The WEREWOLF MYB protein directly regulates *CAPRICE* transcription during cell fate specification in the *Arabidopsis* root epidermis

Kook Hui Ryu[1], Yeon Hee Kang[1], Young-hwan Park[2], Ildoo Hwang[2], John Schiefelbein[3] and Myeong Min Lee[1],*

[1]Department of Biology, Yonsei University, Sinchon 134, Seoul 120-749, Korea
[2]Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Korea
[3]Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA
*Author for correspondence (e-mail: mmlee@yonsei.ac.kr)

Summary

The *Arabidopsis* root epidermis is composed of two types of cells, hair cells and non-hair cells, and their fate is determined in a position-dependent manner. WEREWOLF (WER), a R2R3 MYB protein, has been shown genetically to function as a master regulator to control both of the epidermal cell fates. To directly test the proposed role of WER in this system, we examined its subcellular localization and defined its transcriptional activation properties. We show that a WER-GFP fusion protein is functional and accumulates in the nucleus of the N-position cells in the *Arabidopsis* root epidermis, as expected for a transcriptional regulator. We also find that a modified WER protein with a strong activation domain (WER-VP16) promotes the formation of both epidermal cell types, supporting the view that WER specifies both cell fates. In addition, we used the glucocorticoid receptor (GR) inducible system to show that *CPC* transcription is regulated directly by WER. Using EMSA, we found two WER-binding sites (WBSs; WBSI and WBSII) in the *CPC* promoter. WER-WBSI binding was confirmed in vivo using the yeast one-hybrid assay. Binding between the WER protein and both WBSs (WBSI and WBSII), and the importance of the two WBSs in *CPC* promoter activity were confirmed in *Arabidopsis*. These results provide experimental support for the proposed role of WER as an activator of gene transcription during the specification of both epidermal cell fates.

Key words: WEREWOLF, CAPRICE, EMSA, Direct target, Root epidermis, Cell fate specification, *Arabidopsis*

Introduction

In multicellular organisms, all cells originate from a single cell, the zygote. During development, cells derived from the zygote must adopt distinct fates in a precisely controlled manner. This process relies on the differential expression of many genes, implying that transcription factors play crucial roles.

In plants, the epidermal cells in the *Arabidopsis* root provide a model system to study cell fate specification. The root epidermis is composed of two kinds of cells, hair-bearing cells and non-hair cells, and their fates are determined in a position-dependent manner (Dolan et al., 1994; Galway et al., 1994). The cells located on the periclinal cell wall of the underlying cortex (N-position) differentiate into non-hair cells, and the cells located on the anticlinal cell wall of the underlying cortex (H-position) differentiate into hair-bearing cells. Molecular genetic studies have revealed that many putative transcription factors are involved in this position-dependent cell fate specification. For example, *WEREWOLF* (*WER*), *GLABRA2* (*GL2*) and *TRANSPARENT TESTA GLABRA1* (*TTG1*) are known to promote the non-hair cell fate, so that in their cognate mutants, most of the root epidermal cells differentiate into hair cells, regardless of their

position (Galway et al., 1994; Lee and Schiefelbein, 1999; Masucci et al., 1996). *WER* encodes a putative transcription factor of the MYB family, and it is preferentially expressed in the N-position cells (Lee and Schiefelbein, 1999). The MYB transcription factor family is one of the largest families of transcription factors in the *Arabidopsis* genome (Reichmann et al., 2000). There are more than 180 MYB genes in *Arabidopsis* encoding one to three repeats (R1, R2 and R3) of the MYB domain, which is ~50 amino acids long and has regularly spaced tryptophan residues (Rosinski and Atchley, 1998). *GL2*, which encodes a homeodomain-leucine zipper (HD-Zip) protein, is expressed in the N-position cells (Masucci et al., 1996; Rerie et al., 1994) in a WER-dependent manner (Lee and Schiefelbein, 1999). *TTG1* encodes a WD40-repeat containing protein (Walker et al., 1999) and its expression pattern has not been reported yet. *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*), which encode bHLH putative transcription factors, are also reported to promote the non-hair cell fate in a redundant manner (Bernhardt et al., 2003), although they are expressed in the H-position cells (Bernhardt et al., 2005). The *CAPRICE* (*CPC*) gene encodes a single MYB repeat protein that lacks any discernible

transcriptional activation domain; mutation of this gene causes a reduced number of hair cells, implying that it induces the hair cell fate (Wada et al., 1997). The CPC protein probably acts in a partially redundant manner with the related TRIPTYCHON (TRY) and ENHANCER OF TRY AND CPC1 (ETC1) proteins (Schellman et al., 2001; Kirik et al., 2004).

To explain cell fate specification in the root epidermis, a competition mechanism between the WER and CPC MYB proteins was proposed (Lee and Schiefelbein, 1999). This explanation posits that epidermal cells with a relatively high level of WER adopt the non-hair cell fate, whereas cells accumulating a high level of CPC adopt the hair cell fate. However, the *CPC* gene was found to be expressed in the N-position cells instead of the H-position cells, and this expression is WER dependent (Lee and Schiefelbein, 2002; Wada et al., 2002). These results led to a lateral inhibition model for epidermal cell fate specification (Lee and Schiefelbein, 2002). In this model, *WER* expression is regulated by positional information, which causes slightly greater WER accumulation in the N-position cells than in the H-position cells. This is proposed to lead to greater transcription of *GL2* and *CPC* in the N-position cells. The GL2 induces the non-hair cell fate in these N-position cells, whereas the CPC is proposed to move to the neighboring H-position cells where it inhibits *GL2* (and *CPC*) expression and thereby promotes the hair cell fate (Lee and Schiefelbein, 2002; Wada et al., 2002). Therefore, the WER MYB protein is proposed to be a master regulator, which controls both the non-hair cell fate (by promoting *GL2* transcription) and the hair cell fate (by promoting *CPC* transcription).

Although this model fits the experimental results so far, it is necessary to further test and refine the model with additional experiments. Most importantly, we do not know yet whether the WER protein acts as a transcription factor in this system. This assumption was based solely on its amino acid sequence similarity with previously known MYB transcription factors (Lee and Schiefelbein, 1999) and its ability to transactivate transcription using a yeast one-hybrid assay (Lee and Schiefelbein, 2001). Furthermore, we do not know whether WER influences *CPC* and *GL2* transcription directly or indirectly. To address these issues, we have examined the localization and the transcriptional properties of the WER protein. Taken together, our findings strongly suggest that WER is indeed a transcriptional activator and directly induces *CPC* expression to specify cell fates during root epidermis development.

