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# Functional Analysis of the Epidermal-Specific MYB Genes *CAPRICE* and *WEREWOLF* in *Arabidopsis* [W]

**Rumi Tominaga,[a] Mineko Iwata,[a] Kiyotaka Okada,[a,b] and Takuji Wada[a,1]**

[a] Plant Science Center, RIKEN, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan
[b] Department of Botany, Graduate School of Science, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan

**Epidermis cell differentiation in *Arabidopsis thaliana* is a model system for understanding the developmental end state of plant cells. Two types of MYB transcription factors, R2R3-MYB and R3-MYB, are involved in cell fate determination. To examine the molecular basis of this process, we analyzed the functional relationship of the R2R3-type MYB gene *WEREWOLF* (*WER*) and the R3-type MYB gene *CAPRICE* (*CPC*). Chimeric constructs made from the R3 MYB regions of *WER* and *CPC* used in reciprocal complementation experiments showed that the CPC R3 region cannot functionally substitute for the WER R3 region in the differentiation of hairless cells. However, WER R3 can substantially substitute for CPC R3. There are no differences in yeast interaction assays of WER or WER chimera proteins with GLABRA3 (GL3) or ENHANCER OF GLABRA3 (EGL3). CPC and CPC chimera proteins also have similar activity in preventing GL3 WER and EGL3 WER interactions. Furthermore, we showed by gel mobility shift assays that WER chimera proteins do not bind to the *GL2* promoter region. However, a CPC chimera protein, which harbors the WER R3 motif, still binds to the *GL2* promoter region.**

## INTRODUCTION

Cell fate determination is a critical step in the developmental processes of plants and involves the participation of a large number of transcription factors. The MYB family is one of the largest groups of transcription factors in the *Arabidopsis thaliana* genome, with >125 members (Kranz et al., 1998; Stracke et al., 2001). Members of this gene family encode proteins characterized by two 50- to 52-residue imperfect repeats (R2 and R3 MYB domains). Each of these MYB repeats contains three $\alpha$-helices, with the second and third helices forming a helix-turn-helix structure when bound to DNA (Ogata et al., 1992). It has been proposed that plant R2R3 MYB genes originated from an ancestral gene encoding a three-MYB repeat protein (R1, R2, and R3) that survives in animals today as *c-MYB* and related genes (Lipsick, 1996) and in plants as the small *pc-MYB* gene family (Braun and Grotewold, 1999). After the loss of the R1 repeat, a rapid amplification of the R2R3 MYB gene family apparently occurred 250 to 400 million years ago in plants (Rabinowicz et al., 1999).

Because of its well-characterized genetics and ease of observation, the epidermis of *Arabidopsis* has been used as a model for understanding cell fate determination. Root epidermal cells are generated at the root apical meristem and differentiate into either of two cell types (hair cells or hairless cells) in a cell position–dependent manner (Dolan et al., 1994). Epidermal cells in contact with two cortical cells differentiate into hair cells, whereas cells touching only one cortical cell develop into hairless cells. Wild-type *Arabidopsis* has eight hair cell files aligned longitudinally along the root.

From previous work it is clear that two types of MYB-related transcription factors (R2R3 and R3) are involved in epidermis differentiation. The closely related *WEREWOLF* (*WER*), *GLABRA1* (*GL1*), and *MYB23* genes encode R2R3-type MYB genes. *WER* promotes differentiation to the non-hair cell fate, so that *WER* mutant root epidermal cells mostly differentiate into hair cells (Lee and Schiefelbein, 1999). In contrast with the R2R3-type MYB genes, the *CAPRICE* (*CPC*) gene encodes a single MYB repeat protein that lacks any discernable transcriptional activation domain. Mutation of this gene results in a reduced number of root hairs, implying that it is critical in the induction of hair cell fate (Wada et al., 1997, 2002). Koshino-Kimura et al. (2005) reported that the transcription of *GL2*, which encodes a homeodomain-leucine zipper protein and is thought to act farthest downstream in the root hair regulatory pathway (Lee and Schiefelbein, 1999; Galway et al., 1994; Rerie et al., 1994; Wada et al., 1997; Bernhardt et al., 2005), is controlled by a protein complex that includes WER, GL3, ENHANCER OF GLABRA3 (EGL3), and TRANSPARENT TESTA GLABRA1 (TTG1) proteins. *GL3* and *EGL3* encode basic helix-loop-helix (bHLH) proteins and affect hairless cell differentiation in a redundant manner (Bernhardt et al., 2003; Zhang et al., 2003). Most hairless cells are converted into root hair cells in the *gl3 egl3* double mutant, whereas *gl3* and *egl3* single mutants have only slightly increased numbers of hair cells (Bernhardt et al., 2003). Like WER, these bHLH proteins also regulate *GL2* expression in hairless cells (Bernhardt et al., 2003; Zhang et al., 2003). Using the yeast two-hybrid system, GL3 and EGL3 were shown to interact with WER (Bernhardt et al., 2003) and with a WD40 protein (TTG1) (Payne et al., 2000; Esch et al., 2003; Zhang et al., 2003). The CPC protein was proposed to

[1] Address correspondence to twada@psc.riken.jp.
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disrupt this protein complex by competitive binding with WER, leading to repression of *GL2* expression (Wada et al., 2002; Koshino-Kimura et al., 2005).

In this study, the differences between WER and CPC R3 MYB functions were examined. Chimeras made from the R3 regions of *WER* and *CPC* were designed to identify which residues specify their functional identity because it would have been thought that WER and CPC had opposite functions for root hair formation in *Arabidopsis*. Either the entire WER R3 domain or portions of it were exchanged with the corresponding regions of *CPC* to form a series of chimeric constructs. We then transformed *wer* mutant plants with these chimeras and scored any resulting complementation by counting root hairs. The R3 domains of *CPC* and *WER* were also exchanged and used to complement *cpc* mutant plants. We found that the WER R3 domain can functionally replace CPC R3, but CPC R3 cannot functionally replace WER R3. All of the WER and CPC chimera proteins interact with GL3/EGL3. These results substantially support the competition model for CPC and WER in determining root hair cell fate. In addition, we suggest that CPC evolved from WER as a result of truncation of the activation domain and loss of DNA binding ability.

## RESULTS

### Evolutionary Relationship between WER and CPC

The *WER* gene encodes a MYB protein containing R2 and R3 repeats. The *CPC* gene encodes a single R3 MYB repeat protein that shares 54% sequence identity with the R3 motif of WER (Figure 1A). The R3 motif, like c-MYB, is composed of three helices (h1, h2, and h3) (Figures 1A and 1B; Ogata et al., 1992). A comparison of each helix of WER R3 and CPC R3 indicated that h1 and h2 are similar, but h3 is considerably different. WER R3 h3 is mainly composed of neutral polar residues, whereas the CPC

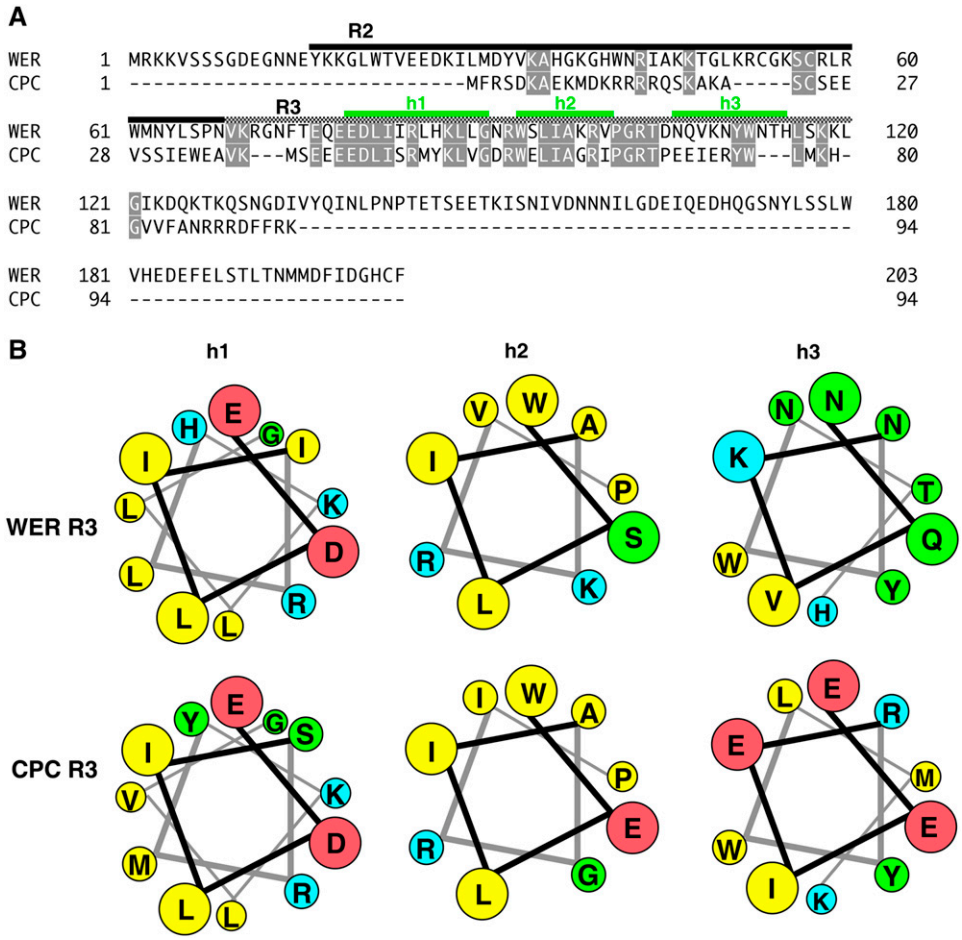


**Figure 1.** WER (R2R3 MYB) and CPC (R3 MYB) Genes in *Arabidopsis*.

**(A)** Sequence alignment of the WER and CPC proteins. Shaded letters indicate identical residues. The MYB DNA binding domains present in each of these proteins are indicated. Although WER has two MYB domains (R2 and R3), CPC has only R3. The positions of the three helices (h) forming R3 MYB are shown with green lines.

