Plant Signaling & Behavior 10:12, e1103407; December 2015; © 2015 Taylor & Francis Group, LLC

TORNADO1 regulates root epidermal patterning through the *WEREWOLF* pathway in *Arabidopsis thaliana*

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Keywords: *Arabidopsis thaliana*, ENHANCER OF GLABARA3 (EGL3), Epidermal patterning, Root hair, TORNADO1 (TRN1), WEREWOLF (WER)

Cell fate in the root epidermis of *Arabidopsis thaliana* is determined in a position-dependent manner. SCRAMBLED (SCM), an atypical leucine-rich repeat receptor-like kinase, mediates this positional regulation via its effect on *WEREWOLF* (*WER*) expression, and subsequently, its downstream transcription factor, *GLABRA2* (*GL2*), which are required for nonhair cell development. Previously, TORNADO1 (TRN1), a plant-specific protein with a leucine-rich repeat ribonuclease inhibitor-like domain, was shown to be required for proper epidermal patterning in Arabidopsis roots. In this work, we analyzed the possible involvement of *TRN1* in the known root epidermal gene network. We discovered that the *trn1* mutant caused the ectopic expression of *WER* and the randomized expression of *GL2* and *EGL3*. This suggests that TRN1 regulates the position-dependent cell fate determination by affecting *WER* expression in Arabidopsis root epidermis. Additionally, the distinct phenotypes of the aerial parts of the *trn1-t* and *scm-2* mutant suggest that TRN1 and SCM might have different functions in the development of aerial parts.

Two types of epidermal cells are found in the epidermis of *Arabidopsis thaliana* roots: root-hair cells and nonhair cells. Interestingly, the type of Arabidopsis root epidermal cells is determined by their position relative to the underlying cells of cortical layer. Epidermal cells contacting 2 cortical cells (designated as the H position) differentiate into root-hair cells. On the other hand, epidermal cells located on a single cortical cell (designated as the N position) adopt the nonhair cell fate.[1,2] Because root initial cells divide in an anticlinal orientation, the epidermal cells form files of root-hair cells and nonhair cells establishing a stripe pattern of root hair.

WEREWOLF (*WER*), a R2R3 MYB transcription factor is expressed in the epidermal cells on the N position preferentially.[3] The WER proteins form a complex with the bHLH transcription factors GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3), and the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1).[3,4] This WER-GL3/EGL3-TTG1 complex activates the expression of *GLABRA2* (*GL2*), a homeodomain transcription factor that is required for the nonhair cell development of the epidermal cells on the N position.[3,5] On the other hand, the WER-GL3/EGL3-TTG1 complex inhibits the expression of *GL3* and *EGL3*.[6] Because of this feedback regulation, *GL3* and *EGL3* are expressed in the epidermal cells on the H position preferentially.[6] SCRAMBLED (SCM; also known as STRUBBELIG), an atypical leucine-rich repeat receptor-like kinase is required for the position-dependent *WER* expression.[7-9] Accordingly, *scm* mutants disturb the stripe pattern of root epidermis development causing the 'scrambled' pattern of root epidermal gene expression and cell types.[7]

QUIRKY (QKY), adenosine dimethyl transferase 1A rRNA dimethylase (DIM1A), TORNADO1 (TRN1) and TORNADO2 (TRN2) are also required for position-dependent epidermal patterning.[10-14] The mutation in DIM1A rRNA dimethylase gene affects the spatial expression pattern of *WER* and *GL2*.[13] QKY, a plasmodesmata-localized C2-domain protein, was shown to be required for the proper *GL2* expression pattern, and interacts with SCM physically.[10-12] Mutations in *TRN1* and *TRN2* cause pleiotropic phenotypes including the spiral (twisted) growth of roots, abnormal root apical meristem organization, and the alteration of root epidermal patterning and radial patterning.[14] However, the mechanism by which TRN1 and TRN2 affect root epidermal patterning is unknown. In this work, we analyzed the effect of the *trn1* mutation on the expression of *GL2, WER* and *EGL3* to understand the molecular role of TRN1 in root epidermal patterning.

First, we studied the spatial expression pattern of *GL2* in the *trn1-t* allele by using the *GL2::GUS* reporter. The *GL2::GUS* reporter is a fusion of the *GL2* promoter and β-glucuronidase (GUS) coding sequence, which accurately reflects the spatial expression of the *GL2* gene.[15] The *trn1-t* allele contains a

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Submitted: 09/02/2015; Revised: 09/23/2015; Accepted: 09/24/2015
http://dx.doi.org/10.1080/15592324.2015.1103407

transfer-DNA insertion in the second exon of the *TRN1* gene (AT5G55540, SALK_065854), and it exhibits the identical phenotypes to other alleles that were described previously (data not shown).[14] In wild-type roots, *GL2::GUS* expression is detected in the cells on the N position showing the stripe pattern in the epidermis (**Fig. 1A**). As described previously, the *scm-2* roots showed the 'scrambled' pattern of *GL2* expression in the epidermis (**Fig. 1B**), indicating that the *scm-2* epidermal cells adopt their fate in a position-independent manner.[7] We observed a similar 'scrambled' pattern of *GL2* expression in the epidermis of the *trn1-t* mutant roots (**Fig. 1C**). This suggests that TRN1 also exerts its role in root epidermal patterning by affecting *GL2* expression.