Materials and methods
Plant lines and growth conditions
The *wer-1* and *cpc* mutant alleles were described previously (Lee and Schiefelbein, 1999; Wada et al., 1997). The *wer-1* mutant line harboring P_{35S}:*WER*, and the plants harboring P_{GL2}:*GUS* (β-glucuronidase) or P_{CPC}:*GUS* have also been previously described (Lee and Schiefelbein, 2002; Masucci et al., 1996; Wada et al., 2002). The P_{GL2}:*GUS* and P_{CPC}:*GUS* reporter constructs were introduced into each line by genetic crosses.

Seeds were germinated and grown vertically on agarose-solidified medium containing mineral nutrients at 22°C under continuous light (Schiefelbein and Somerville, 1990).

Histochemical GUS staining
GUS activity was histochemically defined by staining 4-day-old seedlings as described previously (Lee and Schiefelbein, 2002).

Confocal microscopy
GFP expression in seedlings was examined using a BioRad Radiance 2100 scanning system with excitation at 488 nm and detection at 500-530 nm.

RNA extraction and northern blot analysis
Total RNA was extracted from the root tips of seedlings using the TRIZOL reagent according to the manufacturer's protocol. Northern blot analysis with a *CPC* gene fragment as a probe was described previously (Lee and Schiefelbein, 2002). The resulting signal was visualized with a Bioimage analyzer, BAS-2500 (Fuji film).

Electrophoretic mobility shift assay
Labeling of DNA fragments for the electrophoretic mobility shift assay was carried out using T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP (>3000 Ci/mmol, 10 mCi/ml) as described previously (Sainz et al., 1997) and this end-labeled probe was purified on a gel.

Electrophoretic mobility shift assays were carried out as described (Ausubel et al., 1988), except that the binding reaction included 20 mM NaCl, 10 mM Tris Cl (pH 8.0), 1 mM EDTA, 1 mM DTT, 5% glycerol, 30 μg/ml poly(dI-dC), 20,000 to 40,000 cpm of probe and the WER protein. This reaction was incubated at room temperature for 30 minutes and resolved on 6% polyacrylamide gel in 0.5×TBE buffer. The gel was dried and the signal was visualized using BAS-2500 (Fuji film).

Protein expression and purification
E. coli BL21 (DE3) transformed with pET-WER was treated with 1 mM β-D-thiogalactopyranoside. These bacterial cells were harvested after 3-hour incubation at 37°C, disrupted using a sonicator and were centrifuged at 14,000 *g* for 20 minutes to remove debris. From this extract, the expressed WER protein was purified using His•Bind Quick 900 Cartridges according to the manufacturer's manual (Novagen), and was dialyzed against storage buffer (25 mM Tris-Cl, pH 7.5, 25 mM NaCl, 10 mM EDTA, 0.1% NP-40, 50% glycerol).

Gene constructs and plant transformation
To make the chimeric genes, a genomic DNA fragment of the WER-coding region without the stop codon was PCR amplified, and fused to the N terminus of the GFP (no ER version) gene in frame (*WER-GFP*), to the N-terminus of the transactivating domain of the herpes viral VP16 (Triezenberg et al., 1988) in frame (*WER-VP16*) or to the N-terminus of the hormone binding domain of the glucocorticoid receptor (Picad et al., 1988) in frame (*WER-GR*).

To examine the subcellular localization of the WER protein, the P_{WER}:*WER-GFP* construct was generated by fusing a 2.4 kb 5′ flanking region DNA fragment and a 1.1 kb 3′ flanking region DNA fragment from the *WER* gene to the *WER-GFP* chimeric gene. As a control, the P_{WER}:*GFP* construct was made with the same GFP (no ER version).

For P_{35S}:*WER-VP16* (or P_{35S}:*WER-GR*), the chimeric *WER-VP16* DNA (or *WER-GR*) was inserted between 35S dual promoter and terminator in pRTL2-GUS (Restrepo et al., 1990). For *BD-WER-VP16*, the *WER-VP16* DNA fragment was fused to the GAL4 DNA-binding domain in the vector pGBT9. In this construct, we used the coding region of the *WER* cDNA instead of genomic DNA. Constructions of the *BD-WER* and the *AD-WER* have been described previously (Lee and Schiefelbein, 1999; Bernhardt et al., 2003).

To express and purify WER protein from bacteria, we made pET-WER. The coding region of the WER cDNA excluding the 3′ 72 bp long fragment was PCR amplified. This truncated DNA fragment was inserted downstream of the T7 tag sequence in the vector of pET28(a) using *Eco*RI and *Sac*I.

All the DNA fragments that were PCR amplified using Phusion

DNA polymerase (Finnzyme) were sequenced and confirmed to be error-free.

We used pCB302 vector as a binary vector for plant transformation (Xiang et al., 1999). Plant transformation was achieved by electroporating constructs into the Agrobacterium strain GV3101 followed by introduction into *Arabidopsis* using the floral dip method as previously described (Clough and Bent, 1998). T1 seeds were harvested and transgenic plants were selected by spraying commercially available BASTA three times a week for 2 weeks.

Yeast one-hybrid assay

To compare the transcriptional activation activities of the native WER protein and the WER-VP16 chimeric protein, the yeast one-hybrid assay was employed as described by Sadowski et al. (Sadowski et al., 1992). *BD-WER* construct was transformed into yeast strain Y190 and *lacZ* reporter gene expression was assessed by measuring β-galactosidase activity using chlorophenol red-b-D-galactopyranoside (CPRG; BMS) as a substrate.

To test WBSI and WBSII in vivo, we used the Matchmaker yeast one-hybrid system (Clontech) (Kumar et al., 1966) according to the manufacturer's manual. We synthesized three tandem copies of WBSI, WBSII or point mutated WBSIs. These were inserted upstream of the *HIS3* gene in the vector pHISi and upstream of *lacZ* gene in pLacZi, and these were integrated into yeast strain YM4271 genome. *AD-WER* described above was introduced into this reporter strains and *lacZ* reporter gene expression was assessed.

Arabidopsis protoplast transient expression assay

Arabidopsis mesophyll protoplasts were isolated and transfected as described previously (Hwang and Sheen, 2001). Protoplasts (2×10^4) were transfected with 40 μg plasmid DNA with different combinations of reporter (P_{CPC}:*LUC*), effector (WER) and internal control (UBQ10-GUS). Protoplasts were incubated in WI [0.5 M mannitol, 4 mM MES (pH 5.7), 20 mM KCl]for 9 hours under light conditions at room temperature. The luciferase reporter activity was determined by the Luciferase Assay System (Promega). The GUS assay was performed as described previously (Jang and Sheen, 1994).