**(B)** Helical diagrams of helix 1, helix 2, and helix 3 in WER R3 and CPC R3 with nonpolar residues in yellow, polar uncharged residues in green, acidic residues in red, and basic residues in blue.

R3 h3 is mainly composed of nonpolar and acidic residues (Figure 1B).

To provide a framework for examining *WER* and *CPC* evolution, we estimated the phylogeny of R2R3- and R3-type Myb proteins based on R3 amino acid sequences (Figure 2B). MYB36, MYB37, MYB38, MYB68, MYB84, and MYB87 belong to another R2R3-type Myb clade (Kranz et al., 1998). PAP1 and PAP2 form a side branch of the CPC/WER cluster and are known to interact with bHLH proteins (Zimmermann et al., 2004). The neighbor-joining tree of just the R3 Myb regions (Figure 2A) yields a similar clustering to a tree constructed from the complete sequences (Kranz et al., 1998; Stracke et al., 2001). This neighbor-joining tree of R3 Myb sequences has four distinct branches consisting of the MYB68 (MYB36, MYB37, MYB38, MYB68, MYB84, and MYB87), WER (GL1, WER, and MYB23), PAP (PAP1 and PAP2), and CPC (CPC, TRY, ETC1, ETC2, and At4g10160) subgroups (Figure 2A). The WER subgroup and CPC subgroup branches are statistically supported by having bootstrap values in excess of 90%. Branching of the WER and CPC clusters from a common trunk suggests that the evolution of R3 in WER and CPC began with duplication of a single common ancestor (Figure 2A).

### WER R3 Cannot Be Replaced by the MYB Domain of CPC

In the *wer* mutant, most of the root epidermal cells differentiate into root hairs (Lee and Schiefelbein, 1999). By contrast, the *cpc* mutant has a reduced number of root hairs (Wada et al., 1997). To investigate the functional differences between the R3 motifs of CPC and WER, seven *WER:WER-CPC* chimera constructs under control of the *WER* promoter (*WER:WC1*-*WER:WC7*) were

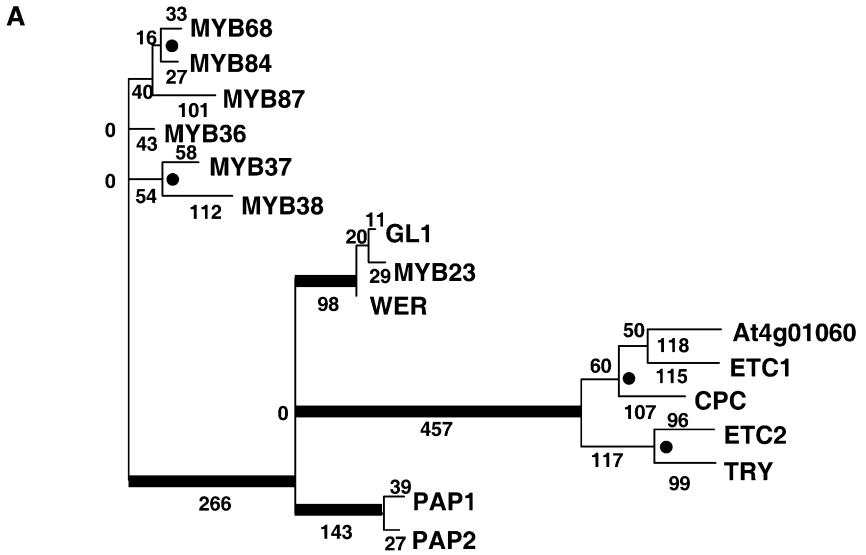


**Figure 2.** Phylogenetic Tree Displaying the Relationship among R3 Myb Regions.

**(A)** A neighbor-joining phylogenetic tree of the amino acid sequences of R3 Myb regions (CPC, TRY, ETC1, ETC2, At4g01060, WER, MYB23, GL1, MYB36, MYB37, MYB38, MYB68, MYB84, MYB87, PAP1, and PAP2). Distances are shown as the p-distance multiplied by $10^3$. Branches with bootstraps of 90% or greater are in bold. Branches with bootstraps between 70 and 90% are marked with a circle. Branches with bootstraps below 70% are unmarked.

**(B)** Amino acid sequences of Myb R3 motifs of CPC, TRY, ETC1, ETC2, At4g01060 WER, GL1, MYB23, MYB36, MYB37, MYB38, MYB68, MYB84, MYB87, PAP1, and PAP2. Boxes outlined in red indicate identical amino acids.

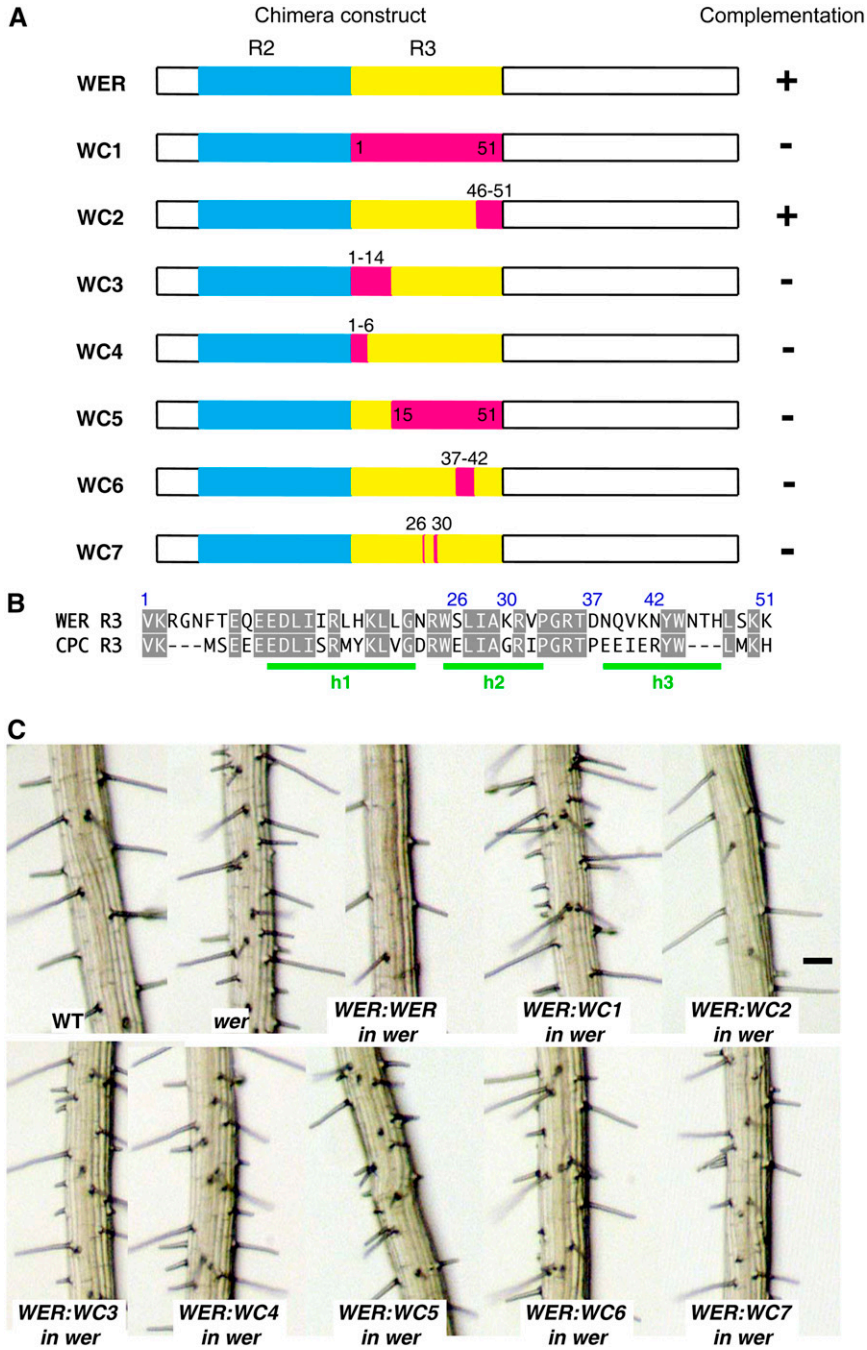


**Figure 3.** Complementation of the *wer* Mutant Phenotype by *WER:WER-CPC* Chimera Constructs.

**(A)** Schematic representation of chimera WER R3 (yellow) and CPC R3 (red) constructs. Complementation results are on the right. Numbers indicate the amino acids removed from WER regions as indicated in **(B)**. Only *WER:WER* and *WER:WC2* could complement the *wer* mutant phenotype.
**(B)** Alignment of the MYB R3 regions of WER and CPC. Shaded letters indicate identical residues. The positions of the three helices forming R3 MYB are indicated with green lines.
**(C)** Phenotypes of Col-0, *wer*, and *wer* transformants. Transformants with *WER:WER* and *WER:WC2* had a decreased number of root hairs compared with *wer*. Bar = 100 μm.

introduced into the *wer* mutant to test their ability to complement the *wer* root hair phenotype (Figure 3). At least four individual homozygous T3 lines were analyzed for each construct (see Supplemental Figure 3B online), among which three typical lines were chosen for root hair assays (Table 1). Only two transgenic lines, *WER:WER* and *WER:WC2*, complemented the *wer* mutant phenotype (Figures 3A and 3C, Table 1), indicating that only the WC2 chimera protein retains the biochemical activity of WER protein. The *WER:WC7* construct, which harbors S26E and K30G substitutions in the WER R3 region, does not rescue the *wer* mutant phenotype (Figures 3A and 3C, Table 1), demonstrating that as few as two amino acid changes are sufficient to disrupt WER function (Figure 3A).

