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New insights into the subcellular localization of tubby-like proteins and their participation in the *Arabidopsis-Piriformospora indica* interaction

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Abbreviations: CLSM, confocal laser-scanning microscopy; GFP, green fluorescing protein; PIP_2, phosphatidylinositol 4,5-bisphophate; dai, days after inoculation; PM, plasma membrane; TLP, Tubby-like protein

Tubby-like proteins (TLPs) have been associated with hormone signaling and responses to abiotic and biotic stress in plants. Recently, *Arabidopsis thaliana* TLP3 was found to translocate from the plasma membrane of cells in response to distinct abiotic stresses, thereby activating cellular signaling. In addition, several AtTLPs were demonstrated to be necessary for normal colonization of roots by the mutualistic fungus *Piriformospora indica*. Here, we present evidence for the involvement of another two AtTLPs in this interaction. Furthermore, we show that plasma membrane targeting of TLPs might be conserved in other plant species, although we did not find it for all members of the protein family. Finally, the position of a GFP-tag influences the localization of AtTLP3, which needs to be considered when working with TLPs.

Introduction

TUBBY, the founding member of the Tubby-like protein (TLP) family, has first been described in mice (*Mus musculus*) where mutations in the gene were associated with obesity, infertility and loss of vision and hearing.[1-5] In addition, four mammalian TLPs (TLP1–4) have been identified.[6-8] Knockout of *MmTLP3* causes embryonic lethality, while mutations in *TLP1* are associated with retinal degeneration, in mice as well as in humans.[9-12] On a molecular level, TUBBY and TLPs were shown to act as transcriptional regulators or to bridge molecules in vesicular trafficking and phagocytosis.[13] Located at the C-terminus of the proteins, the Tubby domain is the hallmark of all TLPs and its specific interaction with the phospholipid phosphatidylinositol 4,5-bisphophate [PtdIns(4,5)P_2] targets MmTUBBY to the plasma membrane (PM).[14,15] G protein-coupled receptor activation by neurotransmitters causes translocation of the protein to the nucleus and a DNA binding activity of the Tubby domain indicated an involvement of TUBBY in transcriptional regulation.[14,15]

TLPs have been found in many eukaryotic species.[16] Although conservation of the Tubby domain in different organisms suggests important functions, little information is available for TLPs in plants. They are present in small gene families with 11 members in *Arabidopsis thaliana* and *Populus trichocarpa*, respectively and 14 members in *Oryza sativa*.[16-18] In *Arabidopsis*, AtTLP9 acts in ABA signaling during germination and plays a role in responses to salt and drought stress.[17,19] Consistent with this, a TLP from chickpea was recently demonstrated to confer increased tolerance to salt, drought and oxidative stress when overexpressed in tobacco.[20] Furthermore, two studies in rice implicated functions of OsTLPs in plant immunity.[21,22] In a recent study, we reported that AtTLPs may act as compatibility factors in the interaction between *Arabidopsis* roots and the mutualistic fungus *Piriformospora indica*.[23] In addition, the subcellular localization of AtTLPs was investigated using green fluorescing protein (GFP) fusions and confocal laser-scanning microscopy (CLSM). The Tubby domains of AtTLP3 and several other AtTLPs were found to be located at the PM in *Arabidopsis* leaf epidermal cells. As reported for MmTUBBY, PM targeting of AtTLP3 was most likely caused by interaction with PtdIns(4,5)P_2.[15,23] Moreover, the protein was released from the PM in response to salt and drought stress as well as hydrogen peroxide, presumably translating the extracellular stress into adaptive intracellular signaling.[23]

Here, T-DNA insertion lines for *AtTLP1* and *AtTLP11* were tested for their colonization by *P. indica*. Furthermore, we show results on the subcellular localization of other plant TLPs, adding

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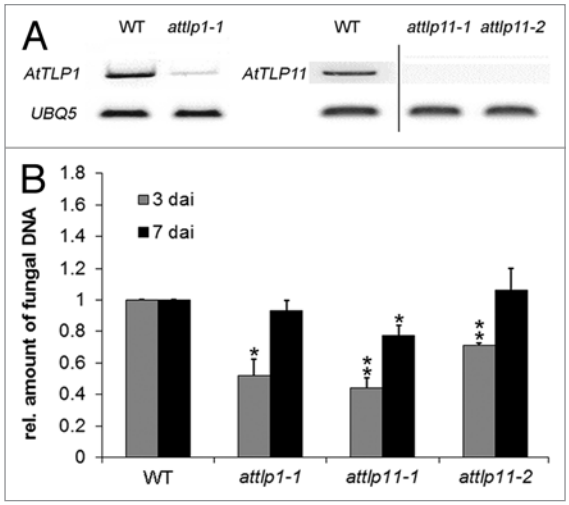

