TITLE

WEREWOLF and ENHANCER OF GLABRA3 are interdependent regulators of the spatial expression pattern of *GLABRA2* in *Arabidopsis*

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ABSTRACT

In multicellular organisms, cell fates are specified through differential regulation of transcription. Epidermal cell fates in the *Arabidopsis thaliana* root are precisely specified by several transcription factors, with the GLABRA2 (GL2) homeodomain protein acting at the farthest downstream in this process. To better understand the regulation of *GL2* expression, we ectopically expressed WEREWOLF (WER) and ENHANCER OF GLABRA3 (EGL3) in various tissues and examined *GL2* expression. Here we show that WER expressed ubiquitously in the root induced *GL2* expression only in the root epidermis, whereas co-expression of *WER* and *EGL3* induced *GL2* expression in the corresponding tissues. We also found that GL3 accumulated in the nucleus at the early meristematic region and EGL3 accumulated later in the nucleus of epidermal cells. We further found that ectopic expression of WER and EGL3 in ground tissues inhibited *GL2* expression in the epidermis. Our results suggest that the co-expression of *WER* and *EGL3* is sufficient for driving *GL2* and *CPC* expression.

1. INTRODUCTION

Plants undergo de novo organogenesis throughout their lives from embryogenesis to senescence to adapt to their environment. During organogenesis, it is critical to define the proper cell fates based on lineage and surrounding positional cues. The plant epidermis is the outermost tissue of which the fate is determined at 16-cell stage embryo by a small number of transcription factors [1]. Once the epidermal fate is determined, the epidermis produces elaborately patterned structures which have been used as model systems for understanding the process of cell fate specification.

The *Arabidopsis* root epidermis consists of two distinct types of cells: root hair cells (hair cells) and non-hair cells. The fate of epidermal cells is determined by their relative positions to the underlying cortical cells. Epidermal cells contacting with two cortical cells (H position) differentiate into hair cells, whereas epidermal cells contacting with one cortical cell (N position) adopt the non-hair cell fate.[2] Molecular genetic studies have revealed that several transcription factors including WEREWOLF (WER), CAPRICE (CPC), TRIPTYCHON (TRY), GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3) and GLABRA2 (GL2), are involved in the specification of the epidermal cell fate [3,4,5,6,7,8]. A WD40-repeat protein, TRANSPARENT TESTA GLABRA1 (TTG1) and a leucine-rich repeat receptor-like kinase SCRAMBLED (SCM) are also known to participate in this position-dependent cell fate determination [9,10]. CPC and TRY specify the hair cell fate, whereas WER, GL3, EGL3, TTG1 and GL2 specify the non-hair cell fate. *WER* is expressed preferentially in the N position epidermal cells (N cell) as a result of the negative regulation of SCM and the communication between epidermal cells [3,11]. WER forms a complex with GL3 (or EGL3) and TTG1 (WER-GL3/EGL3-TTG1 complex) in N cells [5,12,13], and binds to the promoter of *GL2* directly to induce the transcription of *GL2* [14]. The WER-GL3/EGL3-TTG1 complex also induces the expression of *CPC* in N cells by direct binding to the *CPC* promoter [15,16,17]. The CPC protein induced in N cells diffuses into neighboring H cells (epidermal cells on the H position), and competes with WER for GL3/EGL3 binding [14,18]. Eventually, the relative ratio between WER and CPC in a cell determines the

expression of *GL2* and the cell fate [14].

Interestingly, most of the transcription factors involved in cell fate determination in the root epidermis are also involved in the determination of epidermis cell types in aerial organs, although the epidermal cells quite differ in shapes and functions. In the *Arabidopsis* leaf epidermis, *GL3*, *EGL3*, *TTG1*, and *GLABRA1* (*GL1*), a paralog of *WER* in leaves, form the *GL1-GL3/EGL3-TTG1* complex, and induce *GL2* expression [12,13,19]. The expression of *GL2* activates trichome formation in leaf epidermis [20]. The *GL1-GL3/EGL3-TTG1* complex in the leaf epidermis also induces *CPC* and *TRY* expression in developing trichomes. *CPC* and *TRY* protein diffuse into neighboring cells and form an inactive *CPC/TRY-GL3/EGL3-TTG1* complex [19]. As a result, the neighboring cells fail to express *GL2* and remain as pavement cells.