Results

WER is a nuclear protein

WER does not possess a detectable nuclear localization signal based on its amino acid sequence. To examine the subcellular localization of WER, the GFP reporter gene was fused in-frame with the *WER*-coding region, and this fusion was expressed under the control of the WER promoter (P_{WER}:*WER-GFP*)

(Fig. 1A). This construct was introduced into the *wer*-1 mutant in *Arabidopsis* and was able to complement the *wer*-1 mutant phenotype, indicating that the WER-GFP fusion protein functions like the native WER protein in *Arabidopsis* (Fig. 1B). In this stably transformed *Arabidopsis* line, fluorescence was detected in the nucleus of the root epidermal cells preferentially in the N-position cells and the lateral root cap cells (Fig. 1C). By contrast, the P_{WER}:*GFP* alone does not generate a localized intracellular GFP signal (Fig. 1C). Together, these results suggest that the WER protein is localized to the nucleus in *Arabidopsis* cells.

WER is a master gene that controls both of the cell fates

It has been proposed that *WER* regulates the hair cell fate as well as the non-hair cell fate in the *Arabidopsis* root epidermis (Lee and Schiefelbein, 2002). This proposal is largely based on the fact that *wer*-1 plants harboring P_{35S}:*WER* produce a random pattern of both cell types, rather than a single cell type (Lee and Schiefelbein, 2002). However, it is possible that the *wer*-1 P_{35S}:*WER* phenotype is caused by a near-threshold level of WER activity driven by the 35S promoter that is insufficient to induce the non-hair cell fate in all epidermal cells. To test this possibility, we made a construct, P_{35S}:*WER-VP16*, which had a dual CaMV 35S promoter and the *WER*-coding region fused to the activation domain of a potent viral transcriptional activator, *VP16* (Fig. 2A; 81 amino acids) (Triezenberg et al., 1988). Prior to introducing this construct into plants, we tested the ability of the recombinant WER-VP16 protein to activate transcription in a yeast one-hybrid assay (Fig. 2B). When we fused this chimeric gene downstream of the GAL4 DNA-binding domain sequence (BD-WER-VP16) and transformed yeast strain Y190 with this construct, the reporter gene activity (β-galactosidase activity) in this yeast was 831.2±122.7. This activity is 2.5 times higher than the activity in the yeast transformed with a construct in which the native *WER*-coding region was fused downstream of the GAL4 DNA-binding domain sequence (BD-WER) (348.8±81.7), which demonstrates the effectiveness of the VP16 activation domain. To examine the effect of the WER-VP16 protein on epidermal cell specification, we transformed *wer*-1 P_{GL2}:*GUS* plants with the P_{35S}:*WER-VP16* construct. The P_{GL2}:*GUS* marker was used
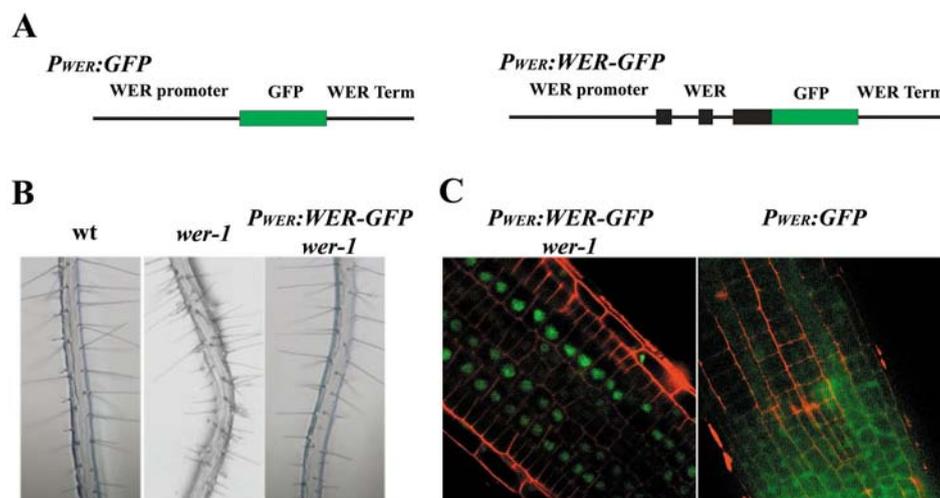
Fig. 1. Subcellular localization of the WER-GFP. (A) Schematic diagrams of P_{WER}:*GFP* and P_{WER}:*WER-GFP* gene constructs. The stop codon of the WER was deleted for the translational fusion with the GFP. (B) Complementation of the *wer* mutation with P_{WER}:*WER-GFP*. The *wer*-1 mutant was transformed with P_{WER}:*WER-GFP*. wt, wild type. (C) WER-GFP accumulation in the *Arabidopsis* root epidermal cells. The *wer*-1 mutant transformed with P_{WER}:*WER-GFP* and the wild-type plant transformed with P_{WER}:*GFP* were grown on agarose-solidified media. Fluorescence was observed in the 4-day-old seedlings using confocal microscopy.
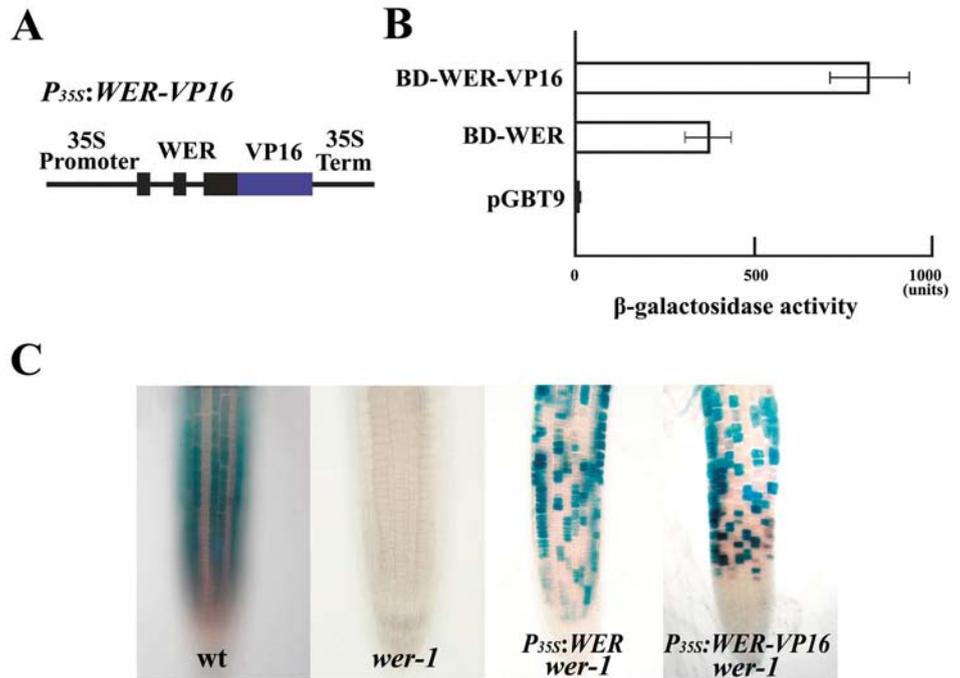
Fig. 2. Effect of P_{35S}:*WER-VP16* on cell fate specification in the root epidermis. (A) Schematic diagram of P_{35S}:*WER-VP16*. The stop codon of the WER was deleted. (B) Comparison of the transcriptional activation activity of the WER-VP16 recombinant protein with the activity of the native WER protein. Error bars indicate s.d. (C) Expression pattern of P_{GL2}:*GUS* in the epidermis of 4-day-old seedlings. Seedlings were stained for 4 hours. wt, wild type.