To determine whether TRN1 affects the epidermal-patterning network generally or only *GL2* specifically, we examined the expression of *WER* and *EGL3* by analyzing the *WER::GFP* and *EGL3::GUS* reporters, which are fusions of the *WER* and *EGL3* promoter to reporter genes. In wild-type roots, preferential expression of *WER* was detected in the epidermal cells on the N position as previously described (**Fig. 2A**).[3] In contrast, most of the epidermal cells of the *trn1-t* roots appeared to express *WER::GFP* (**Fig. 2B**). This suggests that TRN1 is involved in the N-position-preferred *WER* expression, and the lack of TRN1 function causes ectopic expression of *WER* in the epidermis. The *EGL3* expression pattern was also disrupted in the *trn1-t* mutant. The *EGL3::GUS* reporter was detected in the epidermal cells on the H position in the wild-type root, as previously reported (**Fig. 2C**),[6] but *EGL3* expression pattern was 'scrambled' in the *trn1-t* mutant roots (**Fig. 2D**). These results indicate that the expression of *WER* is the likely target of TRN1 action, and it is the de-regulation of *WER* expression that causes the 'scrambled' expression pattern of *EGL3* and *GL2*, which ultimately leads to the disorganized root epidermal patterning.

In previous studies, it was shown that an intermediate level of *WER* over-expression resulted in the 'scrambled' expression pattern of *GL2*, and a strong level of *WER* over-expression caused all epidermal cells to express *GL2*.[5] These results are likely due to the function of the CAPRICE (CPC) and TRIPTYCHON (TRY) R2 MYB transcription factors.[16,17] The WER-GL3/EGL3-TTG1 complex activates the expression of *CPC* and *TRY* as well as *GL2*.[18,19] However, the CPC and TRY proteins act as antagonistic competitors of WER. Therefore, unless the level of the WER protein predominates the level of the CPC/TRY proteins, *GL2* expression pattern is stochastic and random in the root epidermis expressing *WER* ectopically. This suggests that, in the *trn1-t* roots, the expression of *WER* is deregulated, but not strongly over-stimulated. Previous work showed that the *cpc trn1–1* double mutant developed fewer root hairs than the *cpc* single mutant.[14] Our results suggest that this might be due to additive effect of the ectopic expression of *WER* by the *trn1* mutation and the loss of CPC function. Previous analysis of the *ttg1 trn1–1* double mutant revealed that *ttg1* was epistatic to *trn1–1*.[14] This genetic relationship is supported by our findings because TRN1 appeared to affect the expression of *WER* which is a part of the WER-GL3/EGL3/TTG1 complex. However, it is not clear how TRN1 regulate the expression pattern of *WER*. The mechanisms of *WER* expression regulation by TRN1 and SCM might be different. In the *scm* mutants, the expression of *WER* in root epidermis was randomized,[7] but most of the epidermal cells expressed *WER::GFP* in the *trn1-t* mutant roots (**Fig. 2B**). The aerial parts of the *trn1-t* mutant and the *scm* mutant exhibit distinct phenotypes.[14,20] These suggest that SCM and TRN1 both affect the expression pattern of *WER*, however; they have distinct functions in the development of aerial parts, and TRN1 might regulate *WER* expression in different mechanism from the mechanism of SCM. The *scm* mutants exhibit multiple phenotypes including root epidermal patterning defect, twisted root growth and silique dehiscence defect.[20,21] All 3 developmental defects appeared to be implicated in distinct signaling pathways regulated by SCM.[20] Furthermore, we discovered that ANGUSTIFOLIA (AN) mediates SCM signaling pathway specifically involved in the spiral growth of tissues.[21] Interestingly, the *trn1*

