Cell Fate in the Arabidopsis Root Epidermis Is Determined by Competition between WEREWOLF and CAPRICE[1][C][W]

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The root hair and nonhair cells in the Arabidopsis (*Arabidopsis thaliana*) root epidermis are specified by a suite of transcriptional regulators. Two of these are *WEREWOLF* (*WER*) and *CAPRICE* (*CPC*), which encode MYB transcription factors that are required for promoting the nonhair cell fate and the hair cell fate, respectively. However, the precise function and relationship between these transcriptional regulators have not been fully defined experimentally. Here, we examine these issues by misexpressing the *WER* gene using the GAL4-upstream activation sequence transactivation system. We find that *WER* overexpression in the Arabidopsis root tip is sufficient to cause epidermal cells to adopt the nonhair cell fate through direct induction of *GLABRA2* (*GL2*) gene expression. We also show that GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3), two closely related bHLH proteins, are required for the action of the overexpressed *WER* and that WER interacts with these bHLHs in plant cells. Furthermore, we find that CPC suppresses the WER overexpression phenotype quantitatively. These results show that WER acts together with GL3/EGL3 to induce *GL2* expression and that WER and CPC compete with one another to define cell fates in the Arabidopsis root epidermis.

One of the fundamental questions in developmental biology is how a cell adopts its fate, and many studies have revealed that the relative position of a cell, rather than its lineage, plays an important role in cell fate decision (van den Berg et al., 1995; Kidner et al., 2000). Therefore, to adopt their appropriate fate, it is very important that cells communicate properly with neighboring cells and recognize their relative position.

The Arabidopsis (*Arabidopsis thaliana*) root epidermis is a good model system for studying the process of cell fate specification. The root epidermis consists of two types of cells, root hair-bearing cells (hair cells) and nonhair cells. Their cell fates are determined in a position-dependent manner, so that the cells located over a single cortical cell (N-position) adopt the nonhair cell fate while the cells located over the anticlinal wall of the underlying cortical cells, and thereby in contact with two cortical cells (H-position), adopt the hair cell fate (Dolan et al., 1993; Galway et al., 1994).

Several regulators that are involved in this cell fate specification have been identified. MYB-type transcription factors including WEREWOLF (WER; Lee and Schiefelbein, 1999) and MYB23 (Kang et al., 2009), bHLH-type transcription factors including GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3; Bernhardt et al., 2003), a WD-repeat protein, TRANSPARENT TESTA GLABRA1 (Galway et al., 1994; Walker et al., 1999), and a homeodomain-Zip transcription factor, GLABRA2 (GL2; Masucci et al., 1996), have been shown to induce the nonhair cell fate at the N position, while single repeat MYB-type transcription factors CAPRICE (CPC), TRIPTYCHON (TRY), and ENHANCER OF TRY AND CPC1 (ETC1) have been shown to positively regulate the hair cell fate at the H position (Wada et al., 1997; Schellmann et al., 2002; Simon et al., 2007). In addition, a Leu-rich repeat receptor-like kinase, SCRAMBLED (SCM), was also shown to be involved in this epidermal cell patterning (Kwak et al., 2005).

A complex action network of these regulators for root epidermal cell fate specification has been proposed (Kang et al., 2009). SCM is suggested to perceive a positional signal (not yet identified), and it suppresses *WER* expression in the H-position cells, which leads to a

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small difference in the *WER* expression level between the cells at the two positions (Kwak et al., 2005; Kwak and Schiefelbein, 2007). In the N-position cells, a relatively high level of WER, which can form a complex with GL3/EGL3, induces the nonhair cell fate by inducing the expression of *GL2* and *MYB23* (Lee and Schiefelbein, 1999, 2002; Kang et al., 2009). This complex also promotes the hair cell fate by inducing the expression of single-repeat MYB genes *CPC, TRY,* and *ETC1* in the N-position cells (Lee and Schiefelbein, 2002; Koshino-Kimura et al., 2005; Ryu et al., 2005; Simon et al., 2007). These single-repeat MYBs move into the neighboring H-position cells (Kurata et al., 2005) and further downregulate *WER* expression as well as the expression of *CPC* and *GL2*, which results in hair cell fate specification (Lee and Schiefelbein, 2002).

The above model is largely consistent with the available experimental evidence. However, aspects of this model have not been rigorously tested, and there are experimental results that do not appear to fit the model. For example, the overexpression of *WER* using the cauliflower mosaic virus (CaMV) 35S promoter (*35Spro*:*WER*) did not cause any discernible effect in the wild-type root epidermis (Lee and Schiefelbein, 1999, 2002), suggesting that WER may not be sufficient to induce *GL2*, *CPC*, or the nonhair cell fate in the H-position. On the other hand, *35Spro*:*WER* caused a randomized cell fate specification in the *wer* mutant root epidermal cells independent of their position, leading to another explanation in which WER primarily functions in epidermal cell patterning rather than merely in the nonhair cell fate specification (Lee and Schiefelbein, 2002). Also, WER's possible interactions with other cell fate regulators, including GL3/EGL3, CPC, and SCM, to regulate *GL2* expression have not been critically examined.

In this report, we used several molecular genetic approaches to directly test and extend current models for root epidermal cell fate specification. In one line of experiments, we drove *WER* expression using the GAL4-upstream activation sequence (UAS) targeted expression system (Brand and Perrimon, 1993; Haseloff, 1999) and found that WER was able to promote the nonhair cell fate by directly inducing *GL2* expression in every root epidermal cell and that GL3/EGL3 is required in this process. We also showed that the transcriptional repression of *WER* by CPC is not required for the hair cell fate specification in the root epidermis. We further discovered that WER and CPC compete with each other to specify one of the fates by regulating *GL2* expression quantitatively.

RESULTS

WER Expression Is Sufficient to Induce the Nonhair Cell Fate in the Root Epidermis

To determine whether WER is able to induce the nonhair cell fate in the H-position, we expressed *WER* using the GAL4-UAS targeted expression system (Brand and Perrimon, 1993; Haseloff, 1999). We generated an Arabidopsis line harboring a *UASpro*:*WER* construct and crossed it with three GAL4-GFP enhancer trap lines (J2812, J2301, and Q2610; Haseloff, 1999) to induce *WER* expression at the root tip with different tissue specificity (Fig. 1A). *WER* expression is induced in the cortex and the epidermis in J2812 roots, in the epidermis and the lateral root cap in J2301 roots, and in most of the tissues in Q2610 root tips. While WER expressed under the control of the 35S promoter (*35Spro*:*WER*) did not cause any noticeable defect in epidermal cell patterning, as reported earlier (Lee and Schiefelbein, 1999, 2002), *WER* expressed using the enhancer trap lines J2812, J2301, and Q2610 (designated as J2812>>WER, J2301>>WER, and Q2610>>WER) disrupted the epidermal cell patterning to cause some H-position cells to adopt the nonhair cell fate (Fig. 1A; Table I). In particular, the Q2610>>WER line showed the most severe effect on cell fate specification, so that most of the epidermal cells differentiated into a nonhair cell regardless of their position.

Although *GL2* expression is known to require WER function (Lee and Schiefelbein, 1999), it is not clear whether WER induces *GL2* expression quantitatively. To address this, we first examined *GL2pro*:*GUS* reporter gene expression in the root tip of the *WER*-overexpressing lines (Fig. 1B). It is well known that the *GL2pro*:*GUS* reporter gene is specifically expressed in the N-position cell files of the root epidermis (Masucci et al., 1996). However, in the J2812>>WER and J2301>>WER roots, some of the H-position epidermal cells also showed GUS activity, resulting in disruption of the file-specific expression pattern (Fig. 1B). Furthermore, Q2610>>WER caused almost every epidermal cell to express *GL2pro*:*GUS* at a similar level irrespective of their relative position. On the other hand, in the *35Spro*:*WER* plant root, the *GL2pro*:*GUS* expression pattern was similar to the pattern in the wild-type roots (Fig. 1B).