because P_{GL2}:*GUS* expression reflects the pattern of cell fates in the *Arabidopsis* root epidermis. We found P_{GL2}:*GUS*-expressing cells and the P_{GL2}:*GUS* non-expressing cells in each cell position in these P_{35S}:*WER-VP16 wer*-1 plants (Fig. 2C), similar to the P_{35S}:*WER wer*-1 plants (Lee and Schiefelbein, 2002). Thus, even though the transcriptional activation activity of the WER-VP16 protein was much higher than that of the WER native protein, both cell types were specified in the P_{35S}:*WER-VP16 wer*-1 line (Fig. 2C). This result supports the proposal that WER does not only specify the non-hair cell fate, but also specifies the hair cell fate, perhaps through its regulation of the *CPC* gene (Lee and Schiefelbein, 2002).
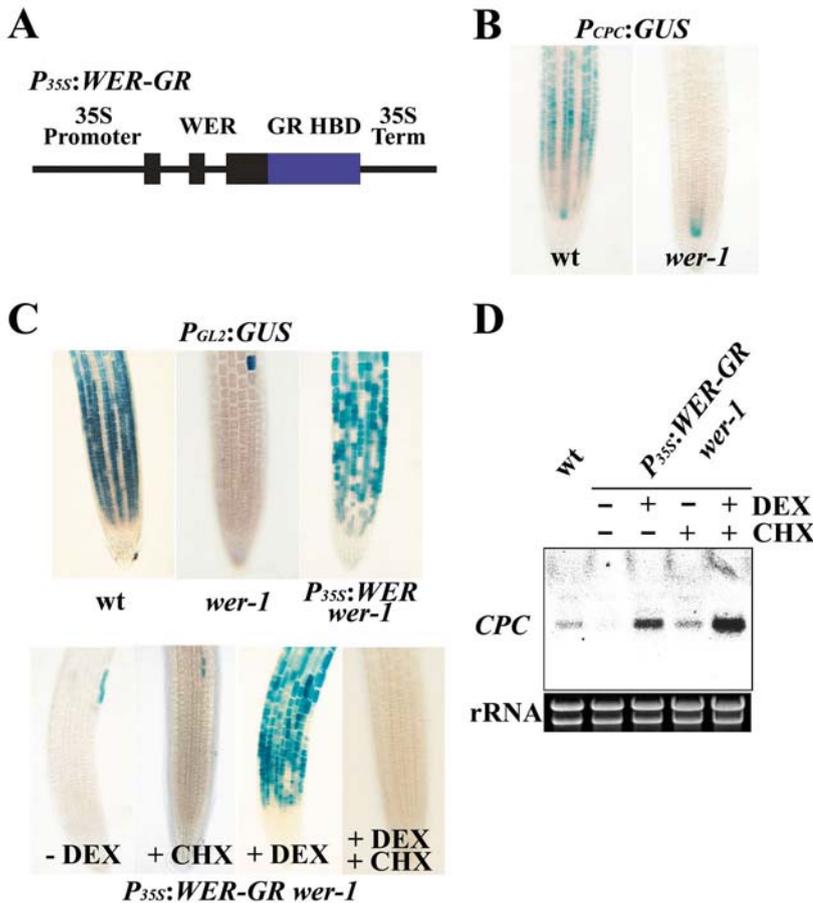
WER directly regulates *CPC* gene transcription

It has been suggested that *WER* promotes the hair cell fate through a lateral inhibition mechanism mediated by CPC, in part because a functional *WER* gene is required for *CPC* promoter activity in the N cell position (Fig. 3B) (Lee and Schiefelbein, 2002). However, it has not been determined yet whether WER directly regulates *CPC* transcription. To address this, we used the glucocorticoid receptor (GR)-mediated inducible system (Lloyd et al., 1994; Picard et al., 1988). The GR inducible system has been used successfully to define direct target genes of several putative *Arabidopsis* transcription factors (Sablowski and Meyerowitz, 1998; Samach et al., 2000; Wagner et al., 1999).

We generated a P_{35S}:*WER-GR* construct in which the DNA sequence for the hormone-binding domain of the glucocorticoid receptor (Picard et al., 1988) was fused to the *WER*-coding region and its expression was driven by the CaMV 35S promoter (Fig. 3A). This construct was stably introduced into *wer*-1 mutant plants. In these P_{35S}:*WER-GR wer*-1 plants, WER activity is expected to depend on glucocorticoid [e.g. dexamethasone (DEX)] because the translocation of the WER-GR recombinant protein into the

nucleus should require glucocorticoid. Furthermore, the addition of cycloheximide, an inhibitor of translation, is expected to prevent indirect transcriptional induction of genes following DEX treatment. We tested these expectations by examining the expression of reporter gene P_{GL2}:*GUS*, which was introduced by crossing into the P_{35S}:*WER-GR wer*-1 line as a means to monitor epidermal cell specification in response to various treatments. In the absence of DEX, GUS activity was almost undetectable in the *wer*-1 mutant harboring P_{35S}:*WER-GR* (Fig. 3C), just as in the *wer*-1 mutant itself (Fig. 3C) (Lee and Schiefelbein, 1999). However, when we applied 1 μM DEX to 4-day-old transgenic seedlings for 3 hours, P_{GL2}:*GUS* was expressed in epidermal cells in a pattern indistinguishable from the *wer*-1 mutant harboring P_{35S}:*WER* (Fig. 3C). Furthermore, when we applied 10 μM cycloheximide (CHX) with 1 μM DEX, we could not detect any GUS activity (Fig. 3C). In addition to the P_{GL2}:*GUS* expression, the epidermal cell fate was also affected by the application of DEX. While the 4-day-old P_{35S}:*WER-GR wer*-1 seedlings grown without DEX showed the typical *wer*-1 mutant phenotype, the seedlings grown on agarose media containing 1 μM DEX showed a random distribution of epidermal cell types (Table 1), similar to the phenotype of the P_{35S}:*WER wer*-1 seedlings previously reported (Lee and Schiefelbein, 2002), implying that the WER-GR can act like the native WER protein and postembryonic functioning of WER is sufficient to change the cell fate. These results show that 1 μM dexamethasone is sufficient for the WER-GR fusion to induce P_{GL2}:*GUS* expression and that 10 μM CHX is effective to suppress translation of the transcribed *GUS* mRNA.