Although it has been well established that *WER* and *GL3/EGL3* form a complex and are required for *GL2* expression, it is still unclear how they co-regulate the exquisitely patterned expression of *GL2*. Interestingly, lateral root cap cells that neighbor the root epidermis do not express *GL2* although strong *WER* expression is detected in the lateral root cap [3]. In this study, we discovered that the ectopic expression of *EGL3* in lateral root cap cells induced *GL2* expression in those cells. To better understand how *WER* and *EGL3* regulate cell-specific *GL2* expression, we co-expressed *WER* and *EGL3* in a cell-type specific way using *GAL4-UAS* enhancer trap lines and cauliflower mosaic virus (CaMV) 35S promoter, and examined the expression pattern of *GL2* and epidermal patterning in roots and leaves.

2. MATERIALS AND METHODS

2.1 Plant materials and growth conditions

The *35Sp:EGL3* transgenic plant, Q2610>>WER line, and J0571>>CPC line were described previously [5,14,21]. The J0571>>WER, J1721>>WER, Q0990>>WER, Q2500>>WER and J0571>>WER;CPC lines were generated as previously described [14]. The *GL2p:GUS*, *CPCp:GUS*, *GL3p:GL3-YFP* and *EGL3p:EGL3-YFP* reporter lines were also described previously [6,8,19,22]. The enhancer trap lines were obtained from the Arabidopsis Biological Resource Center.

Seeds were sterilized and germinated on vertical agarose medium at 22°C under continuous light as described previously [3].

2.2 GUS histochemical analysis, cell type analysis and plastic section preparation

GUS histochemical analysis were done as previously described [3]. Four-day-old seedlings were incubated in GUS staining solution at 37°C or used for cell type analysis. At least ten roots were observed for cell type quantification. The H position and N position were identified under a microscope, and root-hair cells and non-hair cells were counted on each position. The plastic sections of root tissues were obtained as described previously [3].

2.3 Confocal microscopy

The fluorescence from GFP was detected from plant tissues using a Zeiss LSM510 Meta confocal microscope as described previously [23].

3. RESULTS

3.1 *GL2* expression requires both *WER* and *EGL3*

In previous work, we showed that overexpression of *WER* in the root tip using the Q2610 enhancer trap line (Q2160>>*WER*) causes the ectopic expression of *GL2p:GUS* reporter gene and the hairless phenotype in the root epidermis [14,24]. We further analyzed the ectopic expression of *GL2p:GUS* reporter gene and found that the overexpressed *WER* using the Q2610 enhancer trap line induced the ectopic expression of *GL2* only in epidermal cells although *WER* expression was induced ubiquitously in most of the cells in the root tip (Fig. 1A). This strongly suggests that *WER* alone is not sufficient for the induction of *GL2* expression in the developing root. *WER* is known to form the *WER-GL3/EGL3-TTG1* complex to activate *GL2* expression [3,5,13], and the *GL3* and the *EGL3* protein accumulates preferentially in epidermal cells [6,21]. Therefore, we hypothesized that *GL2* expression was induced only in the epidermal cells of the Q2610>>*WER* roots because only epidermal cells contain the endogenous *GL3/EGL3* protein.

To test this idea, we examined the *GL2p:GUS* expression pattern in transgenic plants overexpressing *EGL3* under the regulation of CaMV 35S promoter (*35Sp:EGL3*), which leads to strong hairless phenotype [5]. The overexpression of *EGL3* caused the ectopic expression of *GL2* not only in all epidermal cells but also in the lateral root cap cells (Fig. 1A) where the endogenous *WER* protein is known to be localized [17]. These results suggest that the solitary expression of either *WER* or *EGL3* is not sufficient for *GL2* expression in Arabidopsis roots, but the co-expression of *WER* and *EGL3* is sufficient.