We next examined the *WER* transcript level in the root tip of those lines using quantitative real-time PCR to determine whether these phenotypic differences were caused by a difference in *WER* expression level (Fig. 1C). We discovered that the *WER* transcript level in the *35Spro*:*WER* line was 1.4-fold higher than in the wild type, which seems to be a very small addition to the endogenous *WER* transcript level in the root epidermis. However, the *WER* transcript levels in three different GAL4-UAS transgenic lines (J2812>>WER, J2301>>WER, and Q2610>>WER) were much higher than the levels in the wild type and the *35Spro*:*WER* line (28.6-, 102.3-, and 145.4-fold higher, respectively, than in the wild type). Although it is difficult to compare the expression levels of *WER* in a particular cell type between the enhancer trap lines, the degree of hairless phenotype seemed to largely correlate with the *WER* transcript level, implying a quantitative effect of *WER*.

Altogether, these results show that *WER* expression is able to induce the nonhair cell fate in every epider-

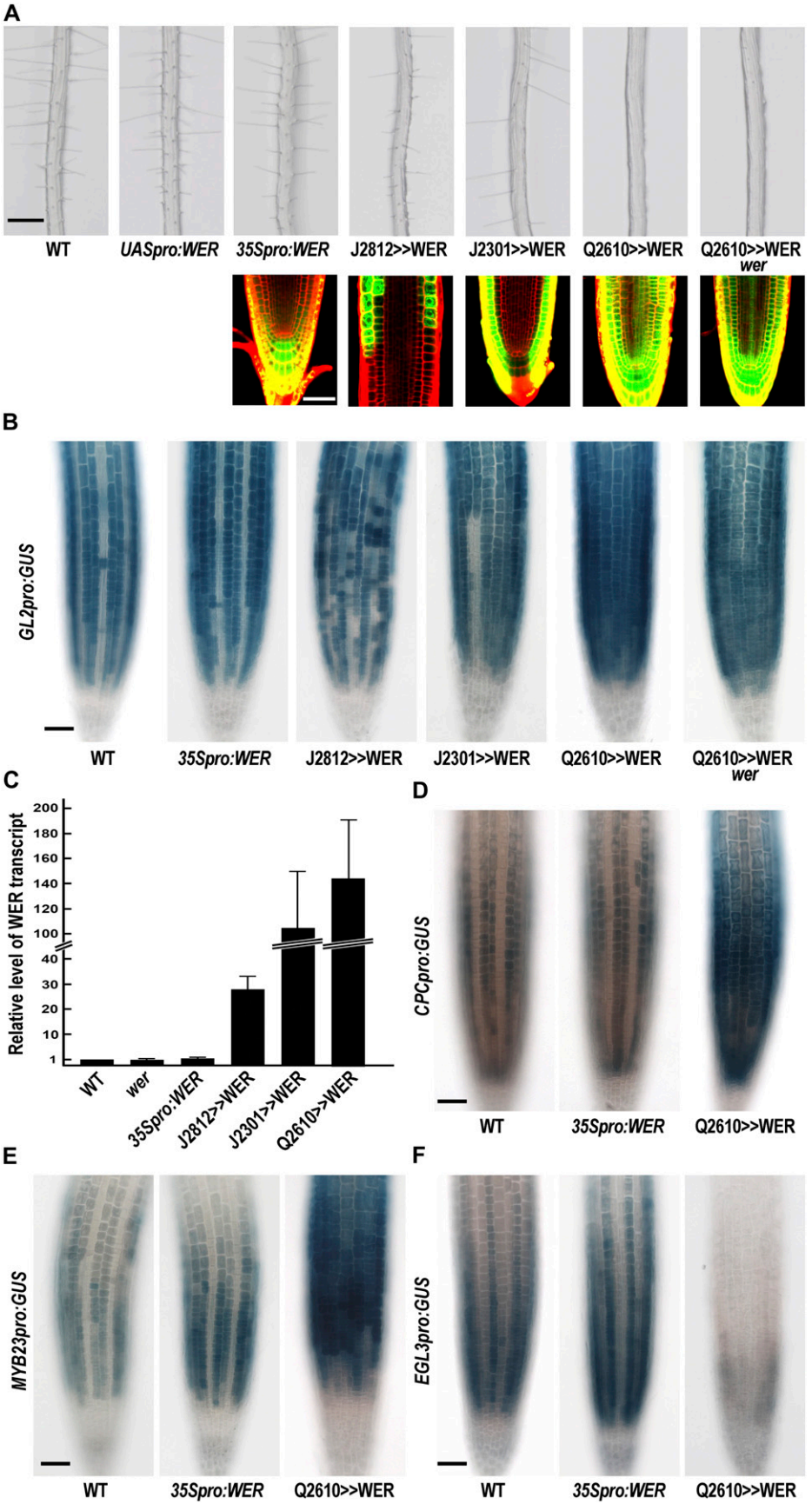

Figure 1. WER induces the nonhair cell fate in the Arabidopsis root epidermis in a dose-dependent manner. A, Root hair phenotype for the wild type (WT) and the *WER*-overexpressing lines. The top panels show the wild-type and transgenic root phenotypes. *WER* was ectopically expressed under the regulation of the CaMV 35S promoter and enhancer trap lines of J2812, J2301, and Q2610 by using the GAL4-UAS transactivation system. A transgenic line harboring *UASpro*:*WER* was selected and crossed to the indicated enhancer trap lines, and F3 seedlings homozygous for both the enhancer trap line and the transgene were screened. The bottom panels show the expression patterns of *35Spro*:*GFP*, J2812, J2301, and Q2610 in the root tip observed using confocal microscopy. Top bar = 200 μm; bottom bar = 50 μm. B, *GL2pro*:*GUS* reporter gene expression pattern in root tips of the wild type and *WER*-overexpressing lines. Wild-type and transgenic roots harboring the *GL2pro*:*GUS* transgene were stained for GUS activity using 5-bromo-4-chloro-3-indolyl-β-GlcA as a substrate. C, Relative level of the *WER* transcript in root tips of the wild type and *WER*-overexpressing lines. The relative level of the *WER* transcript was determined by quantitative real-time PCR analysis. Error bars indicate SD from at least three replicates. D, Wild-type and transgenic roots harboring *CPCpro*:*GUS*. Four-day-old seedlings were stained for GUS activity. E, Wild-type and transgenic roots harboring *MYB23pro*:*GUS*. Four-day-old seedlings were stained for GUS activity. F, Wild-type and transgenic roots harboring *EGL3pro*:*GUS*. Four-day-old seedlings were stained for GUS activity. Bars in B and D to F = 50 μm.

Table I. *Cell type pattern in the root epidermis in various mutants and transgenic plants*
At least 30 4-d-old seedlings were examined for each plant line. Values indicate means ± SD.

Genotype	H-Position		N-Position	
	Hair Cell	Nonhair Cell	Hair Cell	Nonhair Cell
	%			
Wild type (ecotype Columbia)	95.0 ± 2.5	5.0 ± 2.5	2.5 ± 2.2	97.5 ± 2.2
wer	99.7 ± 0.6	0.3 ± 0.6	99.3 ± 0.6	0.7 ± 0.6
gl2	100.0 ± 0.0	0.0 ± 0.0	99.2 ± 0.7	0.8 ± 0.7
gl3 egl3	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0
cpc	34.6 ± 3.1	65.4 ± 3.1	0.4 ± 0.7	99.6 ± 0.7
scm-2	73.3 ± 4.0	26.7 ± 4.0	19.6 ± 11.5	80.4 ± 11.5
35Spro:CPC	100.0 ± 0.0	0.0 ± 0.0	97.0 ± 2.9	3.0 ± 2.9
Q2610>>CPC	99.6 ± 0.6	0.4 ± 0.6	100.0 ± 0.0	0.0 ± 0.0
35Spro:WER	97.7 ± 3.2	2.3 ± 3.2	1.3 ± 1.5	98.7 ± 1.5
J2812>>WER	54.0 ± 13.0	46.0 ± 13.0	7.0 ± 1.0	93.0 ± 1.0
J2301>>WER	47.6 ± 5.7	52.4 ± 5.7	2.9 ± 2.0	97.1 ± 2.0
Q2610>>WER	0.0 ± 0.0	100.0 ± 0.0	0.0 ± 0.0	100 ± 0.0
Q2610>>WER *wer*	6.4 ± 4.0	93.6 ± 4.0	0.9 ± 0.9	99.1 ± 0.9
Q2610>>WER *gl2*	100.0 ± 0.0	0.0 ± 0.0	97.5 ± 1.2	2.5 ± 1.2
Q2610>>WER *gl3 egl3*	100.0 ± 0.0	0.0 ± 0.0	99.6 ± 0.6	0.4 ± 0.6
Q2610>>WER *scm-2*	0.9 ± 1.6	99.1 ± 1.6	0.0 ± 0.0	100.0 ± 0.0
Q2610>>WER *cpc*	0.4 ± 0.6	99.6 ± 0.6	0.0 ± 0.0	100.0 ± 0.0
Q2610>>WER *35Spro:CPC*	15.2 ± 8.7	84.8 ± 8.7	0.6 ± 1.0	99.4 ± 1.0
Q2610>>WER; CPC	100.0 ± 0.0	0.0 ± 0.0	99.7 ± 0.5	0.3 ± 0.5

mal cell, which implies that WER functions primarily in the nonhair cell fate specification.

