Regulation of CAPRICE Transcription by MYB Proteins for Root Epidermis Differentiation in Arabidopsis

Yoshihiro Koshino-Kimura 1,2, Takuji Wada 2, Tatsuhiko Tachibana 1,4, Ryuji Tsugeki 1, Sumie Ishiguro 1,5 and Kiyotaka Okada 1,2,3,6

1 Department of Botany, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto, 606-8502 Japan
2 Plant Science Center, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa, 230-0045 Japan
3 Core Research for Evolutional Science and Technology (CREST) Research Project, Japan Science and Technology Agency, Kawaguchi, Saitama, 322-0012, Japan

Introduction

Cell fate determination is a critical step in the developmental process of multicellular organisms, in which transcription factors are known to play important roles. Because of its well-characterized genetics, short generation time and ease of observation, Arabidopsis epidermal cells have been used as a simple but significant model for understanding cell differentiation. Epidermal cells are generated at the root apical meristem and differentiate into two cell types, hair cells and hairless cells, in a cell position-dependent manner (Dolan et al. 1994). Epidermal cells contacting two cortical cells differentiate into hair cells, whereas cells touching only one cortical cell develop into hairless cells. Wild-type Arabidopsis ecotypes have eight hair cell files aligned longitudinally along the root.

Several mutants defective in epidermal cell differentiation have been isolated. The glabra2 (gl2) and werewolf (wer) mutants show conversion of hairless cells to root hair cells (Masucci et al. 1996, Lee and Schiefelbein 1999). GL2 encodes an HD-Zip protein and WER encodes an R2R3-type MYB protein (Rieke et al. 1994, Di Cristina et al. 1996, Masucci et al. 1996, Lee and Schiefelbein 1999). Expression of GL2 induces differentiation into hairless cells, and WER has been suggested to induce GL2 expression (Lee and Schiefelbein 2002). Two basic helix-loop-helix (bHLH) proteins, GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3), work on hairless cell differentiation in a redundant manner (Bernhardt et al. 2003, Zhang et al. 2003). In the gl3 egl3 double mutant, most hairless cells are converted to root hair cells, though gl3 and egl3 single mutants show a slight increase of hair cells (Bernhardt et al. 2003). Like WER, these bHLH proteins also regulate GL2 expression in hairless cells (Bernhardt et al. 2003, Zhang et al. 2003). Using the yeast two-hybrid system, GL3 and EGL3 were shown to interact with WER (Bernhardt et al. 2003) and with a WD40 protein TRANSPARENT TESTA GLABRA1 (TTG1) (Payne et al. 2000, Esch et al. 2003, Zhang et al. 2003), suggesting that a transcriptional complex including MYB, bHLH and WD40 protein regulates GL2 transcription.

The caprice (cpc) mutant has fewer root hairs apparently because some hair cells are converted to hairless cells (Wada et al. 1997). The CPC gene encodes an R3-type MYB protein.

Keywords: CAPRICE — Cell differentiation — DNA binding — MYB protein — Transcription factor — WEREWOLF.

Abbreviations: bHLH, basic helix–loop–helix; CPC, CAPRICE; DTT, dithiothreitol; GL, GLABRA; GST, glutathione S-transferase; GLS, β-glucuronidase; EGL, ENHANCER OF GLABRA; TTG, TRANSPARENT TESTA GLABRA; WER, WEREWOLF.
The phenotype of cpc suggests that CPC promotes hair cell differentiation. Though CPC is a positive regulator of hair cell differentiation, CPC is preferentially transcribed in hairless cells (Wada et al. 2002). CPC transcription was decreased in root hairless cells in 

\[ \text{tg} \] and \[ \text{wer} \], whereas it was ectopically observed in plants where the maize bHLH gene \[ R \] was constitutively overexpressed (Lee and Schiefelbein 2002, Wada et al. 2002). This genetic evidence has revealed that TTG1, \[ \text{WER} \] and some bHLHs seem to regulate the CPC transcription pattern. Lee and Schiefelbein (2002) proposed that transcriptional feedback loops between the \[ \text{WER}, \text{CPC} \] and \[ \text{GL2} \] genes help to establish the CPC transcription pattern.

