

Nuclear trapping by GL3 controls intercellular transport and redistribution of TTG1 protein in *Arabidopsis*

Rachappa Balkunde*, Daniel Bouyer^{*,†} and Martin Hülskamp[§]

SUMMARY

Trichome patterning on *Arabidopsis* leaves is one of the best-studied model systems for two-dimensional de novo patterning. In addition to an activator-inhibitor-related mechanism, we previously proposed a depletion mechanism to operate during this process such that GLABRA3 (GL3) traps the trichome-promoting factor TRANSPARENT TESTA GLABRA1 (TTG1) in trichomes that, in turn, results in a depletion of TTG1 in trichome neighbouring cells. In this manuscript we analyze the molecular basis underlying this trapping mechanism. We demonstrate the ability of GL3 to regulate TTG1 mobility by expressing *TTG1* and *GL3* in different tissue layers in different combinations. We further show that TTG1 trapping by GL3 is based on direct interaction between both proteins and recruitment in the nucleus.

KEY WORDS: TTG1, GL3, Nuclear trapping

INTRODUCTION

The regular distribution of trichomes on the leaf surface of *Arabidopsis* is particularly suited to study two-dimensional de novo patterning processes (Hülskamp, 2004; Ishida et al., 2008; Pesch and Hülskamp, 2009). At the basal part of young leaves, trichome cells are singled out from apparently equivalent protodermal cells. Clonal analysis rendered it unlikely that a cell lineage mechanism is responsible for trichome spacing but rather relies on cellular interactions of initially equivalent cells (Larkin et al., 1996; Schnittger et al., 1999).

The genetic and molecular analysis of trichome patterning has suggested two distinct patterning processes that are likely to operate in parallel as the key regulators are involved in both of them (Pesch and Hülskamp, 2009). The first mechanism is similar to an activator-inhibitor-based model (for a general introduction, see Meinhardt and Gierer, 2000). According to this model, trichome-promoting factors turn on their own inhibitors that can move into neighbouring cells to mediate lateral inhibition. Three activators, the WD40 protein TRANSPARENT TESTA GLABRA1 (TTG1) (Koorneef, 1981; Galway et al., 1994; Walker et al., 1999), the R2R3 MYB related transcription factor GLABRA1 (GL1) (Oppenheimer et al., 1991) and the helix-loop-helix (bHLH)-like transcription factor GLABRA3 (GL3) (Koorneef et al., 1982; Hülskamp et al., 1994; Payne et al., 2000) act together by forming a trimeric transcriptional activator complex (Payne et al., 2000). They trigger the expression of the R3 single-repeat MYB inhibitor TRIPTYCHON (TRY) and probably also five redundantly acting homologs (Wada et al., 1997; Schellmann et al., 2002; Kirik et al., 2004a; Kirik et al., 2004b; Digiuni et al., 2008; Tominaga et al., 2008; Wang et al., 2008; Wester et al., 2009). Some of the inhibitors were shown to move between cells

and can repress the function of the activators by competitive complex formation (Esch et al., 2003; Kurata et al., 2005; Wang et al., 2007; Digiuni et al., 2008; Wester et al., 2009).

A second model explains de novo trichome patterning by a depletion of the activator TTG1 in non-trichome cells that is brought about through trapping of TTG1 by GL3 in trichome initials, causing lateral inhibition (Bouyer et al., 2008; Pesch and Hülskamp, 2009). This model is based on several findings. First, TTG1 protein was found to be able to move between cells. As TTG1 movement results in a co-movement of marker molecules that are immobile on their own, a plasmodesmatal transport is postulated (Bouyer et al., 2008). Translational TTG1-YFP fusion protein distribution was shown to be markedly reduced in cells next to trichome initials, though the initial expression pattern exhibited the same level of expression in all epidermal cells of the trichome-patterning zone (Bouyer et al., 2008). This depletion of TTG1 in trichome neighbouring cells was lost in *gl3* mutants, suggesting that TTG1 is trapped in trichomes where *GL3* is strongly expressed (Bouyer et al., 2008). Most aspects of the activator-depletion model, however, remain speculative. In particular, it is not known whether a direct interaction between GL3 and TTG1 is important or whether depletion is indirectly dependent on GL3.

Here, we have analysed the molecular details of the proposed trapping mechanism. We demonstrate that GL3 can regulate TTG1 mobility by expressing *TTG1* and *GL3* in different tissue layers in different mutant combinations. We further show that GL3 mediates the nuclear localization of TTG1 by direct interaction between GL3 and TTG1, indicating that TTG1 trapping is mediated by intracellular compartmentalization.

MATERIALS AND METHODS

Constructs

TTG1-YFPpEN was described previously (Bouyer et al., 2008). TTG1ΔC26-YFPpEN and C26TTG1-YFPpEN constructs were created by inverse PCR using primers flanking the deletion region and TTG1-YFPpEN as a template. To create CFP-GL3pEN, the full-length coding sequence of GL3 was amplified by PCR introducing *SalI* restriction sites and was cloned in pBluescript vector. The *SalI* fragment was then cloned into pEN1a vector digested with *SalI* and *XhoI* restriction enzymes to obtain GL3pEN. CFP was cloned at the *XmnI* site N-terminally to GL3 to create CFP-GL3pEN. CFP-GL3Δ78pEN and CFP-GL3ΔNLSpEN were

University of Cologne, Botanical Institute III, Zùlpicher Strasse 47b, 50674 Kùln, Germany.

*These authors contributed equally to this work

[†]Present address: Institut de Biologie Moléculaire des Plantes du CNRS, BMP-CNRS – UPR2357, 12 rue du Général Zimmer, 67084 Strasbourg, France

[§]Author for correspondence (martin.huelskamp@uni-koeln.de)

created by inverse PCR using primers flanking the deletion region and CFP-GL3pEN as a template. An internal 78 amino acid fragment (360-437 amino acids) from GL3 was amplified by PCR using both forward and reverse primers attached with *NcoI* restriction sites. The PCR product was cloned N-terminally to GUS at the *NcoI* restriction site in GUSpEN (Invitrogen) to obtain 78GL3-GUSpEN.

TTG1pAMPAT-GW, RbcS2b/RBCpAMPAT-GW and AtML1-GW binary vectors were described previously (Bouyer et al., 2008; Wester et al., 2008). 35S:GL3, 35S:TTG1-YFP, RBC:TTG1-YFP, RBC:GFP-GL3, AtML1:GFP-GL3, 35S:CFP-GL3, 35S:CFP-GL3Δ78, 35S:CFP-GL3ΔNLS, 35S:NLS-TTG1-YFP, TTG1:NLS-TTG1-YFP, RBC:NLS-TTG1-YFP, 35S:TTG1Δ26-YFP and 35S:C26TTG1-YFP constructs were created by gateway LR reaction system (Invitrogen).