### *GL2* Expression Is Positively Regulated Only by WER and WC2

To determine whether *wer* complementation (Figures 3A and 3C, Table 1) was due to the epistatic effects of the WER chimeras on *GL2* promoter activity, we introduced *GL2:*β*-glucuronidase* (*GUS*) into these transgenic lines (Figure 4). In a wild-type background, the *GL2* promoter drove GUS expression within differentiating root epidermal cells located in the hairless cell file position (Figure 4A; Masucci et al., 1996). This position-dependent *GL2:GUS* expression was abolished in the *wer* mutant (Figure 4B; Lee and Schiefelbein, 1999). *GL2:GUS* was expressed in *WER:WER*-complemented *wer* mutant lines about the same as the wild type but was somewhat higher in *WER:WC2*-complemented *wer* lines under these experimental conditions (Figures 4A, 4C, and 4E). By contrast, *GL2:GUS* was not expressed in *WER:WC1*-, *WER:WC3*-, *WER:WC4*-, *WER:WC5*-, *WER:WC6*-, or *WER:WC7*-complemented *wer* background lines, which also did not complement the *wer* mutant phenotype (Figures 4B, 4D, and 4F to 4J). These results indicate that positive regulation of *GL2* in hairless cells is limited to *WER:WER*- and *WER:WC2*-complemented *wer* lines.

**Table 1.** Root Hair Numbers of *WER:WER-CPC* Chimeras in *wer* Transgenic *Arabidopsis* Lines

| Root Hairs per mm | | | | | |
|---|---|---|---|---|---|
| Col-0 | 43.8 ± 1.0 | | | | |
| *wer* | 72.0 ± 1.8 | | | | |
| *WER:WER* in *wer* | | *WER:WC4* in *wer* | | | |
| Line 1 | 36.8 ± 2.6 | | Line 1 | 76.2 ± 2.6 | |
| Line 2 | 15.9 ± 2.4 | | Line 2 | 74.4 ± 2.0 | |
| Line 3 | 26.0 ± 2.9 | | Line 3 | 82.0 ± 2.5 | |
| *WER:WC1* in *wer* | | *WER:WC5* in *wer* | | | |
| Line 1 | 85.3 ± 1.5 | | Line 1 | 77.0 ± 2.6 | |
| Line 2 | 80.5 ± 4.5 | | Line 2 | 66.4 ± 2.6 | |
| Line 3 | 81.1 ± 3.2 | | Line 3 | 70.1 ± 2.5 | |
| *WER:WC2* in *wer* | | *WER:WC6* in *wer* | | | |
| Line 1 | 45.4 ± 1.3 | | Line 1 | 91.0 ± 2.6 | |
| Line 2 | 46.4 ± 1.5 | | Line 2 | 86.6 ± 2.5 | |
| Line 3 | 45.8 ± 2.0 | | Line 3 | 73.5 ± 2.5 | |
| *WER:WC3* in *wer* | | *WER:WC7* in *wer* | | | |
| Line 1 | 83.8 ± 2.8 | | Line 1 | 66.5 ± 4.3 | |
| Line 2 | 89.2 ± 2.9 | | Line 2 | 66.1 ± 2.7 | |
| Line 3 | 87.2 ± 2.9 | | Line 3 | 66.8 ± 2.5 | |

Data, including SD, were obtained from at least 10 5-d-old seedlings from each line.

### The MYB Domain of CPC Can Be Replaced by the WER R3 Domain

To determine whether WER R3 could substitute for CPC R3, we exchanged *CPC:CPC* with the corresponding *WER* R3 regions (*CPC:CW1-CPC:CW5*) (Figure 5A). These chimeric constructs were introduced into a *cpc-2* mutant under the control of the *CPC* promoter. For each construct, at least five individual T2 lines were analyzed (see Supplemental Figure 4 online). Typical lines were chosen and root hairs assayed (Table 2). *CPC:CPC* complemented the *cpc-2* mutant (Columbia-0 [Col-0] background) phenotype (Figures 5A and 5C, Table 2) just as in *cpc-1* (Wassilewskija background) (Wada et al., 1997). All of the chimeric constructs rescued the reduced-hair *cpc-2* phenotype, though the degree of rescue differed. *CPC:CPC* in the *cpc* background had an increased number of root hairs compared with the wild-type background. *CPC:CW1* and *CPC:CW2* in *cpc* restored the number of root hairs to wild-type levels. *CPC:CW3* and *CPC:CW4* in *cpc* transformants had somewhat fewer than the wild type. The number of root hairs in the *CPC:CW5 cpc* transformant was the least (Figure 5C, Table 2). These results suggest that although the entire R3 structure is required for strong function, CPC can act without strict structural conservation of the R3 domain (Figures 5A and 5C, Table 2).

### *GL2* Expression Is Repressed by CPC and CPC Chimera Proteins

*GL2:GUS* expression is normally limited to hairless cell files (Figure 6A; Masucci et al., 1996). The *GL2:GUS* gene is expressed in almost all epidermal cells of the *cpc* mutant but was not expressed in *35S:CPC* transgenic plants (Figure 6B; Lee and Schiefelbein, 2002; Wada et al., 2002). To define the effect of *CPC:CPC-WER* chimeras on *GL2* expression, we introduced *GL2:GUS* into *CPC:CPC-WER* chimera transgenic lines with a *cpc* background (Figures 6C to 6H). As described above, all five chimeric constructs (*CPC:CW1-CPC:CW5*) rescued the *cpc* mutant phenotype (Figures 5A and 5C, Table 2). Like *35S:CPC*-complemented *cpc* transformants, no appreciable *GL2:GUS* expression was observed in any of these transgenic lines when roots were incubated in X-Gluc solution at 37°C for 3.5 h (see Supplemental Figure 1 online). With the exception of the *CPC:CPC*-complemented *cpc* transgenic line, which had no discernable expression (Figure 6C), after incubation overnight they exhibited weak wild-type-like GUS activity in the hairless cell file position (Figures 6D to 6H). The strength of *GL2:GUS* activity in these transgenic lines was directly correlated with the degree of complementation provided by the chimera construct. The *CPC:CPC* construct in the *cpc* background produces a greater number of root hairs than the wild type. *CPC:CW1*- and *CPC:CW2*-complemented *cpc* transgenic lines have almost the same number of root hairs as the wild type, and *CPC:CW3*-, *CPC:CW4*-, and *CPC:CW5*-complemented *cpc* transgenic lines produce lower numbers of root hairs than the wild type (Figures

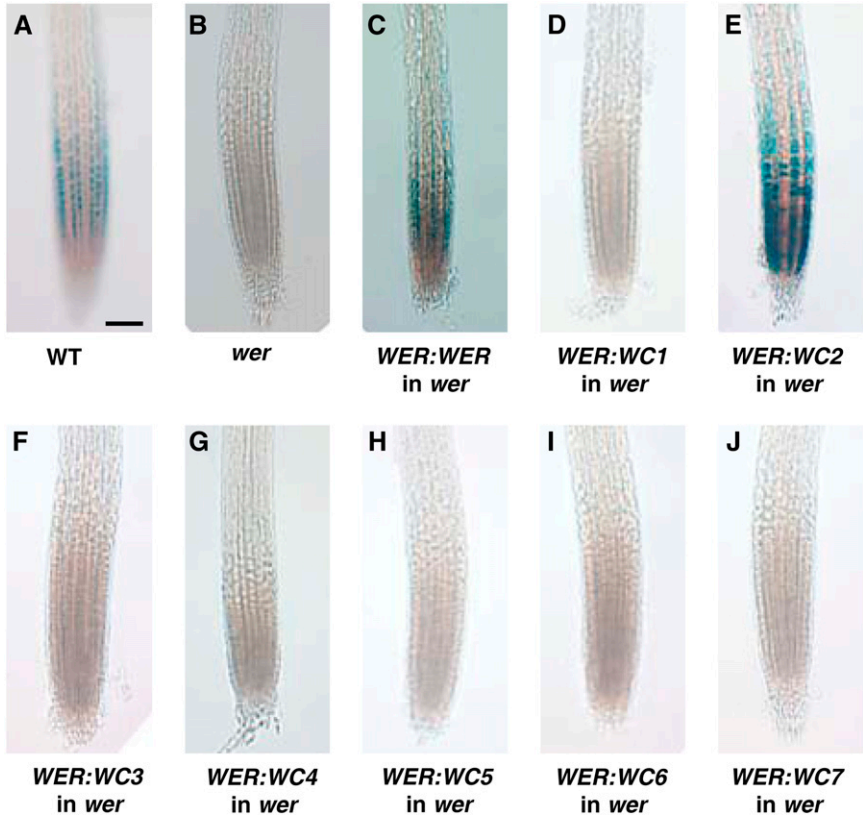


**Figure 4.** Regulation of *GL2:GUS* in the *wer* Mutant Background.

Expression of the *GL2:GUS* reporter in the developing root epidermis of 5-d-old seedlings in Col-0, *wer*, and *wer* transformants. *GL2* promoter activity is reduced in the epidermis of the *wer* line. Transformants *WER:WER* and *WER:WC2* had increased *GL2* promoter activity compared with *wer*. Bar = 100 μm.

