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SHORT COMMUNICATION

Dimerization of GTR1 regulates their plasma membrane localization

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ABSTRACT

Members of the nitrate transporter 1/peptide transporter family (NPF) are multifunctional transporters of various compounds including plant hormones and play important roles in plant growth and responses to environmental stress. Recently, we found that Arabidopsis GTR1 (also known as NPF2.10) takes up gibberellic acid and jasmonoyl-L-isoleucine in addition to glucosinolates. For normal plant growth, *GTR1* is regulated at the gene expression level; however, it is unclear whether post-translational regulation also occurs. Here, we found that dimerization of GTR1, possibly induced by dephosphorylation of the Thr residue located between the possible transmembrane regions, regulates its plasma membrane localization, leading to transport of glucosinolates and gibberellic acid in *Xenopus* oocytes. These findings suggest that dimerization of multifunctional transporters contributes to their activities at the plasma membrane.

Abbreviations: NPF, Nitrate transporter 1/peptide transporter family; GTR1, glucosinolate transporter 1; GA_3, Gibberellin A3

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The NPF is responsible for the transport of various compounds including plant hormones.[1,2] In Arabidopsis, 53 members of the NPF have been identified, which transport nitrate, amino acids, peptides, glucosinolates, auxin, abscisic acid, gibberellin, and jasmonoyl-L-isoleucine.[3-9] Recently, we found that GTR1/NPF2.10 is a multifunctional transporter of gibberellic acid and jasmonoyl-L-isoleucine in addition to glucosinolates.[4,7,9] GTR1 has several physiologic roles in stamen development, systemic wound responses, and glucosinolate translocation in Arabidopsis.[4,7,9] According to its multifunctionality and physiologic importance, expression of *GTR1* is tightly regulated according to the growth stage and in response to environmental changes.[7,9] However, the regulatory mechanism remains unclear.

The nitrate dual-affinity transporter NRT1.1 (also known as CHL1 and NPF6.3) takes up nitrate over a wide range of concentrations.[10,11] The de/phosphorylation status of Thr at position 101 located in the loop between the third and fourth transmembrane (TM) regions determines its affinity for nitrate. Substitution of Ala/Asp for the Thr residue (the nonphosphorylated form T101A or the phosphorylation-mimicking form T101D) results in monophasic low-affinity (T101A) or high-affinity (T101D) transport activity.[11] Fluorescence resonance energy transfer assays concluded that the T101A mutant forms a dimer and the T101D mutant remains as a monomer.[12] In the GTR1 amino sequence, the Thr residue (T135) is preserved between the third and fourth TM regions. GTR1 imports glucosinolates, gibberellin A3 (GA_3), and jasmonoyl-L-isoleucine into *Xenopus* oocytes.[4,7,9] To examine the effect of phosphorylation at the Thr residue, substrate uptake rates were plotted against glucosinolate or GA_3 concentrations for GTR1-WT, T135A, and T135D (Fig. 1). In terms of glucosinolate transport, K_m values did not significantly differ between GTR1-WT and these mutants (WT = 61 μM, T135A = 62 μM, and T135D = 27 μM); however, the V_{max} of WT, T135A, and T135D was 282, 350, and 18 pmol/h, respectively (Table 1). In terms of GA_3 transport, the K_m values of WT and T135A were 1 mM or higher, whereas that of T135D was lower than the detectable level (Table 1). The uptake rate of jasmonoyl-L-isoleucine was too small to estimate K_m and V_{max} (data not shown). The results suggest that non-phosphorylated GTR1 plays an important role in the transport of these compounds in the plasma membrane.

To elucidate whether GTR1 can form a dimer, we performed a bimolecular fluorescence complementation assay. Co-expressed GTR1-WT:vyce and GTR1-WT:vyne or GTR1-T135A:vyce and GTR1-T135A:vyne localized in the plasma membrane and cytosol (Fig. 2). By contrast, co-expressed GTR1-T135D:vyce and GTR1-T135D:vyne did not localize in either location (Fig. 2). GTR1-T135D:VENUS did not localize in the plasma membrane, suggesting that lack of plasma membrane localization signal may not be due to lack of expression (Fig. 2). These data suggested that dephosphorylated GTR1 forms a dimer in the plasma membrane and that phosphorylation regulates its subcellular localization.

Dephosphorylation-dependent dimerization of NRT1.1 is necessary for its dual-affinity transporter activity over a wide range of concentrations.[11] GTR1-mediated transport activities accompanying its plasma membrane localization may be regulated by phosphorylation of T135 located between the third and fourth TM regions. Although dimerization of GTR1 and NRT1.1 is similarly regulated by de/phosphorylation, this post-

