

The Non-canonical Tetratricopeptide Repeat (TPR) Domain of Fluorescent (FLU) Mediates Complex Formation with Glutamyl-tRNA Reductase*

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Background: FLU negatively regulates glutamyl-tRNA reductase (GluTR) during chlorophyll biosynthesis.

Results: The structures of the TPR domain of FLU in its uncomplexed form and the GluTR dimeric domain-bound form were solved.

Conclusion: The non-canonical TPR domain of FLU mediates complex formation with GluTR.

Significance: The work enables a delineation of the spatial regulation of GluTR.

The tetratricopeptide repeat (TPR)-containing protein FLU is a negative regulator of chlorophyll biosynthesis in plants. It directly interacts through its TPR domain with glutamyl-tRNA reductase (GluTR), the rate-limiting enzyme in the formation of δ -aminolevulinic acid (ALA). Delineation of how FLU binds to GluTR is important for understanding the molecular basis for FLU-mediated repression of synthesis of ALA, the universal tetrapyrrole precursor. Here, we characterize the FLU-GluTR interaction by solving the crystal structures of the uncomplexed TPR domain of FLU (FLU^{TPR}) at 1.45-Å resolution and the complex of the dimeric domain of GluTR bound to FLU^{TPR} at 2.4-Å resolution. Three non-canonical TPR motifs of each FLU^{TPR} form a concave surface and clamp the helix bundle in the C-terminal dimeric domain of GluTR. We demonstrate that a 2:2 FLU^{TPR}-GluTR complex is the functional unit for FLU-mediated GluTR regulation and suggest that the formation of the FLU-GluTR complex prevents glutamyl-tRNA, the GluTR substrate, from binding with this enzyme. These results also provide insights into the spatial regulation of ALA synthesis by the membrane-located FLU protein.

Fluorescent (FLU)³, a plastid membrane protein, directly interacts with glutamyl-tRNA reductase (GluTR), the initial enzyme in plant tetrapyrrole biosynthetic pathway (1, 2). The

mature FLU protein is about 27 kDa and contains an N-terminal hydrophobic region that may form a transmembrane structure, a coiled-coil domain, and a C-terminal tetratricopeptide repeat (TPR) domain. Amino acid sequence analysis predicted that the C-terminal TPR domains of *Arabidopsis* FLU and *Chlamydomonas* FLU-like protein consist of two TPR motifs (1, 3), whereas barley Tigrina d protein, an ortholog of *Arabidopsis* FLU, consists of three TPR motifs (4). Mutations in the TPR domain, such as A262V in *Arabidopsis* FLU (*flu1-1*) and frameshift at Ala²⁴⁹ in barley Tigrina d (*tigrina d*), cause defect in the regulation of chlorophyll biosynthesis (1, 4, 5). The *flu* and *tigrina d* mutants lose their ability to suppress protochlorophyllide (PChlide) accumulation upon a dark/light shift and resemble phenotypes similar to wild-type plants fed with exogenous δ -aminolevulinic acid (ALA). GluTR is the rate-limiting enzyme for endogenous ALA production (6), and hence the physical interaction between FLU and GluTR suggests an inhibitory role of FLU on GluTR activity (2). Indeed, an up-regulation of ALA synthesis was observed for the *flu1-1* mutant under continuous light (7). In a yeast two-hybrid system, it was shown that the C-terminal 43 residues of GluTR were required for interacting with the TPR domain of FLU (7). However, in the crystal structures of GluTR, instead of being exposed, this C-terminal region packs inside the cleft of the V-shaped GluTR dimer (8, 9). Therefore, the FLU-GluTR interaction needs to be characterized at the atomic level.