Plant materials and growth conditions

The mutant lines used in this study, *ttg1-1*, *gl3-1*, *gl3 egl3*, *gl3 ttg1* (Ler background), *ttg1-13* (RLD background) and *gl3 egl3 tt8* have been described previously (Koornneef et al., 1982; Walker et al., 1999; Larkin et al., 1999; Payne et al., 2000; Zhang et al., 2003; Bouyer et al., 2008). *ttg1* TTG1:TTG1-YFP has been described previously (Bouyer et al., 2008). The *ttg1* RBC:TTG1-YFP, *gl3* RBC:GFP-GL3, *ttg1 gl3* RBC:TTG1-YFP, *ttg1 gl3* RBC:GFP-GL3, *ttg1 gl3* AtML1:GFP-GL3, Ler 35S:GL3, *gl3 egl3* TTG1:TTG1-YFP, *gl3 egl3 tt8* TTG1:TTG1-YFP, *ttg1* TTG1:NLS-TTG1-YFP and *ttg1* RBC:NLS-TTG1-YFP lines were generated by Agrobacterium-mediated transformation in the corresponding backgrounds using the floral dip method described previously (Clough and Bent, 1998). Transformants were selected in the T1 generation on soil using 0.1% BASTA solution. Homozygous lines were used to make the genetic crossings and F1 plants were selfed to identify the progeny with the desired background among the segregating F2 population. *gl3* TTG1:TTG1-YFP, 35S:GL3 TTG1:TTG1-YFP, *ttg1 gl3* RBC:TTG1-YFP RBC:GFP-GL3 and *ttg1 gl3* RBC:TTG1-YFP AtML1:GFP-GL3 were created by genetic crossings. *Arabidopsis* plants were grown on soil at 22°C in continuous light conditions.

Yeast two hybrid assay

The *Saccharomyces cerevisiae* strain AH109 was used for the yeast two-hybrid assays. Yeast transformation was performed as described before (Gietz et al., 1995). pC-ACT2 and pAS2 plasmids (Clontech) were used for the fusion with GAL4 activation domain and GAL4 DNA-binding domain, respectively. GL3, GL3Δ78, GL3ΔNLS, 78GL3-GUS, EGL3, TT8 and GUS were fused to the GAL4 activation domain, and TTG1, TTG1ΔC26, C26TTG1, 78GL3-GUS and NLS-TTG1 were fused to the DNA-binding domain by the gateway LR reaction system (Invitrogen). Yeast were grown on synthetic dropout media lacking leucine, tryptophan and histidine (Leu⁻/Trp⁻/His⁻) and supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT) to analyze the protein-protein interactions.

Nuclear transportation trap (NTT) assay

Plasmid vectors pNH2 (NES-LexAD) and pNH3 (NES-LexAD-NLS) and pNS (modified pNH2) have been described previously (Ueki et al., 1998). pNS-TTG1 and pNS-TTG1ΔC26 were constructed by cloning the *SalI/XhoI* fragments of TTG1 and TTG1ΔC26 into the *SalI* restriction site in the pNS vector. pNS-GL3 and pNS-GL3Δ78 were constructed by cloning the *SalI* fragment of GL3 and GL3Δ78 into the *SalI* restriction site of the pNS vector. *SalI/Ecl36I* and *SalI/PvuII* fragments of GL3 and GL3Δ78, respectively, were cloned into the *XhoI/PvuII*-digested pVT-U vector to obtain pVT-U-GL3 and pVT-U-GL3Δ78.

The nuclear transportation trap (NTT) assay was carried out as described previously (Ueki et al., 1998) using the yeast strain EGY 48 (Clontech). NES-LexAD [pNH2], NES-LexAD-NLS [pNH3], NES-LexAD-TTG1 [pNS-TTG1], NES-LexAD-TTG1ΔC26 [pNS-TTG1ΔC26], NES-LexAD-GL3 [pNS-GL3] and NES-LexAD-GL3Δ78 [pNS-GL3Δ78] plasmids were transformed individually and grown on synthetic dropout media lacking leucine and histidine (Leu⁻/His⁻) for 4-7 days at 30°C to detect the expression of the LEU2 reporter gene expression for the transport of the fusion protein into the nucleus. Similarly, pNS-TTG1 and pNS-TTG1ΔC26 were separately co-transformed with either pVT-U-GL3 or pVT-U-GL3Δ78.

Transient expression

The biolistic PDS – 1000/He system (Bio-Rad) was used for the transient expression studies. Gold particles (1.0 μm) were coated with 300 ng of each DNA and were co-bombarded into onion or *Arabidopsis* cotyledon epidermal cells with 900 psi rupture discs under a vacuum of 26 inches of Hg. Fluorescence was analyzed 12-15 hours after the bombardment.

Microscopy and quantification of YFP signal

Fluorescent images were captured using the Leica TCS-SP2 confocal microscope equipped with the LCS software. Images were made using 40× water immersion objective. The z-stack images were obtained and merged to one plane. Raw images were used for quantifying the YFP fluorescence using the histogram quantification tool of the LCS software. Young rosette leaves were stained with 5 μg/ml of propidium iodide (PI) for 1-2 minutes to mark the cell walls. Fluorescent pictures of onion epidermal cells were captured using the LEICA-DMRE microscope equipped with a high-resolution KY-F70 3-CCD JVC camera and DISKUS software. Images were processed with Adobe Photoshop CS2.

Scanning Electron Microscopy (SEM)

Twelve to 15-day-old seedlings were mounted on stubs with silver adhesive (Electron Microscopy Sciences) and kept cold on ice until viewing in a Hitachi S-3500N environmental scanning electron microscope (ESEM) using high vacuum mode. Images were taken quickly (within 15 minutes) to minimize damage by the electron beam on the live sample, using an accelerating voltage of 5.0 kV and a working distance of 10-20 mm.

RESULTS

GL3 counteracts TTG1 mobility

The previous finding that TTG1 protein depletion is lost in *gl3* mutants led to the hypothesis that TTG1 binding to GL3 leads to a trapping of TTG1 in trichomes due to elevated GL3 levels in these cells (Bouyer et al., 2008; Zhao et al., 2008). One prediction of this hypothesis is that mobility of TTG1 can be altered depending on the presence or absence of GL3. We took advantage of the previous finding that *TTG1* can rescue the *ttg1* mutant trichome phenotype when expressed in the sub-epidermis. If the hypothesis were correct, one would expect that tissue-specific *GL3* expression modulates the rescue efficiency of sub-epidermal expressed *TTG1*. The experiments were designed to test this in two directions, by either providing GL3 exclusively in the epidermis or in the sub-epidermis.

Does epidermal GL3 promote the rescue by trapping TTG1 in the upper layer? In order to address this question, we tested the rescue ability of sub-epidermal TTG1 in the absence or abundance of epidermal GL3. As shown before using the

Table 1. Mobility between cell layers

Genotype	Number of trichomes on leaves 3 and 4 ±s.d.	n
Ler	86±7.8	29
<i>gl3</i>	37±12.8	17
<i>ttg1</i>	0	30
<i>ttg1 gl3</i>	0	30
<i>ttg1</i> RBC:TTG1-YFP	70±8.8	16
<i>ttg1 gl3</i> RBC:TTG1-YFP	2±2.2	34
<i>gl3</i> RBC:GFP-GL3	36±10.2	19
<i>ttg1 gl3</i> RBC:GFP-GL3	0	30
<i>ttg1 gl3</i> RBC:GFP-GL3 RBC:TTG1-YFP	0	30
<i>ttg1 gl3</i> AtML1:GFP-GL3 RBC:TTG1-YFP	>300	15
<i>ttg1 gl3</i> AtML1:GFP-GL3	70±12.6	21

s.d., standard deviation; n, number of plants analyzed.