5A, 5C, and 6C to 6H, Table 2). These results strongly suggest that each of the CPC chimera proteins has the ability to inhibit *GL2* expression.

### WER and WER Chimera Proteins Interact Equally with GL3 or EGL3

WER protein physically interacts with either of the GL3 or EGL3 proteins in yeast cells (Bernhardt et al., 2003). To examine the possibility that the WER chimera proteins physically associate with GL3 or EGL3, we employed the yeast two-hybrid assay (Fields and Sternglanz, 1994). The Myb regions of the WER, WC1, and WC7 constructs were fused to the binding domain (BD) of GAL4, and GL3 or EGL3 was fused to the activation domain (AD) of GAL4. Yeast containing either empty pBridge (BD) or pGAD424 vectors in conjunction with any of the corresponding protein fusions did not exhibit significant β-gal activity, whereas yeast containing WER-BD, WC1-BD or WC7-BD, and GL3-AD or EGL3-AD exhibited β-gal activity (Figure 7A). There were no significant differences in β-gal activity among yeast isolates containing WER-BD GL3-AD, WC1-BD GL3-AD, or WC7-BD GL3-AD (Figure 7A). Although EGL3-AD exhibited one-sixth the level of binding activity as GL3-AD (Figure 7A; Bernhardt et al., 2003), yeast isolates containing each of the EGL3 fusions had approximately equal β-gal activities (Figure 7A). Thus, WER-BD, WC1-BD, and WC7-BD interacted equally well with either GL3-AD or EGL3-AD (Figure 7A).

### CPC and CPC Chimera Proteins Compete with WER for Binding Sites on GL3 or EGL3

As presented in a previous model, TRY and GL1 compete for a GL3 protein binding site to form different types of complexes that are involved in *Arabidopsis* trichome development (Marks and Esch, 2003). TRY also prevents the interaction between GL1 and GL3 (Esch et al., 2003). CPC protein has also been found to physically interact with both GL3 and EGL3 in yeast cells (Bernhardt et al., 2003), suggesting a competition model for CPC and WER (Lee and Schiefelbein, 1999; Bernhardt et al., 2003). To determine whether CPC and the CPC chimera proteins compete with WER for a binding site on GL3 or EGL3 equally, *CPC*, *CW1*, and *CW5* were cloned into the free site of the WER-BD constructs to form the WER-BD/CPC-free, WER-BD/CW1-free, and WER-BD/CW5-free constructs for the yeast three-hybrid assay. Under conditions of low Met concentrations, in which CPC-free, CW1-free, and CW5-free expression would be

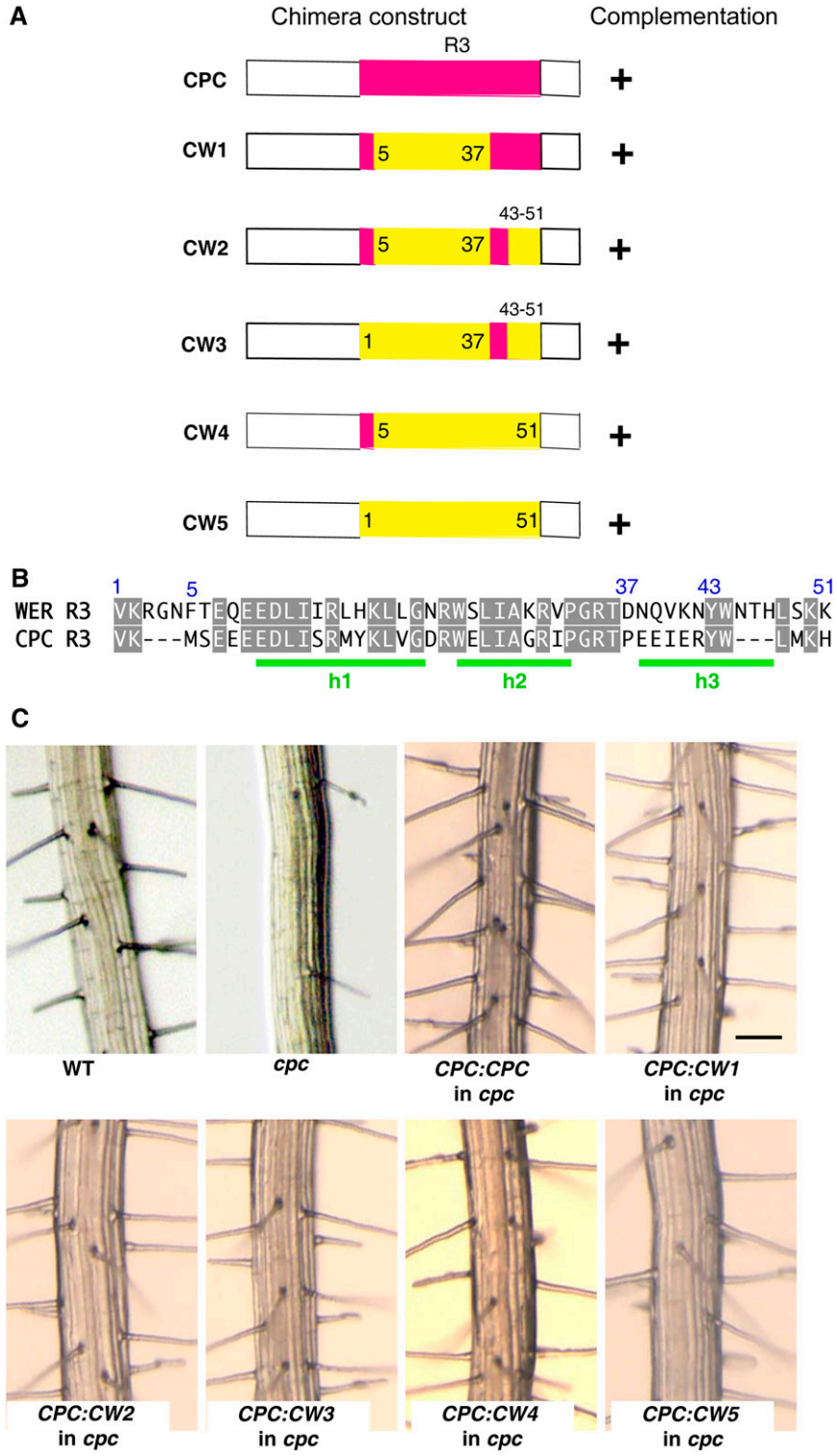


**Figure 5.** Complementation of the *cpc* Mutant by *CPC:CPC-WER* Chimera Constructs.

**(A)** Schematic representation of chimera CPC R3 (red) and WER R3 (yellow) constructs. Complementation results are on the right. Each of the constructs complemented the *cpc* mutant phenotype. Numbers indicate replaced WER regions as shown in **(B)**.

**(B)** Alignment of the MYB R3 regions of WER and CPC. Shaded letters indicate identical residues. The positions of the three helices forming R3 MYB are shown with green lines.

**(C)** Phenotypes of Col-0, *cpc*, and *cpc* transformants. All transgenic plant lines had an increased number of root hairs compared with *cpc*. Bar = 100 μm.

**Table 2.** Root Hair Numbers of *CPC:CPC-WER* Chimeras in *cpc-2* Transgenic *Arabidopsis* Lines

| Root Hairs per mm | | | |
|---|---|---|---|
| Col-0 | 43.8 ± 1.0 | | |
| *cpc-2* | 12.0 ± 1.6 | | |
| *CPC:CPC* in *cpc-2* | | *CPC:CW3* in *cpc-2* | |
| Line 1 | 74.6 ± 4.9 | Line 1 | 39.8 ± 2.8 |
| Line 2 | 82.2 ± 7.4 | Line 2 | 39.0 ± 2.0 |
| Line 3 | 85.4 ± 2.3 | Line 3 | 35.2 ± 1.4 |
| *CPC:CW1* in *cpc-2* | | *CPC:CW4* in *cpc-2* | |
| Line 1 | 44.8 ± 2.6 | Line 1 | 38.0 ± 1.5 |
| Line 2 | 43.6 ± 3.0 | Line 2 | 36.8 ± 3.4 |
| Line 3 | 42.2 ± 3.3 | Line 3 | 37.2 ± 2.7 |
| *CPC:CW2* in *cpc-2* | | *CPC:CW5* in *cpc-2* | |
| Line 1 | 43.2 ± 1.2 | Line 1 | 31.8 ± 2.2 |
| Line 2 | 43.4 ± 1.1 | Line 2 | 30.2 ± 3.0 |
| Line 3 | 41.8 ± 1.3 | Line 3 | 31.8 ± 2.6 |

Data, including SD, were obtained from at least 10 5-d-old seedlings from each line.

at maximum levels, yeast containing GL3-AD or EGL3-AD and WER-BD/CPC-free, WER-BD/CW1-free, or WER-BD/CW5-free had decreased β-gal activity (Figure 7B). As the concentration of Met was increased, which should depress CPC-free, CW1-free, and CW5-free expression, the levels of β-gal activity increased (Figure 7B). Although GL3-AD had a higher level of binding activity to WER than EGL3-AD (Figure 7A) (Bernhardt et al., 2003), free CPC, CW1, and CW5 showed the same inhibitory levels on WER-BD and GL3-AD or EGL3-AD interactions (Figure 7B). Although a decrease in Met concentration decreased β-gal activity in the GL3-AD and WER-BD binding assay, there was essentially no reduction of β-gal activity as shown in the GL3-AD WER-BD/CPC-free three-hybrid assay (Figure 7B; see Supplemental Figure 2 online).