As a hub for the post-transcriptional regulation of tetrapyrrole biosynthesis, GluTR is controlled by multiple mechanisms besides the above mentioned FLU-mediated down-regulation (10, 11). Metabolic feedback inhibition is a common strategy to repress GluTR activity, although how the tetrapyrrole metabolites, including heme and, particularly interestingly, PChlide (12, 13), affect GluTR awaits to be elucidated. An immunoprecipitation/mass spectrometry study has identified a chloroplast membrane complex containing FLU and several key enzymes devoted to chlorophyll synthesis (14). This complex is proposed to interact with GluTR upon accumulation of fluorescent

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The atomic coordinates and structure factors (codes 4YVO and 4YVQ) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

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³ The abbreviations used are: FLU, Fluorescent; GluTR, glutamyl-tRNA reductase; TPR, tetratricopeptide repeat; ALA, δ -aminolevulinic acid; PChlide, protochlorophyllide; GluBP, GluTR-binding protein; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; DD, dimeric domain.

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PChlide. Although the above model explains how FLU mediates GluTR activity, the spatial organization of such a membrane complex is unclear. A delineation of the FLU-GluTR interaction should provide a structural insight into how FLU exerts its regulatory role in this complex.

Here we describe the 1.45 Å structure of TPR domain of FLU (FLU^{TPR}) consisting of three non-canonical TPR motifs. To crystallize a complex containing the components for direct FLU-GluTR interaction, we constructed GluTR C-terminal truncations that preserve dimerization ability but with different polypeptide size and successfully obtained a 2.4 Å structure of the dimeric domain of GluTR (GluTR^{DD}) in complex with FLU^{TPR}. The FLU^{TPR}-GluTR^{DD} complex has a dissociation constant similar to that of FLU^{TPR} with the full-length GluTR, which indicates that this complex structure can represent the

FLU-GluTR interaction adequately. Together with an enzymatic ALA synthesis assay, these findings allow an elucidation of the molecular basis for FLU-mediated GluTR regulation.

Experimental Procedures

Protein Expression and Purification—The PCR-amplified cDNA fragments containing the TPR domain of *Arabidopsis* FLU (*At3g14110*) and the dimeric domain of *Arabidopsis* GluTR (*At1g58290*) were inserted into expression vector pET-22b(+) (Novagen). Recombinant N-His₆-tagged FLU^{TPR} and GluTR^{DD} were overexpressed in *Escherichia coli* BL21(DE3) and purified to homogeneity by nickel affinity column (Ni²⁺-nitrilotriacetic acid, Qiagen) and size-exclusion chromatography (GE Healthcare). For preparation of the FLU^{TPR}-GluTR^{DD} complex, pET-22b(+) vectors containing N-His₆-tagged FLU^{TPR} and the non-tagged form of GluTR^{DD} were co-transferred into *E. coli* BL21(DE3) cells. The purification procedure was the same as described above. Protein purity was monitored by SDS-PAGE. Proteins for crystallization were concentrated to ~12 mg ml⁻¹ in buffer containing 20 mM Tris-HCl, 100 mM NaCl, 5% glycerol, and 1 mM DTT (pH 7.5) and then stored at -80 °C.

Isothermal Titration Calorimetry—Experiments were performed on a MicroCal iTC200 calorimeter (GE Healthcare). The purified proteins for ITC measurement were in buffer containing 200 mM NaCl, 20 mM Tris-HCl, 5% glycerol, and 1 mM DTT (pH 7.5) and were degassed before titration. Each titration series consisted of 20 injections of 2 μl of FLU^{TPR} into 200 μl of GluTR or GluTR^{DD}. Control experiments were carried out by injecting FLU^{TPR} into the buffer, and the resulting heat of dilution was subtracted from the binding isotherm data. The data were analyzed with the Origin software (OriginLab) using a single-site binding model.

Crystallization and Structure Determination—Crystals of FLU^{TPR} and FLU^{TPR}-GluTR^{DD} were grown by the hanging-drop vapor diffusion method. Crystals of FLU^{TPR} were formed within 1 week in 0.2 M NaCl, 0.1 M Bis-Tris (pH 6.5), and 25% (w/v) polyethylene glycol 3350. Crystals of FLU^{TPR}-GluTR^{DD}

TABLE 1
Data collection and structure refinement statistics

Values in parentheses are for the data in the highest-resolution shell.