Figure 1. Colonization of *attlp* lines by *P. indica*. (**A**) *AtTLP1* and *AtTLP11* transcript levels in wild type (Col-0) and respective *attlp* mutant lines. Semi-quantitative PCR was performed with cDNA generated from 3-wk-old plants. Amplification of an *AtUBI5* fragment was included as a control. (**B**) *P. indica* colonization in wild type and *attlp* lines. Three-wk-old aseptically grown plants were inoculated with *P. indica*. Genomic DNA was isolated from roots harvested at 3 and 7 dai. qRT-PCR was performed using plant (*AtUBI5*) and *P. indica* (*ITS*) specific primers. Results were analyzed using the $2^{-\Delta CT}$ method to compare colonization in wild type and mutant plants.[37] Data are averages (+SE) from at least three independent biological experiments. About 200 plants were analyzed per *Arabidopsis* line and time point in each experiment. Asterisks indicate significance at $p < 0.05$ (*), 0.01 (**), 0.001 (***) as analyzed by Student's t-test.

a note of caution when generating TLP-GFP fusions and discuss the possible influence of mutations in the PtdIns(4,5)P_2 binding site on the subcellular localization of TLPs.

Results and Discussion

AtTLP1 and -11 are required for normal colonization of *Arabidopsis* roots by the mutualistic fungus *Piriformospora indica*. In contrast to many mutualistic microbes, *P. indica* is able to colonize the roots of a great number of plant species, thereby conferring various beneficial traits to its hosts. Plants living in association with the fungus displayed increased growth, higher seed yield and enhanced tolerance to biotic and abiotic stresses.[24] As *P. indica* has been sequenced and is accessible for genetic modifications, it is considered as suitable model for the study of mutualistic plant-microbe interactions.[25,26]

In *Arabidopsis*, the mutualist employs a biphasic colonization strategy.[27,28] Whereas living root cells are colonized by *P. indica* during the first 3 d after inoculation (dai), fungal growth depends on plant cell death at later time points. Several factors have been identified to influence this colonization process. For example, signaling through the phytohormones jasmonic acid and gibberellins generally promote root colonization, while it is inhibited by salicylic acid signaling.[27] A delayed colonization by *P. indica* was recently demonstrated for mutant lines with reduced transcription of *AtTLP* genes, suggesting significant roles for members of this protein family in the interaction.[23] These experiments comprised lines for seven of the 11 *AtTLPs*.

Here, fungal colonization was determined for T-DNA insertion lines of two additional *AtTLP* genes. Transcription of *AtTLP1* was strongly reduced in *attlp1-1*, while *attlp11-1* and *attlp11-2* were complete knockouts (**Fig. 1A**). To test for fungal proliferation, roots of 3-wk-old mutant and wild type plants were inoculated with *P. indica* spore suspension and harvested at different time points. The relative amount of fungal DNA was determined by quantitative real-time (qRT) PCR (**Fig. 1B**). A significantly reduced fungal colonization could be detected in all three lines at 3 dai (biotrophic phase), while wild type levels were reached in mutant plants at 7 dai (cell death dependent phase). In line *attlp11-1*, a significantly reduced colonization was found at 7 dai.

The results suggest that like other *AtTLP* genes *AtTLP1* and *AtTLP11* support *Arabidopsis* colonization by *P. indica*. In addition, it demonstrates that individual AtTLP proteins contribute to the colonization success, which is in clear contrast to a suggested redundant function in abiotic stress signaling (e.g., salt stress).[23] The suggested plastidial localization of certain AtTLPs after PM dislodgement indicates a possible function of plastids in *P. indica* colonization.[23] Although our localization experiments have been restricted to leaves so far, *AtTLPs* have been reported to be expressed in *Arabidopsis* roots and root plastids have been implicated to function in symbiotic interactions.[17,23,29,30] Arbuscules, intracellular hyphae of mycorrhizas, are surrounded by plastids.[29] Moreover, cells hosting arbuscules are enriched in plastids that form extensions. These so called stromules connect plastids and probably other organelles (e.g., nucleus) and are hypothesized to support the exchange of metabolites and proteins thereby facilitating organellar communication and cell signaling.[30] It is tempting to speculate that TLPs support *P. indica* colonization by participating in stromule development or enrichment.