### WER Chimera Proteins Do Not Bind to the *GL2* Promoter

The *GL2* promoter has a putative MYB binding site, which is required for expression in trichome cells and root hairless cells (Hung et al., 1998; Szymanski et al., 1998). Previously, it has been shown by gel mobility shift experiments that WER binds to the *GL2* MYB binding site (GL2MBS1 [GAC**TAACGG**TAAG]) (Koshino-Kimura et al., 2005). We used a gel mobility shift assay to determine whether the WER chimeric proteins bind to this site or not. When the WER protein was added, a band shift was observed and free probe decreased (Figure 8A, lane 2; Koshino-Kimura et al., 2005). However, neither WC1 nor WC7 chimera proteins caused any significant gel shift or decrease in free probe (Figure 8A, lanes 4 and 6). These results indicate that WER chimera proteins WC1 and WC7 do not bind to the Myb binding site (GL2MBS1) of the *GL2* promoter (Figure 8A), although they can bind to GL3/EGL3 proteins (Figure 7A).

### CW5 Protein Binds to the *GL2* Promoter

We also used a gel mobility shift assay to determine whether the CW5 chimera protein, which gives complementation but contains the WER R3 domain (Figure 5A), binds to the *GL2* MYB binding site. Intact CPC protein did not cause any significant gel shift or decrease in free probe (Figure 8B, lane 2). However, when CW5 protein was added, a band shift was observed and free probe decreased (Figure 8B, lane 4). These results indicate that CW5 chimera protein binds to the Myb binding site of the *GL2* promoter.

## DISCUSSION

We designed chimeric constructs (Figures 3A and 5B) based on a sequence comparison of WER and CPC R3 variable residues (Figure 1). Areas of the R3 domain that contain functionally similar amino acids are less likely than more variable regions to have a major impact on protein functionality. Thus, we have concentrated on the domains that are more likely to provide insights into R3 functionality (Figure 1B). Complementation analyses within the *wer* mutant background with *WER:WER-CPC* chimeras (*WER:WC1-WER:WC7*) revealed that WER R3 could not be replaced by CPC R3 (Figures 3A and 3C, Table 1). Two amino acid residue substitutions at positions 26 and 30 inactivated WER (WC7) (Figures 3A and 3C, Table 1). A nuclear magnetic resonance study showed that h3 is a DNA recognition helix and that the h3 Asn and Lys residues are probably the key residues for DNA recognition in c-Myb (Ogata et al., 1994). Although WC3, WC4, and WC7 have these residues, they lost WER function (Figures 3A and 3C, Table 1). It was reported that a single residue substitution within MYB.Ph3 from *Petunia* causes a drastic change in binding specificity (Solano et al., 1997). For WER to function normally, accurate structural maintenance is necessary (Figures 3A and 3C, Table 1). We also showed that consistent with their phenotype, *GL2:GUS* expression is positively regulated only in *WER:WER* and *WER:WC2* transgenic lines (Figure 4).

In contrast with the results of the *wer* complementation analyses, *cpc-2* complementation tests with *CPC:CPC-WER* chimeras (*CPC:CW1-CPC:CW5*) revealed that WER R3 could substitute for CPC R3 function (Figures 5A and 5C, Table 2). Consistent with their phenotype (Figure 5C, Table 2), *GL2:GUS* expression is negatively regulated by CPC-WER chimeras (Figure 6). *CPC:CPC*-complemented *cpc* transgenic plants did not show the same GUS staining pattern as the wild type (Figure 6A; Masucci et al., 1996), possibly because of position effects and/or an overwhelming effect of CPC protein, which may strongly repress *GL2:GUS* (Figure 6C).

Payne et al. (2000) proposed a model for the regulation of trichome development, in which the GL1-GL3-TTG1 complex binds to promoters of downstream genes and CPC or TRY can inhibit its activation. Szymanski et al. (2000) proposed a model in which CPC inhibits physical interactions between GL1 and GL3. This model was subsequently modified to allow for TRY inhibition of physical interactions between GL1 and GL3 (Marks and Esch, 2003). WER and CPC have been reported to interact with GL3/EGL3 (Bernhardt et al., 2003). Thus, we examined the interactions between either WER or WER chimeras WC1 and WC7 with either GL3 or EGL3 (Figure 7A). There was no noticeable difference in the protein–protein binding properties between WER and the WER chimeras (Figure 7A). Competitive yeast interaction assays demonstrated that CPC and CPC chimera proteins CW1

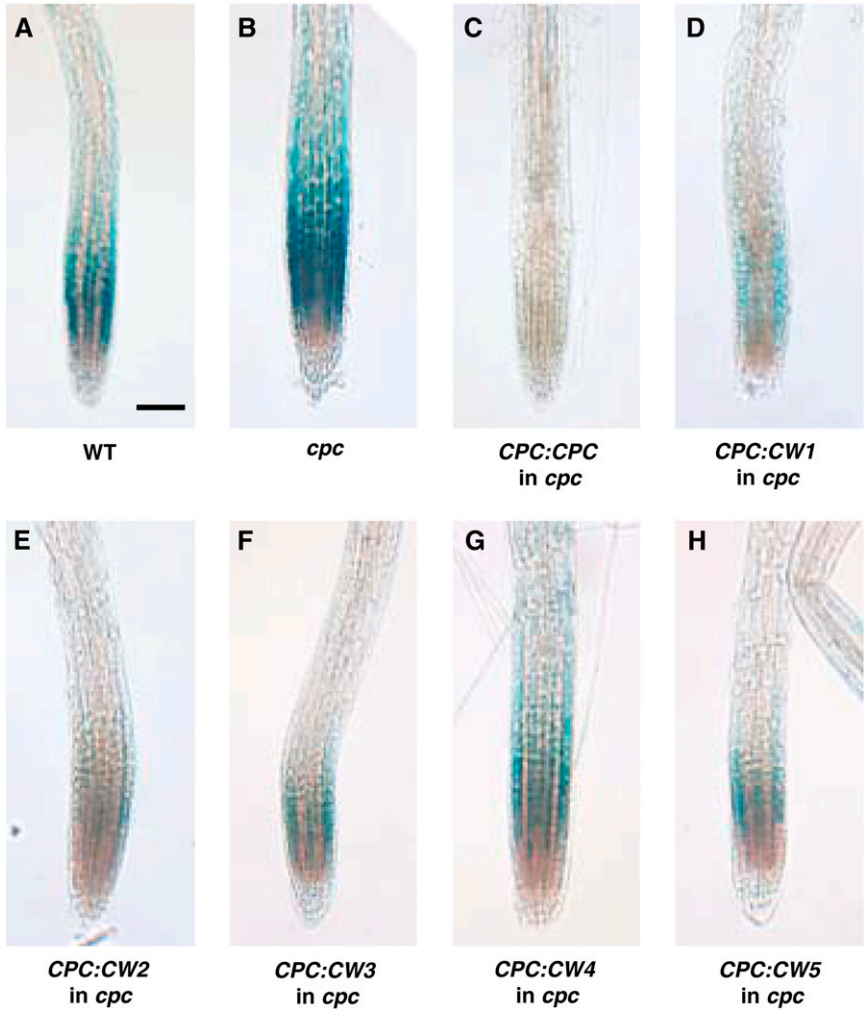


**Figure 6.** Regulation of *GL2:GUS* in the *cpc* Mutant Background.

Expression of the *GL2:GUS* reporter in the developing root epidermis of 5-d-old seedlings in Col-0, *cpc*, and *cpc* transformants. *GL2* promoter activity is increased in the epidermis of the *cpc* line. All transformant lines had reduced *GL2* promoter activity compared with *cpc* with overnight incubation in X-Gluc solution. Bar = 100 μm.

and CW5 similarly prevent WER–GL3/EGL3 interactions (Figure 7B). Thus, it is apparent that all of the WER and CPC chimera proteins have the ability to interact with GL3/EGL3. Because the R3 domains of both WER and CPC contain the conserved [DE]$Lx_2$[RK]$x_3Lx_6Lx_3R$ predicted bHLH interaction motif (Zimmermann et al., 2004), any point of exchange might be possible. We were also able to demonstrate that WER chimeras WC1 and WC7 lose their DNA binding ability to GL2MBS1 in the *GL2* promoter (Figure 8A), although a faint band, which was not retarded to the same degree as with intact WER, was observed in WC1 (Figure 8A, lane 4). Because high protein concentrations can alter target recognition (Andersson et al., 1999), the high purity of WC1 protein (60% purity for WER, 82% for WC1, and 53% for WC7) could have increased the actual concentration to the point where nonspecific DNA binding occurred at a low level (Figure 8A, lane 4). These data together suggest a regulatory cascade model in which WER and CPC competitively bind to GL3/EGL3 (Figure 9A). Once the WER-GL3/EGL3-TTG1 complex is formed, it binds to the *GL2* promoter and promotes expression of GL2 protein, which leads to the hairless cell fate (Figure 9B; Koshino-Kimura et al., 2005). On the other hand, WER chimera proteins lost their ability to bind to the *GL2* promoter; thus, chimera proteins do not result in a hairless cell fate (Figure 9B). CPC and the CPC chimeras bind to the TTG1-GL3/EGL3 complex to prevent activation of the *GL2* promoter, eventually resulting in root hair formation (Figure 9C).