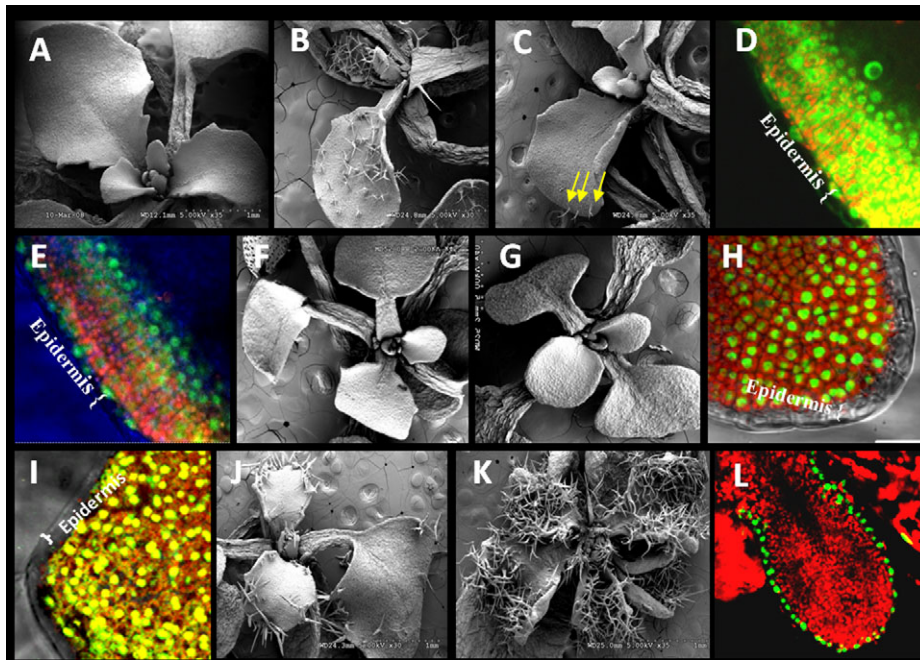


Fig. 1. GL3 controls TTG1 movement. (A–C, F, G, J, K) Scanning electron microscopy micrographs of rosette leaves. (D, E, H, I, L) Confocal images. (A) *ttg1 gl3*. No trichomes are found. (B) *ttg1* RBC:TTG1-YFP. Trichome phenotype is restored. (C) *ttg1 gl3* RBC:TTG1-YFP. Weak rescue of trichome phenotype. Arrows indicate trichomes. (D) *ttg1* RBC:TTG1-YFP. YFP fluorescence is found in the sub-epidermis, as well as in the epidermis. (E) *ttg1 gl3* RBC:TTG1-YFP. YFP fluorescence is found in the sub-epidermis, as well as in the epidermis. (F) *ttg1 gl3* RBC:GFP-GL3. No trichomes are found. (G) *ttg1 gl3* RBC:GFP-GL3 RBC:TTG1-YFP. No trichomes are found. (H) *ttg1 gl3* RBC:GFP-GL3. GFP fluorescence is found exclusively in the sub-epidermis. (I) *ttg1 gl3* RBC:GFP-GL3 RBC:TTG1-YFP. No YFP fluorescence is found in the epidermis. (J) *ttg1 gl3* AtML1:GFP-GL3. Moderate trichome rescue mostly at the leaf margin. (K) *ttg1 gl3* AtML1:GFP-GL3 RBC:TTG1-YFP. Strong over-production of trichomes. (L) *ttg1 gl3* AtML1:GFP-GL3. GFP fluorescence is exclusively found in the epidermis.

heterologous PPCA1 promoter, sub-epidermal *TTG1* expression driven by the widely used Rubisco small subunit 2B promoter (RBC) also rescues the *ttg1* mutant trichome phenotype (Table 1; Fig. 1A,B). In these lines, TTG1-YFP is clearly found in both cell layers, the sub-epidermis as well as the epidermis, demonstrating the movement between cell layers (Fig. 1D). The influence of GL3 in the epidermis on TTG1-YFP movement from the sub-epidermis was assayed by studying the rescue ability of sub-epidermal TTG1 in *ttg1 gl3* double mutants. If GL3 does not interfere with TTG1 mobility, then *ttg1 gl3* double mutants expressing sub-epidermal TTG1 should show the same number of trichomes as seen in *gl3* single mutants. However, the rescue of the *ttg1 gl3* double mutant by RBC:TTG1-YFP was much less effective. In spite of this, the intercellular transport of TTG1-YFP per se is not affected as seen by the presence of TTG1-YFP fluorescence in all cell layers comparable with the situation in the *ttg1* single mutant (Table 1; Fig. 1C,E). Although *gl3* mutants show on average 37 trichomes on leaves 3 and 4, the corresponding RBC:TTG1-YFP lines display on average only two trichomes. This suggests that *GL3* strongly promotes the rescue efficiency of sub-epidermal TTG1. In order to distinguish between the possibilities that GL3 promotes the rescue either through co-movement from the sub-epidermis and/or by modification of TTG1 function, or by the proposed trapping mechanism, we created several lines in which GFP-GL3 is expressed in the sub-epidermis or the epidermis. GFP-GL3 was found exclusively in the layers in which it was expressed, indicating that it cannot move between the layers in either direction in the leaf. This result also confirms the tissue specificity of the used promoters (Fig. 1H,L). Both, the co-movement and modification hypotheses are ruled out by the findings that no rescue is observed in *gl3* RBC:GFP-GL3, *ttg1 gl3* RBC:GFP-GL3 and *ttg1 gl3* RBC:TTG1-YFP RBC:GFP-GL3 lines (Table 1; Fig. 1F,G). In fact, when crossing low efficiently rescuing RBC:TTG1-YFP in the *gl3 ttg1* double homozygous background together with RBC:GFP-GL3 in the

same background, trichome development is completely abolished (Table 1). As the fluorescence of GFP/YFP is exclusively detected in the nuclei of the sub-epidermis (Fig. 1I), this strongly suggests that the presence of GL3 restricts the mobility of TTG1 rather than modifying its function or being co-transported.

In a complementary experiment, we increased the epidermal GL3 levels using an epidermis-specific AtML1:GFP-GL3 line. As GL3 is able to partially complement *ttg1*, these lines show restoration of trichomes mainly at the margin of the leaves (Table 1; Fig. 1J). However, these trichomes show strong distortions as previously described for lines overexpressing *GL3* in a *ttg1* background (Fig. 1J) (Zhang et al., 2003). When crossing this line with the *ttg1 gl3* RBC:TTG1-YFP line, we observed a drastic increase in trichome number, suggesting that GL3 promotes TTG1-triggered sub-epidermal rescue by capturing TTG1 in the epidermis (Table 1; Fig. 1K). Together, these data demonstrate that GL3 modulates TTG1 mobility in a manner consistent with the hypothesis that it traps TTG1 in cells with high *GL3* expression.