The position of GFP influences the subcellular localization of AtTLP3 fusion proteins. AtTLP3, the best studied plant TLP regarding subcellular localization, consists of a short leading sequence of about 50 amino acids followed by an F-box domain, a short 10 amino acid linker and the C-terminal Tubby domain (**Fig. 2**). In our previous study, we could not detect fluorescence from an AtTLP3-GFP chimera in *Arabidopsis*.[23] We therefore generated truncated versions of AtTLP3 and studied their subcellular localization. The N-terminal part of AtTLP3 (AtTLP3Δ116-406-GFP) located to plastids, the cytosol and the nucleus, while its C-terminal part (GFP-AtTLP3Δ1-115) was detected at the PM. PM localization was also observed for the full-length polypeptide when expressed in *Nicotiana benthamiana* with GFP inserted between the linker sequence and the Tubby domain.[23]

Translational fusions with fluorescing proteins like GFP are common tools to study the subcellular localization of proteins. However, when inserted close to a functional domain, fluorescing protein tags may interfere with subcellular targeting, resulting in mislocalization of the fusion protein. For example, AtNDPK2 showed plastidial targeting when fused C-terminally

to GFP while a nucleo-cytosolic localization was reported when GFP was placed N-terminal to AtNDPK2.[31,32] Therefore, we examined possible positional effects of GFP on the localization of AtTLP3. For this, GFP was placed in front of the N-terminal part of AtTLP3 (GFP-AtTLP3Δ116-406), or C-terminal to its Tubby domain (AtTLP3Δ1-115-GFP), reversing its position in comparison to the AtTLP3 versions described above. The chimeras were transiently expressed in *Arabidopsis* leaf epidermal cells and their subcellular localization was observed using CLSM. No plastidial localization was found for GFP-AtTLP3Δ116-406. Instead, the protein was detected solely in the cytosol and the nucleus. Thus, similar to AtDNKP2, N-terminal GFP apparently inhibited plastidial localization of GFP-AtTLP3Δ116-406. Interestingly, AtTLP3Δ1-115 could not be observed at the PM, when GFP was attached to its C-terminus, but showed a nucleo-cytosolic localization instead. Presumably, GFP blocked the interaction of the Tubby domain with PtdIns(4,5)P_2. The data presented, therefore, demonstrate restrictions of GFP fusion constructs to study TLP localization in plants. Our results further show that the mislocalization of proteins in dependency of the intramolecular position of GFP tags needs to be considered for functional studies. Accordingly, previous analyses indicated the effect of tags on the function of BAK1 in immune signaling.[33]