We also show that the CW5 chimera protein, which has WER R3 motif sequences, retains its DNA binding ability (Figure 8B). These results suggest a couple of possible evolutionary scenarios. The *CPC* and *CPC*-like R3-type MYB genes could have arisen from the R2R3-type MYB gene family, including *WER* (Figure 2A), with the functional novelty of CPC and CPC-like MYB

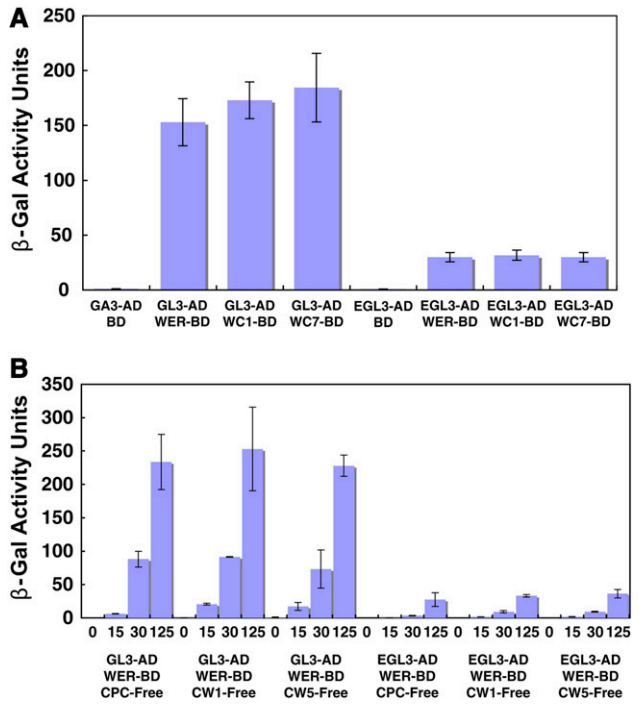


**Figure 7.** Protein Interactions with Native Myb versus Chimeric Myb Proteins.

**(A)** Comparison of protein interactions using a yeast two-hybrid assay between WER, WC1, and WC7 with GL3 and EGL3. The WER, WC1, and WC7 proteins were compared as GAL4 binding domain (BD) fusions, whereas GL3 and EGL3 were expressed as GAL4 activation domain (AD) fusions.

**(B)** CPC, CW1, and CW5 competition for the WER binding sites of GL3 and EGL3. Using the pBridge vector (Clontech), a third protein (CPC, CW1, or CW5) under the control of a Met-repressible promoter was expressed in a yeast interaction assay at varying Met concentrations (0, 15, 30, and 125 μM). CPC, CW1 or CW5 were expressed as a free protein (no AD or BD domains). Samples were normalized by $OD_{550}$. The strength of the interaction was determined by β-gal activity.

proteins arising from loss of the acidic region activation domain and/or loss of DNA binding though residue change. In the former case (Figure 10B), the proto-CPC chimeras may be analogous to *C1-I* in maize (*Zea mays*), in which C1-I protein lacking the activator domain acts as an inhibitor (Paz-Ares et al., 1990). Because this initial truncation would have maintained its DNA binding activity, it would act as a dominant repressor in competition with WER (Figure 10B). Because repression is the only function subject to selection, any biochemical properties that didn't contribute to repression activity would be free to drift. Thus, DNA binding ability could be lost or modified without consequence. All that would remain is a conversion to bHLH binding at the same spot as WER (Figure 10C).

Alternatively, loss of DNA binding activity in a WER-like duplication due to amino acid residue changes would release the proto-CPC molecule from having regulatory consequences (Figure 10D), thus freeing it for whole-scale truncations (Figure 10C). In addition, there are incomplete repetitions in the *WER* genome sequence presumed to cause a simple loop crossing-over event that would cause deletion of N-terminal region and/or C-terminal activation domain of WER (see Supplemental Figure 5 online). These provide the mechanical possibilities for truncations to make proto-CPC. Newly evolved CPC would have thus lost the ability to bind DNA and activate transcription (Figure 10C), allowing it the freedom to evolve a specialized competitive inhibitory function through the selective loss of other functions important for gene activation.

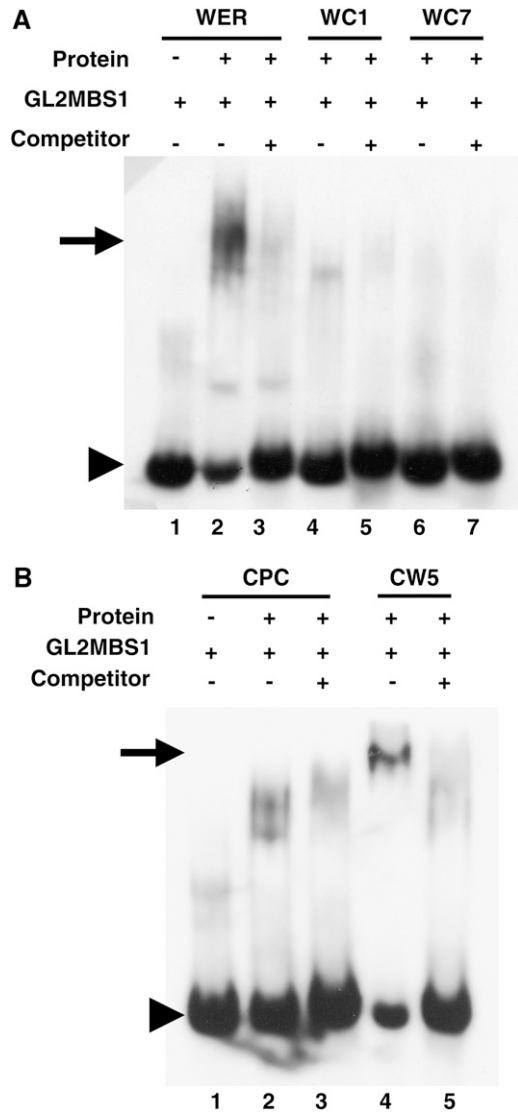


**Figure 8.** The DNA Binding Properties of WER and CPC Chimera Proteins.

**(A)** WER, WC1, or WC7 protein was added with or without a 200-fold excess of competitor.

**(B)** CPC or CW5 protein was added with or without a 200-fold excess of competitor.

Arrows indicate shifted bands, and arrowheads indicate free probe. Digoxigenin-labeled DNA of GL2MBS1 was used as the probe.

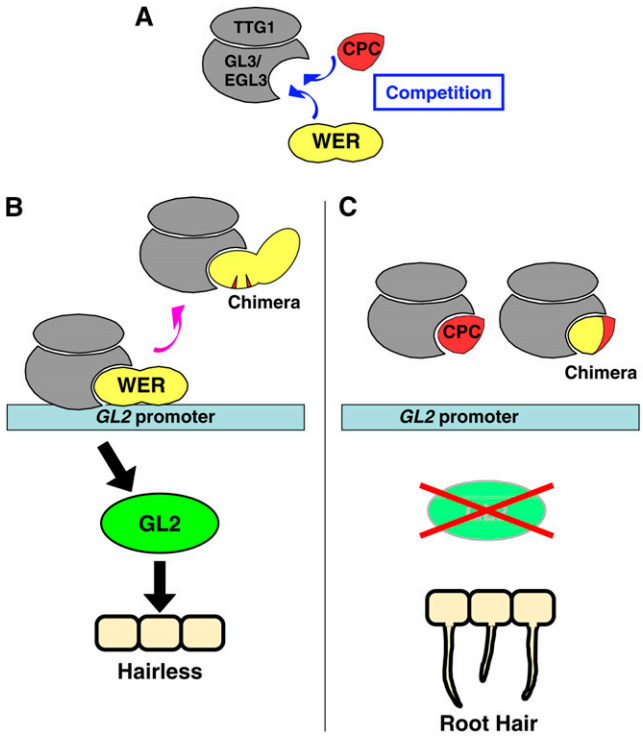


**Figure 9.** Regulatory Cascade Models for WER and CPC Chimeras.

**(A)** WER and CPC proteins competitively bind to the GL3/EGL3-TTG1 complex.

**(B)** The WER-GL3/EGL3-TTG1 complex can bind the *GL2* promoter to promote *GL2* expression, which leads to the hairless cell fate, whereas the WER chimera-GL3/EGL3-TTG1 complex cannot bind to the *GL2* promoter, thus preventing *GL2* expression.

**(C)** Both CPC-GL3/EGL3-TTG1 and CPC chimera-GL3/EGL3-TTG1 complexes prevent expression of *GL2*. The absence of GL2 results in root hair formation.

## METHODS

### Plant Materials and Growth Conditions

*Arabidopsis thaliana* Col-0 ecotype and cognate *cpc-2* and *wer-1* mutant plants were used. Seeds were sterilized with 10% (v/v) bleach with 0.02% (v/v) Triton X-100 for 5 min. After seeds were rinsed five times in sterile water, seeds were germinated and grown on square Petri dishes containing half-strength Okada and Shimura medium [2.5 mM $KNO_3$, 1 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 25 μM Fe-EDTA, 1.25 mM K-$PO_4$, pH 5.5, 35 μM $H_3BO_3$, 7 μM $MnCl_2$, 0.25 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 0.1 μM $NaMo_4$, 5 μM NaCl, and 0.05 μM $CoCl_2$] (Okada and Shimura, 1990) with 1.5% (w/v) agar. After sowing, dishes were wrapped with Micropore surgical tape (3M Healthcare) to prevent desiccation. The dishes were then kept in darkness at 4°C for 2 d and then transferred to a growth chamber at 22°C under constant white light (white fluorescent lamp model FL20S-EXNH; Toshiba).

### Gene Constructs

#### *Primers*

All primer sequences used in this article are listed in Supplemental Table 1 online.