Many of the same or similar components involved in root epidermis differentiation also regulate shoot epidermis differentiation. MYB protein, GL1 and bHLH proteins, GL3 and EGL3, are predominantly expressed in trichome cells and positively regulate GL2 expression (Bernhardt et al. 2003, Zhang et al. 2003). TTG1 is also involved in trichome differentiation. \[ \text{TRIPTYCHON} \] (TRY) encodes a CPC-homologous MYB-related transcription factor and regulates leaf trichome distribution (Schnittger et al. 1998, Schnittger et al. 1999, Schellmann et al. 2002). In the cpc mutant, the number of trichomes is increased, suggesting that CPC has a role in long-range lateral inhibition of trichome formation in leaves (Schellmann et al. 2002).

Genetic evidence suggests that CPC and GL2 transcription are regulated positively by \[ \text{WER} \] and negatively by CPC. However, little is known about the details of these transcriptional relationships; in particular, there is no evidence of whether \[ \text{WER} \] and CPC directly regulate transcription of the target genes or not. To understand the transcriptional regulation of CPC, we analyzed the CPC promoter region and identified a cis-acting element. We also evaluated the binding affinity of transcription factors for this element.

**Results**

*Deletion analyses of the CPC promoter*

In order to understand the cell file-specific expression of CPC, we examined the structure of the CPC promoter sequence by constructing a series of deletions (Fig. 1B). First, the transcription start site was identified in position \(-156\) relative to the translational start codon by screening of an \[ \text{Arabidopsis} \] cDNA library of the root and by \(5'\) rapid amplification of cDNA ends (\(5'\) RACE) (Fig. 1A, Wada et al. 1997). Then, we used an approximately 1.2 kb fragment from position \(-1,252\) to \(-63\) as a control to examine the CPC promoter activity, [designated \(pCPC(-1,252)\); Fig. 1A, B], because this fragment is sufficient to complement the \(cpc\) mutation when it was fused to the CPC coding sequence (Wada et al. 1997).

When the \(pCPC(-1,252)\) fragment was fused to the \(\beta\)-glucuronidase (GUS) gene and introduced into wild-type \[ \text{Arabidopsis} \], GUS staining was observed in hairless cells at the root meristem (Fig. 2A, F). Weak GUS staining was observed in root stefle cells (including the pericycle and vascular tissue) (Fig. 2F). In leaves, GUS staining was observed in trichome cells exclusively (Fig. 2K). On younger developing leaves, GUS staining was observed more broadly (Fig. 2K arrow). These GUS staining patterns were consistent with the results of in situ RNA hybridization (Schellmann et al. 2002, Wada et al. 2002).

When the promoter was truncated up to \(-681\) \([pCPC(-681)]\), GUS staining was observed in hairless cells and stele cells in the root, and in the trichome cells in leaves (Fig. 2B, G, L). This is the same pattern observed for full-length promoter up to \(-1,252\). When using the \(-492\) promoter, GUS staining was observed in hairless cells and stele cells, as well as trichome cells (Fig. 2C, H, M). In \(pCPC(-492)\):GUS, however, the staining was not observed in epidermal cells near the root tip where GUS staining was observed in \(pCPC(-1,252):GUS\) and \(pCPC(-681):GUS\) plants (Fig. 2A–C). About 10 epidermal cells close to their initial cells are not stained (Fig. 2C). This result suggests that the region between \(-492\) and \(-681\) of the CPC promoter is required for CPC transcription in the early stage of root epidermis development. When the promoter was deleted up to \(-423\) \([pCPC(-423)]\), GUS staining was detected neither in hairless cells at the root (Fig. 2D, I) nor in leaf trichome cells (Fig. 2N). However, the staining remained in the stele (Fig. 2D, I). With the \(-394\) promoter, the GUS staining pattern was similar to that of the \(-423\) promoter (Fig. 2E, J, O). These results indicate that the 69 bp region between \(-492\) and \(-423\) of the CPC promoter (termed the CWB region; Fig. 1B, C) is required for epidermis-specific transcription, specifically in root hairless cells and leaf trichome cells. The GUS staining in root stele cells, which was observed with the full-length CPC promoter, was retained in all of the truncated promoters. The region that is responsible for CPC transcription in stefle cells should be located downstream of \(-394\).