3.2 Co-expression of *WER* and *EGL3* is sufficient for *GL2* expression in all types of cells in roots

To confirm that *WER* and *EGL3* are sufficient for *GL2* expression, we co-expressed *WER* and *EGL3* in various layers of the root tip using several enhancer trap lines, and examined the effect on *GL2* expression. For the co-expression of *WER* and

EGL3, we crossed the *35Sp:EGL3* line with available enhancer trap lines ectopically expressing *WER*. Q2610>>*WER* overexpresses *WER* in the root tip ubiquitously, J0571>>*WER* in the ground tissues, J1721>>*WER* in protoxylem and columella cells, Q0990>>*WER* in stele, and Q2500>>*WER* in cortex, endodermis, and pericycle, specifically (Fig. 1B, C). In all of these lines, *GL2* was expressed in cells that expressed *WER* ectopically. These data confirm that the co-expression of *WER* and *EGL3* is sufficient for *GL2* expression in any types of cells in Arabidopsis roots.

3.3 *GL3* protein accumulates in the quiescent center and its surrounding cells

Next, we examined the effect of J0571>>*WER* and Q2500>>*WER* without *35Sp:EGL3* (Fig. 2A). Unexpectedly, we found *GL2p:GUS* expression inside the root tip of seedlings harboring J0571>>*WER* or Q2500>>*WER* without *35Sp:EGL3*, which we were not able to observe in the wild type root without those transgenes. Through careful examination via transverse sections of the root tip, we discovered that J0571>>*WER* or Q2500>>*WER* caused *GL2p:GUS* expression in cells surrounding the quiescent center.

This result led us to examine the expression pattern of *EGL3* and its redundant gene, *GL3* in the root tip. It has been reported that the *GL3* and the *EGL3* proteins accumulate in the nucleus of the epidermal cells [6,21], but their accumulation pattern inside the root has not been examined before. We examined the accumulation pattern of the *GL3* and the *EGL3* protein, using the yellow fluorescent protein (YFP) translationally fused to these proteins and expressed under the control of their own promoter (*GL3p:GL3-YFP* and *EGL3p:EGL3-YFP*) (Fig. 2B). The *GL3-YFP* protein accumulated in cells surrounding quiescent center including cortex/endodermis initials, epidermis/lateral root cap initials, and columella initials as well as in the root epidermal cells. Unlike the *GL3-YFP* protein, the *EGL3-YFP* protein accumulated in the nucleus of the root epidermal cell as reported earlier [21]. These results imply that *GL3* may act in conjunction with *WER* (as *EGL3* does) to induce *GL2* expression in the initial cells of the root.

3.4 Co-expression of *WER* and *EGL3* in the aboveground organ induces trichome development

Overexpression of *WER* (*35Sp:WER*) causes trichome-like structures to form in the hypocotyl, but it doesn't cause any significant phenotypic difference from the wild type in leaves [3]. The J0571 enhancer has been shown to be active in the epidermis of sepals, carpels and hypocotyls [25]. The J0571>>WER plant showed a few trichome-like structures in sepals and carpels, and also in the hypocotyl similar with the *35Sp:WER* plant (Fig. 3A, B) [3]. On the contrary, overexpression of *EGL3* (*35Sp:EGL3*) did not cause ectopic trichome formation in these organs. Ectopic expression of *WER* in the *35Sp:EGL3* plant (*35Sp:EGL3* J0571>>WER) induced many trichomes in the epidermis of the hypocotyl, sepal and carpel to cover the surface of these organs (Fig. 3A, B). These results further suggest that *WER* and *EGL3* are sufficient to ectopically induce *GL2* expression and trichome formation in the epidermis of hypocotyls, sepals and carpels.