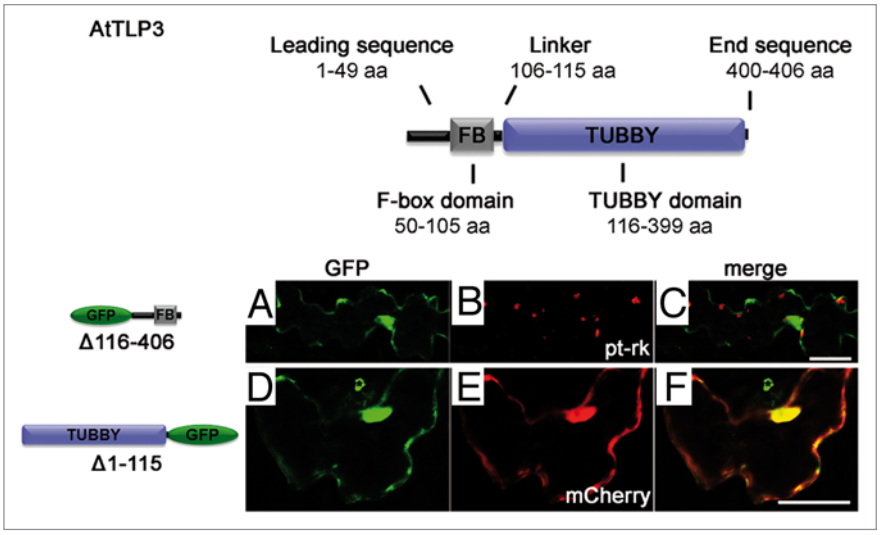

Figure 2. Subcellular localization of AtTLP3 variants fused to GFP. Top: Domain organization of AtTLP3 (aa: amino acids). (**A–F**) Subcellular co-localization of GFP-AtTLP3 deletion variants and red fluorescing cellular markers (pt-rk, mCherry) in single *Arabidopsis* leaf epidermal cells transiently transformed using a biolistic approach. Proteins were expressed under control of the cauliflower mosaic virus 35S promoter. Yellow color in merged images (**C and F**) indicates co-localization of green and red fluorescing proteins. Constructs used for transformation are indicated on the left. (**A–C**) Nuclear and cytosolic localization of (**A**) GFP-AtTLP3Δ116-406 in cells co-transformed with (**B**) the plastidial marker pt-rk.[38] (**D–F**) Nuclear and cytosolic localization of AtTLP3Δ1-105-GFP in cells co-transformed with the nucleo-cytosolic marker mCherry. Experiments were repeated three times with similar results. Bars = 20 μm.

Natural changes in the PtdIns(4,5)P_2 binding cavity influence the subcellular localization of TLPs. PM targeting of TLPs is conserved across species and kingdoms.[15,23] Nevertheless, in plants it has only been described for TLPs from *Arabidopsis* so far.[23] Therefore, we fused GFP N-terminally to the Tubby domain of a barley (*Hordeum vulgare*) TLP (GenBank: AK251904.1) designated HvTLP12 for its similarity to rice TLP12.[18] The chimera (GFP-HvTLP12Δ1-123), was observed at the PM when expressed in *Arabidopsis* leaf epidermal cells (**Fig. 3D**). This demonstrated that PM targeting of TLPs might be conserved across plant species.

A detailed study of the PtdIns(4,5)P_2 binding site in MmTUBBY revealed lysine (K) 330 and arginine (R) 332 as the most essential amino acids for the interaction.[15] In addition, a change of the corresponding amino acids (K187 and R189) in AtTLP3 to alanine abrogated PM targeting of the polypeptide, underlining the crucial role of these amino acids for PtdIns(4,5)P_2 binding also in plant TLPs.[23] The same amino acids and the PtdIns(4,5)P_2 binding site in general are also conserved in HvTLP12 (**Fig. 3**). However, although the PtdIns(4,5)P_2 binding site is well conserved in all mammalian TLPs, not all of them have been detected at the PM.[13] Thus, although PM targeting of TLPs cannot be inferred from the presence of the conserved PtdIns(4,5)P_2 binding cavity alone, the amino acid sequence of the binding site might still give useful hints toward the localization of the proteins. In AtTLP8, for example, the respective K and R residues are replaced by serine (S) and threonine (T) (**Fig. 3**), making PtdIns(4,5)P_2 binding unlikely. Consistent with this, the Tubby domain of AtTLP8 was not detected at the PM when fused to GFP.[23] Other AtTLPs show more subtle changes in the KxR sequence. AtTLP5 features $K_{190}L_{191}K_{192}$ (**Fig. 3**). Although K and R are both positively charged amino acids, a change from R to K can have dramatic effects on binding to phosphates.[34] Therefore, the subcellular localization of the AtTLP5 Tubby domain fused to GFP was investigated (**Fig. 3A–C**). Although fluorescence obtained from this chimera was generally very low, no hints for PM targeting were found. Instead the polypeptide was observed in the cytosol and nucleus. However, AtTLP5 has been described not to be expressed in leaves.[17] This might give rise to concerns about an artificial localization in our experiments caused by expressing the protein in cells where it generally does not occur. Nevertheless, in our studies, promoter activity for *AtTLP5* was detected in rosette leaves in *AtTLP5*$_{Promoter}$::*GUS* lines, suggesting that the protein actually is present in this tissue (**Fig. 3**, right panel).

Taken together, the composition of the PtdIns(4,5)P_2 binding site indicates the PM binding ability of TLPs. Future studies will show if and which other changes in the binding cavity have an influence on the subcellular localization of TLPs and how this is linked to their function. This would not only enhance our knowledge on this interesting protein family but will also