The CWB region is sufficient for hairless cell- and trichome cell-specific transcription

To clarify whether the CWB region between \(-492\) and \(-423\) of the CPC promoter was sufficient for hairless cell- and trichome cell-specific transcription, we constructed an 8-fold tandem repeat of the CWB fragment fused to the \(35S\) minimal promoter and GUS gene, designated \(pCPC(CWBx8):mp::GUS\) (Fig. 1D). GUS activity was preferentially observed in trichome cells and hairless cells (Fig. 3A–C), and was not observed in stelae cells (Fig. 3B). These results indicate that the CWB region is sufficient for specific expression in trichome cells and hairless cells.

When the same promoter was connected to the CPC coding region, designated \(pCPC(CWBx8):mp::CPC\), and the resulting construct was used to transform the \(cpc\) mutant, the transformant complemented the \(cpc\) phenotype (Fig. 3E, Table 1). This result suggests that the CWB region has an important role for the activity of CPC that leads to the development of normal root hairs. It is also suggested that CPC transcription in stefle cells is not related to the CPC function in root hair formation.
Putative MYB-binding sites are required for epidermis-specific transcription

In the sequence of the CWB region, we found two putative MYB-binding sites (termed CPCMBS1 and 2; see Materials and Methods). To determine whether these CPCMBSs were related to the epidermis-specific transcription of CPC, we introduced base substitutions in both CPCMBSs (m1), in CPCMBS2 (m2) or in CPCMBS1 (m3) to the pCPC(-492)::GUS construct, and checked their promoter activity (Fig. 1C, 4A–F). When pCPC(m1) or pCPC(m2) was used, GUS staining was not observed in epidermal cells of roots (Fig. 4A, B, D, E). In the case of pCPC(m3), only very weak GUS staining could be detected in some root epidermal cells (Fig. 4F). In leaves, we were not able to detect GUS staining in trichome cells or in any other cells in any of the transgenic plants (Fig. 4G–I). These results indicate that the two CPCMBSs have important roles for transcription in hairless and trichome cells.

WER binds to the CWB region of the CPC promoter

Previous genetic studies using mutants suggested that CPC transcription was regulated by two MYB proteins, WER and CPC itself (Lee and Schiefelbein 2002, Wada et al. 2002). In the wer mutant, GUS staining was reduced and rarely observed in epidermal cells (Fig. 4J, N), whereas it was ectopically observed in hair cells in the 35S::WER plant (Fig. 4K, O) (Lee and Schiefelbein 2002). On the contrary, the GUS staining was observed in all of the epidermal cells in the cpc mutant (Fig. 4L, P), whereas it disappeared from the epidermis in the 35S::CPC transformant (Fig. 4M, Q) (Lee and Schiefelbein 2002, Wada et al. 2002). These results suggest that CPC transcription is positively regulated by the WER protein and is negatively regulated by the CPC protein itself.

To determine whether WER can directly bind to the CWB region of the CPC promoter, we carried out a gel mobility shift assay using WER recombinant protein. Because the full-length WER protein could not be obtained as a glutathione S-trans-

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**Fig. 1** Sequence of the CPC promoter and constructions for generating transgenic plants. (A) The sequence of the CPC promoter region. The number indicates the distance from initiation codon ATG. The CWB region is underlined and CPCMBS1 and CPCMBS2 are indicated in bold. (B) A series of deletions in the 5′ regulatory region of the CPC gene. Each fragment is connected to the GUS coding region and nopaline synthase terminator. (C) The CWB region sequence located from position –492 to –423 and the modified promoters of the –492 fragment which are mutated in both or either CPCMBSs. Red stripes in pCPC(m1), pCPC(m2) and pCPC(m3) indicate the position where the base substitutions were introduced. Wild-type CWB fragment and m1–m4 fragments were used for gel mobility shift assay. (D) pCPC(CWBx8) has eight tandem repeats of the region from –492 to –423 (arrows) connected to the minimal CaMV 35S promoter from –46 to –1 (box).
ferase (GST)-fused recombinant protein (data not shown), we used the N-terminal fragment of WER (residues 1–126; including two MYB domains), which was expressed as a GST-fused protein in *Escherichia coli* (GST–WERMYB). The DNA-binding activity of this fusion protein was tested by gel mobility shift assays using fragments of the wild-type CWB region (Fig. 5A). A shifted band was observed (Fig. 5A, lane 2), indicating that the MYB domain of the WER protein directly binds to the wild-type CWB fragment. Any shifted bands were not observed in the absence of dithiothreitol (DTT) (Fig. 5A, lane 4), indicating that the reduced state of the WER protein is required for binding to the CWB region.