GL3 modulates the intracellular localization of TTG1

In wild-type leaves, TTG1 is typically found in the cytoplasm and the nucleus of epidermal cells (Fig. 2A) (Bouyer et al., 2008; Zhao et al., 2008). This intracellular localization appears to be altered in *gl3* mutants or in plants overexpressing *GL3*. In *gl3* mutants, the TTG1-YFP distribution appears to be more diffuse, indicating a shift of the protein from the nucleus to the cytoplasm (Fig. 2C). By contrast, TTG1-YFP appears to be localized mainly into the nucleus in the 35S:GL3 overexpression background (Fig. 2B). A quantitative analysis using confocal laser scanning microscopy (CLSM) confirmed this impression. The intracellular distribution pattern was found to be significantly different with respect to the level of GL3 (Table 2). Whereas, in wild type, 69% of the TTG1-YFP fluorescence was found in the nucleus, *gl3* mutants show a statistical significant ($P=5.7 \times 10^{-66}$) reduction to 56% nuclear

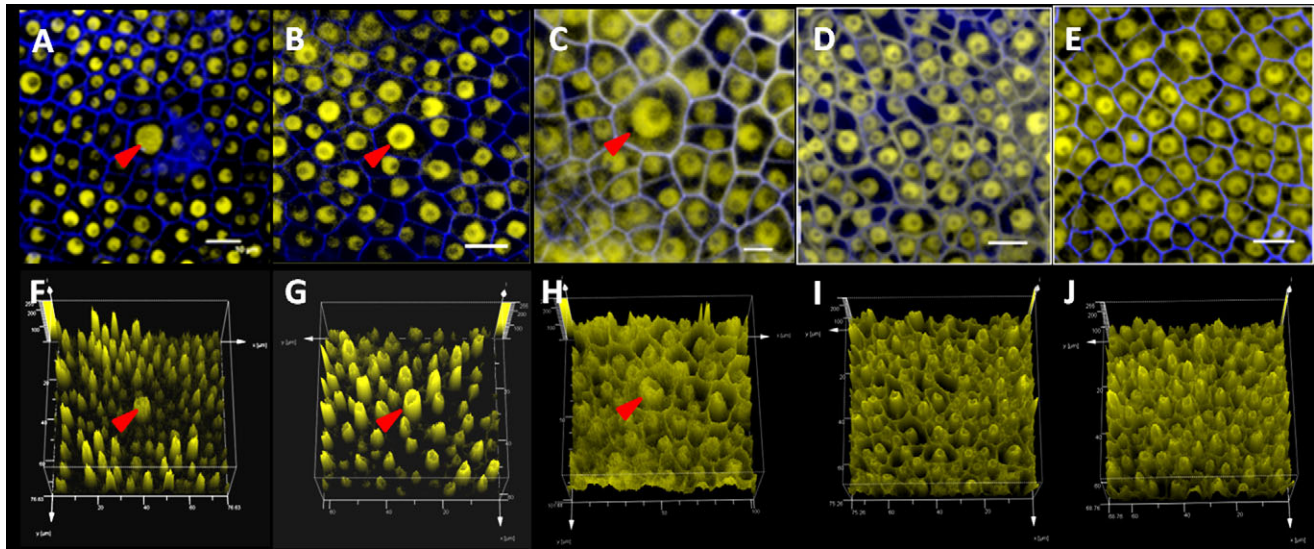


Fig. 2. Cellular distribution pattern of TTG1-YFP fusion protein in the trichome patterning zone in the leaf epidermis. (A-E) The upper row shows TTG-YFP in yellow and propidium iodide staining the cell wall in blue. Trichome cells are marked by red arrowheads. (F-J) The bottom row shows intensity projection images of confocal stacks of TTG1:TTG1-YFP lines. The fluorescence intensity is indicated by size of the peaks. (A,F) *ttg1* TTG1:TTG1-YFP. The YFP fluorescence intensity is much weaker in the nuclei of trichome neighbouring cells. (B,G) 35S:GL3 TTG1:TTG1-YFP. YFP fluorescence intensity in trichome neighbouring cells is comparable with that in trichome cells. (C,H) *gl3* TTG1:TTG1-YFP. The YFP signal is also found in the cytoplasm and shows similar strength in all cells. (D,I) *gl3 egl3* TTG1:TTG1-YFP. YFP signal distribution is similar to that in *gl3* (C,H). (E,J) *gl3 egl3 tt8* TTG1:TTG1-YFP. YFP signal distribution is similar to that in *gl3* (C,H). Scale bars: 10 μ m.

distribution and in 35S:GL3 lines the nuclear localized TTG1-YFP is increased to 89% (Table 2). During the course of these experiments, we noted that 35S:GL3 lines showed a subtle but significant depletion of the TTG1-YFP signal. We found 77% of the trichome fluorescence in the first tier of cells around the trichome initials, 85% in the second and 90% in the third with a significant difference between the first and the other two tiers (*t*-test, $P<0.05$) (Fig. 2F-H).

Our *TTG1* misexpression data in different cell layers indicated that GL3 plays an important role in TTG1 trapping. In order to test the relevance of other bHLH homologs of GL3 in TTG1-YFP localization, we tested also *gl3 egl3* double mutants and *gl3 egl3 tt8* triple mutants as GL3 and EGL3 act in a partially redundant manner and differ in their expression pattern in leaves; furthermore, TT8 is required for the development of leaf marginal trichomes (Zhang et al., 2003; Zhao et al., 2008). We saw a significant change in the *gl3 egl3* double mutant compared with the *gl3* single mutant, but the additional removal of *TT8* (*gl3 egl3 tt8*) had no additional effect (Table 2; Fig. 2D,E,I,J).

Taken together, we conclude that the intracellular localization of TTG1 depends largely on the bHLH proteins GL3 and EGL3 in a partially redundant manner, which is reflected in the mutant phenotype of single and double mutants of *gl3 egl3* (Zhang et al., 2003).

GL3 promotes the nuclear transport of TTG1

The regulation of nuclear targeting of GL3 and TTG1 was analysed using a heterologous yeast-based nuclear transportation trap (NTT) assay (Ueki et al., 1998). In this system the protein of interest is expressed as a translational fusion to an artificial transactivator LexAD (consisting of LexA DNA-binding domain and GAL4AD transactivation domain) fused to a nuclear export signal (NES) from HIV Rev protein (NES-LexAD-‘Protein-of-interest’). Owing to the presence of the NES, non-nuclear-targeted proteins that lack a functional nuclear localization signal (NLS) are excluded from the nucleus. However, nuclear-targeted proteins can overcome the NES-mediated nuclear export and enter the nucleus, thereby activating the LexAD-responsive *LEUCINE2*

Table 2. Quantification of nuclear concentration of TTG1-YFP expressed under TTG1 promoter in wild-type, *gl3*, *gl3 egl3*, *gl3 egl3 tt8* and 35S:GL3 lines

	Wild type	p35S:GL3*	<i>gl3</i> [‡]	<i>gl3 egl3</i> ^{§,¶}	<i>gl3 egl3 tt8</i> ^{¶,**}
Percentage of total YFP fluorescence in nucleus \pm s.d. ^{††}	69 \pm 6.9	89 \pm 4.4	56 \pm 6.9	48 \pm 6.8	49 \pm 6.8
<i>n</i>	175	188	345	385	290

WT, wild type; s.d., standard deviation; *n*, number of single cells in the patterning zone used for measurement.

*The values for 35S:GL3 are statistically significantly different from wild type ($P=2.03\times10^{-132}$).

[‡]The values for *gl3* are statistically significantly different from wild type ($P=5.7\times10^{-66}$).

[§]The values for *gl3 egl3* are statistically significantly different from wild type ($P=2\times10^{-133}$).

[¶]The values for *gl3 egl3* are statistically significantly different from *gl3* ($P=4.8\times10^{-46}$).

^{††}The values for *gl3 egl3 tt8* are statistically significantly different from wild type ($P=3\times10^{-109}$).

**The values for *gl3 egl3 tt8* are statistically not significantly different from *gl3 egl3* ($P=0.056$).

^{†††}Nuclear YFP fluorescence is expressed as a percentage of the total YFP fluorescence in the cell.