WER Regulates *GL2* Expression to Induce the Nonhair Cell Fate by Binding to Two Sites in the *GL2* Promoter

We questioned whether GL2 mediates the nonhair cell fate specification by WER, which is an issue that has not been examined directly. To test this, we introduced Q2610>>WER into the *gl2* mutant background by a genetic cross. The resulting Q2610>>WER *gl2* plant showed a hairy root phenotype similar to the phenotype of the *gl2* single mutant (Fig. 2A; Table I).

Next, we examined whether WER induces *GL2* expression directly by using the glucocorticoid receptor (GR)-mediated inducible system (Picard et al., 1988; Lloyd et al., 1994; Ryu et al., 2005). We used the *35Spro:WER-GR wer-1* line, which was generated and verified previously (Ryu et al., 2005), and induced WER function with the same conditions (Fig. 3A). Whereas the seedling roots not exposed to dexamethasone (DEX) showed a very low level of *GL2* transcript, the seedling roots treated with 10 μM DEX for 6 h accumulated a greater level of *GL2* transcript. Furthermore, this increase was not diminished when cycloheximide was treated together with DEX, which suggests that de novo synthesis of proteins is not required and therefore that WER directly induces *GL2* expression.

To test whether WER binds to the *GL2* promoter, electrophoretic mobility shift assays (EMSAs) were performed using several small DNA fragments in the *GL2* promoter region including 2.1 kb upstream of the translation start codon as a probe (Fig. 3B), which is sufficient to induce proper *GL2* expression (Lin and Schiefelbein, 2001). We identified two WER-binding DNA fragments, and the binding regions were further defined to two small segments located at 909 to 890 bp (GWBSI) and 932 to 915 bp (GWBSII) upstream from the translation start codon (Fig. 3B). Several point-mutated versions of these two DNA fragments were used in the EMSA to define the nucleotides important for WER binding (Fig. 3C). WER did not bind to some of the mutated versions of DNA fragments, which revealed that the nucleotides shown as uppercase letters in aaaTgcgGTTgg for GWBSI and in aaGTTaGTTga for GWBSII are important for WER binding. Next, these binding sites were validated in yeast using a yeast one-hybrid assay with these two binding sites and the WER protein (Supplemental Fig. S1). In addition, we examined the importance of these sites for *GL2* expression in plants and found that these two sites are involved in the position-specific expression of *GL2* (Fig. 3D). When the *GL2* promoter had the mutated GWBSI (m1; aaCCTcgTCCgg), the promoter activity was greatly reduced in all of the individual transgenic plants compared with the activity of the wild-type *GL2* promoter. The mutations at GWBSII (m2; aaTCCaTCCga) or at both of the sites completely abolished the promoter activity in the root epidermis of every individual transgenic plant examined.

These results show that WER causes epidermal cells to adopt the nonhair cell fate through direct induction of *GL2* expression by binding to its promoter.

Expression of Other Cell Fate Regulators in the *WER*-Overexpressing Lines

It is well known that WER is necessary for *CPC* and *MYB23* expression in the N-position epidermal cells

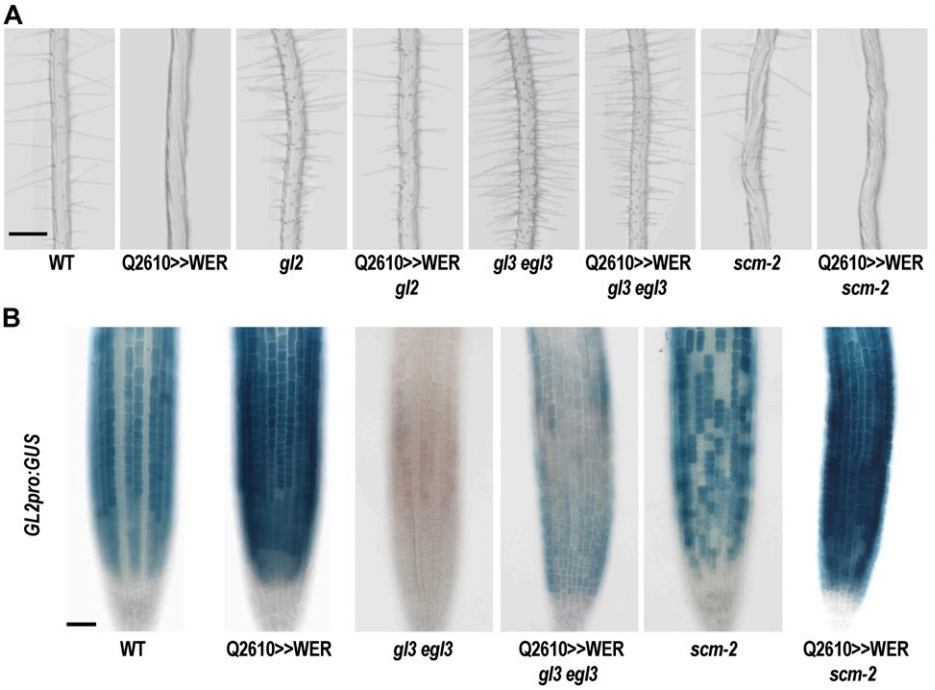

Figure 2. WER functions upstream of GL2 and GL3/EGL3 and downstream of SCM. A, Root hair phenotypes for the wild type (WT), mutants, Q2610>>WER, and mutants harboring Q2610>>WER. Bar = 200 μm. B, *GL2pro*:*GUS* reporter gene expression pattern in the root tip. Four-day-old seedling roots of the wild type, mutants, Q2610>>WER, and mutants bearing Q2610>>WER were stained for GUS activity after *GL2pro*:*GUS* was introduced into the backgrounds by genetic crosses. Bar = 50 μm. [See online article for color version of this figure.]

(Lee and Schiefelbein, 2002; Kang et al., 2009) and that WER directly induces *CPC* and *MYB23* expression by binding to their promoter (Koshino-Kimura et al., 2005; Ryu et al., 2005; Kang et al., 2009). However, it is not clear whether *WER* expression is sufficient to induce *CPC* and *MYB23* in the root epidermis. To test this, we examined *CPC* and *MYB23* expression in the *WER*-overexpressing plant roots using the reporter genes *CPCpro*:*GUS* and *MYB23pro*:*GUS* (Fig. 1, D and E). As expected, *35Spro*:*WER* did not cause any noticeable change in *CPCpro*:*GUS* and *MYB23pro*:*GUS* expression in the root epidermis. However, Q2610>>WER was able to increase their expression levels and made almost every epidermal cell express these reporter genes regardless of its position relative to the underlying cortical cells. Furthermore, Q2610>>WER was able to reduce *EGL3pro*:*GUS* expression in the root epidermis, while *35Spro*:*WER* was not (Fig. 1F). This is consistent with the report that *EGL3pro*:*GUS* expression was increased in the *wer* mutant root epidermis (Bernhardt et al., 2005).

Taken together, these results show that *WER* expression is sufficient to alter the expression of three cell fate regulators, *CPC*, *MYB23*, and *EGL3*, in the root epidermis.