Binding of WER to the CWB region was also assayed using the yeast one-hybrid system. Eight tandem repeats of the CWB region were fused to the *LacZ* gene, and then served as a reporter construct, designated *CWBx8::LacZ*. Three types of WER corresponding to full-length WER, the N-terminal fragment (including MYB domains) and the C-terminal fragment of WER were fused at the C-terminus of the yeast GAL4 activation domain, and then served as effector constructs. When the full-length WER or the N-terminal fragment of WER was expressed, the LacZ expression level was increased approximately 9-fold or 5-fold compared with the vector control, respectively (Fig. 6). These results indicate that the MYB domain of the WER protein recognizes and binds to the CWB region of the *CPC* promoter.
We also estimated the effect of base substitutions in CPC-MBSs of the CWB region on binding of WER by competition gel mobility shift assay. The m1 fragment did not compete with the labeled CWB fragment as effectively as the wild-type competitor (Fig. 5B). As well as the m1 fragment, the competition was less effective when we used the m4 fragment, in which base substitutions were introduced more broadly than in m1 (data not shown). These results indicate that WER recognizes the CPC-MBSs of the CWB region. The m3 fragment did not compete effectively but the m2 fragment did, indicating that CPC-MBS1 is more important for the recognition of WER than is CPC-MBS2 (Fig. 5B).

CPC does not bind to the CPC promoter but suppresses WER binding

As described previously, the CPC protein has been suggested to be a negative regulator of CPC transcription (Fig. 4L, M, P, Q). We examined whether the CPC protein could bind to the CWB region of the CPC promoter. When GST-fused CPC protein was added to the CWB fragment in gel mobility shift assays, no shifted bands were observed (Fig. 5C, lane 4). Yeast one-hybrid assays using CWB×8::LacZ and GAL4 activation domain-fused CPC showed no significant increase of β-galactosidase activity (Fig. 6). These results suggest that CPC could not bind to the CWB region of the CPC promoter.

However, when both full-length WER protein and CPC protein were co-expressed in yeast, LacZ activity was lower than in yeast with WER only (Fig. 6), suggesting that the existence of CPC interferes with the binding of WER to the CWB region. We also tested this interference using gel mobility shift assay. The efficiency of the shift was not affected when the CPC protein was added in the binding solution of WER and the CWB fragment, suggesting that CPC does not directly interfere with the binding of WER to the CPC promoter. We discuss later the mechanism of interference by the CPC protein.

WER also binds to GL2 promoter

Another key player in epidermis differentiation, GL2, is transcribed in hairless cells and trichome cells (Hung et al. 2019). The ratio of root hair formation per cell (%) in H position cells (that make contact with two cortical cells) and in N position cells (that make contact with only one cortical cell) in wild-type, cpc mutant and transformant [pCPC(CWB×8):mp::CPC in cpc].