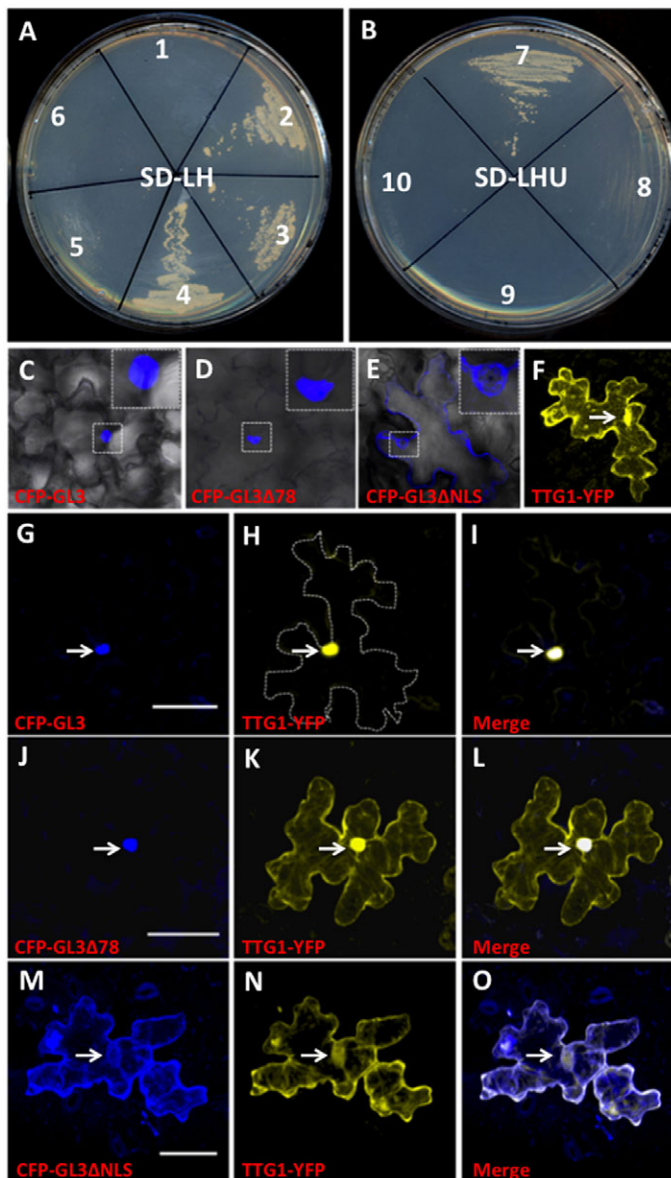


Fig. 3. Nuclear targeting of TTG1 by GL3. (A,B) Nuclear transportation trap (NTT) assay in yeast. When fusion protein (NES-LexAD-‘gene-of-interest’) is in the nucleus, the reporter gene is expressed, which allows growth on the medium lacking leucine. (A) NES-LexAD-SV40NLS (2), NES-LexAD-GL3 (3) and NES-LexAD-GL3Δ78 (4) activates the reporter gene, allowing growth on medium lacking leucine, whereas yeasts containing NES-LexAD (1), NES-LexAD-TTG1 (5) or NES-LexAD-TTG1ΔC26 (6) are unable to activate the reporter and cannot grow on medium lacking leucine. (B) When co-expressed with GL3, NES-LexAD-TTG1 allows growth on medium lacking leucine (7), whereas co-expression of GL3Δ78 with NES-LexAD-TTG1 (8), GL3 with NES-LexAD-TTG1ΔC26 (9) and GL3Δ78 with NES-LexAD-TTG1ΔC26 (10) did not result in the activation of reporter and cannot grow on medium lacking leucine. SD-LH, synthetic dropout medium lacking leucine and histidine; SD-LHU, synthetic dropout medium lacking leucine, histidine and uracil. (C-O) Transient expression in *Arabidopsis* cotyledon epidermal cells. (C-F) Subcellular localization of CFP-GL3 (C), CFP-GL3Δ78 (D), CFP-GL3ΔNLS (E) and TTG1-YFP (F). Deletion of TTG1 interaction domain in GL3 (GL3Δ78) does not affect its subcellular localization; deletion of NLS from GL3 shifts GL3ΔNLS protein completely to cytoplasm. (G-I) Co-expression of TTG1-YFP (G) and CFP-GL3 (H). The bulk of the TTG1-YFP is accumulated in the nucleus. (J-L) Co-expression of TTG1-YFP (J) and CFP-GL3Δ78 (K). Subcellular distribution of TTG1-YFP is similar to the situation when TTG1-YFP is expressed alone. (M-O) Co-expression of TTG1-YFP (M) and CFP-GL3ΔNLS (N). The bulk of the TTG1-YFP is in the cytoplasm and is co-localized with CFP-GL3. Insets in C-E show higher magnification of the nucleus. Scale bars: 50 μm. Arrows indicate the position of the nucleus.

(LEU2) reporter gene. In this assay, we found that TTG1 fused to the NES did not enter the nucleus, suggesting that it is not actively transported into the nucleus (Fig. 3A). By contrast, GL3 behaved as a nuclear protein when expressed as a fusion to NES in this assay (Fig. 3A). We extended the NTT assay using co-expression of NES-fused proteins and its interaction partners without NES attachment to test their interaction with respect to intracellular distribution. In this experiment, we found that co-expression of NES-LexAD-TTG1 fusion protein with GL3 protein resulted in a re-localization of TTG1 to the nucleus (Fig. 3B). This indicates that the nuclear localization of TTG1 is mediated by its direct interaction with GL3.

To demonstrate that the specific interaction between TTG1 and GL3 is responsible for nuclear targeting, we created TTG1 and GL3 variants that disturb their interaction. A TTG1 variant lacking the C-terminal 26 amino acids has been shown to lose the interaction with GL3 (Payne et al., 2000) and can no longer be targeted by GL3 to the nucleus in the yeast NTT assay (Fig. 3B). In the case of GL3, we used a deletion corresponding to an EGL3

deletion previously shown not to interact with TTG1 in yeast two hybrid screens (I. Zimmermann, PhD Thesis, University of Cologne, 2003). The resulting GL3 protein (hereafter referred as GL3Δ78) has an internal 78 amino acid deletion between 360-437 amino acids and showed no interaction with TTG1 in yeast two-hybrid analysis (Table 3). In the next experiment, we fused this 78 amino acid fragment from GL3 (hereafter referred as 78GL3) N-terminally to GUS in order to ensure stability and found binding to TTG1 in a yeast two-hybrid system. Control experiments revealed no binding of this fragment to GL3 itself, showing that the TTG1-GL3 interaction domain can be separated from the GL3-self-dimerization domain (Table 3) (Payne et al., 2000). Although the GL3Δ78 variant localizes to the nucleus, it is unable to trigger TTG1 nuclear localization in co-expression assays in yeast (Fig. 3B).

These findings were confirmed in plants using transient expression analysis following biolistic transformation of *Arabidopsis* epidermal cells. In one set of experiments, we co-expressed TTG1-YFP and CFP-GL3 in *Arabidopsis* cotyledon epidermal cells. While TTG1-YFP alone is localized in the nucleus and the cytoplasm (Fig. 3F), co-expression with CFP-GL3 (Fig. 3G) causes a nuclear localization of the majority of TTG1-YFP (Fig. 3H). CFP-GL3Δ78 was still found in the nucleus (Fig. 3D,J), but had no influence on the subcellular distribution of TTG1-YFP (Fig. 3K). Here, TTG1-YFP localization was similar to TTG1-YFP expressed alone.

