS staining was detected at a low level when the *CPC::GUS* gene was introduced in transgenic plants carrying the *35S::CPC* gene (Fig. 3L).

The expression pattern of *CPC::GUS* was not changed in *rhd6*, *ctr1* mutants (Fig. 3E,F), or wild-type seedlings treated with an ethylene precursor ACC (1-amino-cyclopropane-1-carboxylic acid) or an ethylene synthesis inhibitor, AVG (aminoethoxyvinylglycine) (data not shown). Similarly, the expression pattern of *GL2::GUS* was not affected in *axr2*, *rhd6* or *ctr1* mutants or by treatment with ACC or AVG (Masucci

and Schiefelbein, 1996). These results suggest that the steps controlled by plant hormones lie downstream of those where *CPC* and *GL2* act in the process of root hair patterning.

CPC and *GL2* expression have similar controls

Although the genetic roles of *CPC* and *GL2* are opposite, the expression pattern of the two genes in different mutant or transgenic backgrounds revealed that both genes have similar controls. Expression of both genes is promoted by *TTG* and *R*, but repressed by *CPC*. The almost identical patterns of expression indicate that the promoter region of the two genes share common *cis* elements responsive to the regulator proteins.

This notion was supported by the promoter substitution experiment. When a chimeric gene of *GL2* promoter::*CPC* was introduced into the *cpc* mutant, the transgenic plants showed normal pattern of root hairs. A detailed analysis of the *CPC* promoter and comparison with the *GL2* promoter will be necessary to clarify the regulatory mechanism.

Role of the CPC and R complex

CPC is a small protein carrying a Myb domain but no other domains that might activate transcription. The Myb region is known to be a DNA binding motif in mammals and plants, and also to be a protein-protein interaction domain in plants (Goff et al., 1991; Goff et al., 1992; Szymanski et al., 1998). The two-hybrid assay in yeast cells and the direct coprecipitation experiment showed that the Myb region of the CPC protein interacts with the N-terminal region of R. This is supported by reports that two plant Myb proteins carrying a transcriptional activation domain, maize C1 and *Arabidopsis* GL1, interact with R and promote the expression of anthocyanin biosynthesis genes and *GL2*, respectively (Goff et al., 1991; Goff et al., 1992; Larkin et al., 1994; Szymanski et al., 1998). *WER*, another Myb homolog controlling root-hair differentiation, also interacts with R in yeast (Lee and Schiefelbein, 1999).

In a dominant inhibitor allele of maize C1, C1-I, asparate (D) at position 101 was changed to glutamate (E) in a dominant inhibitor allele of c1, C1-I (Paz-Arez et al., 1990). C1:D101E was able to interact with the maize Myc gene, *B* (Goff et al., 1992). In contrast, C1:D101E was not able to bind a1 promoter, which is one of the enzymes involved in anthocyanin biosynthesis (Sainz et al., 1997). These two results lead to the prediction that C1:D101E is a DNA binding mutant. The amino acid in CPC corresponding to position 101 in C1 is proline. Therefore, the CPC Myb domain is thought to act only as a protein-protein interaction region.

It is important to identify the functional homolog of *R* in *Arabidopsis* that forms a complex with the CPC protein to regulate root-hair differentiation. There are several Myc-like genes in *Arabidopsis* (Abe et al., 1997; de Pater et al., 1997; Urao et al., 1996). Further analysis is required for determining the true functional R homolog involved in root hair development.

Model of root hair development

Based on the results presented here and previous reports on the regulation of the root hair development, we propose a model explaining the patterning of hair-forming cells and hairless cells. The hairless cells express *GL2*, which leads to the repression of the formation of root hairs. *GL2* expression is induced by the transcriptional activation function of the R homolog and repressed by CPC. The expression of *CPC* and the R homolog in the hairless cells could be induced by *TTG*, whereas in hair cells, the expression of *GL2* is repressed. Because the repression in hair cells is lost in the *cpc* mutant, the CPC protein synthesized in hairless cells is postulated to be responsible for the repression. Our results from *CPC* promoter::*GFP* transgenic plants suggested that the repression can be explained as follows. CPC mRNA and possibly the protein are synthesized abundantly in hairless cells and then transferred to the neighboring hair cells where CPC protein represses GL2 expression. NLS:GFP data confirm that the CPC protein moves from the hairless cell to the hair cell.