WER Acts Together with GL3/EGL3 to Induce *GL2* Expression

Although WER is required for GL3/EGL3 function in nonhair cell fate specification in the N position (Bernhardt et al., 2003), it is not known whether GL3/EGL3 is necessary for WER function in the root epidermis. To test this, we introduced the *gl3 egl3* mutations into the Q2610>>WER line by a genetic cross. In the Q2610>>WER *gl3 egl3* plant, the hairless phenotype caused by *WER* overexpression disappeared and most of the epidermal cells adopted the hair cell fate similar to the *gl3 egl3* double mutant (Fig. 2A; Table I). Consistent with this, *GL2pro*:*GUS* expression in this line was very low, much lower than in the wild type and slightly higher than in the *gl3 egl3* double mutant (Fig. 2B).

We also examined the interaction between WER and GL3 in regulating *GL2* promoter activity. We expressed *WER* and *GL3* transiently in Arabidopsis leaf protoplasts as effectors using the 35S promoters (*35Spro*:*WER* and *35Spro*:*GL3*), and the reporter gene activity (*GL2pro*:*LUC*) was analyzed (Fig. 4A). WER alone was able to induce *GL2* promoter activity slightly (1.6-fold), and GL3 alone did not induce. On the contrary, *GL2* promoter activity was increased dramatically (32-fold) when *WER* and *GL3* were coexpressed.

Next, we examined the physical interaction between WER and GL3/EGL3 in plant cells using fluorescence resonance energy transfer (FRET) analysis. We expressed WER-CFP and the YFP-GL3 (or YFP-EGL3) chimeric proteins in tobacco (*Nicotiana tabacum*) leaf epidermal cells, and these colocalized in the nuclei (Fig. 4B). We analyzed the change in the cyan fluorescent protein (CFP) signal intensity after photobleaching of the yellow fluorescent protein (YFP) and discovered that, after photobleaching of YFP-GL3 and YFP-EGL3, WER-CFP signal intensity increased, with FRET efficiency of 11.6% and 33.5%, respectively, while FRET efficiencies of negative controls employing CONSTANS (CO)-CFP and YFP-GL3 or CO-CFP and YFP-EGL3 were negligible (0.8 and 0.9, respectively; Fig. 4,

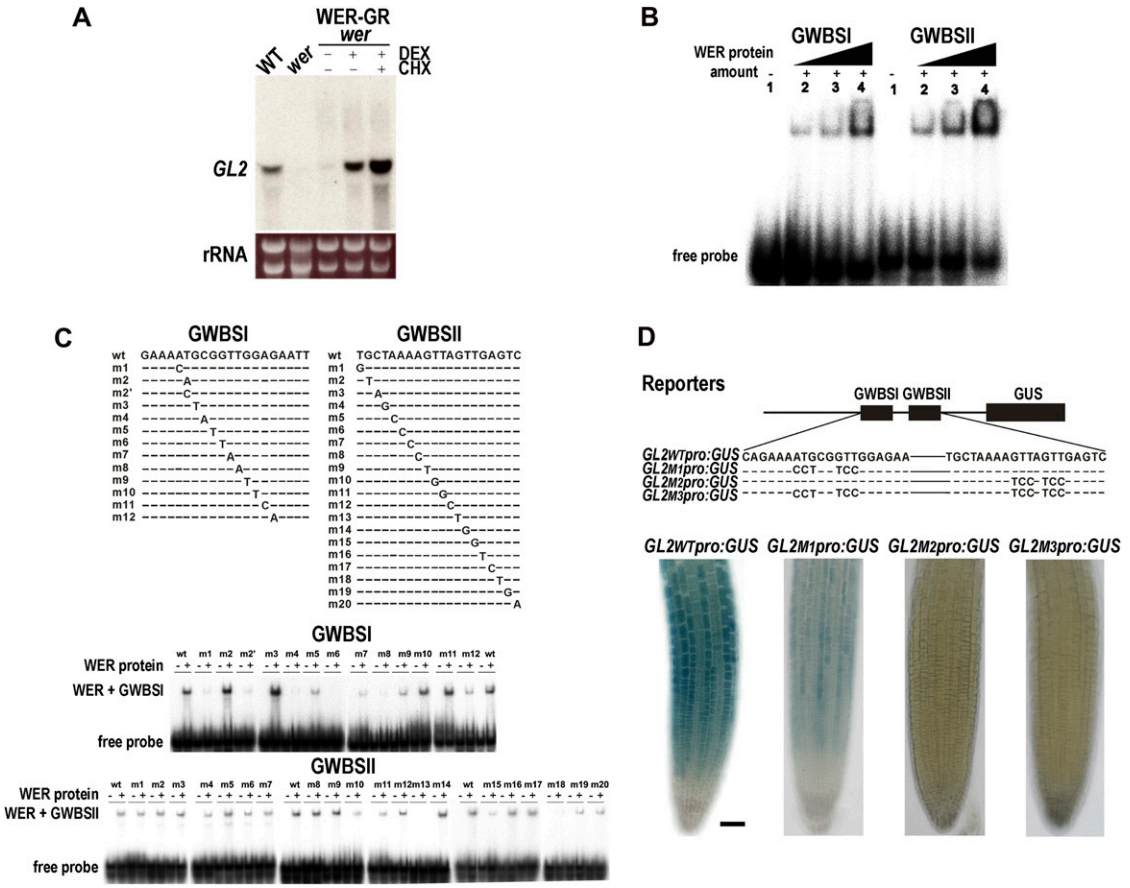

Figure 3. WER directly regulates *GL2* expression. A, Direct induction of *GL2* expression by WER. The *GL2* transcript was accumulated in the *wer* mutant seedlings harboring the *35Spro*:*WER-GR* transgene when DEX and cycloheximide (CHX) were given. RNA gel-blot analysis was performed with total RNA extracted from the root tips using the *GL2* fragment as a probe. WT, Wild type. B, Binding of WER to the *GL2* promoter. EMSA was performed using the purified WER protein and 20-bp-long DNA fragments (GWBSI and GWBSII screened from the *GL2* promoter). Lane 1, no WER; lanes 2 to 4, with increased amounts of WER (1×, 3×, and 6×, respectively). C, EMSA using the purified WER protein and the mutated probes. Sequences of mutated GWBSI and GWBSII used in this experiment are shown at the top. Each double-stranded probe contains a single base substitution as indicated. Dashes indicate no base change. A core sequence for GWBSI and GWBSII was deduced from these EMSA results. D, Importance of GWBSI and GWBSII in the proper expression of *GL2* in the Arabidopsis root. The top panel shows schematic diagrams of the wild-type and mutated *GL2pro*:*GUS* reporter genes used for the stable transgenic lines. The bottom panel shows the GUS activity of the transgenic roots. Bar = 50 μm. [See online article for color version of this figure.]

B and C). We also included a positive control of this FRET analysis with CO-YFP and AtHAP5a-CFP, which were shown to physically interact using FRET analysis (Wenkel et al., 2006).

SCM Does Not Affect the WER Function

To determine the genetic relationship between WER and SCM, we analyzed the effect of *WER* overexpression in the *scm* mutant background. In the Q2610>> WER *scm* root, every epidermal cell adopted the non-hair cell fate, which implies that SCM is not required for the WER overexpression effect (Fig. 2A; Table I). Furthermore, *GL2pro*:*GUS* expression in the Q2610>> WER *scm* plant root was indistinguishable from that in the Q2610>>WER plant root, while the *scm* mutant root showed a largely random expression pattern (Fig. 2B).