In order to test whether targeting is mediated by the GL3 NLS signal, we also tested a CFP-marked GL3 variant lacking the NLS nuclear targeting sequence. GL3ΔNLS still interacts with TTG1 in

Table 3. Yeast two hybrid interaction between TTG1 and GL3 mutant proteins

	BD-TTG1	BD-TTG1ΔC26	BD-78GL3-GUS	BD-NLS-TTG1	BD-C26TTG1
AD-GL3	+	–	–	+	–
AD-GL3Δ78	–	–	n.d.	n.d.	n.d.
AD-78GL3-GUS	+	–	n.d.	n.d.	n.d.
AD-GUS	–	–	n.d.	n.d.	n.d.
AD-GL3ΔNLS	+	–	n.d.	n.d.	n.d.
AD-EGL3	+	–	n.d.	+	n.d.
AD-TT8	+	–	n.d.	+	n.d.

+, positive interactions; –, no interaction; BD, GAL4 DNA-binding domain; AD, GAL4 DNA activation domain; n.d., not determined.

yeast-two-hybrid assays (Table 3). The CFP-GL3ΔNLS was found in the cytoplasm (Fig. 3E,M) and co-expression experiments revealed that TTG1-YFP is not targeted to the nucleus anymore (Fig. 3N). The co-expression of TTG1-YFP with CFP-GL3 variants in onion epidermal cells showed the same results (supplementary material Fig. S1).

As deletions may cause aberrant protein functions, we aimed to demonstrate the relevance of TTG1-GL3 binding by an aptamer approach (Fig. 4). The aptamer approach uses a small protein fragment to compete with the binding of the two proteins under consideration (Rudolph et al., 2003). For this experiment, we used the 78 amino acids of the GL3 protein known to be relevant for the interaction with TTG1 (Table 3). To ensure the stability of the protein fragment and to enable its visualization, we fused GUS to the C terminus and RFP to the N terminus. This RFP-78GL3-GUS protein was co-expressed with CFP-GL3 and TTG1-YFP in onion epidermal cells as described above and the RFP-78GL3-GUS fluorescence was observed in the cytoplasm as expected (Fig. 4B). TTG1-YFP was observed both in the cytoplasm and the nucleus (Fig. 4C), and comparatively more signal was observed in the cytoplasm when compared with experiments carried out using RFP-GUS without the 78GL3 aptamer (Fig. 4D-F). This result suggests that RFP-78GL3-GUS interfered with the ability of GL3 to recruit TTG1-YFP to the nucleus (Fig. 4C). In order to demonstrate that this also occurs in trichomes, we analyzed the distribution of a TTG1-YFP variant not

binding to GL3 (Table 3), TTG1ΔC26-YFP. As expected, TTG1ΔC26-YFP localization was shifted to the cytoplasm. This redistribution, however, was much more pronounced than in *gl3 egf3* double mutants, indicating additional regulatory complexity for the intracellular localization of TTG1 (Fig. 5A). Attempts to show that the deleted 26 amino acids alone can bind to GL3 failed (Table 3; supplementary material Fig. S2)

NLS-TTG1 is able to move and does not affect TTG1 depletion

In a next step, we aimed to analyze whether nuclear targeting affects the intercellular mobility of TTG1. Towards this end, we created NLS-TTG1-YFP construct. In transient expression assays, the NLS efficiently targeted the fusion protein to the nucleus (Fig. 6A). Protein-protein interactions of NLS-TTG1-YFP with GL3 are indistinguishable from TTG1-YFP in yeast two hybrid interaction assay (Table 3). When this fusion protein was expressed under the *TTG1* promoter, *ttg1* mutants were rescued, indicating that the NLS fusion does not interfere with TTG1 function (Table 4). To test whether TTG1 mobility between cells is affected by NLS-mediated nuclear targeting, we expressed the NLS-TTG1-YFP fusion protein under the RBC promoter in the sub-epidermis of *ttg1* mutants. These plants showed a rescue of the trichome phenotype, indicating that sub-epidermal nuclear-targeted TTG1 can move from the sub-epidermis into the epidermis.

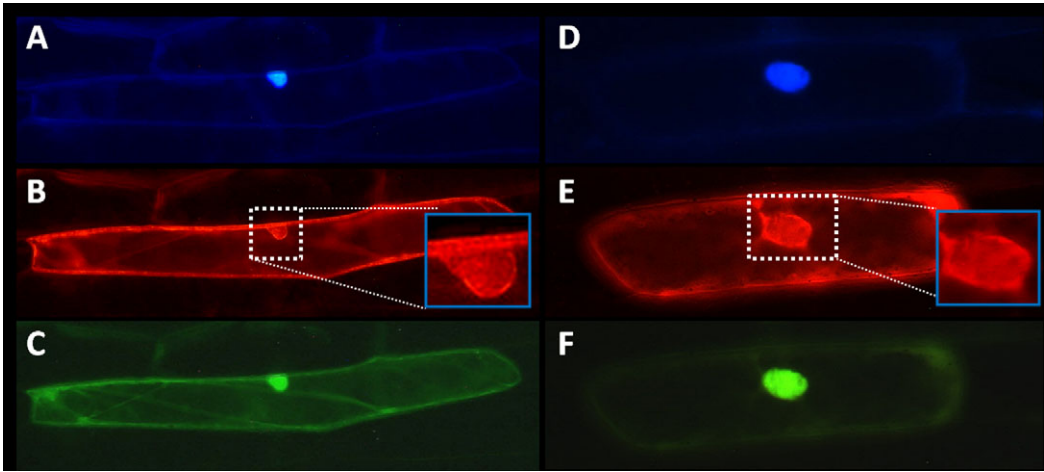


Fig. 4. GL3 fragment (78GL3) competes with GL3 for binding to TTG1 in aptamer approach. (A-C) Co-expression of TTG1-YFP, RFP-78GL3-GUS and CFP-GL3. (A) Localization of CFP-GL3 in the nucleus. (B) Localization of RFP-78GL3-GUS in the cytoplasm and weakly in the nucleus. (C) TTG1-YFP is localized in the cytoplasm and in the nucleus. (D-F) Co-expression of TTG1-YFP, RFP-GUS and CFP-GL3. (D) CFP-GL3 is localized in the nucleus. (E) RFP-GUS is localized in the cytoplasm and weakly in the nucleus. (F) Bulk of the TTG1-YFP is trapped in the nucleus. Inset in B and E shows a higher magnification of the nucleus.

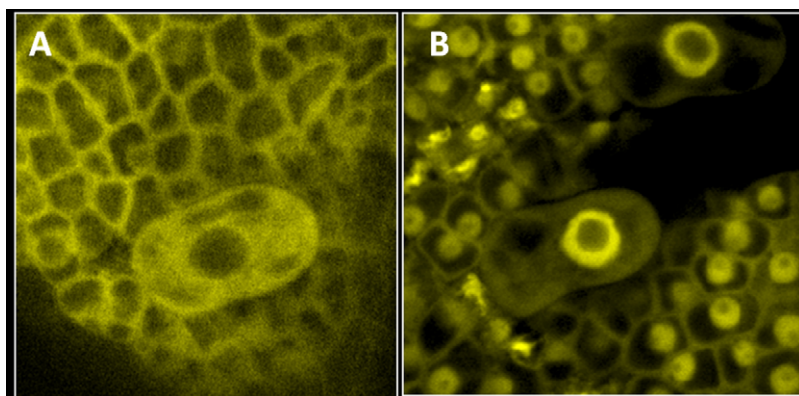


Fig. 5. TTG1ΔC26YFP shifts to the cytoplasm.