In *35S::CPC* transgenic plants, the high-level expression of CPC produces free CPC proteins that could repress *GL2* expression in both hairless cells and hair cells. Repression of *GL2* would induce root hair formation in both types of epidermal cells. When *35S::R* is introduced into wild type cells, the large amount of R may promote expression of both *CPC* and *GL2*. In transgenic plants expressing the N-terminal region of R, truncated R would quench the free CPC protein by forming a complex with it. The reduction in the level of free CPC would help the endogenous R homolog and activate the expression of *GL2*.

The initial step in the differentiation of the two types of cells might be the perception of some positional information related to the arrangement of the cortical cells underneath the epidermis. Although the molecular nature of this information is not known, it could be postulated that it induces the expression of *TTG* in the hairless cells, which leads to the differentiation and maintenance of the two types of cells. This idea could be tested by examining the expression pattern of *TTG*.

A maize homeobox protein, KNOTTED1, that controls leaf formation was shown to move from inner cells to the epidermal cells possibly through plasmodesmata (Lucas et al., 1995). Recently, an *Arabidopsis* protein, SHORTROOT, was shown to move from stele cells to the surrounding endodermis cells, possibly through plasmodesmata (Nakajima et al., 2001). Because the CPC protein is small, it may be transferred through such structures.

Root hair development is parallel to trichome development

Recent genetic and molecular analyses have revealed that the initial step in the development of trichomes, which are branched, outgrowths of epidermal cells on the surface of leaves and stems, is controlled by a genetic mechanism similar to that operating in root hair development. The *gl2* mutant forms a few non-branched trichomes (Koornneef et al., 1982), and the *ttg* mutant fails to form trichomes (Koornneef, 1981). The mutant phenotypes indicate that both *GL2* and *TTG* act as positive regulators of the trichome development. Expression of the maize *R* gene in the *ttg* mutant induced trichome formation (Lloyd et al., 1992). The genetic complementation of the *ttg* mutation by *R* strongly indicates that some R homolog(s) of *Arabidopsis* are working in the process of trichome development, too. Recently, *GLABRA3* (*GL3*) was shown to encode a bHLH protein (Payne et al., 2000). GL3 interacts with the N-terminal portion of GL1 in yeast (Payne et al., 2000). In addition, formation of the trichome-forming cells is also positively controlled by *GL1*, a gene encoding a Myb domain and an acidic region (Oppenheimer et al., 1991). Plants that ectopically express both GL1 and R initiate ectopic trichomes (Larkin et al., 1994). *GL2::GUS* analysis indicated that *GL2::GUS* expression is reduced in mature leaves of *gl1* and *ttg* mutants and strong and ectopic expression of *GL2::GUS* resulted in ectopic expression of both GL1 and R (Szymanski et al., 1998). These results are interpreted to indicate that *GL1* and *GL3* cooperatively promote the expression of *GL2* and that *GL2* initiates the trichome development.

As shown above, CPC protein is likely to be a repressor of *GL2* expression, because this protein does not have an activation domain. This notion is supported by the phenotypes of *35S::CPC* transgenic plants that failed to develop trichomes on leaves and stems (Wada et al., 1997), possibly because overexpression of CPC protein in the trichome-forming epidermal cells competitively blocked the action of the GL1 protein, and repressed the expression of *GL2*. The similarity between the genetic control of root hair development and that of the trichomes may indicate that the two processes are derived from a basic cell differentiation process that accompanies oriented cell growth.

This study showed that interaction of a set of transcription factors determines the initial step in cell differentiation. It strongly suggests that a signaling between neighboring cells is important in the process. The genetic regulatory system of root hair development appears to be a good model system of cell differentiation in plants.

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