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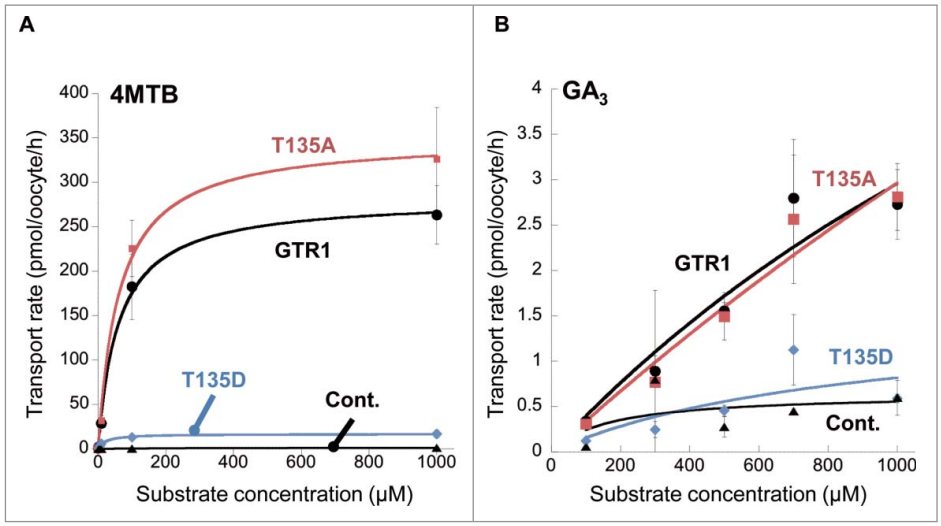

Figure 1. Glucosinolate or GA_3 transport by GTR1-WT, -T135A and -T135D in *Xenopus* oocyte (A) Glucosinolate (4-methylthiobutyl glucosinolate: 4MTB) transport properties of GTR1-WT, -T135A and -T135D. Values are the means with SD (n = 3–7). (B) Gibberellin A3 (GA_3) transport properties of GTR1-WT, -T135A and -T135D. Values are the means with SD (n = 3–7).

translational modification has different roles, i.e., regulation of the transport activities of NRT1.1 and the plasma membrane localization of GTR1.

Material and methods

Plasmid construction

We used the described previously plasmid[13] as the template plasmids for cRNA synthesis. For substrate uptake assay, we used the GTR1 construct described previously.[7] We performed site-directed mutagenesis to add GTR1 to T135A and T135D mutations with following primers: 5′-AAGGCTCT-CAGTGTCGCTGTCAT-3′ (forward)/5′- GTAGCGAC-CAAAGTAAGTGTCGCAA-3′ for T135A mutation, and 5′-AAGGCTCTCAGTGTCGCTGTCAT-3′ and 5′- GTAGC-GACCAAAGTAAGTGTCGCAA-3′ for T135D mutation. For constructing VENUS fused protein, VENUS[14] sequence were amplified with following primers: 5′-AAAGAATTCGCAG-CAGCGATGGTGAGCAAGGG-3′ and 5′-AAAGGATCCT-CACGCTTCCTTGTACAGCTC-3′ and subcloned into *Eco*R I and *Bam*H I sites of the plasmid. Plasmids for bimolecular fluorescence complementation assay were constructed using following primers: 5′-AAAGAATTCGCAGCTATGGAGCAAAA GT -3′ and 5′-AAAGGATCCCTACTCGATGTTGTG-3′ for vyce construct, and 5′-AAAGAATTCGCAGCTATGTACCCA TACG-3′ and 5′-AAAGGATCCTTACTTGTACAGCTCGTC-3′ for vyne construct. The amplified PCR products were subcloned into *Eco*R I and *Bam*H I sites of the plasmid. Coding sequence of *GTR1* without stop codon was amplified with 5′-A AAGAGCTCGCAGCGAAGAGCAGAGTCATT-3′ and 5′-AA AGAATTCTGCGGCGACAGAGTTCTTGTC-3′, and subcloned into *Sac* I and *Eco*R I site of the plasmids for VENUS-fusion or bimolecular fluorescence complementation assay. T135A and T135D mutations were added using above-mentioned primer combinations.

Table 1. Transport properties of GTR1-WT and mutants in *Xenopus* oocyte.

	4MTB			GA_3		
	V_0 (pmol/h)	K_m	V_{max} (pmol/h/oocyte)	V_0 (pmol/h)	K_m	V_{max} (pmol/h/oocyte)
WT	1.9	60	282	1.6	2.5	11
T135A	3.0	62	350	2.0	5.9	21
T135D	1.0	27	17	—	—	—

Preparation of frog oocytes

Ovary lobes were surgically removed from female *Xenopus laevis* and washed with Barth's solution w/o Ca^{2+} (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 5 mM Tris/HCl (pH 7.4)). Ovaries were then gently shaken with Barth's solution w/o Ca^{2+} containing 0.05%(w/v) type IA collagenase at 18°C for 60 min. After washing them using Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 5 mM Tris/HCl (pH 7.4)), oocytes were gently shaken with Barth's solution at 18°C for 10 min. After washing, oocytes were shaken again with Barth's buffer w/o Ca^{2+} at 18°C for 10 min. Defolliculated oocytes were stored in Barth's solution containing 50 mg/mL gentamicin at 18°C.