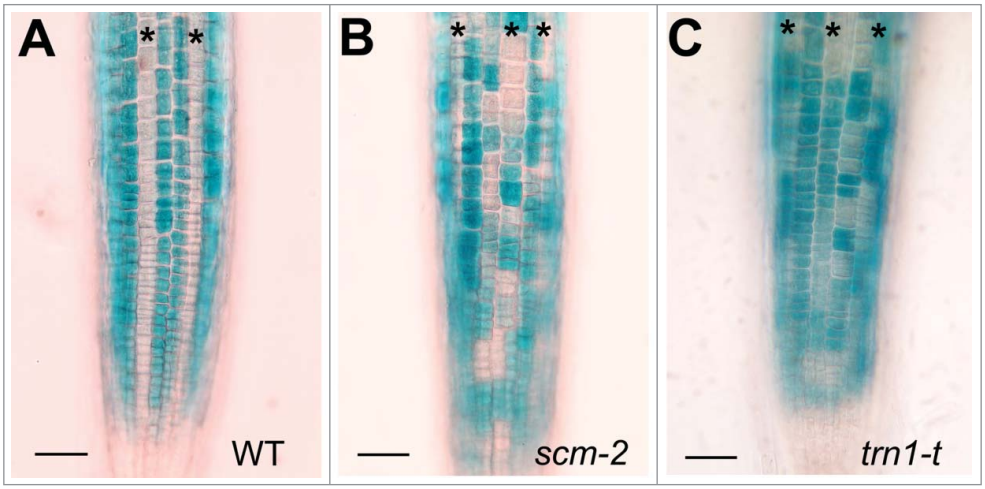

Figure 1. Expression of the *GL2::GUS* reporter in the root epidermis of the *scm-2* and *trn1-t* mutants. The roots of 4-day-old wild-type (**A**), *scm-2* (**B**) and *trn1-t* (**C**) seedlings were analyzed for *GL2::GUS* expression as described previously.[15] The blue-stained cells represent the cells expressing *GL2* and adopting the nonhair cell fate. The Arabidopsis Biological Resource Center (ABRC; Columbus, OH) provided the SALK_086357 (*scm-2*) and SALK_065854 (*trn1-t*) insertion line. Asterisks indicate files of epidermal cells on the H position. The scale bars represent 50 μm.

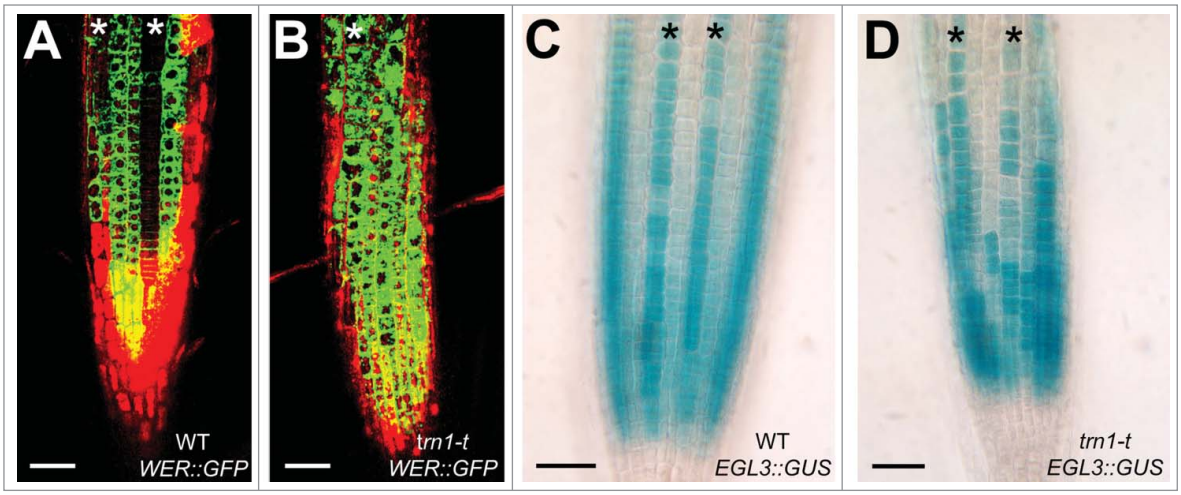

Figure 2. Expression of *WER::GFP* and *EGL3::GUS* reporters in the root epidermis of the *trn1-t* mutants. The roots of 4-day-old wild-type (**A, C**) and *trn1-t* (**B, D**) seedlings were analyzed for *WER::GFP* (**A, B**) and *EGL3::GUS* (**C, D**) expression as described previously.[3,6] (**A, B**) Fluorescence of GFP (green) and cell-wall-staining propidium iodide (red) was taken from the epidermis of the wild-type *WER::GFP* and *trn1-t WER::GFP* roots using confocal microscopy. Green fluorescence represents the transcriptional activity of the *WER* promoter. (**C, D**) The blue staining in the cells represents transcriptional activity of *EGL3* promoter. Asterisks indicate files of epidermal cells on the H position. The scale bars represent 50 μm

mutants exhibit both epidermal patterning defect and spiral root growth.[14] These suggest a possibility that TRN1 and SCM might be involved in the same pathways that regulate root epidermal patterning and spiral root growth although their action mechanisms might be different. The genetic and molecular interactions between TRN1 and SCM, and TRN1 and AN will be studied in future research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We acknowledge the Arabidopsis Biological Resource Center (ABRC; Columbus, OH) for providing the SALK_086357 (*scm-2*) and SALK_065854 (*trn1-t*) insertion lines.

Funding

This work was supported by the US. National Science Foundation (grant no. IOS-1121602 and IOS-1444400 to J.S. and S.H.K), and the faculty start-up fund from Long Island University, Brooklyn Campus (to S.H.K).

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