	FLU ^{TPR}	FLU ^{TPR} -GluTR ^{DD}
Data collection		
Wavelength (Å)	0.9793	0.9793
Space group	P3 ₁ 21	P6 ₅ 22
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	60.2, 60.2, 67.5	74.7, 74.7, 161.7
α, β, γ (°)	90, 90, 120	90, 90, 120
Resolution (Å)	50 – 1.45 (1.50 – 1.45)	50 – 2.4 (2.49 – 2.40)
<i>R</i> _{sym} or <i>R</i> _{merge}	0.091 (0.292)	0.106 (0.756)
<i>I</i> / <i>σ</i> <i>I</i>	24.9 (5.5)	49.8 (5.4)
Completeness (%)	98.0 (89.2)	100 (100)
Redundancy	10.7 (5.8)	33.7 (34.9)
Unique reflections	25,072 (2247)	11,105 (1063)
Refinement		
Resolution (Å)	26.1 – 1.45 (1.51 – 1.45)	34.28 – 2.40 (2.64 – 2.10)
No. of reflections	24,973	11,051
<i>R</i> _{work} / <i>R</i> _{free}	0.138/0.178	0.208/0.243
No. of atoms		
Protein	987	1624
Water	152	66
Mean <i>B</i> -factors (Å ²)	13.5	47.8
r.m.s.d. ^a		
Bond lengths (Å)	0.008	0.004
Bond angles (°)	1.097	0.675
PDB code	4YVO	4YVQ

^a r.m.s.d., root mean square deviation.

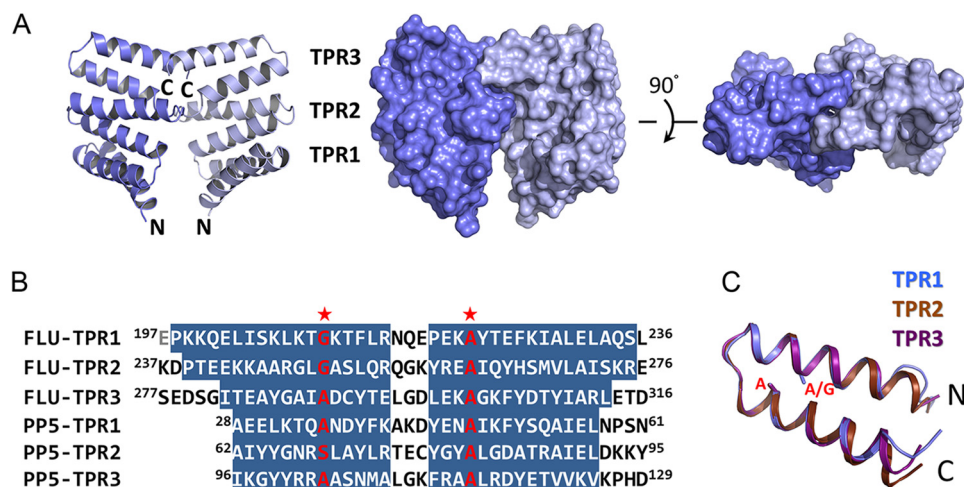


FIGURE 1. Structure of FLU^{TPR} at 1.45 Å. *A*, left, a ribbon representation showing overall structure of FLU^{TPR} dimer. One protomer is colored *indigo*, and the other is *light blue*. Middle and right, two perpendicular views in surface representation. *B*, alignment of TPR motifs from FLU and protein phosphatase 5 (PP5). Residues in α -helix are highlighted. Glu¹⁹⁷ is colored gray because it is not observed in the FLU^{TPR} structure. Red stars indicate the strictly conserved residues in TPR motif. *C*, superimposition of the three TPR motifs. The individual motifs are color-coded. Side chains of residues at the two strictly conserved positions are shown as sticks.

were formed over 3 weeks in 0.15 M KBr and 30% (w/v) polyethylene glycol monomethyl ether 2000. Prior to data collection, crystals were cryo-protected in the above crystallization solution supplemented with 20% (v/v) glycerol and flash-frozen in liquid nitrogen.