CPC Overexpression Does Not Inhibit *WER* Expression

In the *cpc* mutant root epidermis, *WER* is expressed at a high level in some of the H-position cells, which suggested that CPC is able to suppress *WER* expression (Lee and Schiefelbein, 2002). To test this hypothesis, we examined *WER* expression in the root epidermis in *CPC*-overexpressing lines using the *WERpro*:*GUS* transgene as a reporter. This transgene has a shorter 5′ *WER* flanking DNA sequence (1.4 kb) than the reporter gene previously described (4 kb; Lee and Schiefelbein, 1999), but this shorter version was still able to drive *WER*

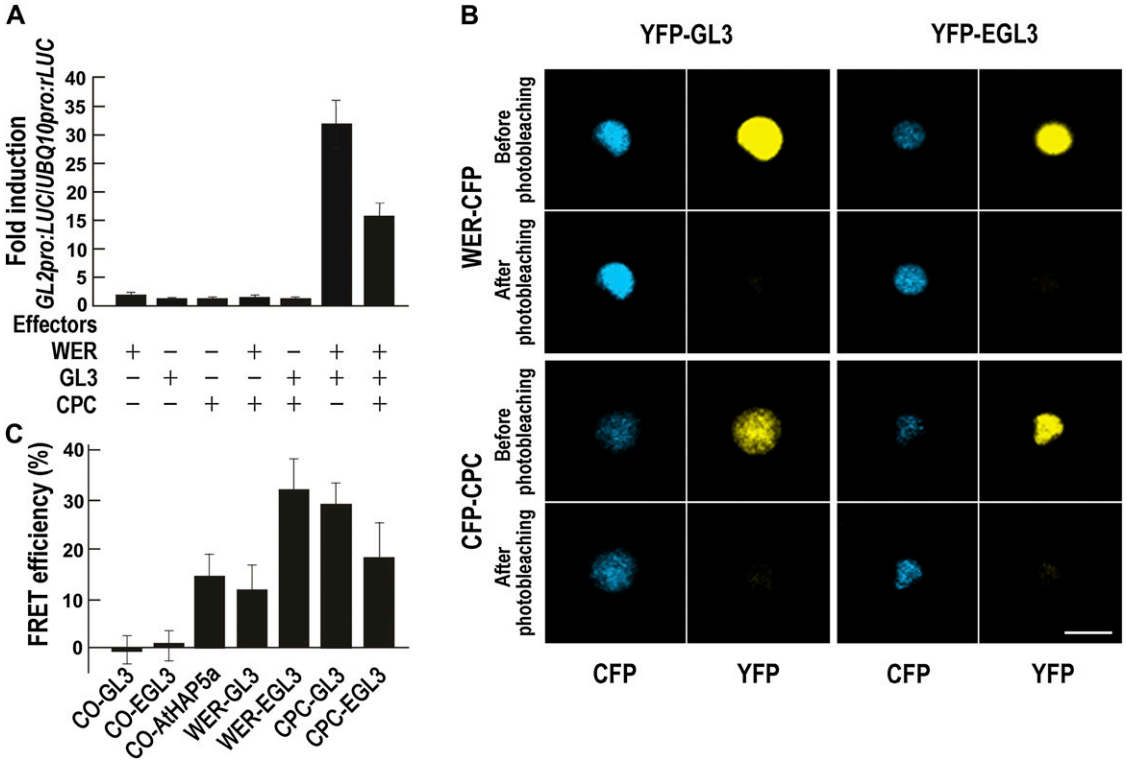

Figure 4. WER interacts with GL3/EGL3 in the regulation of *GL2* expression. A, Interactions among WER, GL3, and CPC in the regulation of *GL2* expression in the Arabidopsis protoplast transient expression assay. Protoplasts were transfected with *35Spro*:*WER*, *35Spro*:*GL3*, *35Spro*:*CPC*, and their combinations as indicated together with *GL2pro*:*LUC* as a reporter and *UBQ10pro*:*rLUC* as an internal control. B, In vivo FRET analysis for the interaction of WER and CPC with GL3 and EGL3 in plant cells. WER (top panels) and CPC (bottom panels) interaction with GL3 and EGL3 was visualized as an increase in the WER-CFP and CFP-CPC fluorescence after photobleaching of YFP-GL3 and YFP-EGL3. Images display the CFP and YFP channels in false colors before and after photobleaching. Blue color indicates CFP signal, and yellow color indicates YFP signal. Bar = 10 μm. C, Quantification of FRET efficiency after acceptor photobleaching. FRET efficiency was calculated from the formula FRET efficiency = {(CFP signal after photobleaching − CFP signal before photobleaching)/CFP signal after photobleaching} × 100. CO-CFP was used as a negative control. Error bars indicate SD from 10 to 14 independent FRET analyses through two independent experiments.

expression sufficient to complement the *wer* mutant phenotype (Supplemental Fig. S2).

Overexpression of *CPC* using the 35S CaMV promoter (*35Spro*:*CPC*) was not able to change the *WER* expression pattern (Fig. 5A), even though it was sufficient to cause N-position cells to adopt the hair cell fate (Wada et al., 1997). Quantitative real-time PCR analysis revealed that the *WER* transcript level in the *35Spro*:*CPC* plant root was not significantly different from its level in the wild-type root (Fig. 5B). In a separate experiment, we generated a Q2610>>CPC line and examined its effect on *WER* expression. Like the *35Spro*:*CPC* line, we found that, in the root tip of the Q2610>>CPC line, the *WER* expression pattern was not altered and the *WER* transcript level was not reduced (indeed, perhaps slightly increased; Fig. 5), even though the hair cell fate was induced in every epidermal cell (Fig. 6A; Table I) and *CPC* expression was much higher than in the *35Spro*:*CPC* root or the wild-type root (Fig. 6C). Because *WER* expression was not reduced in any of these *CPC* overexpression lines, these results suggest that CPC is not sufficient to inhibit *WER* gene expression.

CPC Can Inhibit WER Function Quantitatively

We conducted a series of experiments to investigate the proposed competition between WER and CPC in cell fate specification. First, we examined the effect of CPC on *GL2* promoter activation by WER and GL3. When *CPC* was transiently expressed together with *WER* and *GL3* in protoplasts, we observed a 50% decrease in *GL2* promoter activation by WER and GL3 (Fig. 4A). Next, we analyzed the possible competition using stably transformed Arabidopsis plants. We introduced the *cpc* mutation, *35Spro*:*CPC*, or *UASpro*:*CPC* into the Q2610>>WER plant by genetic crosses to express *CPC* at different levels (Fig. 6C). *35Spro*:*CPC* has been known to inhibit *GL2* expression so that most of the epidermal cells adopt the hair cell fate (Fig. 6, A and B; Table I; Wada et al., 1997, 2002; Lee and Schiefelbein, 2002). Q2610>>CPC strongly inhibited

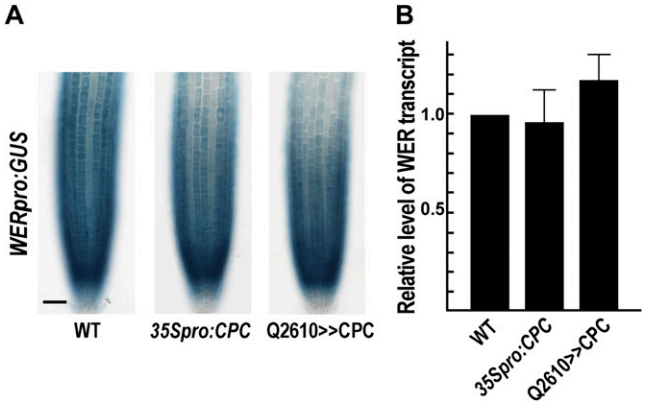

Figure 5. The ectopic expression of *CPC* does not affect *WER* expression. A, Wild-type (WT) and transgenic roots harboring *WERpro*:*GUS* were stained for GUS activity. Bar = 50 μm. B, Relative level of the *WER* transcript in wild-type, *35Spro*:*CPC*, and Q2610>>CPC transgenic roots determined by quantitative real-time PCR analysis. Error bars indicate SD from at least three replicates. [See online article for color version of this figure.]