Using these established conditions, we examined *CPC* RNA accumulation using Northern blot analysis. Treatment of P_{35S}:*WER-GR wer*-1 seedlings with DEX for 3 hours resulted in a large increase in the accumulation of *CPC* transcript, when compared with seedlings not exposed to DEX (Fig. 3D). To

A

P_{35S}:*WER-GR*

35S Promoter   WER   GR HBD   35S Term

B

P_{CPC}:*GUS*

wt   *wer-1*

C

P_{GL2}:*GUS*

wt   *wer-1* P_{35S}:*WER wer-1*

- DEX   + CHX   + DEX   + DEX + CHX

P_{35S}:*WER-GR wer-1*

D

wt P_{35S}:*WER-GR*   *wer-1*

– + – + DEX
– – + + CHX

CPC

rRNA

Fig. 3. Direct regulation of *CPC* transcription by the WER. (A) Schematic diagram of P_{35S}:*WER-GR* gene construct. The stop codon of the WER was deleted for the translational fusion with the hormone-binding domain of the glucocorticoid receptor (GR HBD; from the 519th amino acid to the end). (B) Expression of the P_{CPC}:*GUS* reporter gene in the epidermis of 4-day-old seedlings. Seedlings were stained for 8 hours. (C) Expression of the P_{GL2}:*GUS* reporter gene in the epidermis of 4-day-old seedlings. (Top) The seedlings were stained for 6 hours without any treatment. wt, wild type. (Bottom) The seedlings harboring P_{35S}:*WER-GR* (P_{35S}:*WER-GR wer-*1) were untreated (–DEX), treated with 10 μM of cycloheximide (CHX) alone, treated with 1 μM of dexamethasone (+DEX) alone, or treated with both (+DEX, +CHX). Seedlings were pre-treated with 10 μM of CHX for 20 minutes before treatment with CHX and DEX. Seedlings were stained for 6 hours. (D) Accumulation of CPC transcripts in transgenic plants harboring P_{35S}:*WER-GR*. P_{35S}:*WER-GR wer-*1 seedlings (4-day-old) were treated as described in C. Northern blot analysis was carried out with total RNA extracted from the root tips using radiolabeled *CPC* DNA fragment as a probe (Lee and Schiefelbein, 2002). Resulting signal was detected using BAS-2500 (Fujifilm).

test whether this induction required de novo protein synthesis, we treated the P_{35S}:*WER-GR wer-*1 seedlings with DEX plus CHX. In this case, the steady state level of *CPC* transcript was not reduced, rather it increased slightly (Fig. 3D). This means that translocation of the WER-GR from the cytosol to the nucleus was sufficient to induce transcription of *CPC* gene in the absence of de novo protein synthesis, strongly suggesting that WER is a direct regulator of *CPC* transcription.

Determination of the WER binding site in the *CPC* promoter

To identify the region that WER binds to the *CPC* promoter, we made several small DNA fragments from the *CPC* promoter region and used them as probes in electrophoretic mobility shift assays (EMSA). The *CPC* promoter region

includes ~0.7 kb of DNA upstream of the translation start codon which is known to be sufficient to complement the *cpc* mutant phenotype when fused to the *CPC*-coding region (data not shown). For the EMSA experiments, we used a truncated WER protein lacking its C-terminal 24 amino acids as we were not able to produce a detectable amount of the full-length WER protein in *E. coli*. We have previously shown that the C-terminal 24 amino acids are responsible for the transcriptional activation activity of WER (Lee and Schifelbein, 2001), and we note that the MYB domain is sufficient for DNA-binding specificity in animal MYB protein (Howe et al., 1990) and plant MYB proteins, including P (Willams and Grotewold, 1997) and C1 (Sainz et al., 1997). In our EMSA experiments, we found that WER could bind to two *CPC* DNA fragments, and we narrowed down these regions to 20 nucleotide

Table 1. Specification of cell types in the root epidermis treated with DEX

Genotype	DEX	H cell position		N cell position	
		Hair cells (%)	Non-hair cell (%)	Hair cells (%)	Non-hair cell (%)
Wild type (Columbia)	+	94.1±7.4	5.9±7.4	2.4±4.0	97.6±4.0
wer-1	+	96.8±8.6	3.2±8.6	90.1±8.2	9.9±8.2
P_{35S}:*WER-GR wer*-1	+	82.4±11.0	17.6±11.0	48.3±16.4	51.7±16.4
	–	95.9±7.3	4.0±7.3	86.2±10.4	13.8±10.4

We examined 10 to 20 4-day-old seedlings for each line.
+, DEX was treated in the media.
Values represent mean±s.d.

Development

fragments (Fig. 4A), which were located at –425 to –406 and –468 to –459 upstream of the translation start codon, respectively. We synthesized several mutant versions of these 20 nucleotide fragments and used them in the EMSA (Fig. 4B). Some of the point-mutated DNA fragments were not recognized by WER, while others showed the same binding affinity for the WER as the wild-type fragments (Fig. 4C). Using this information, we were able to deduce a core sequence for the WER-binding site I (WBSI) and WER-binding site II (WBSII) as AgtaGTTa and CAACtg, respectively (Fig. 4C). If the WER-DNA binding was significantly affected when the base was mutated, the bases are shown as capital letters. Because we changed a base to only one different base, rather than all three possible different

bases, we could not tell whether the bases shown as lower case letters were also important.

To test the binding specificity of WBSI and WBSII, we performed a competitive gel shift assay with a wild-type version of WBSI or WBSII as a probe and two mutated versions of each as a competitor (Fig. 4D). Binding of the WER protein with WBSI and WBSII was determined to be nucleotide sequence specific, because the mutated versions of WBSI and II did not significantly inhibit the WER-WBSs binding whereas the wild-type versions of WBSs did inhibit the WER-WBSs binding (Fig. 4D).