X-ray diffraction data were collected at beamline BL17U of the Shanghai Synchrotron Radiation Facility (SSRF) at a wavelength of 0.9793 Å at 100 K and then processed using HKL2000 (HKL Research). The model of FLU^{TPR} was determined by molecular replacement using the program Phaser (15), and the search model was the TPR domain of the mouse Leu-Gly-Asn repeat-enriched protein (16) (Protein Data Bank (PDB) code 3RO3). The resulting model was rebuilt using ARP/wARP (17). Manual correction was performed in Coot (18) according to the $2F_o - F_c$ and $F_o - F_c$ electron density maps, and further refinement was done with phenix.refine (19). The structure of the FLU^{TPR}-GluTR^{DD} complex was determined by molecular replacement using the FLU^{TPR} structure as the search model, and the dimeric domain of GluTR was fitted into the density manually and then refined as described above. The overall quality of the final structural models was assessed by MolProbity (20) with 99.2 and 0.8% in favored and additional allowed regions for the FLU^{TPR} structure, and 99.5 and 0.5% in favored and disallowed regions for the FLU^{TPR}-GluTR^{DD} complex structure (see Table 1). The protein structure figures were prepared using the program PyMOL (31).

ALA Synthesis Assay—The assay was performed as described previously (9), except that an equal volume of buffer was replaced by FLU^{TPR} for measurement with FLU^{TPR}. The molar ratio of FLU^{TPR}:GluTR was kept as 1.2:1. The reaction time was 60 min.

Results

Crystal Structure of FLU^{TPR}—The final model of the recombinant FLU^{TPR} contains 119 residues (Pro¹⁹⁸–Asp³¹⁶) and is refined to 1.45 Å resolution (Table 1). FLU^{TPR} consists of three TPR motifs (TPR1–TPR3) and forms dimer through TPR3 (Fig. 1A). When compared with the prototype of TPR motif (21), these three FLU TPR motifs are composed of 40 residues rather than the canonical 34 residues (Fig. 1B). The three non-canonical TPR motifs are structurally conserved and can be well superimposed (Fig. 1C). The *flu1-1* mutation (A262V) occurs on the absolutely conserved alanine of TPR2, and the *tigrina d* mutation, a frameshift at Ala²⁴⁹ (corresponding to Ala²⁴⁶ of FLU), results in loss of TPR2 and TPR3. The structure of FLU^{TPR} clarifies the discrepancy among the predicted TPR domains from different species (1, 3, 4).

The FLU^{TPR}-GluTR^{DD} Complex—FLU directly interacts through its TPR domain with GluTR (2). To gain a structural insight into the FLU-GluTR interaction, we purified the full-length GluTR in complex with FLU^{TPR} (Fig. 2A). However, the FLU^{TPR}-GluTR complex failed to crystallize. We speculated that the two relatively flexible arms of the V-shaped GluTR dimer could prevent crystal formation. Thus, GluTR truncations that constitute only the stem of the V shape were constructed, and a fragment corresponding to the C-terminal 104 residues (Leu⁴⁴⁰–Lys⁵⁴³) was able to form stable complex with FLU^{TPR} (Fig. 2B). The functional integrity of GluTR^{DD} was