GL2pro:*GUS* expression to an undetectable level and caused almost every root epidermal cell to adopt the hair cell fate (Fig. 6, A and B; Table I). The *35Spro*:*CPC* root tip showed about 25-fold higher accumulation of *CPC* transcript than the wild-type root tip, and the Q2610>>CPC root tip showed even higher accumulation of *CPC* transcript (108-fold higher than the wild type; Fig. 6C). Increased accumulation of *CPC* transcript was also observed in the Q2610>>WER background (Q2610>>WER *35Spro*:*CPC* and Q2610>>WER; CPC; Fig. 6C). The *cpc* mutation did not alter the action of Q2610>>WER, based on the complete hairless phenotype of the Q2610>>WER *cpc* plant root (Fig. 6A). *35Spro*:*CPC* in the Q2610>>WER plant made 15.2% of the epidermal cells differentiate into a hair cell, while none of the epidermal cells in the Q2610>>WER plant differentiated into a hair cell (Fig. 6A; Table I). Accordingly, some epidermal cells in the Q2610>>WER *35Spro*:*CPC* roots were not expressing the *GL2pro*:*GUS* reporter gene (Fig. 6B). Furthermore, Q2610>>CPC in the Q2610>>WER background completely changed the hairless root phenotype into a hairy phenotype (Fig. 6A). A detailed analysis of the cell type pattern revealed that almost every epidermal cell differentiated into a hair cell (Table I). Although the epidermal cell type pattern in Q2610>>WER; CPC was almost the same as the cell type pattern found in Q2610>>CPC, *GL2pro*:*GUS* expression was detected in the Q2610>>WER; CPC plant after overnight staining (Fig. 6D). We have never been able to detect *GL2pro*:*GUS* expression in the Q2610>>CPC plant root, even after prolonged staining.

CPC Interacts with GL3/EGL3 in Plant Cells

To test for possible protein-protein interaction between CPC and GL3/EGL3 in plant cells, we used FRET analysis. CPC-CFP and the YFP-GL3 (or YFP-EGL3) chimeric proteins were expressed in tobacco leaf epidermal cells and were detected in nuclei (Fig. 4B). We analyzed the change in CFP signal intensity after photobleaching of the YFP. After photobleaching of YFP-GL3 and YFP-EGL3, the CPC-CFP signal intensity was increased with FRET efficiency of 29.1% and 18.2%, respectively (Fig. 4, B and C).

DISCUSSION

The Ratio of CPC to WER Specifies the Cell Fate in the Root Epidermis

Although *35Spro*:*WER* did not affect the epidermal cell patterning in the wild-type root, it caused a novel pattern phenotype in the root epidermis in the *wer* mutant background (Lee and Schiefelbein, 2002). Specifically, the position-dependent cell fate specification was disrupted, so that both of the cell types were found in each position. It was suggested that this phenotype was due to increased and equivalent *CPC* and *GL2* expression by *35Spro*:*WER* in all epidermal cells. In the Q2610>>WER plant roots, however, every epidermal cell adopted the nonhair cell fate, even though *CPC* expression is high in every root epidermal cell (Fig. 1). This shows that CPC induced by the WER in this plant was not sufficient to induce the hair cell fate and generate a pattern of both cell types. This raised two possible explanations. One possibility is that the negative regulation of *WER* expression by CPC is critical for the hair cell fate specification, which was proposed previously (Lee and Schiefelbein, 2002). The second possibility is that the level of CPC protein in the Q2610>>WER plant might not be sufficient to inhibit the WER function in nonhair cell fate specification. CPC overexpressed using the 35S CaMV promoter or the Q2610 enhancer trap line did not suppress *WER* expression in the root, while it inhibited *GL2* expression and made the root epidermal cells adopt the hair cell fate regardless of their relative positions (Figs. 5 and 6). Also, the more *CPC* was expressed in the Q2610>>WER plant, the fewer root epidermal cells expressed the *GL2* gene and the more cells adopted the hair cell fate (Fig. 6; Table I). Furthermore, transiently expressed CPC suppressed the increase in *GL2* promoter activity by WER and GL3 without affecting their expression (Fig. 4A). Together, these results strongly indicate that the most important factor in hair cell fate specification is not negative regulation of *WER* expression by CPC but the ratio of the level of CPC to the level of WER.

It has been shown that the *CPC* promoter is activated in the N-position cells by WER (Lee and Schiefelbein, 2002; Wada et al., 2002; Koshino-Kimura et al., 2005; Ryu et al., 2005) and that the CPC protein accumulates in the epidermal cells at both of the positions (Wada et al., 2002; Kurata et al., 2005). On the contrary, the WER protein was shown to prefer-

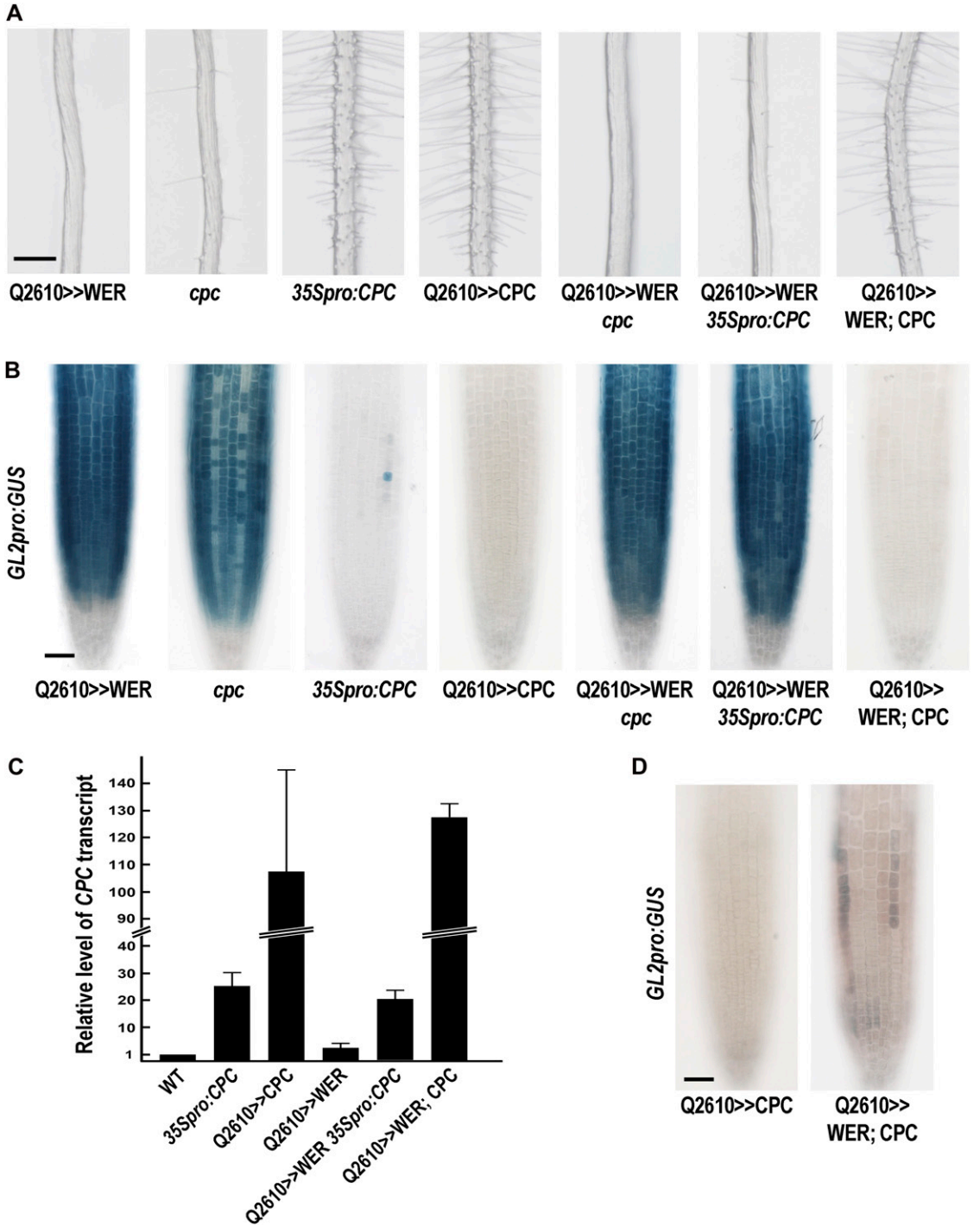

Figure 6. CPC inhibits the WER function quantitatively. A, The phenotypes of root hair pattern in *cpc* and transgenic plants ectopically expressing CPC, WER, or both of them. Bar = 200 μm. B, *GL2pro:GUS* reporter gene expression pattern in the root tips of *cpc* and transgenic roots bearing *GL2pro:GUS*. Four-day-old seedlings were stained for GUS activity. Bar = 50 μm. C, Relative levels of the *CPC* transcript in wild-type (WT) and various transgenic roots determined by quantitative real-time PCR analysis. Error bars indicate SD from at least three replicates. D, *GL2pro:GUS* reporter gene expression pattern in the root tip of Q2610>>CPC and Q2610>>WER; CPC harboring *GL2pro:GUS*. Four-day-old seedlings were stained overnight for GUS activity to examine the effect of *WER* overexpression. Bar = 50 μm. [See online article for color version of this figure.]