#### *WER:WER-CPC Chimeric Constructs*

To define the relationship between the R3 motifs of *WER* and *CPC*, based on published data (Lee and Schiefelbein, 2001), we first tested a construct harboring *WER*. This construct (designated *WER:WER*; Figure 3A, Table 1) was introduced into the *wer* mutant background. To make the *WER:WER* chimera constructs, we used a 6.1-kb PCR-amplified *WER:WER* genome fragment that includes the 4.0-kb 5′ region, the 1.0-kb coding region, and the 1.1-kb 3′ region as well as *pBS-gCPC*, including the 1.3-kb 5′ region, the 0.9-kb coding region, and the 0.45-kb 3′ region (Wada et al., 1997) as amplification templates. The *WER:WER* region, amplified using primers RT11/RT12, and the *CPC* region, amplified using primers TW1149/TW1150, were ligated (*WER:WC1*). The *WER:WER* region was amplified using primers RT32/RT34, the *WER:WC1* fragment was amplified using primers RT37/RT11, and the products were ligated to form *WER:WC2*. The *WER:WER* region, amplified with primers RT33/RT35, and the *WER:WC1* fragment, amplified with primers RT36/RT12, were ligated to make *WER:WC3*. The *WER:WER* fragment was amplified with primers RT101/RT102 and was self-ligated to form *WER:WC4*. The *WER:WER* region, amplified with primers RT103/RT12, and the *CPC* region, amplified by primers RT104/TW1150, were ligated to make *WER:WC5*. The *WER:WER* fragment amplified with primers WERCPC6-1/WERCPC6-2 was also self-ligated to form *WER:WC6*. To create *WER:WC7*, PCR-mediated mutagenesis was performed on *WER:WER* using the QuickChange site-directed mutagenesis kit (Stratagene), with the primers WERCPC7-1/WERCPC7-2. PCR-generated constructs were completely sequenced following isolation of the clones to check for amplification-induced errors. Finally, the amplified and ligated constructs were cloned into the transformation vector pJHA212K (Yoo et al., 2005).

#### *CPC:CPC-WER Chimera Constructs*

To create *CPC:CPC-WER* chimera constructs (Figure 5A), we used *pBS-gCPC* and *pBS-WER* as templates. The *CPC:CPC* region, amplified by primers CPCWER-a-1/CPCWER-a-2, and the *WER* region, amplified by primer pair CPCWER-a-3/CPCWER-a-4, were ligated to form *CPC*:*CW1*. The *CPC*:*CW1* fragment, amplified by primers CPCWER-b-1/CPCWER-b-2, was self-ligated to make *CPC*:*CW2*, which was then amplified using primers CPCWER-c-1/CPCWER-c-2 and self-ligated to form *CPC*:*CW3*. The *CPC*:*CW3* fragment, amplified with primers CPCWER-d-1/CPCWER-d-2, was self-ligated to create *CPC*:*CW4*. The *WER* region, amplified with primer pair RT32/RT35, and the *CPC:CPC* region, amplified with the primer pair NEKO16/RT86, were ligated to make *CPC*:*CW5*. PCR-generated constructs were completely sequenced following isolation of the clones to check for amplification-induced errors. These amplified and ligated constructs were also cloned into the transformation vector pJHA212K (Yoo et al., 2005).

#### *Transgenic Plants*

Plant transformation was performed by a vacuum transformation procedure (Bechtold et al., 1993) or floral dip method (Clough and Bent, 1998), and transformants were selected on a 0.5× Murashige and Skoog agar plate containing 50 $mg^{-1}$ kanamycin. Homozygous transgenic lines were selected by kanamycin resistance. For *CPC:CPC-WER* and *WER:WER-CPC* chimera constructs, we isolated at least 24 T1 lines for each construct and selected at least five T2 and T3 lines on the basis of their segregation ratios for kanamycin resistance (see Supplemental Figures 4 and 5 online). For each transgenic line, at least 10 individual 5-d-old seedlings were assayed for root hair numbers. Some outliers were eliminated from the data because of the possibility that positional or other aberrant effects would distort the data. However, outliers may be examined in the future and may provide additional clarification of the data.

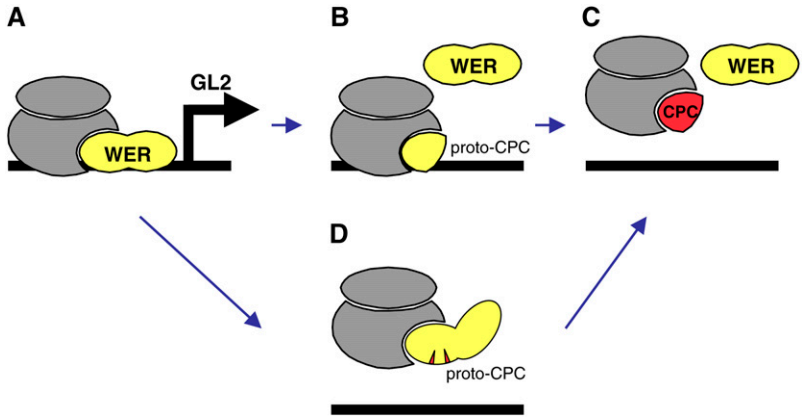


**Figure 10.** Evolutionary Models of CPC and WER.

**(A)** WER binds the *GL2* promoter to promote *GL2* expression.
**(B)** Proto-CPC protein derived from WER (yellow) truncation can bind the promoter but prevents expression of *GL2*.
**(C)** CPC cannot bind to the *GL2* promoter, strongly preventing *GL2* expression.
**(D)** Proto-CPC protein derived from WER amino acid substitution cannot bind to the *GL2* promoter.

A *GL2:GUS* construct (Wada et al., 2002) was introduced into transgenic lines by crossing plants and analyzing F2 seedlings for *GL2:GUS* by PCR (GUS+00+/GUS+09-). For each transgenic line, at least ten individual seedlings were assayed for GUS activity.

### Histology

Primary roots of 5-d-old transgenic seedlings were excised and immersed in X-Gluc solution containing 1.0 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-glucuronide), 1.0 mM $K_3Fe(CN)_6$, 1.0 mM $K_4Fe(CN)_6$, 100 mM NaPi, pH 7.0, 100 mM EDTA, and 0.1% Triton X-100. Excised primary roots were incubated at 37°C for 3.5 h or overnight.

### Microscopy

For observation of the root hairs, root images were obtained with a three-dimensional digital fine microscope (VC4500-PC; Omron) or with a digital microscope (VH-8000; Keyence). For each transgenic line, at least 10 individual 5-d-old seedlings were analyzed for root hair number and root GUS activity.

### Construction, Transformation, and Analysis of Yeast Constructs

p*GL3-AD* and p*EGL3-AD* were constructed by cloning *GL3* and *EGL3* coding regions from Col-0 cDNA into pGAD424 (Clontech). p*WER-BD*, p*WC1-BD*, and p*WC7-BD* were constructed by cloning *WER*, *WC1*, and *WC7* coding regions from Col-0, *WER:WC1* transformants, or *WER:WC7* transformants, respectively, into pBridge MCSI (Clontech). The constructs used for WER-CPC, -CW1, or -CW5 competition assays, p*WER-BD/CPC-free*, p*WER-BD/CW1-free*, or p*WER-BD/CW5-free*, were generated by cloning *CPC*, *CW1*, or *CW5* coding regions into pBridge MCSII (Clontech). The appropriate pGAD424- and pBridge-based constructs were transformed into the yeast strain Y187 using the Yeastmaker 2 transformation system (Clontech). Cells were selected on plates containing SD synthetic medium (2% glucose and 1× yeast nitrogen base) lacking Leu and Trp. Liquid cultures of SD synthetic medium lacking Leu and Trp were used to measure β-galactosidase (β-gal) activity (Ausubel et al., 1995). Cells were grown to an $OD_{600}$ of 0.7 to 1.0, pelleted by centrifugation, and suspended in z-buffer (60 mM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol, pH 7.0). Cells were permeabilized by adding a final concentration of 0.005% SDS and 3.5% (v/v) chloroform. *o*-nitrophenyl-D-galactopyranoside (Sigma-Aldrich) was added as a substrate. After incubation at 30°C, the reaction was stopped with sodium carbonate and measured for activity at $OD_{420}$. β-gal activity was determined using the equation $U = 1000 \times [OD_{420}]/\text{time (in seconds)} \times \text{volume (in mL)} \times [OD_{600}]$. For each comparison, three independent yeast isolates were tested three times.

### Bacterial Expression of Proteins and Purification of His-Tagged Recombinant Proteins

The WER coding sequence was amplified from pGEX_WER (Koshino-Kimura et al., 2005) as a template. The WC1 and WC7 coding sequences were amplified from total root cDNA of *WER:WC1* transformants and *WER:WC7* transformants, as described above. The CPC and CW5 coding sequences were amplified from pWER-BD/CPC-free and pWER-BD/CW5-free as templates. The fragments were cloned into expression vector pColdTF (TaKaRa Bio). DNA sequences were checked, and the constructs were transformed into *Escherichia coli* strain Rosetta 2 (DE3) (Novagen). These transformed bacteria were used for purifying the recombinant proteins (custom-made by TaKaRa Bio).

### Gel Mobility Shift Assay

Oligonucleotides for gel mobility shift assays were labeled with a Roche DIG gel shift kit (2nd Generation; Roche). The sequence of GL2MBS1 is the same as reported previously (Koshino-Kimura et al., 2005). DNA–protein binding reactions were basically performed by incubating 32 fmol of digoxigenin-labeled oligonucleotide with 100 ng of each protein in 20 μL of binding buffer [10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% 80 $\mu g^{-1}$ poly(dI-dC), and 100 $\mu g^{-1}$ BSA] at 22°C for 15 min, and then free and bound complexes were resolved by electrophoresis through 1-mm 5% native polyacrylamide gels (Real Gel Plate; BIO CRAFT) in 0.5× TBE buffer at 8 V $cm^{-1}$ for 60 min.

### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *WER* (AL391149), *GL1* (AF495524), At *MYB23* (Z95747), *CPC* (AB004871), *TRY* (AY519523),

*ETC1* (AY519518), *ETC2* (AY234411), At4g01060 (AY519522), *MYB36* (AF062878), *MYB37* (AF062879), *MYB38* (AF062880), *MYB68* (AF062901), *MYB84* (Y14209), *MYB87* (AF062914), *PAP1* (AF325123), *PAP2* (AF087936), *GL2* (AB117767), *GL3* (AF246291), and *EGL3* (AF027732).

### Supplemental Data

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

**Supplemental Figure 1.** Regulation of the *GL2:GUS* Expression Pattern in Col-0, *cpc*, or *cpc* Transformants.

**Supplemental Figure 2.** Protein Interactions between WER-BD and GL3-AD or EGL3-AD.

**Supplemental Figure 3.** Complementation of the *wer* Mutant by *WER*:*WER-CPC* Chimera Constructs.

**Supplemental Figure 4.** Complementation of the *cpc* Mutant by *CPC*:*CPC-WER* Chimera Constructs.

**Supplemental Figure 5.** Homologous Recombination in the *WER* Genome Sequence.

**Supplemental Table 1.** Primer Sequences Used in This Study.

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## REFERENCES

**Andersson, K.B., Berge, T., Matre, V., and Gabrielsen, O.S.** (1999). Sequence selectivity of c-Myb in vivo. Resolution of a DNA target specificity paradox. J. Biol. Chem. **274:** 21986–21994.

**Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K.** (1995). Current Protocols in Molecular Biology. (New York: John Wiley & Sons).

**Bechtold, N., Ellis, J., and Pelletier, G.** (1993). In planta Agrobacterium-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris **316:** 1194–1199.

**Bernhardt, C., Lee, M.M., Gonzalez, A., Zhang, F., Lloyd, A., and Schiefelbein, J.** (2003). The bHLH genes GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) specify epidermal cell fate in the Arabidopsis root. Development **130:** 6431–6439.

**Bernhardt, C., Zhao, M., Gonzalez, A., Lloyd, A., and Schiefelbein, J.** (2005). The bHLH genes GL3 and EGL3 participate in an intercellular regulatory circuit that controls cell patterning in the Arabidopsis root epidermis. Development **132:** 291–298.

**Braun, E.L., and Grotewold, E.** (1999). Newly discovered plant c-myb-like genes rewrite the evolution of the plant myb gene family. Plant Physiol. **121:** 21–24.

**Clough, S.J., and Bent, A.F.** (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidodpsis thaliana*. Plant J. **16:** 735–743.

**Dolan, L., Duckett, M.D., Grierson, C., Linstead, P., Schneider, K., Lawson, E., Dean, C., Poethig, S., and Roberts, K.** (1994). Clonal relationships and cell patterning in the root epidermis of Arabidopsis. Development **120:** 2465–2474.

**Esch, J.J., Chen, M., Sanders, M., Hillestad, M., Ndkium, S., Idelkope, B., Neizer, J., and Marks, M.D.** (2003). A contradictory GLABRA3 allele helps define gene interactions controlling trichome development in Arabidopsis. Development **130:** 5885–5894.

**Fields, S., and Sternglanz, R.** (1994). The two-hybrid system: An assay for protein-protein interactions. Trends Genet. **10:** 286–292.

**Galway, M.E., Masucci, J.D., Lloyd, A.M., Walbot, V., Davis, R.W., and Schiefelbein, J.W.** (1994). The TTG gene is required to specify epidermal cell fate and cell patterning in the Arabidopsis root. Dev. Biol. **166:** 740–754.

**Hung, C.Y., Lin, Y., Zhang, M., Pollock, S., Marks, M.D., and Schiefelbein, J.** (1998). A common position-dependent mechanism controls cell-type patterning and GLABRA2 regulation in the root and hypocotyl epidermis of Arabidopsis. Plant Physiol. **117:** 73–84.

**Koshino-Kimura, Y., Wada, T., Tachibana, T., Tsugeki, R., Ishiguro, S., and Okada, K.** (2005). Regulation of CAPRICE transcription by MYB proteins for root epidermis differentiation in Arabidopsis. Plant Cell Physiol. **46:** 817–826.

**Kranz, H.D., et al.** (1998). Towards functional characterisation of the members of the R2R3-MYB gene family from *Arabidopsis thaliana*. Plant J. **16:** 263–276.

**Lee, M.M., and Schiefelbein, J.** (1999). WEREWOLF, a MYB-related protein in Arabidopsis, is a position-dependent regulator of epidermal cell patterning. Cell **99:** 473–483.

**Lee, M.M., and Schiefelbein, J.** (2001). Developmentally distinct MYB genes encode functionally equivalent proteins in Arabidopsis. Development **128:** 1539–1546.

**Lee, M.M., and Schiefelbein, J.** (2002). Cell pattern in the Arabidopsis root epidermis determined by lateral inhibition with feedback. Plant Cell **14:** 611–618.

**Lipsick, J.S.** (1996). One billion years of Myb. Oncogene **13:** 223–235.

**Marks, M.D., and Esch, J.J.** (2003). Initiating inhibition. Control of epidermal cell patterning in plants. EMBO Rep. **4:** 24–25.

**Masucci, J.D., Rerie, W.G., Foreman, D.R., Zhang, M., Galway, M.E., Marks, M.D., and Schiefelbein, J.W.** (1996). The homeobox gene GLABRA2 is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*. Development **122:** 1253–1260.

**Ogata, K., Hojo, H., Aimoto, S., Nakai, T., Nakamura, H., Sarai, A., Ishii, S., and Nishimura, Y.** (1992). Solution structure of a DNA-binding unit of Myb: A helix-turn-helix-related motif with conserved tryptophans forming a hydrophobic core. Proc. Natl. Acad. Sci. USA **89:** 6428–6432.

**Ogata, K., Morikawa, S., Nakamura, H., Sekikawa, A., Inoue, T., Kanai, H., Sarai, A., Ishii, S., and Nishimura, Y.** (1994). Solution structure of a specific DNA complex of the Myb DNA-binding domain with cooperative recognition helices. Cell **79:** 639–648.

**Okada, K., and Shimura, Y.** (1990). Reversible root tip rotation in Arabidopsis seedlings induced by obstacle-touching stimulus. Science **250:** 274–276.

**Payne, C.T., Zhang, F., and Lloyd, A.M.** (2000). GL3 encodes a bHLH protein that regulates trichome development in Arabidopsis through interaction with GL1 and TTG1. Genetics **156:** 1349–1362.

**Paz-Ares, J., Ghosal, D., and Saedler, H.** (1990). Molecular analysis of the C1-I allele from *Zea mays*: A dominant mutant of the regulatory C1 locus. EMBO J. **9:** 315–321.

**Rabinowicz, P.D., Braun, E.L., Wolfe, A.D., Bowen, B., and Grotewold, E.** (1999). Maize R2R3 Myb genes: Sequence analysis reveals amplification in the higher plants. Genetics **153:** 427–444.

**Rerie, W.G., Feldmann, K.A., and Marks, M.D.** (1994). The GLABRA2 gene encodes a homeo domain protein required for normal trichome development in Arabidopsis. Genes Dev. **8:** 1388–1399.

**Solano, R., Fuertes, A., Sanchez-Pulido, L., Valencia, A., and Paz-Ares, J.** (1997). A single residue substitution causes a switch from the

dual DNA binding specificity of plant transcription factor MYB.Ph3 to the animal c-MYB specificity. J. Biol. Chem. **272:** 2889–2895.

**Stracke, R., Werber, M., and Weisshaar, B.** (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. Curr. Opin. Plant Biol. **4:** 447–456.

**Szymanski, D.B., Jilk, R.A., Pollock, S.M., and Marks, M.D.** (1998). Control of GL2 expression in Arabidopsis leaves and trichomes. Development **125:** 1161–1171.

**Szymanski, D.B., Lloyd, A.M., and Marks, M.D.** (2000). Progress in the molecular genetic analysis of trichome initiation and morphogenesis in Arabidopsis. Trends Plant Sci. **5:** 214–219.

**Wada, T., Kurata, T., Tominaga, R., Koshino-Kimura, Y., Tachibana, T., Goto, K., Marks, M.D., Shimura, Y., and Okada, K.** (2002). Role of a positive regulator of root-hair development, CAPRICE, in Arabidopsis root epidermal cell differentiation. Development **129:** 5409–5419.

**Wada, T., Tachibana, T., Shimura, Y., and Okada, K.** (1997). Epidermal cell differentiation in Arabidopsis determined by a Myb homolog, CPC. Science **277:** 1113–1116.

**Yoo, S.Y., Bomblies, K., Yoo, S.K., Yang, J.W., Choi, M.S., Lee, J.S., Weigel, D., and Ahn, J.H.** (2005). The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene. Planta **221:** 523–530.

**Zhang, F., Gonzalez, A., Zhao, M., Payne, C.T., and Lloyd, A.** (2003). A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. Development **130:** 4859–4869.

**Zimmermann, I.M., Heim, M.A., Weisshaar, B., and Uhrig, J.F.** (2004). Comprehensive identification of Arabidopsis thaliana MYB transcription factors interacting with R/B-like BHLH proteins. Plant J. **40:** 22–34.