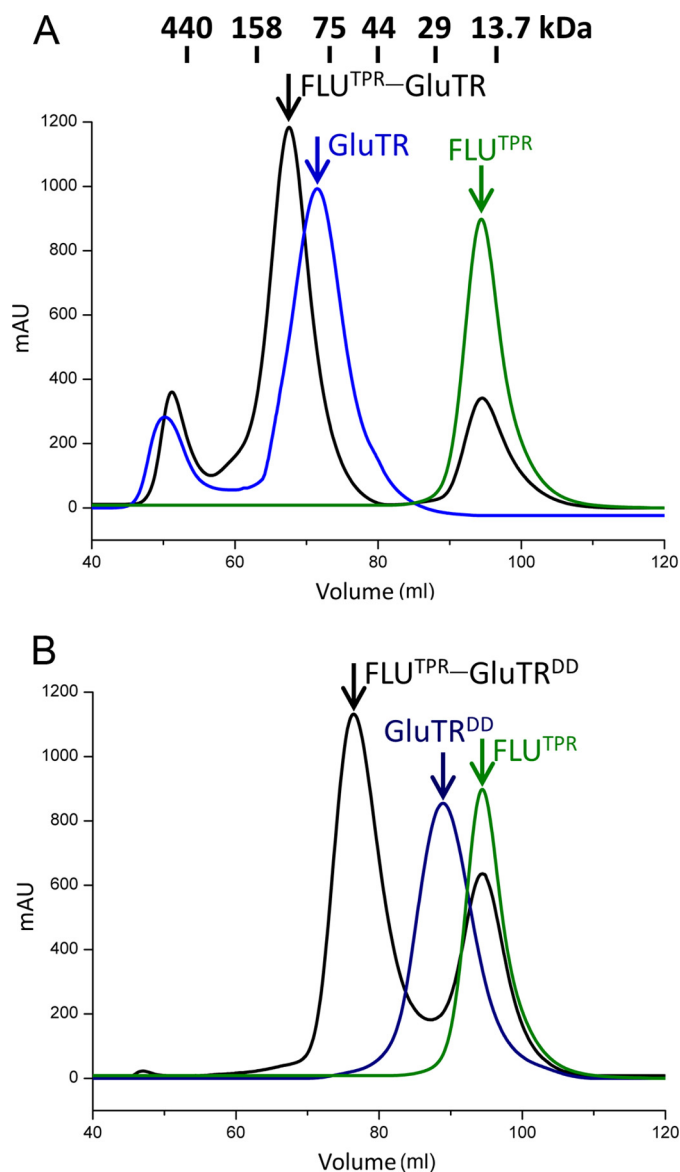


FIGURE 2. Size-exclusion chromatography profiles on a HiLoad 16/60 Superdex 200 column. A, elution profiles of FLU^{TPR}, GluTR, and mixture of FLU^{TPR} and GluTR. B, elution profiles of FLU^{TPR}, GluTR^{DD}, and mixture of FLU^{TPR} and GluTR^{DD}. mAU, milli-absorbance units.

tested by comparison of its binding affinity to FLU^{TPR} with that of the full-length GluTR (Fig. 3). The K_d value, as measured by isothermal titration calorimetry, is $2.03 \pm 0.10 \mu\text{M}$ for FLU^{TPR} and GluTR^{DD}, similar to that for FLU^{TPR} and GluTR ($K_d = 1.66 \pm 0.13 \mu\text{M}$), indicating that GluTR^{DD} maintains binding affinity to FLU^{TPR}.

The crystal structure of the FLU^{TPR}-GluTR^{DD} complex was solved at 2.4 Å resolution (Table 1). The 2:2 complex resembles two hands holding up a cup (Fig. 4), with two FLU^{TPR} molecules clamping the GluTR^{DD} dimer through their concave face. Because the concave face is a conventional site for TPR-mediated protein-protein interaction (22), the complex structure shows that despite its non-canonical TPR motifs, FLU uses a canonical binding mode for GluTR recognition. Because the GluTR^{DD} structure starts from the middle of a long α -helix (Fig. 5), we attempted to crystallize GluTR truncations including this