entially accumulate in the N-position cell of the wild-type root epidermis (Ryu et al., 2005). Taken together, this suggests a possible mechanism based on a competition between WER and CPC quantitatively to specify the cell fate in the root epidermis. In this view, the N-position cells express *WER* and *CPC*, and CPC moves to the neighboring H-position cells while WER does not. This results in different ratios of the CPC level to the WER level between the cells at the two positions. In the H-position cell, the high ratio of CPC to WER inhibits *GL2* expression, leading to the hair cell fate, while the low ratio of CPC to WER in the N-position cell induces *GL2* expression, leading to the nonhair cell fate. This mechanism explains why CPC does not induce the hair cell fate in the N-position cell where it is expressed.

Possible Competition between WER and CPC in Interacting with GL3/EGL3

The maize (*Zea mays*) *R* gene encodes a bHLH protein, and it was shown to affect cell fate specification in the Arabidopsis root epidermis when it was overexpressed (Galway et al., 1994). WER was shown to interact with the maize R protein (Lee and Schiefelbein, 1999) and two Arabidopsis bHLH proteins, GL3 and EGL3 (Bernhardt et al., 2003), using yeast two-hybrid analysis. GL1, which is functionally equivalent to WER (Lee and Schiefelbein, 2001), was shown to interact with GL3 and EGL3 using yeast two-hybrid analysis (Payne et al., 2000; Zhang et al., 2003) and coimmunoprecipitation assay (Gao et al., 2008). Also, CPC and TRY, negative regulators of *GL2* expression, were found to bind to GL3 and EGL3 in the yeast two-hybrid assay (Bernhardt et al., 2003; Esch et al., 2003) and in plant protoplasts using bimolecular fluorescence complementation (Wester et al., 2009). Furthermore, it was demonstrated that CPC and CPC-like proteins interfere with the interaction between GL1 and GL3 in yeast (Esch et al., 2003; Wester et al., 2009).

Here, we demonstrated that WER and GL3/EGL3 interact in plant cells using FRET analysis and that GL3/EGL3 is required for WER function using genetic analysis and a transient expression system in Arabidopsis protoplasts (Figs. 2 and 4; Table I). We also demonstrated the interaction between CPC and GL3/EGL3 in plant cells using FRET analysis (Fig. 4). Our results confirm that those protein-protein interactions previously suggested are able to occur in plant cells, and they further indicate that WER and CPC compete for binding to GL3/EGL3 to regulate *GL2* expression quantitatively. A dose-dependent competition mechanism, especially for the interaction with a partner protein, has been also proposed in other biological processes. For example, it has been suggested that APETALA3 (AP3) and PISTILLATA (PI) antagonize AGAMOUS (AG) functions by competing for the MADS box protein complex formation in Arabidopsis and, therefore, that the balance between AP3/PI and AG is important in floral organ specification and floral meristem termination (Prunet et al., 2009). In the photoreceptor specification of the *Drosophila melanogaster* retina, SOC36E and Drk compete with each other for the physical interaction with Sevenless (Sev) to repress and activate the Sev pathway, respectively (Almudi et al., 2010).

TRY was demonstrated to bind to GL1 in a glutathione *S*-transferase pull-down experiment, and this direct interaction was proposed as another possible mechanism for the inhibition of GL1 function (Digiuni et al., 2008). However, in our experiment using the yeast two-hybrid assay and FRET analysis, we were not able to detect an interaction between WER and CPC (data not shown). This discrepancy might indicate the difference between WER and GL1 and/or between CPC and TRY.

In our FRET analyses, WER interacted with EGL3 more efficiently than with GL3, while CPC interacted more efficiently with GL3 than with EGL3 (Fig. 4C). Also, it was shown that overexpression of *GL3* and *EGL3* promotes the nonhair cell fate specification and that *EGL3* overexpression caused a stronger hairless phenotype than overexpression of *GL3* (Bernhardt et al., 2003). One possible explanation for these phenotypic differences could be the difference in the transgene expression level. Another possible explanation raised by our FRET results is a difference in the preference for an interacting partner.

WER Directly Induces the Expression of Three Different Genes in the N-Position Cell for Epidermal Cell Patterning in the Arabidopsis Root

It has been suggested that WER positively regulates the expression of three genes, *GL2*, *CPC*, and *MYB23*, in the N-position cells of the root epidermis based on the reduced level of their transcript in the *wer* mutant root epidermis (Lee and Schiefelbein, 1999, 2002; Kang et al., 2009). WER was shown to directly induce *CPC* expression using a DEX-inducible expression system and was further shown to bind to three sites in the *CPC* promoter (WBSI, WBS/CPCMBS1, and CPCMBS2; Koshino-Kimura et al., 2005; Ryu et al., 2005). These sites were found to be necessary for the proper expression of *CPC* in the Arabidopsis root epidermis. WER also binds to multiple sites in the *MYB23* promoter to directly induce its expression in the root epidermis (Kang et al., 2009). *GL2* was reported to have two WER-binding sites in its promoter (GL2MBS1 and GL2MBS2) based on an EMSA result (Koshino-Kimura et al., 2005). Recently, it was reported that GL1 is associated with the *GL2* promoter using chromatin immunoprecipitation analysis (Zhao et al., 2008; Morohashi and Grotewold, 2009). However, it is not known whether *GL2* expression is directly regulated by WER or whether these two binding sites reported are important for *GL2* expression. Here, we show that WER specifies the nonhair cell

fate in the root epidermis through the induction of *GL2* expression (Fig. 2) and that this induction is a direct effect ofWER by binding to two sites (GWBSI and GWBSII) in the *GL2* promoter that are different from the previously reported sites (GL2MBS1 and GL2MBS2; Fig. 3, A and C). Importantly, these binding sites were tested in vivo as well as in vitro (Fig. 3C; Supplemental Fig. S1). Also, both of these two WER-binding sites are important for the position-specific expression of the *GL2* gene (Fig. 3D). These binding sites are located within the region that was reported to be important for the appropriate expression of the *GL2* gene (Hung et al., 1998) and to be a putative GL1-binding region based on a chromatin immunoprecipitation experiment (Zhao et al., 2008). These two binding sites are located approximately 350 to 550 bp downstream of the previously reported GL2MBS1 and GL2MBS2 (Koshino-Kimura et al., 2005), and we were unable to detect these GL2MBSs in our analysis. Furthermore, we were unable to find any significant disruption of the *GL2* promoter activity in the root epidermis when we introduced a mutation in these binding sites, GL2MBS1 or GL2MBS2. Also, a *GL2* reporter gene driven by a shorter promoter (1.2 kb) without these two sites had no significant change in promoter activity (Supplemental Fig. S3), whereas the *GL2* reporter genes with a mutation in one of the GWBSs showed very low levels of expression in the root epidermis (Fig. 3D).

Analysis of the WER-binding sites confirmed in these three genes shows a shared core sequence, (C/T) DGTT(G/A), which is similar to the vertebrate MYB-binding site, CNGTTR (Howe and Watson, 1991). Plants possess a particularly large number of MYB genes in their genomes, and they appear to regulate many different cellular processes (Stracke et al., 2001). Therefore, their DNA-binding sites might be expected to differ considerably among themselves. Among the plant MYB-binding sites, the WER-binding site is similar to one of the binding sites of MYB.Ph3 from petunia (*Petunia hybrida*; Solano et al., 1995). In addition to this core sequence, the flanking sequence also seems to play an important role in vivo, because GL2MBS1 and GL2MBS2 are not involved in *GL2* gene regulation by WER, in spite of their same core sequence, (C/T)DGTT(G/A) (Koshino-Kimura et al., 2005).

