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 (atriochoblasts). 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 WEREWOLF (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
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 ecotopically 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 Rsechew 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 ctrl1 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 HindIII fragment obtained from pgl2gen into pBluescript SK+ (Stratagene). After digestion of this plasmid with Nhel and Apol, the larger fragment was purified and ligated into the XbaI and Apol sites of CPC cDNA prepared from SK+::CPC (SK+GL2::CPC). The GL2::CPC region was removed with HindIII and Apol from SK+::CPC and ligated into the HindIII and SacI sites of a binary vector, pARK5. For 35S::RN construction, an XbaI-SnaI8387I fragment including the 5' UTR region and the N-terminal region of R was ligated to a Psol and EcoRV-digested fragment including the 3' UTR region of R, and inserted into pBluescript SK+. This plasmid DNA was digested with XbaI and HindII, and subcloned into the XbaI and HpaI sites of pMAT137Hm (Matsuoka and Nakamura, 1991).

The GL2 promoter::GUS chimeric gene was constructed by ligation of a Xhol-Sall fragment of 4 kb at the 5' upstream region of GL2 into a Sall site of pH101 (Masucci et al., 1996; Szymanski et al., 1998). For construction of the CPC promoter::GUS, a Psol-Bbsf fragment of 1.2 kb was blunted by T4 DNA polymerase and subcloned into a Smal site of pH101-Hm3 (provided by H. Hirano and K. Nakamura, Nagoya University, Nagoya, 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 F2 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-D-glucoride), 1.5 mM K3Fe(CN)6, 1.5 mM K4Fe(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 (Doahmm EM, Kyoto, Japan).

In situ hybridization

RNA probes used for detecting CPC transcripts in situ were prepared by PCR using the following primers: CPCF1, 5' TTAAGCTTCTCGATGTTCCT TTCTTTT 3'; CPCB2, 5' GGAATTCTTTTCAAAAAATGCTC 3'. A PCR fragment (400 bp) was digested with HindIII and EcoRI, 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 HindIII or EcoRI, respectively, prior to adding them to the in vitro transcription mixture (Trans Probe kit, Pharmacia) containing T3 RNA polymerase and 32P-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 Ncol and into the Smal site pGBT9 after blunting with a Klenow Fragment.

R deleted of residues 1-371. pSPUTK-R was digested with Ncol and EcoRI, and cloning into the Smal site pGBT9 or of pGAD424.

R deleted of residues 1-298 or 1-206 combined to the GAL-AD.
CPC: a positive regulator of root hair formation

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 promoter::CPC in 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 plants.

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 pSPUTK with MluI and Clal 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 pSPUTK with KsAI and NcoI. After the ends had been blunted by a Klenow fragment, the fragment was inserted into the NcoI and Clal 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 EcoRI and SalI. 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 [35S]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.

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 plants.

Table 1. Phenotype of root epidermal cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of root hairs per mm (a)</th>
<th>Length of epidermal cell (μm) (b)</th>
<th>Relative hair number*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (WS)</td>
<td>43.2±1.0</td>
<td>257.1±9.9</td>
<td>11.1</td>
</tr>
<tr>
<td>WT (Rschew)</td>
<td>53.1±2.1</td>
<td>261.5±5.2</td>
<td>13.9</td>
</tr>
<tr>
<td>cpc</td>
<td>10.6±0.6</td>
<td>289.0±12.0</td>
<td>3.1</td>
</tr>
<tr>
<td>GL2::CPC in cpc</td>
<td>39.6±1.4</td>
<td>269.3±8.5</td>
<td>10.6</td>
</tr>
<tr>
<td>35S::R†</td>
<td>0.6±0.4</td>
<td>161.1±6.2</td>
<td>0.1</td>
</tr>
<tr>
<td>35S::Nterminal region of R (1-298)</td>
<td>1.3±0.5</td>
<td>261.5±5.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*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.
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 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. 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
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 \( GL2 \) 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. 3L, 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 \( R \) 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 \( R \). As a control, \( CPC \) interacted with residues 372-525 of \( R \) to a similar degree. However, \( CPC \) interacted with residues 1-525 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 \( R \), 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 GAL4-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...
CPC a positive regulator of root hair formation

**A**

CPC

Myb region

region required for binding to R

maize R

acidic region

Myc (bHLH) region

region required for binding to CPC

**B**

bound to GAL4-DB

CPC 1-94
CPC 1-94
CPC 1-94
R 1-371
no proteins
no proteins
no proteins
SV40 large T antigen

bound to GAL4-AD

R 1-525
R 1-371
R 372-525
R 1-94
R 1-525
R 1-371
no proteins
cPC 1-94
no proteins
no proteins
no proteins
p53

β-galactosidase activity (units)

Colony Formation on his+ Plates

+ + - - + - -

**C**

truncated R bound to GAL4-AD

1-271
1-321
1-381
1-291
1-291
1-241
1-239
1-239
23-268
30-268

β-galactosidase activity (units)

**D**

truncated CPC bound to GAL4-AD

1-94
4-94
1-63
32-94
32-63
1-75
1-65
44-94

β-galactosidase activity (units)

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 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 protein (Wada et al., 1997). These results show that the Myb-homologous domain of CPC, residues 33-83, is sufficient for binding to 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).

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...
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::GUS 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, ctrl 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 ctrl 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, asparagine (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 al 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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