3.5 *WER* expression in ground tissue suppresses non-hair cell fate specification in epidermis

Normally, *WER* and *EGL3* induce *GL2* expression to make root epidermal cells adopt the non-hair cell fate in the H position [3,5]. Overexpression of *WER* or *EGL3* in the epidermis of Arabidopsis roots caused all epidermal cells to express *GL2*, which resulted in hairless roots (Fig. 1A) [5,14]. However, J0571>>WER expressing *WER* in the ground tissue of the *35Sp:EGL3* plant, caused a reduced expression of *GL2* in epidermis compared with the expression level in the root epidermis of the plant harboring *35Sp:EGL3* alone (Fig. 1A). In particular, the expression level of *GL2p:GUS* in the H-position cells was greatly decreased. We also analyzed the root epidermal patterning of J0571>>WER and found a significant reduction of the non-hair cell number in both H position and N position (Table 1), which is in accord with the reduction in *GL2* expression level.

3.6 CPC is involved in the down-regulation of *GL2* expression by *WER* and *EGL3* co-expression in the ground tissues

It is well known that *WER* and *GL3/EGL3* also induce the expression of *CPC*, a negative regulator of *GL2* expression and non-hair cell fate specification [15]. In contrast

to the WER or GL2 proteins that function in a cell autonomous manner, the CPC protein diffuses between epidermal cells, and from the inner tissues to epidermis of Arabidopsis roots [18,21]. It is likely that the ectopic co-expression of *WER* and *EGL3* induces ectopic *CPC* expression in the ground tissue of *35Sp:EGL3 J0571>>WER* plants. Ectopically expressed CPC protein in ground tissue would diffuse into epidermis, resulting in inhibition of *GL2* expression and non-hair cell fate specification. To test the involvement of CPC in the inhibition of *GL2* expression and non-hair cell fate specification in the epidermis of *35Sp:EGL3 J0571>>WER* roots, we first analyzed the *CPC* expression pattern using *CPCp:GUS* reporter gene (Fig. 3C). We detected a strong ectopic induction of *CPCp:GUS* expression in the ground tissues, suggesting the ground tissue-specific expression of *WER* and *EGL3* induced *CPC* expression in ground tissue. On the contrary, *CPC* expression in the epidermis was greatly reduced compared with that in the root epidermis of wild type as well as *GL2* expression. This could be explained by CPC acting antagonistically to WER, therefore, CPC inhibits not only *GL2* expression, but also its own expression in epidermis. Next, we ectopically expressed *CPC* along with *WER* in ground tissue using J0571 enhancer trap line (*35Sp:EGL3 J0571>>WER;CPC*) or *CPC* alone (*J0571>>CPC*), and examined the *GL2p:GUS* expression in the root epidermis. In the epidermis of both *35Sp:EGL3 J0571>>WER;CPC* and *J0571>>CPC* roots, the *GL2p:GUS* expression was dramatically reduced (Fig. 3D). These results suggest that the co-expression of *WER* and *EGL3* is sufficient for the ectopic induction of *CPC* expression in the ground tissue of the root tip, and this CPC protein is able to inhibit *GL2* expression in the epidermis non-cell autonomously.

Interestingly, the *J0571>>WER* plant inhibited trichome formation in leaves, to become glabrous, while it showed ectopic trichomes in hypocotyls (Fig. 3E). This glabrous leaf was also seen in the *J0571>>CPC* plant (Fig. 3E). We observed that the J0571 enhancer is highly active in the ground tissue of leaves (mesophyll cells), and its activity was not detected in the leaf epidermis except for the guard cell (Fig. 3F). These results suggest that the WER protein expressed in ground tissue induced the expression of *CPC* and the CPC protein diffused into leaf epidermis resulting in glabrous leaves.