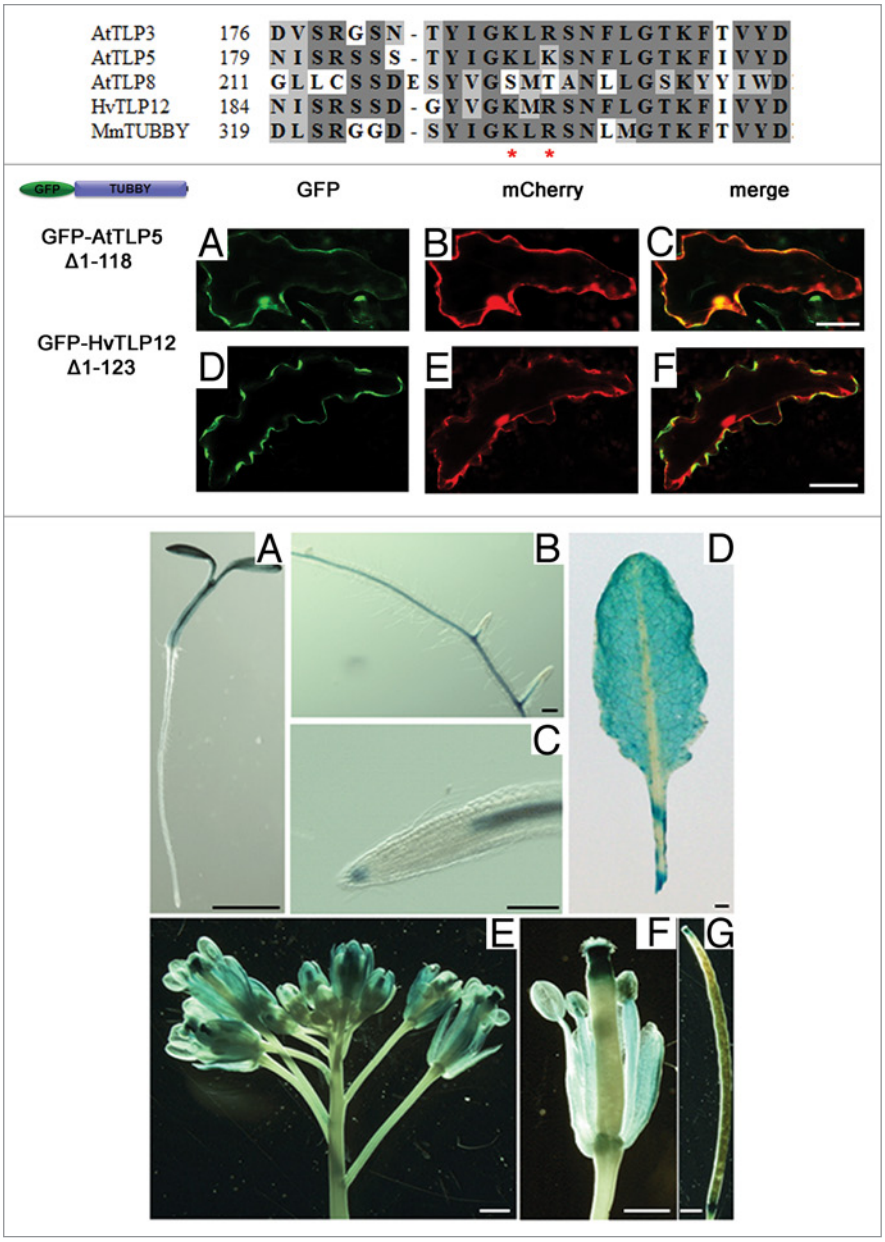

Figure 3. PtdIns(4,5)P_2 binding site composition influences the localization of TLPs. Top panel: Amino acid sequence alignment of MmTUBBY and several plant TLPs, showing a part of the Tubby domain. Red asterisks: amino acids essential for PtdIns(4,5)P_2 binding in AtTLP3 and mouse TUBBY. Middle panel: Subcellular localization of the Tubby domain of AtTLP5 and HvTLP12. Cells were co-transformed with (**A and D**) a TLP-GFP chimera and (**B and E**) the red-fluorescing nucleo-cytosolic marker mCherry. Yellow color in merged images (**C and F**) indicates co-localization of green and red fluorescing proteins. (**A–C**) Nuclear and cytosolic localization of GFP-AtTLP5Δ1–108. (**D–F**) PM localization of GFP-HvTLP12Δ1-123. Experiments were repeated three times with similar results. Bars = 20 μm. Bottom panel: Histochemical analysis of GUS activity in *AtTLP5*$_{Promoter}$::*GUS* transgenic plants. (**A**) Four-d-old seedling, (**B–C**) roots of 18-d-old seedlings, (**D**) rosette leaf, (**E**) anthotaxy, (**F**) flower with some sepals and petals removed, (**G**) mature silique. Bars: 1 mm in (**A and D–G**); 0.1 mm in (**B–C**). Similar results were found for two independent transgenic lines.

be useful in predicting the subcellular localization of TLPs from other plant species.