Next, we tested whether these two binding sites had different affinities for the WER protein. When we used radiolabeled WBSI as a probe and the cold WBSII as a competitor, we were

Fig. 4. Binding of the WER to *CPC* promoter. (A) EMSA using the purified WER protein and two 20 bp long DNA fragments (WBSI and WBSII) from the *CPC* promoter. Lane 1 of each gel contains the free DNA probe without the WER protein, and lane 2, 3 and 4 of each gel contain increasing amount of the WER protein (1×, 3× and 6×). (B) Sequences of mutated WBSI and WBSII used in this experiment. Each double-stranded probe contains a single base substitution as indicated. Lines indicate no change. (C) EMSA using the purified WER protein and the mutated probes as shown in B. (D) Competitive EMSA. Competitions were performed using increasing amounts of wild-type DNA fragments or some mutated derivatives as shown in B. Lane 1 of each gel contains the free DNA probe without the WER protein and lane 2 contains the WER protein and the radiolabeled wild-type DNA fragment (WBSI or WBSII) as a probe without a competitor. Increasing amounts (1×, 10× and 50×) of the unlabeled wild type DNA fragments (lanes 3, 4 and 5), and the unlabeled mutated versions of WBSI or WBSII (lanes 6, 7, 8 and lanes 9, 10, 11) were added as a competitor. (E) Binding competition between WBSI and WBSII. Increasing amounts of the wild-type or mutated derivative (m10) of WBSII, and increasing amounts of the wild-type or mutated derivative (m6) of WBSI were used as an unlabeled competitor for the binding of the WER protein to radiolabeled WBSI and to radiolabeled WBSII, respectively. Lane 1 contains only free probe (radiolabeled WBSI or WBSII) and lane 2 contains the WER protein and the radiolabeled wild-type DNA fragment (WBSI or WBSII) as a probe without a competitor. Lanes 3, 4 and 5 of each gel contain increasing amounts of the unlabeled wild-type DNA fragments (unlabeled WBSI for radiolabeled WBSII, and unlabeled WBSI for radiolabeled WBSII) as a competitor (1×, 10× and 50×). Lanes 6 and 7 contain the same components as lane 3, 4 and 5, except that they contain increasing amounts of unlabeled mutated versions of WBSII (m10) or WBSI (m6) instead of the unlabeled wild-type DNA fragments as a competitor.

able to detect a slight reduction in WBSI-WER binding with increasing amounts of the cold competitor (Fig. 4E). By contrast, when we used WBSII as a probe and WBSI as a cold competitor, the WBSII-WER binding was decreased sharply with increasing amounts of the cold competitor (WBSI) (Fig. 4E). These reductions were not caused by competition with mutated sequences (Fig. 4E, lanes 6 and 7). This competitive gel shift assay suggests that the affinity of WBSI for the WER protein is much stronger than that of WBSII in vitro.

In vivo tests of the WER-binding sites in the *CPC* promoter

To validate these WER-binding sites in vivo, a yeast one-hybrid assay was employed with these two binding sites and the WER protein. For the reporter constructs, we made three tandem repeats of WBSI and WBSII, respectively, inserted them upstream of the *lacZ* reporter gene promoter, and introduced these constructs into yeast (Fig. 5A). We also made yeast reporter strains that have three tandem copies of two mutated versions of WBSI in the *lacZ* promoter (Fig. 5A). For the effector construct, a DNA fragment including the *WER*-coding region was fused to the GAL4 activation domain sequence (*AD-WER*) and expressed in these reporter strains. We found that the AD-WER was able to increase the β-galactosidase activity by threefold in the yeast harboring WBSI on its reporter gene promoter (Fig. 5B). By contrast, there was no increase in the β-galactosidase activity when *AD-WER* was expressed in the yeast harboring the two mutated versions of

Fig. 5. Binding of the WER protein to WBSI in yeast. (A) Schematic diagrams of the reporter genes and the effectors used in this yeast one-hybrid assay. Three tandem repeats of each DNA fragment were inserted upstream of a reporter gene (*lacZ*) in the vector pLacZi and then yeast reporter strains were made by genomic integration of these reporters. (B) Yeast one-hybrid assay with WBSI as a cis-acting element. AD-WER was expressed in the yeast reporter strains harboring a reporter gene that has the wild-type or one of the mutant derivatives of WBSI in its upstream as shown in A. A β-galactosidase assay is performed to verify the DNA-protein interaction using CPRG as a substrate.

WBSI (WBSI-M1 and WBSI-M2). These data clearly show that WBSI, which we identified in vitro using EMSA (Fig. 4), functions as a WER-binding site in vivo. We were not able to detect a significant increase in reporter expression when the AD-WER was expressed in yeast harboring WBSII on its reporter gene promoter, perhaps because the β-galactosidase activity in this yeast without the WER protein was already very high (data not shown). It is possible that an endogenous yeast transcriptional activator is able to bind to this WBSII in yeast cells.