(A) TTG1:TTG1ΔC26-YFP. YFP fluorescence in the nucleus is drastically reduced as a result of the deletion of the GL3 interaction domain from TTG1. (B) TTG1:TTG1-YFP. YFP fluorescence is observed both in the nucleus and the cytoplasm.

If nuclear targeting does not change the intercellular mobility, one would expect that NLS-TTG1-YFP distribution should not be changed when compared with TTG1-YFP in a wild-type background. Consistent with this, we found a depletion around trichome in TTG1:NLS-TTG1-YFP lines (Fig. 6C-F).

The first tier of cells around the trichomes exhibited 45% of the YFP fluorescence found in trichomes, the second tier 62% and the third tier 74%. Statistical analysis showed that these values were highly significant (t -test, $P < 0.001$) similar to TTG1-YFP without NLS fusion. Together, these data show that the nuclear localization of TTG1 by GL3 is not as important as the trapping of TTG1 in the nucleus.

DISCUSSION

The TTG1 depletion model was initially derived from three observations (Bouyer et al., 2008): first, TTG1 protein can move between cells; second, the depletion of TTG1 in trichome neighbouring cells is not found in *gl3* mutants; and, third, GL3 can bind to TTG1. Because *GL3* is expressed in trichomes, it was proposed that TTG1 movement is regulated by GL3 such that the elevated GL3 levels in trichomes result in an accumulation of TTG1. However, the molecular nature that triggers TTG1 depletion by GL3 have not been elucidated so far and here we show that the direct interaction between TTG1 and GL3 give rise to nuclear translocation and trapping in trichomes, which is a prerequisite in the computational modelling that we proposed previously (Bouyer et al., 2008).

Is TTG1 movement controlled by GL3?

The depletion model predicts that TTG1 can freely move between cells and that its movement is retained by GL3 in trichomes. If this were the case, one would expect that GL3 should also be able to modulate TTG1 movement when expressed in a cell-type-specific manner. We tested this by analyzing the movement behaviour of TTG1-YFP between cell layers. This assay is based on the observation that TTG1-YFP can complement the epidermal trichome phenotype of *ttg1* mutants when expressed in the sub-epidermis (Bouyer et al., 2008). This rescue ability depends strongly on the presence of *GL3* as sub-epidermally provided TTG1 is no longer able to compensate for the loss of endogenous *TTG1* in a *gl3 ttg1* double mutant. Two additional findings are particularly relevant: first, the observation that exclusive expression of *GL3* in the sub-epidermis further precludes the rescue and prevents TTG1-YFP mobility from this layer; and, second, exclusive expression of *GL3* in the epidermis conversely enhances the RBC:TTG1-YFP-mediated trichome production dramatically.

Notably, GL3 seems to differ with respect to its intercellular mobility in the leaf and in the root. In contrast to leaves (Digiuni et al., 2008; Zhao et al., 2008), it has been suggested that GL3 moves in the root as the protein is found in atrichoblasts, whereas its RNA expression pattern is confined to trichoblasts in the root epidermis (Bernhardt et al., 2005).

GL3 itself has no obvious influence on the mobility of TTG1 between the sub-epidermis and the epidermal layer as the TTG1-YFP signal can clearly be detected in the L1 in *gl3* mutants when expressed from the L2. It is, however, possible that a redundant function of EGL3 masks a requirement of GL3 for TTG1 mobility. Yet, GL3 is essential to prevent further movement owing to recruitment of TTG1 to the nucleus and its binding within a immobile protein complex in a trichome initial. This is demonstrated by the retention of TTG1-YFP in the sub-epidermis when GL3 is co-expressed there (RBC:GFP-GL3). Together, these data demonstrate that the mobility of TTG1 is controlled by GL3.

Intracellular localization of TTG1 and its regulation by GL3

Whether TTG1 is present and/or required predominantly in the nucleus or in the cytoplasm is not clear from the current data. On the one hand, cell fractionation studies showed that the ortholog of TTG1 from *Petunia*, AN11, is cytoplasmic (Vetten et al., 1997). On the other hand, molecular data indicate that TTG1, together with GL3, directly controls the expression of a common set of target genes and that GL3 protein forms subnuclear speckles in the absence of TTG1, indicating a role for TTG1 in the nucleus (Gonzalez et al., 2008; Zhao et al., 2008; Yoshida et al., 2009). As TTG1 does not contain any obvious NLS motif, an active nuclear translocation seems unlikely (Walker et al., 1999). A direct assessment of its localization in *Arabidopsis* using TTG1-YFP fusions revealed nuclear and cytoplasmic fluorescence (Bouyer et al., 2008; Zhao et al., 2008). Because GL3 was shown to control the distribution of TTG1-YFP in the epidermis, we speculated that the intracellular

Table 4. Trichome rescue efficiency of nuclear-targeted TTG1-YFP

Genotype	Number of trichomes \pm s.d.	<i>n</i>
<i>ttg1-13</i> TTG1:NLS-TTG1-YFP	128 \pm 18	26
<i>ttg1-13</i> RBC:NLS-TTG1-YFP	124 \pm 30	26
<i>ttg1-13</i> TTG1:TTG1-YFP	127 \pm 20	26
<i>ttg1-13</i> RBC:TTG1-YFP	98	1

Trichome number is the average number of trichomes on leaves 3 and 4 in T1 plants; s.d., standard deviation; *n*, number of plants analyzed.

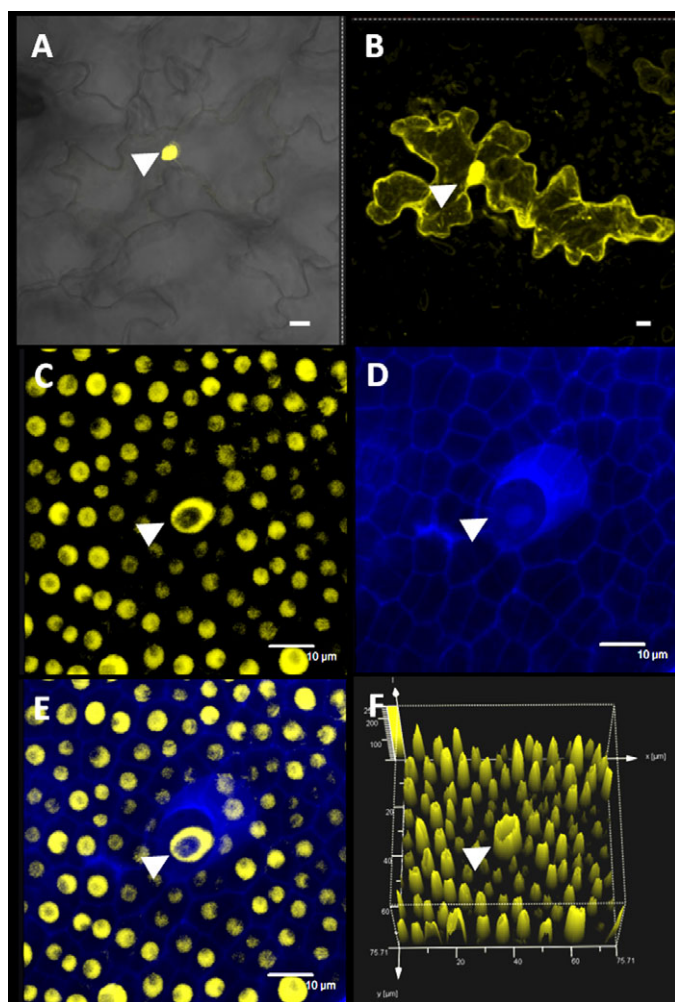


Fig. 6. Cellular distribution of nuclear targeted TTG1-YFP.