Materials and Methods

Plant growth conditions and determination of *AtTLP* transcript abundance. *Arabidopsis* lines were obtained from the Nottingham *Arabidopsis* Stock Centre.[35] Presence of T-DNA insertions in *attlp1-1* (N674227), *attlp11-1* (N662879) and *attlp11-2* (N663172) was confirmed by PCR using primers flanking putative insertion sites (*attlp1-1*: 5'-TTT GAT TTC ATC TTT GGG CAG-3'/5'-GAA ACA AAG ACT CCA GAT TCT GG-3'; *attlp11-1*: 5'-TAG CGT CGG TTG AAA CAA AAG-3'/5'-AGG CTG CAG AAG ATA CAC ACC-3'; *attlp11-2*: 5'-AAA AGG GAC CTT TCC ACA CAC-3'/5'-CAT CTC TCT CAA GCA GGT TCG-3') and a T-DNA specific primer (LBb1.3 5'-ATT TTG CCG ATT TCG GAA C-3'). Plants were grown aseptically on vertically placed squared Petri.[23] Semi-quantitative reverse transcription PCR was performed as described before using the following primers: *AtTLP1* fwd/rev: 5'-ATG TCG TTC CGT AGC ATA-3'/5'-TTA TTC GCA AGC AAG TTT TGT G-3'; *AtTLP11* fwd/rev: 5'-GTA TGA CCT TAC GTA GCT TAA TCC-3'/5'-TCA TTC ACA AGC GAT TCT AGT C-3'. The *Arabidopsis Ubiquitin 5* (*AtUBI5*) transcript was used as constitutive control.[23]

Quantification of *P. indica* colonization. Roots of 3-wk-old plants were inoculated with *P. indica* isolate DSM11827 spore suspension (500,000 spores/ml) and harvested at 3 and 7 d after inoculation. Fungal colonization was analyzed by qRT-PCR as described by us before.[23]

Microscopic studies. For generation of GFP constructs, the respective parts of the *TLP* coding sequences were amplified from Col-0 cDNA by PCR (AtTLP3Δ116-406 fwd/rev: 5'-GCG GCC GCG ATG TCC TTC AAG AGT CTC ATT CAG-3'/5'-CCC GGG TCA CTG TTT GAG GGA GAT AGG AAA AGT G-3'; AtTLP3Δ1-105 fwd/rev: 5'-GTC GAC ATG CCG GGT CCT AGA GGA TCA C-3'/5'-GCG GCC GCT TCA CAT GCT ATC TTG GTG TCG-3'; AtTLP5Δ1-108 fwd/rev: 5'-GCG GCC GCA CCC GGG CCT AGG GAT-3'/5'-ACT AGT TTA TTC ACA TGC CAA TTT AGT ATC AAA GGT G-3') adding terminal restriction sites. Fragments were ligated in frame with *GFP* in a modified pAMPAT-MCS vector backbone (AY436765) behind a cauliflower mosaic virus 35S promoter.

HvTLP12Δ1-123 was amplified from *Hordeum vulgare* L. var Golden Promise cDNA (fwd/rev: 5'-GCG GCC GCT CCA GGA CCT CGA GAT GG-3'/5'-CCC GGG TCA TTC ACA CGC CAG CTT-3'). Terminal restriction sites were used as follows: *AtTLP3Δ116-406*: SalI/*Not*I; *AtTLP3Δ1-105*: *Not*I/XmaI; *AtTLP5Δ1-108*: *Not*I/*Spe*I; *HvTLP12Δ1-123*: *Not*I/XmaI. An ATG start codon was added for *AtTLP3Δ1-105* between the 5' restriction site and the coding sequence. Transformation of *Arabidopsis* leaf epidermal cells and confocal laser scanning microscopy were performed as described before.[23]

Generation of $AtTLP5_{Promoter}$::*GUS* lines and histochemical GUS-staining. 1,231 bp upstream of the translational start site of *AtTLP5* (AT1G43640) were amplified from genomic DNA by PCR (fwd/rev: 5'-TTT TGG TGA AAA ATG GTA TAC ATC-3'/5'-TTT TCC GAA TAC CAA AGA TTC TA-3'). The amplification product was ligated to *Xcm*I digested pCXGUS-P in front of the β-*glucuronidase* gene to generate the vector $AtTLP5_{Promoter}$::*GUS*.[36] Generation of stable transgenic *Arabidopsis* lines with $AtTLP5_{Promoter}$::*GUS* and analysis of GUS activity was performed as reported previously in reference 23.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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