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<tr>
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<tr>
<td>Wild-type(WS)</td>
<td>90.0 (n = 10)</td>
<td>0.0 (n = 14)</td>
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<tr>
<td>cpc</td>
<td>11.9 (n = 42)</td>
<td>0.0 (n = 39)</td>
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<tr>
<td>Transformant</td>
<td>89.9 (n = 148)</td>
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Fig. 4 The effects of base substitutions in CPC-MBSs in epidermis and the involvement of MYB-related mutants and transgenic plants in CPC transcription. (A–I) The GUS staining pattern of transgenic plants that carried a mutated promoter and GUS coding sequences was observed. In pCPC(m1)::GUS (A, D and G) and pCPC(m2)::GUS (B, E ad H), we could not detect GUS staining either in hairless cells in root or in leaves. Faint staining was observed in the pCPC(m3)::GUS plant, though not in its leaves (C, F and I). (J–Q) GUS staining of pCPC(-1252)::GUS in wer (J and N), 35S::WER (K and O), cpc (L and P) and 35S::CPC (M and Q). Ectopic expression in hair cells is indicated by the arrowhead in (O) and (P). Bars = 100 µm.
Gene regulation in root epidermis differentiation

1998, Szymanski et al. 1998). Genetic analyses showed that CPC and GL2 expression is controlled by same or similar components (Wada et al. 2002). As well as the CPC promoter, the GL2 promoter has two putative MYB-binding-sites [GL2MBS1 (GACTAACGGTAAAG) and GL2MBS2 (TACTACAGTATA)], which are required for expression in trichome cells and root hairless cells (Hung et al. 1998, Szymanski et al. 1998). To estimating whether the WER protein can also bind to these sites, we carried out the gel mobility shift assay. When the GST-fused MYB domain of WER protein was added, a shifted band was observed (Fig. 5D). This indicates that WER directly binds to the GL2MBSs in the GL2 promoter.

Discussion

CPC promoter can be divided into three regions

As shown in Fig. 2, expression of CPC is regulated in a spatial and temporal manner. CPC transcription is restricted to hairless cells of root epidermis and to trichome cells of leaves at relatively early stages, and expression was not observed in fully developed cells. A series of promoter truncations clearly showed that the CPC promoter includes at least three functionally distinctive regions. The first is the CWB region located between –492 and –424, which is responsible for hairless cell and trichome cell-specific expression. When we deleted this region, epidermis-specific expression was completely absent. The second is a region between –681 and –492 which is involved in determining at which stage of hairless cell CPC transcription starts. When this region was absent, expression was not observed in hairless cells of very early stages, but was detected in cells at later stages (Fig. 2A, C). With the full-length promoter [pCPC(-1252)], the expression level gradually decreased in fully developed epidermal cells. The timing of the decrease was, however, not affected by the presence or absence of this region (data not shown). The third region between –423 and –1 is apparently not responsible for expression in epidermal cells. It is suggested that this region is responsible for expression in stelar cells because the GUS staining was observed in pCPC(-423):::GUS.

An artificial promoter combining repeats of the CWB region and a minimal promoter [pCPC(CWBx8):mp] showed exactly the same expression pattern as the control promoter.
Insufficient and binding to both sites is required for hairless cell- and trichome cell-specific expression, although the base sequences of the two sites are not identical. In addition, these results strongly suggest that it is less likely that sequences outside the MYB-binding sites have some role in epidermal cell-specific expression.

We showed that WER could bind to two CPCMBs and two GL2MBs. A comparison of their base sequences has identified the octameric sequence (C^12/AACNG) as the WER protein-binding consensus sequence. This sequence is similar to the previously reported MYB-binding sites of maize P1 protein (CC^12/ACC) (Grotewold et al. 1994, Williams and Grotewold 1997) and vertebrate MYB protein v-MYB (C^12/AACGG) (Howe and Watson 1991, Weston 1992). Recently, Heine et al. (2004) showed that two cysteines in the R2 domain of the P1 MYB protein seem to form a disulfide bond in the non-reduced state and the reduction of these two cysteines is required before the P1 protein binds to DNA. These same two cysteines are conserved in the R2 domain of WER and, furthermore, DTT is required for the binding of WER to the CPC CWB region. Therefore, it is reasonable to assume that these cysteines in WER protein have a critical role for the ability to bind to the CPC CWB region. In addition, it is highly likely that the GL1 protein recognizes and binds to CPCMBs sites, because a chimeric gene combining GL1 coding sequence with the WER promoter could complement the wer mutation (Lee and Schiefelbein 2001). The WER RNA could not be detected in leaves by Northern blot analysis (Lee and Schiefelbein 1999). Therefore, the GL1 protein could replace the WER protein as a transcriptional regulator of the CPC gene in leaves though the binding sequence of the GL1 protein is not known.