(A,B) Transient expression of NLS-TTG1-YFP(A) and TTG1-YFP (B) in *Arabidopsis* cotyledon epidermal cells. (C-F) Distribution of NLS-TTG1-YFP in the epidermal cells of the trichome patterning zone on leaves in lines carrying TTG1:NLS-TTG1-YFP. (C) NLS-TTG1-YFP distribution. (E) Cell walls counterstained with propidium iodide. (E) Overlay image of B and C. (F) Intensity projection images of confocal stacks of TTG1:NLS-TTG1-YFP lines. The fluorescence intensity is indicated by size of the peaks. Arrowheads indicate the nucleus. Scale bars: 10 μ m.

localization of TTG1 is also controlled by GL3 such that nuclear localized GL3 targets TTG1 to the nucleus. We confirmed this hypothesis in several experiments. First, in planta analysis of TTG1-YFP expressed under the *TTG1* promoter in wild-type, *gl3* mutant and 35S:GL3 lines demonstrated that the fraction of nuclear TTG1-YFP depends on the amount of GL3. Second, co-expression of CFP-GL3 and TTG1-YFP in *Arabidopsis* cotyledon epidermal cells, as well as in onion epidermal cells, showed that TTG1-YFP is targeted by GL3 to the nucleus and that GL3 mutations interfering with the GL3-TTG1 interaction cannot trigger nuclear targeting anymore. Third, yeast nuclear targeting assays showed that TTG1 has no functional nuclear localization sites. Instead, GL3 recruits TTG1 into the nucleus and this depends on the direct interaction between GL3 and TTG1. These data are in line with the observation that the TTG1 homolog PFW1 in *Perilla frutescens* accumulates in the nucleus when co-expressed with bHLH gene MYC-RP (Sompornpailin et al.,

2002), suggesting an evolutionary conservation of this mechanism but offering the possibility that different nuclear and cytoplasmic functions might exist because AN11 has been shown to be cytoplasmic in petunia (Vetten et al., 1997).

bHLH redundancy in the regulation of TTG1 nuclear localization

The bHLH genes involved in TTG1-regulated pathways have partial functional redundancy. *TT8* regulates seed coat mucilage production, seed coat pigment production, anthocyanin biosynthesis and trichome development (Zhang et al., 2003; Baudry et al., 2004; Maes et al., 2008). *EGL3* controls seed coat pigmentation, seed coat mucilage production, anthocyanin biosynthesis, trichome and root hair development, and *GL3* is involved in anthocyanin biosynthesis, trichome and root hair development (Nesi et al., 2000; Bernhardt et al., 2003; Zhang et al., 2003; Baudry et al., 2004; Bernhardt et al., 2005; Baudry et al., 2006; Gonzalez et al., 2008; Maes et al., 2008). All three bHLH proteins interact with TTG1 in yeast two hybrid assays and it is therefore conceivable that they all can sequester TTG1 in the nucleus. Our analysis of double and triple mutants revealed that GL3 has a major role and that EGL3 acts redundantly in this respect, whereas TT8 cannot further shift the TTG1-YFP nuclear/cytoplasm balance in the *gl3 egl3 tt8* triple mutant.

A comparison of the TTG1-YFP nuclear/cytoplasm balance between the *gl3 egl3 tt8* triple mutant and the TTG1 Δ C26-YFP protein revealed a noteworthy discrepancy. About 50% of the TTG1-YFP is found in the cytoplasm in the triple mutant. By contrast the TTG1 Δ C26-YFP protein that does not interact with the bHLH proteins anymore is exclusively found in the cytoplasm. One possibility to explain this is the involvement of additional factors in the regulation of TTG1 localization. This may be caused by a loss of interaction between TTG1 Δ C26-YFP and further nuclear translocation regulators or by promoting its interaction with components that direct its cytoplasmic distribution, or a combination of both. As GFP on its own or even as double- and triple-fusion still appears both nuclear and cytoplasmic (Kim et al., 2005), a passive mechanism is unlikely to account for the exclusively cytoplasmic compartmentalization of TTG1 Δ C26-YFP.

The second explanation would introduce the additional assumption that movement into the nucleus is actively mediated by the deleted C-terminal 26 amino acids. However, such an active nuclear transport mechanism seems unlikely, as TTG1 does not seem to contain any NLS in the yeast NTT assay (Fig. 3A).

Nuclear trapping and intercellular transport

In most experimental systems, nuclear targeting and intercellular transport are interrelated. Mutations or modification in CAPRICE (CPC), KNOTTED1 (KN1) and SHORT ROOT (SHR) proteins that reduce their nuclear localization also affect the mobility between cells (Lucas et al., 1995; Prochiantz and Joliot, 2003; Gallagher et al., 2004; Kurata et al., 2005). Whereas these transcription factors have been shown to require a nuclear localization signal for intercellular transport, nuclear targeting of GFP partially reduced its passive transport ability (Crawford and Zambryski, 2000). These examples suggest that nuclear targeting can prevent, but might also be required for, the movement of proteins into neighbouring cells. A more detailed analysis of SHR protein movement indicated that nuclear as well as cytoplasmic localization are relevant for intercellular movement (Gallagher and Benfey, 2009). By contrast, steady cytoplasmic localization of TTG1 does not seem to be relevant for intercellular protein movement. This is suggested by the

finding that plants expressing a NLS-TTG1-YFP fusion protein exhibit exclusively a nuclear localization signal and when expressed in the sub-epidermis can fully rescue the epidermal trichome phenotype (Table 4; Fig. 6).

Principles of protein movement regulation during patterning

The intercellular movement of transcription factors has been shown to play an important role in various patterning processes in plants, including cortex/endodermis specification, root hair and trichome patterning. In such cases, protein movement provides positional information to other cells, which makes it extremely important to control their distribution in the tissue. This can, in principle, be achieved in two ways. One principle is the local production of a protein that in turn can move into the surrounding tissue thereby forming a gradient. This is thought to be realized during trichome patterning where the R3 single repeat inhibitors are produced in trichomes from where they can move into the neighbouring cells (Kurata et al., 2005; Diguni et al., 2008; Wester et al., 2009). A second principle is the trapping of moving proteins. This has been elegantly demonstrated for the SHR protein, which is sequestered by SCARECROW (SCR) in the nucleus (Cui et al., 2007). In this system, SHR is produced in the stele and its movement into outer tissue layers is restricted by SCR trapping. Thus, trichome patterning relies on a similar molecular mechanism, although the systems in which these different patterning processes take place are strikingly different. Here, trapping is thought to create a depletion pattern from an initial ubiquitous distribution, whereas root patterning is strongly based on positional cues that give rise to a highly regular radial and epidermal distribution of distinct cell types. Here, we could show that the protein interaction between TTG1 and GL3 is necessary for the intracellular and epidermal distribution pattern of TTG1, which in mathematical simulations is required and sufficient to generate a spacing pattern (Bouyer et al., 2008).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.072454/-/DC1>

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