We also examined the importance of the WER-binding sites in *Arabidopsis*. First, we co-transformed *Arabidopsis* protoplasts transiently with reporter and effector constructs. For the reporter constructs, the luciferase gene was fused to the wild-type and several mutant versions of the *CPC* promoter (Fig. 6A). *WER* was expressed under the control of the CaMV 35S promoter (P_{35S}:*WER*) as an effector (Fig. 6A). Protoplasts were prepared from leaf mesophyll cells were transfected with various combinations of reporter and P_{35S}:*WER* effector constructs, as well as a UBQ10-GUS construct as an internal control (Fig. 6B). When protoplasts were transfected with the wild-type reporter gene and the P_{35S}:*WER*, there was a fivefold increase in the relative luciferase activity. However, when protoplasts were transfected with the mutated reporter constructs (which have mutations at WBSI, WBSII, or both) and P_{35S}:*WER*, there was only a small increase in the relative luciferase activity (Fig. 6B). We also co-transformed protoplasts with a WER-VP16 effector construct (P_{35S}:*WER-VP16*) and the wild-type reporter construct (P_{CPCWT}:*LUC*), and this combination yielded approximately threefold higher luciferase activity than the P_{35S}:*WER* + P_{CPCWT}:*LUC* combination (data not shown), which further shows that the induction is WER dependent and that the VP16 domain is able to enhance the transcription activation ability of the WER protein in *Arabidopsis*.
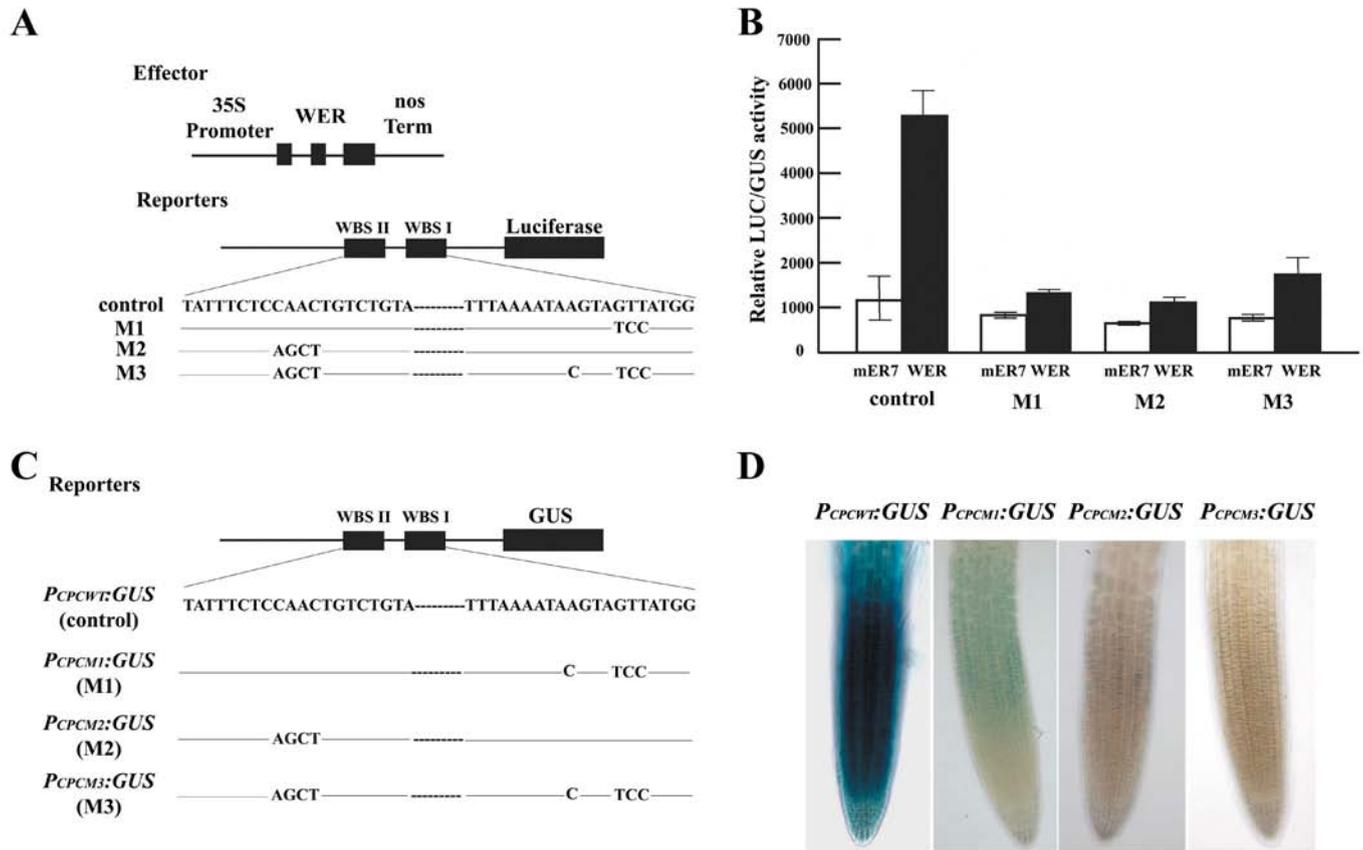
Next, we made stable transgenic lines with the wild-type and three mutated versions of the CPC promoter fused to the GUS reporter gene (Fig. 6C). Although the wild-type version (P_{CPCWT}:*GUS*) showed high level of GUS activity in the root epidermis (preferentially in the N-position cells), the mutant versions (P_{CPCM1}:*GUS*, P_{CPCM2}:*GUS* or P_{CPCM3}:*GUS*, which have mutations at WBSI, WBSII or at both, respectively) showed very little GUS activity (Fig. 6D). Specifically, after 4 hours of incubation, no detectable GUS staining could be detected in these mutant versions, whereas the wild-type version showed strong GUS staining. After 24 hours of incubation, some of the seedlings harboring the mutant reporter genes showed very low GUS staining. These results show that the WER-binding sites are important for WER-dependent CPC promoter activity in the *Arabidopsis* root epidermis.

Discussion

The *Arabidopsis* root epidermis has been used as a model system for many studies on cell fate specification. Several putative transcription factors have been identified in this process, including at least four MYB genes (*WER*, *CPC*, *TRY* and *ETC1*), two bHLH genes (*GL3* and *EGL3*) and a homeobox gene, *GL2* (Bernhardt et al., 2003; Kirik et al., 2004; Lee and Schiefelbein, 1999; Masucci et al., 1996; Schellmann et al., 2002; Wada et al., 1997). Although molecular genetic

Fig. 6. Effect of the WER protein on *CPC* expression in *Arabidopsis*. (A,C) Schematic diagrams of the reporter genes and the effectors used in this transient expression assay (A) and stable transgenic study (C). For reporter constructs, several versions of *CPC* promoters that are 700 bp long (from the translational start site) were inserted upstream of the luciferase gene (A) or β-glucuronidase gene (C). Each mutant reporter contains mutations at WBSI or WBSII, or both. For the effector construct, the genomic DNA fragment of WER-coding region was inserted downstream of the 35S promoter (A). (B) Importance of WBSI and WBSII (refer to A) in *CPC* expression in the *Arabidopsis* protoplast transient expression assay. Protoplasts were transfected with UBQ10-GUS as an internal control, each of the reporters (wild type, M1, M2 or M3) and an effector (WER). (D) Importance of WBSI and WBSII (refer to C) in the proper expression of *CPC* in the *Arabidopsis* root epidermis. The seedlings were stained for 24 hours.

approaches have been used successfully to identify players in this system, relatively little biochemical analyses have been conducted to examine the function of these putative transcription factors and their proposed roles in the cell fate specification mechanism. We provide here new biochemical and molecular evidence to support aspects of the patterning model proposed previously (Bernhardt et al., 2005; Lee and Schiefelbein, 1999; Lee and Schiefelbein, 2002).

WER is a transcriptional activator

Most putative transcription factors are named because their amino acid sequence is similar to other proteins that are known to act as transcription factors. *WER* was cloned by a map-based method, and it was designated as a MYB gene because its deduced amino acid sequence contains two repeats similar to the MYB DNA-binding domain found in MYB-type transcription factors (Lee and Schiefelbein, 1999). Although MYB genes are generally thought to encode transcription factors, several MYB-related genes do not function in transcription. As an unusual case, the *Arabidopsis* homolog of the yeast MYB gene *Cdc5* [required for G2/M phase

progression in yeast (Nurse et al., 1976)] was found to possess DNA-binding activity (Hirayama and Shinozaki, 1996), but it actually functions in pre-mRNA splicing instead of transcriptional regulation (Burns et al., 1999). In addition, telomere-binding proteins from rice (RTBP1) (Yu et al., 2000) and mammals (TRF1 and TRF2) (Brocolli et al., 1997) contain a single MYB domain, yet they play a role in telomere function rather than in transcription. Therefore, the major goal of this study was to determine whether WER acts as a transcription factor, by studying its protein localization, its DNA-binding ability, and its transcriptional regulatory properties.