**Mechanism of the self-regulation of the CPC transcription**

Previous studies reported that the epidermal cell-specific expression of CPC is controlled by a number of proteins including two MYB proteins, WER and CPC itself (Lee and Schiefelbein 2002, Wada et al. 2002), two bHLH proteins, GL3 and EGL3 (Bernhardt et al. 2003), and a WD40 protein, TTG1 (Wada et al. 2002). Lee and Schiefelbein (2001) reported that the C-terminal region 24 amino acid sequence of WER promotes transcription as an activation domain in yeast. Here we have shown that WER protein binds to the CPC CWB region in the CPC promoter, indicating that the WER protein has functions of both DNA binding and transcriptional activation.

bHLH proteins are also involved in activation of CPC transcription. Wada et al. (2002) showed that CPC transcription was observed in both hairless and hair cell files when maize bHLH protein R was ectopically expressed in epidermal cells, indicating that bHLH promotes CPC expression. Recently, Arabidopsis bHLH proteins GL3 and EGL3 were shown to promote CPC transcription (Bernhardt et al. 2003), and both bHLH proteins interacted with WER in yeast (Bernhardt et al. 2003). Therefore, it could be proposed that the bHLH proteins act as co-activators of WER through protein–protein interactions.

Two MYB protein-binding sites in the CWB region are responsible for epidermal cell-specific expression

We showed that the CPC region includes two MYB-binding sites, CPCMBs1 and 2. These sites are responsible for epidermal cell-specific expression, because the specific expression pattern was absent when the binding consensus sequence was disrupted at either site (Fig. 4). The disruption of one site resulting in almost complete loss of transcription in vivo suggests that binding of MYB protein to one of the two sites is insufficient and binding to both sites is required for hairless cell- and trichome cell-specific expression, although the base sequences of the two sites are not identical. In addition, these results strongly suggest that it is less likely that sequences outside the MYB-binding sites have some role in epidermal cell-specific expression.

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protein association. There are several reports that bHLH proteins directly bind to DNA, but it is not known whether GL3 and EGL3 could directly bind to DNA and help to form the protein–DNA complex.

WD40 domains are known to aid in protein–protein interactions. A WD40 protein, TTG1, has a role in promoting CPC transcription, because CPC expression was decreased in the tig mutant (Walker et al. 1999, Wada et al. 2002). Recent reports showed that TTG1 interacts with bHLH proteins GL3 and EGL3 (Payne et al. 2000, Esch et al. 2003, Zhang et al. 2003). These results strongly suggest that hairless cell-specific transcription of CPC is promoted by a protein complex including WER, GL3, EGL3 and TTG1. Similar transcription-promoting complexes of MYB, bHLH and WD40 proteins were reported in the pigment synthesis of Arabidopsis (Baudry et al. 2004). In this system, a MYB (TT2), a bHLH (TT8) and a WD40 (TTG1) form a protein complex, and synergistically specify the expression of BANYULS, a key gene of proanthocyanidin biosynthesis.

As described above, expression of CPC is enhanced in the cpc mutant, and is decreased in CPC overexpression lines, suggesting that CPC expression is negatively self-regulated. It is of considerable interest how CPC controls its own expression. The binding activity of the WER protein to the CBW region was decreased when the CPC protein was co-expressed in yeast, suggesting that CPC has some roles in interfering with WER function on promoting CPC transcription. From its amino acid sequence, the CPC protein is not predicted to have DNA binding activity. The 37th Asp, 41st Lys and 42nd Asn in the MYB R3 domain are not conserved in the CPC R3 domain though these amino acids in MYB proteins are essential for sequence-specific DNA recognition (Ogata et al. 1994, Sainz et al. 1997, Wada et al. 2002). Indeed, we showed that CPC could not bind to the CBW region of the CPC promoter, therefore, it is less likely that CPC competes with WER for the binding to the CBW region. On the other hand, CPC was shown to interact with the N-terminal region of GL3 and EGL3, with which the WER homolog GL1 protein also interacts (Payne et al. 2000, Zhang et al. 2003). These indicate that CPC would compete with WER for the binding to bHLH proteins and would dissociate the WER-associated protein complex that activates CPC expression. In yeast, we observed the interference with the binding between the WER protein and the CBW region by the CPC protein, and it is possible that some endogenous proteins, such as bHLH or WD40 proteins, mediate this interfering mechanism. CPC protein lacks the transcription activation domain, thus the CPC–bHLH complex could not promote CPC expression. For the function of the CPC protein, it is also possible that some modifications are required. In addition, a recent report showed that a CPC-like R3-type MYB protein, TRY, inhibited binding of MYB (GL1) and a bHLH (GL3) in yeast (Esch et al. 2003). These results suggest that a small MYB protein such as CPC or TRY works as an inhibitor of the active transcription complex.