4. DISCUSSION

GL2, a HD-ZIP homeodomain transcription factor, is the key regulator for the non-hair cell fate specification in roots and trichome development in leaves. Accordingly, *GL2* is expressed in epidermal cells of roots and leaves. In this work, ectopic co-expression of *WER* and *EGL3* sufficiently induced *GL2* expression in Arabidopsis roots, leaves and flowers, however; either *WER* or *EGL3* alone appeared to be insufficient for *GL2* expression. In the wild-type Arabidopsis roots, *WER* is expressed in lateral root cap cells and root epidermal cells [3]. *EGL3* expression is limited to the epidermis [21], and *GL3* was expressed in epidermis and the cells surrounding QC. Our results suggest that *GL2* expression is normally induced specifically in the epidermis because the domains of *WER* and *EGL3/GL3* expression overlap in epidermis, suggesting combinatorial transcription regulation determines developmentally important spatial patterns. A similar sort of combinatorial transcription regulation is observed in the ABC model of floral organ development in Arabidopsis. Specific combinations of A, B, C and E function transcription factors determine the fate of specific floral organs [26].

The *EGL3* and *GL3* proteins are closely related bHLH-type transcription factors and have been reported to be functionally redundant [5,12]. However, their expression patterns are slightly different [19]. The *GL3* protein accumulates in the trichome at the basal region of leaves and its level decreases as trichomes mature. On the contrary, *EGL3* protein is detected in the nontrichome cells of leaves as well as in the developing trichome. Furthermore, we have reported previously that the *GL3* protein preferentially accumulates in the N position cell while the *EGL3* protein accumulates preferentially in the H position cell [6,21]. Here we also showed that, in the root, *GL3* was expressed in the cells near the QC, while *EGL3* was expressed later only in the epidermis. Also, *GL3* expression is regulated by an auto-regulatory loop, but *EGL3* is not [27]. Previously we showed that *WER* and *GL1* encode functionally equivalent proteins and divergences in their functions mainly are caused by the difference in their expression domains [28]. These findings provide new examples of the ability of duplicated genes to change their expression profiles during the evolution of a gene family [29]. The resulting

combinatorial functions and the functional divergences are likely important to enable sessile plants to develop and respond to various internal and external signals with the limited number of transcription factors.

Our previous work showed that the overexpression of *WER* in the root epidermis increased *GL2* expression and resulted in hairless roots [14]. We also showed that the *WER* expressed in leaf epidermis is able to induce trichome formation [28]. In this work, we found that expression of *WER* in the ground tissue of roots reduced *GL2* expression in the root epidermis and increased the number of root hair cells. We also found that the ectopic *WER* expression in the ground tissue of leaves prevented trichome development from leaf epidermis. These show that the same transcription factor can cause the opposite phenotypes depending on where it is expressed. The CPC protein seems to mediate the inhibitory effect of the *WER* protein expressed in ground tissue on epidermal *GL2* expression. First, the ectopic expression of *CPC* in ground tissues also inhibited *GL2* expression of root epidermis. Second, the ectopic expression of *WER* and *EGL3* in ground tissues induced *CPC* expression. The CPC proteins are known to diffuse between cells and layers of roots and leaves [16,18,21,30]. Therefore, the *WER* protein expressed in ground tissues could induce *GL2* and *CPC* expression directly in ground tissues but inhibited *GL2* and *CPC* expression indirectly in epidermis by sending the CPC protein. In the *35Sp:EGL3 J0571>>WER* line, the CPC protein in ground tissue might not be able to inhibit *GL2* and *CPC* expression because the ratio of *WER* to *CPC* is high enough for *GL2* and *CPC* expression.

The ectopic expression of *WER* alone or together with *EGL3* in hypocotyl, carpel and sepal epidermis caused trichome formation. These suggest that the lack of *WER* and *GL1* expression in the epidermis of these organs might be the reason that trichomes do not develop in these organs. The transcriptome profile from the Arabidopsis electronic fluorescence pictograph (eFP) browser shows no expression of *WER* and *GL1* in Arabidopsis flower, but an intermediate expression level of *EGL3* in carpels [31]. This profile is consistent with our hypothesis, which helps explain the observed distribution of these epidermal cell types during plant development.