Each of these direct WER target genes (*GL2*, *CPC*, and *MYB23*) has multiple WER-binding sites in its promoter, and each binding site is important for proper expression except for some of the sites in the *MYB23* promoter, which may function redundantly (Fig. 3D; Koshino-Kimura et al., 2005; Ryu et al., 2005; Kang et al., 2009). It has been reported that GL3 and EGL3 interact with each other as well as with themselves in yeast (Payne et al., 2000; Zhang et al., 2003). Taken together with the interaction between WER and GL3/EGL3, it seems that WER acts in a protein complex including at least two GL3/EGL3 proteins and two WER proteins. Therefore, multiple binding sites may be helpful for this protein complex to bind effectively to their promoter and induce appropriate transcription.

CONCLUSION

Through this work, we are able to define the primary function of WER in cell fate specification. WER primarily acts to specify the nonhair cell fate rather than as a master regulator in generating a pattern. Furthermore, this nonhair cell fate specification by WER is genetically mediated by *GL2*, which is a direct target gene of WER. GL3/EGL3 is also genetically shown to be necessary for the proper function of WER in *GL2* expression, and interaction between WER and GL3/EGL3 is demonstrated in plant cells using FRET analysis. CPC also interacts with GL3/EGL3 in plant cells, suggesting a possible competition with WER in interacting with GL3/EGL3. We are able to show that negative regulation of *WER* expression by CPC is not required for hair cell fate specification and that the critical factor in epidermal cell fate specification is the ratio of the level of CPC to the level of WER.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The following mutant lines have been described in Arabidopsis (*Arabidopsis thaliana*): *wer-1* (Lee and Schiefelbein, 1999), *cpc* (Wada et al., 1997), *gl3-1* (Koornneef et al., 1982), *egl3-1* (Zhang et al., 2003), *gl2-1* (Koornneef, 1981), and *scm-2* (Kwak et al., 2005). Plants harboring *35Spro*:*CPC*, *WERpro*:*GFP*, *MYB23pro*:*GUS*, *GL2pro*:*GUS*, and *CPCpro*:*GUS* were also described previously (Masucci et al., 1996; Wada et al., 1997, 2002; Lee and Schiefelbein, 1999; Kirik et al., 2001). The enhancer trap lines were obtained from the Arabidopsis Biological Resource Center.

For plant growth, seeds were surface sterilized, germinated, and grown vertically on agarose-solidified medium containing mineral nutrients at 22°C under continuous light conditions (Schiefelbein and Somerville, 1990).

Histochemical GUS Staining

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

Quantitative Real-Time Reverse Transcription -PCR

Quantitative real-time PCR was performed using SYBR Premix EX Taq (Takara) with an Mx3000P real-time PCR machine (Stratagene) as described previously (Kang et al., 2009). One microgram of total RNA extracted from the root tips of 4-d-old seedlings was used for the reverse transcription. Each experiment was repeated three to six times, and each time the experiment included triplicate samples.

EMSA

EMSA was carried out as described earlier (Ryu et al., 2005).

Yeast One-Hybrid Assay

To test GWBSI and GWBSII in vivo, we used the Matchmaker yeast one-hybrid system (Clontech) according to the manufacturer's manual. Three tandem copies of GWBSI, GWBSII, or point-mutated GWBSs were synthesized and inserted into the reporter vectors pHISi and pLacZi. A reporter strain was made by integrating these reporter genes into the yeast strain

YM4271 genome. *WER* was expressed in this reporter strain, and the reporter gene activity was assessed as described previously (Ryu et al., 2005).

Protein Expression and Purification

Expression of the WER protein in *Escherichia coli* BL21 (DE3) and purification of the protein using His·Bind Quick 900 cartridges were described previously (Ryu et al., 2005).

Confocal Microscopy

Seedlings were counterstained with 5 μg mL^{-1} propidium iodide for 5 min, and GFP expression was examined using a LSM510 Meta confocal microscope (Carl Zeiss) as described (Lee and Schiefelbein, 1999).

FRET Analysis

The coding regions of *GL3*, *EGL3*, and *CO* were PCR amplified and fused to *YFP*, and then these recombinant genes were fused to 35S promoter (*35Spro*:*YFP-GL3*, *35Spro*:*YFP-EGL3*, and *35Spro*:*CO-YFP*). The coding regions of *WER*, *CPC*, *CO*, and *AtHAP5a* were also PCR amplified and fused to CFP and then fused to 35S promoter (*35Spro*:*WER-CFP*, *35Spro*:*CPC-CFP*, *35Spro*:*CO-CFP*, and *35Spro*:*AtHAP5a-CFP*). The sequences of the PCR-amplified genes were confirmed. One of the YFP-fused genes and one of the CFP-fused genes were introduced together into 4-week-old *Nicotiana benthamiana* leaves as described previously (Ratcliff et al., 2001). After 48 h of incubation, leaf epidermal cells exhibiting coexpression of both fluorescent proteins were bleached five times in the acceptor YFP channel with a 514-nm argon laser. Before and after photobleaching, CFP fluorescence intensity was monitored by confocal microscopy (LSM510 Meta; Carl Zeiss), and FRET efficiency was calculated as follows: E = {(CFP signal after photobleaching – CFP signal before photobleaching)/CFP signal after photobleaching} $\times$ 100.

Arabidopsis Protoplast Transient Expression Assay

Single cell-based functional analyses were conducted using transient expression of the Arabidopsis mesophyll protoplast system as described previously (Yoo et al., 2007). For the reporter construct, the 2,032-bp genomic DNA region immediately upstream from the translation start site of the *GL2* gene was PCR amplified. The promoter was fused to a luciferase reporter gene (*GL2pro*:*LUC*). We used three effectors: WER, GL3, and CPC. Genomic DNA fragments of their coding regions were PCR amplified and inserted into a plant expression vector containing 35S promoter. Twenty thousand protoplasts were transfected with 40 μg of plasmid DNA and different combinations of the reporter (*GL2pro*:*Luc*), effectors (*35Spro*:*WER*, *35Spro*:*GL3*, and *35Spro*:*CPC*), and the internal control (*UBQ10pro:rLUC*). An empty vector was used as a negative control. The luciferase reporter activity was determined by the Dual Luciferase Assay System (Promega).

The sequences of the PCR-amplified DNA fragments were confirmed.

Overexpression of *WER* and *CPC*

Genomic DNA fragments of the *WER* and *CPC* coding regions were PCR amplified and cloned into pJET1.2/blunt cloning vector (Fermentas). Their sequences were confirmed. Their genomic DNA fragments were inserted into pCB302 containing 5$\times$ UAS promoter and the nos terminator (Song et al., 2008). Plant transformation was achieved by electroporating constructs (*UASpro*:*WER* and *UASpro*:*CPC*) into the *Agrobacterium tumefaciens* strain GV3101 followed by introduction into Arabidopsis using the floral dip method as described previously (Clough and Bent, 1998). *UASpro*:*WER* and *UASpro*:*CPC* were introduced into the enhance trap lines by genetic crosses.

Other Constructs

For *WERpro*:*GUS* and *WERpro*:*WER*, the GUS gene or the genomic DNA fragment of the WER coding region was inserted between a 1.4-kb 5′ flanking region DNA fragment or a 1.1-kb 3′ flanking region DNA fragment, respectively, from the WER coding region. The resulting construct was introduced into Arabidopsis as described previously (Clough and Bent, 1998).

Primers

The sequences of the primers used in this experiment are listed in Supplemental Table S1.

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *CPC* (At2g46410), *EGL3* (At1g63650), *GL2* (At1g79840), *GL3* (At5g41315), *MYB23* (At5g40330), and *WER* (At5g14750).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. WER interacts with GWBSI and GWBSII for transcriptional activation in yeast.

Supplemental Figure S2. Complementation of the *wer* mutant phenotype using the *WER* gene including the 1.4-kb 5′ region DNA fragment and the 1.1-kb 3′ flanking region DNA fragment.

Supplemental Figure S3. The 1.2-kb *GL2* promoter region containing GWBSI and GWBSII is sufficient for the proper expression of *GL2*.

Supplemental Table S1. Primer sequences used in these experiments.

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