Regulation of GL2 transcription resembles the CPC regulatory system

Previous analyses using mutants and overexpression lines showed that hairless cell-specific expression of GL2 is controlled positively by WER, TTG1, GL3 and EGL3, and negatively by CPC (Lee and Schiefelbein 2002, Wada et al. 2002, Bernhardt et al. 2003, Zhang et al. 2003), which is similar to the situation of CPC expression. In this study, we demonstrated that WER protein binds to GL2MBSs in the GL2 promoter. These results indicate that control of GL2 transcription is likely to be similar to CPC, namely by a protein complex including the same MYB, WD40 and bHLH proteins. CPC protein would disrupt the protein complex by competitive binding with WER, and repress GL2 expression.

The question of why both GL2 and CPC are predominantly expressed in hairless cells, though CPC controls the development of hair cells, has frequently arisen. However, this expression pattern would be reasonable if expression of both genes is dependent on the specific expression of WER in hairless cells and on the inability of the WER protein to move to neighboring cells. CPC protein moves from the hairless cells to the neighboring hair cells and represses GL2 expression in root hair cells (Wada et al. 2002).

Considering that the negative function of CPC on the expression of CPC and GL2 is based on binding competition with WER, the ratio of available protein between CPC and WER would be important to determine the fate of epidermal cells. Therefore, quantitative regulation of CPC expression would be an important addition to cell type regulation. Moreover, it was shown that GL2 regulates cell differentiation in a dose-dependent manner (Ohashi et al. 2002), suggesting that GL2 expression is tightly regulated. Therefore, the existence and function of the CPC protein not only in root hair cells but also in hairless cells are required for precise control of GL2 transcription. Further analyses of components of the transcription complex and their dynamics will clarify mechanisms of the epidermis determination.

Materials and Methods

Plant materials and growth conditions

We used Arabidopsis Wassilewskija (Ws) ecotype as wild type. The seeds of the wer mutant and 35S::WER transgenic plants were provided by Dr. John Schiefelbein. Seeds were surface-sterilized and planted in square Petri dishes containing 1.5% agar medium as described previously (Okada and Shimura 1990). Seeds on plates were incubated at 4°C for 3–4 d and then the plates were placed vertically in an incubator at 22°C under continuous white light.