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FIGURE LEGENDS

Fig. 1. Spatial expression patterns of *GL2* induced by the ectopic expression of *EGL3*, *WER*, and *EGL3/WER*. 4-day-old seedlings were stained for GUS activity and plastic sections were taken from the root tips. (A) Transverse (upper panels) and longitudinal (lower panels) sections of wild type, *35Sp:EGL3* and *Q2610>>WER*. (B) Transverse (upper panels) and longitudinal (lower panels) sections of *35Sp:EGL3* co-expressing *Q2610>>WER*, *J0571>>WER*, *J1721>>WER*, *Q0990>>WER* or *Q2500>>WER*. (C) The green signal in the transverse composite images (upper panels) and median section (lower panels) represents the layers where the enhancers are active. Cell wall stained with propidium iodide is represented by red. C, cortex; E, endodermis; Ep, epidermis; LRC, lateral root cap; P, pericycle; Px, protoxylem; S, stele. Scale bars = 20 μ m.

Fig. 2. Ectopic expression of *WER* in the inner tissues induces locally restricted *GL2* expression. (A) The whole mount (upper panels) and transverse section (lower panels) images of *GL2p:GUS* expression pattern of wild-type, *J0571>>WER*, and *Q2500>>WER* seedlings. Insets in B and C are focused on the median region. Scale bars = 50 μ m (upper panel) and 20 μ m (lower panel). (B) The expression pattern of *GL3p:GL3-YFP* and *EGL3p:EGL3-YFP* in the median section visualized by confocal microscopy. Green represents fluorescence from YFP. Scale bar = 20 μ m.

Fig. 3. Ectopic expression of *WER* enhances or suppresses the epidermal *GL2/CPC* expression depending on its expression region. (A) The trichome development in the epidermis of floral organs of *35Sp:EGL3*, *J0571>>WER* and *35Sp:EGL3 J0571>>WER*. Scale bar = 1 mm. (B) The trichome development together with *GL2p:GUS* expression is shown on the hypocotyls of *35Sp:EGL3*, *J0571>>WER* and *35Sp::EGL3 J0571>>WER* seedlings at 5 day-after germination. Scale bar = 0.4 mm. (C) Transverse plastic section images for *CPCp:GUS* of wild-type and *35Sp:EGL3 J0571>>WER* root tips. Scale bar = 20 μ m. (D) The whole

mount *GL2p:GUS* images of *35Sp:EGL3*, *35Sp:EGL3 J0571>>WER*, *35Sp:EGL3 J0571>>WER;CPC* and *J0571>>CPC*. Scale bars = 50 μ m. (E) The trichome development on leaves of 10-day-old wild-type, *J0571>>CPC* and *J0571>>WER* plants. Scale bar = 1 mm. (F) *J0571* is active in palisade mesophyll cells as shown in transverse section (upper panel) and composite cross-section (lower panel) of leaves. White arrow, guard cell; Ep, epidermis; Pa, palisade. Scale bar = 20 μ m.

Table 1. Quantification of epidermal cell types in the wild-type and *J0571>>WER* roots.

Epidermis

Cortex

Table 1. Quantification of epidermal cell types in the wild-type and J0571>>WER roots.^a

Genotype	H position		N position	
	Hair Cell (%)	Non-hair cell (%)	Hair cell (%)	Non-hair cell (%)
Wild type	94.8 ± 1.4	5.2 ± 1.4	2.4 ± 0.9	97.6 ± 0.9
J0571>>WER	99.7 ± 0.3	0.3 ± 0.3	18.2 ± 2.1	81.8 ± 2.1

^a Values indicate the mean ± standard deviation of at least three independent experiments. In each experiment, at least 10 roots were included per plant line.

Fig. 1.

A

GL2p:GUS

B

Fig. 2.

Fig. 3.