Substrate uptake assay using Xenopus oocytes

cRNA synthesis, oocyte microinjection and substrate quantification were performed as described previously.[15] cRNA-injected oocytes were incubated in Barth's solution at 18°C for 24 h for subsequent experiments. For determination of V_0, 2 oocytes were incubated in 50 μl of kulori-based solution (pH 5.0) containing each substrate at 18°C. For assay with 4MTB (Chromadex, USA), substrate concentration was 1 μM and incubation times were 0, 1, 2, 3 and 6 h. For assay with GA_3 (Wako, Japan), substrate concentration was 1 mM and incubation times were 0, 2, 4, 6 and 24 h. For determination of K_m and V_{max}, 2 oocytes were incubated in 50 μl of kulori-based

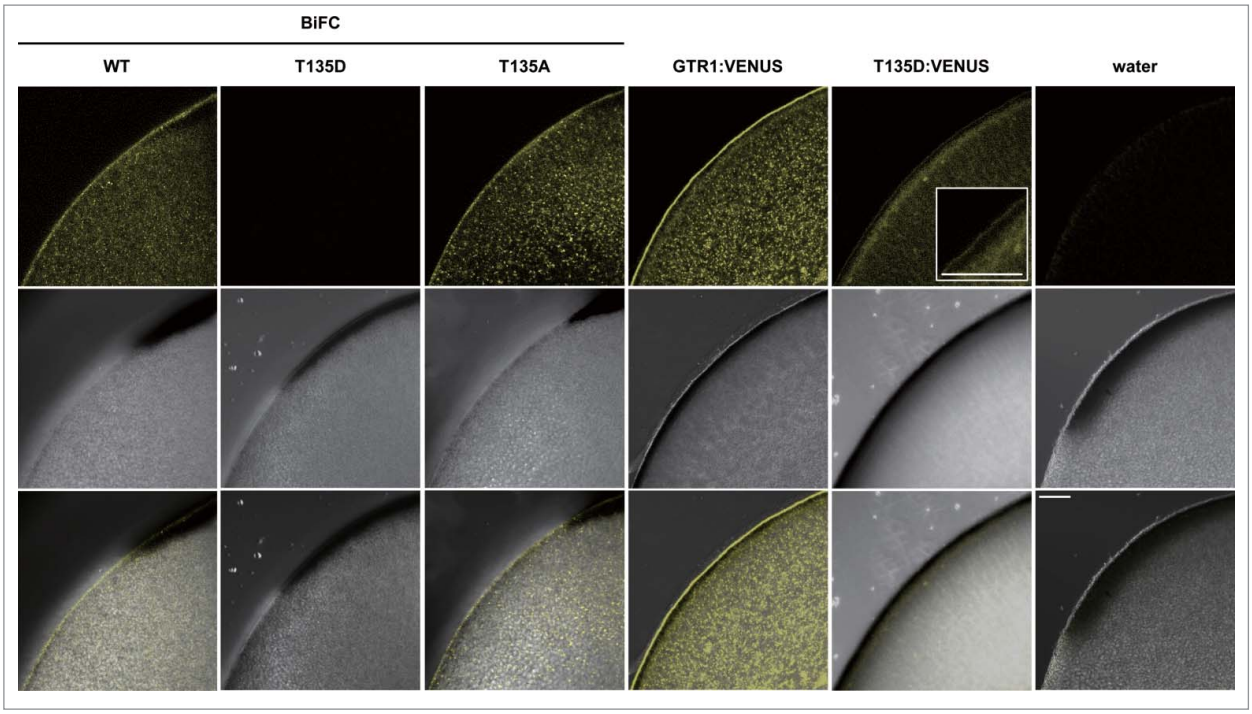

Figure 2. Subcellular localizations of GTR1-WT, -T135A and -T135D by bimolecular fluorescence complementation in *Xenopus* oocytes Pictures of oocytes, taken with a confocal laser scanning microscope (100 μm slice of an oocyte) in GTR1-WT:vyce and GTR1-WT:vyne (WT), GTR1-T135A:vyce and GTR1-T135A:vyne (T135D), GTR1-T135D:vyce and GTR1-T135D:vyne (T135A), GTR1:VENUS or T135D:VENUS. YFP fluorescence (top), transmitted light (middle) and overlay (bottom). Scale bars = 50 μm.

solution (pH5.0) containing each substrate at 18°C. For assay with 4MTB, substrate concentrations were 0, 0.1, 1, 10, 100 and 1000 μM (GTR1, GTR1-T135A)/0, 1, 5, 10, 100 and 1000 μM (GTR1-T135D), and incubation time was 1 h. For assay with GA_3, substrate concentrations were 100, 250, 500, 750 and 1000 μM, and incubation time was 2 h. Data were plotted and fitted with Michaelis-Menten equation to elucidate K_m and V_{max} values.

Bimolecular fluorescence complementation assay in Xenopus oocytes

After injection of each cRNA, oocytes were incubated in Barth's solution at 18°C for 24 h, subsequently incubated in kulori-based solution (pH 5.0) at 18°C for 6 h. Fixation was performed using 1.25% (w/v) paraformaldehyde in PBS solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4) at 4°C. Fixed oocytes were embedded in 4% (w/v) agarose gel and then sliced into 100 μm using MicroSlicer Zero 1 (DOSAKA EM). Fluorescence images were obtained using confocal laser scanning microscopy LSM700 (Zeiss).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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