Construction of chimeric genes and transgenic plants

To make a series of CPC promoter::GUS constructs, we amplified a series of the deleted CPC promoters by PCR using primer sets CPC-RIF (5’-ATAAAGCCTGAATTCTCAGACTTTATAC-3’) and CPCRB1B (5’-ATATCTAGAAGAAGCCTTGGCTTTGCTC-3’). To construct CPC promoter::GUS, we amplified a series of the deleted CPC promoters by PCR using primer sets CPC-D1F (5’-ATAAAGCTTCCAGAGGAGAAGC-3’), CPC-D1R (5’-ATAAACGGTGATTTTATAGGAGCAT-3’) and CPCD1-2F (5’-ATAAACGGTGATTTTATAGGAGCAT-3’).
CTTAAAAATAATGGTTAAGG-3') and CPCRB2 for pCPC(-423), and CPC-2D2 (5'-ATAAAACTTTCTGCTTCTTCTTCTATG-3') and CPCRB2 for pCPC(-394). Amplified fragments were digested with HindIII and XbaI and ligated into pBluescript SK+ (Stratagene, CA, USA) as pBS 681, pBS 492, pBS 423 and pBS 394, respectively. Clones were digested with HindIII–XbaI and subcloned into binary vector pBI101 (Clontech Laboratories, Inc., CA, USA). To create pCPC(m1):GUS, pCPC(m2):GUS and pCPC(m3):GUS, the pBS 492 plasmid described above was used as a template in PCR amplification with the following primer sets; pPCDcm296FF (5′-AGGAATTCTTACAGACAGCGATAGAAATAGTAGCT-3′) and pPCDcm296RR (5′-CTTACAGACAGCCTACGACACAAAGGAC-3′) for pCPC(m1), pPCDc296RR (5′-CTTTACAGACAGTTGGA-3′) and pPCDcm296FF for pCPC(m2), and pPCDcm296RR and pPCDcm296FF for pCPC(m3). The fragments obtained by the PCR were self-ligated and checked with these sequences. Obtained plasmids were then digested with HindIII–XbaI and subcloned into binary vector pBI101. To create a tandem repeat of the CWB region of the wild-type CPC promoter, CWB regions were produced by recursively cloning the same orientation. These binary plasmids were introduced into plants by vacuum infiltration (Bechtold and Pelletier 1998) with pGV2260 or pMP90 by electroporation using strain C58C1 (pGV2260) or C58C1 (pMP90) by electroporation using strain C58C1 (pGV2260) or C58C1 (pMP90). To create a tandem repeat of the CWB region obtained above were subcloned into a vector pBI221 (Clontech). Histological analysis

Gluc (5-bromo-4-chloro-3-indolyl–β-D-glucuronide), 1.0 mM KCl, 10 mM EDTA and 0.1% Triton X-100. They were incubated under reduced pressure for 5 min at room temperature, and then at 37°C for 5 h. For transverse sections, the samples were fixed with ice-cold 90% acetone, stained as described above, and cleared with 70% ethanol.

Bacterial expression of proteins and purification of GST-fused proteins

The DNA–protein binding reaction was basically performed by incubating 32 fmol of digoxigenin (DIG)-labeled oligonucleotide with 100 ng of each protein in 20 μl of binding buffer [10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol, 80 μg ml–1 poly(dI–dC) and 100 μg ml–1 bovine serum albumin (BSA)]. The reaction solution was incubated at 22°C for 15 min, and then free and bound complexes were resolved by electrophoresis through 1 mm thick 6% native polyacrylamide gels (80 : 1 acrylamide : bisacrylamide) in 0.25 TBE buffer at 8 V cm–1 for 60 min.

Yeast one-hybrid analysis

Yeast one-hybrid analyses were carried out based on a MATCH-MAKER One-Hybrid System (Clontech). To make the reporter construct, pBS_pCPC(CWBx8) was digested with XbaI and blunted, then digested with SalI and subcloned into the SacI and SalI sites of pLaCZi (Clontech). To make the effector construct, the pW and CPC cDNA fragments were amplified by PCR using primers WERNco1F (5′-GGCGCATTGGGAGAAAGAAGTAA-3′) and WERNco2R (5′-ATACCATGGGTTCTTCCATCTAC-3′) or CPCXhoR (5′-ATACCATGGGTTCTTCCATCTAC-3′) and CPCNcoF (5′-CCCTCCAGTCTAAACAGTCTCATAT-3′) of binding buffer (Sambrook and Russell 1994). The reaction solution was incubated at 22°C for 15 min, and then free and bound complexes were resolved by electrophoresis through 1 mm thick 6% native polyacrylamide gels (80 : 1 acrylamide : bisacrylamide) in 0.25 TBE buffer at 8 V cm–1 for 60 min.
Acknowledgments

We would like to acknowledge funding from the Japanese Ministry of Education, Culture, Sports, Science and Technology and from the Core Research of Science and Technology (CREST) Research Project. K.O. and Y.K. were supported by The Grant for the Biodiversity Research of the 21st Century COE (A14). Y.K. was supported as a Junior Research Associate by RIKEN from 2000 to 2004.

References


(Received February 21, 2005; Accepted March 23, 2005)