In this paper, we report that the WER protein is localized to the nucleus in the *Arabidopsis* root epidermis (Fig. 1C), that it is required to directly induce CPC RNA accumulation (Fig. 3), that it binds to specific sites in the *CPC* promoter in vitro (Fig. 4) and that these WER-binding sites are important for WER-dependent CPC promoter activation in vivo (Figs 5, 6). Together with the previous finding of a transcriptional activation domain in the C terminus of WER (Lee and Schiefelbein, 2001), the results provided in this paper strongly suggest that *WER* indeed encodes a transcriptional activator.

CPC is a direct target gene of WER

In animals, MYB proteins generally contain three MYB repeats (R1, R2 and R3), and they bind to the consensus DNA sequence, YAACKG (Bidenkapp et al., 1988; Golay et al., 1994; Howe and Watson, 1991). The R2 and R3 domains are important in this sequence specific binding and the R1 domain seems to stabilize the MYB-DNA interaction (Howe et al., 1990; Saikumar et al., 1990; Tanikawa et al., 1993). In plants, two-repeat MYB proteins (R2R3 MYBs) are predominant (Reichmann et al., 2000; Romero et al., 1998), and they differ in their DNA-binding specificity from the R1R2R3 MYB proteins from animals (Solano et al., 1995; Williams and Grotewold, 1997). Plant R2R3 MYB proteins also show different binding specificity between themselves. For example, maize C1 and P which are involved in flavonoid biosynthesis show specific binding to a DNA sequence, CC(T/A)ACC (Sainz et al., 1997), which is not related to the animal MYB binding sequence, YAACKG (MBSI). AtMYB2, which functions in abscisic acid signaling in *Arabidopsis* (Abe et al., 2003), binds to a DNA sequence, TGGTTAG, which is somewhat related to a DNA sequence, CNGTTR, complementary to the MBSI (Abe et al., 1997). MYB.Ph3 from petunia shows dual DNA-binding specificity to the DNA sequences consensus I (aaaAaaC(G/C)GTTA), which is similar to the sequence CNGTTR (MBSI), and consensus II (aaaAGTTAGTTA) (MBSII) (Solano et al., 1995). This consensus II cannot be recognized by animal c-MYB. Although MYB proteins are believed to exert their roles by activating specific target genes that contain MYB-binding sequences in their promoter regions, there are relatively few studies that provide direct evidence for this assumption. One of the best known examples involves P and C1, maize MYB genes. Using EMSA and a transient expression system, P has been shown to regulate the *a1* gene, one of the flavonoid biasynthetic genes, and C1 has been shown to regulate many flavonoid biosynthetic genes, *a1*, *bz1* and *a2* (Grotewold et al., 1994; Lesnick and Chandler, 1998; Sainz et al., 1997). *GaMYB2/FIBER FACTOR 1* (*FIF1*), a MYB gene from cotton, is expressed in developing cotton fibers and has been shown to regulate *RD22-like1* (*RDL1*) using the yeast one-hybrid assay and heterologous transgenic plant analyses (Wang et al., 2004). In addition, one of the MYB proteins in *Arabidopsis*, AtMYB2, has been shown to regulate a dehydration-responsive gene, *rd22*, and an alcohol dehydrogenase gene, *AtADH1*, using a transient expression experiment (Abe et al., 1997; Hoeren et al., 1998).

In this paper, we used the GR-inducible system to identify *CPC* as a direct target of the WER protein, because *CPC* transcripts increased significantly in response to WER-GR induction (in the *wer*-1 mutant background) in the presence of cycloheximide. We then showed that the WER protein has binding specificity to two sequences in the *CPC* promoter (Fig. 4B). These two sequences, WBSI (AgtaGTTa) and WBSII (CAACtg), are imperfectly complementary to each other, but are move closely related to MBSII and MBSI, respectively. The binding affinities of WER to these two sites are different at least in vitro. Similarly, the maize *A1* gene has two P binding sites in its promoter (Grotewold et al., 1994), and these two sites have different affinities for P protein in vitro (Sainz et al., 1997). Both of these sites are functional in vivo and either site is sufficient for P activation in vivo (Sainz et al., 1997). In the

CPC promoter, we find that the two WER-binding sites also function in vivo (Figs 5, 6); however, both of them are required for the proper transcriptional activation of the gene, in contrast to the activation of *a1* gene expression by P. Apparently, WER binds to these two sites separately as we could not detect any physical interaction between WER proteins in yeast (data not shown).

WER influences the fate of H-position cells

Previously, we proposed a lateral inhibition model in the cell fate specification mechanism in the *Arabidopsis* root epidermis (Lee and Schiefelbein, 2002). In that model, WER is responsible to specify the hair cell fate as well as the non-hair cell fate. Here we obtain further support for this model. In the *wer-1* mutant plants harboring P_{35S}:*WER-VP16*, the P_{GL2}:*GUS*-expressing cells and non-expressing cells could be found in both positions (Fig. 2C). This is similar to the P_{GL2}:*GUS* expression pattern in the *wer-1* mutant harboring P_{35S}:*WER* or P_{35S}:*WER-GR* (+DEX) (Fig. 3C) (Lee and Schiefelbein, 2002). In prior studies, it has been found that the inhibition of P_{GL2}:*GUS* expression is partly dependent on CPC (Lee and Schiefelbein, 2002). Transcription of *CPC* takes place in the N-position cells and a CPC-GFP fusion protein accumulates in both-position cells (Lee and Schiefelbein, 2002; Wada et al., 2002). Taken together, these findings suggest that WER influences the fate of cells in the H position through its effect on CPC. Specifically, it appears that WER induces transcription of *CPC* directly in the N-position cells, CPC protein moves to the neighboring H-position cells, and this CPC protein induces the hair cell fate in these H-position cells.

Conclusion

In root epidermal cell fate specification, a cascade of several transcription factors plays an important role. This report provides direct evidence regarding the regulatory interactions between some of these genes. Other than *CPC*, *GL2* is another strong candidate for a direct target of WER. The *GL2* promoter contains several putative MYB-binding sites and bHLH-binding sites, and some of these are located close, like the sites in the *CPC* promoter. It will be interesting to see whether WER binds to these sites and directly regulates *GL2* expression. In addition, it will be interesting to see how *GL2* expression is induced by the WER and suppressed by the CPC using biochemical and molecular approaches. Finally, it will be important to understand how the CPC protein suppresses the expression of some genes (*GL2*, *CPC* and *WER*), while it induces the expression of *GL3* and *EGL3*.

Note added in proof

While this manuscript was under review, another article was published that indicates WER is a regulator of *CPC* transcription (Koshino-Kimura et al., 2005).

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