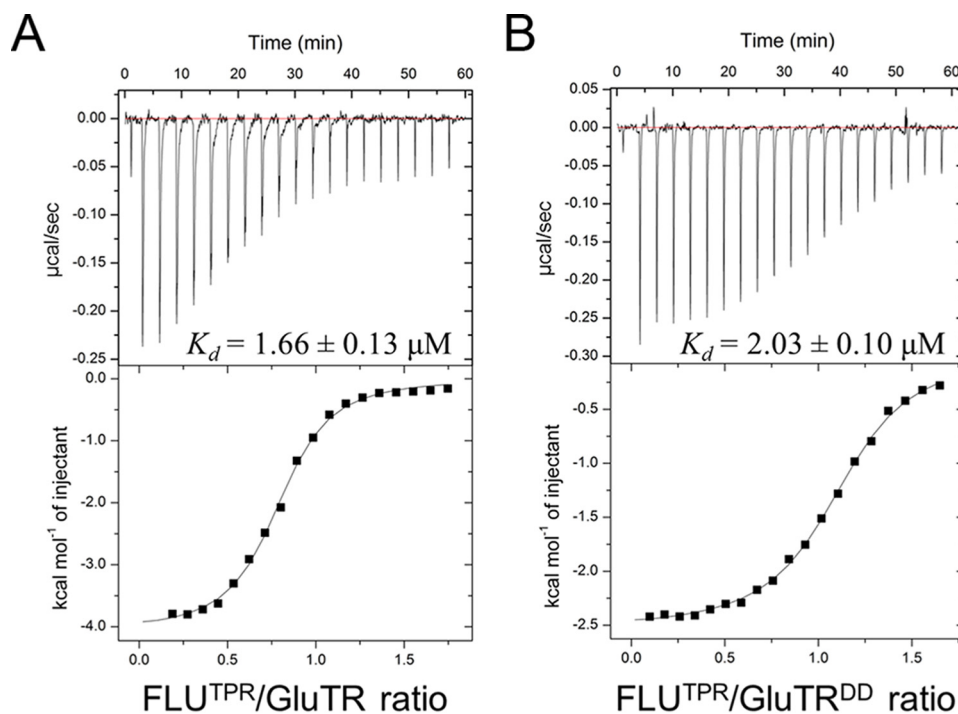


FIGURE 3. **ITC analysis of the interaction between FLU^{TPR} and GluTR.** *A*, titration of full-length GluTR with FLU^{TPR}. The *top panel* shows the heat response to injections, and the *bottom panel* shows the integrated heats of each injection (■) and the fit (—) to a single-site binding model. *B*, titration of GluTR^{DD} with FLU^{TPR}.

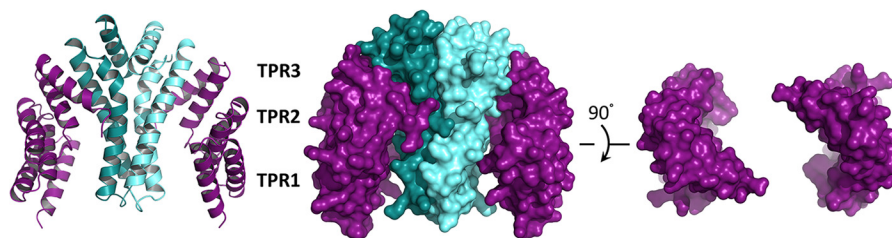


FIGURE 4. **Structure of the FLU^{TPR}-GluTR^{DD} complex at 2.4 Å.** *Left*, overall structure in ribbon representation; *Middle*, overall structure in surface representation. Two FLU^{TPR} molecules are colored *purple*; one GluTR^{DD} protomer is colored *teal*, and the other is in *cyan*. *Right*, FLU^{TPR} structure in surface representation with GluTR^{DD} not shown.

whole α -helix but did not succeed. Notably, this long α -helix could undergo a wobbling motion (8, 9), which suggests that the compact GluTR^{DD} structure reported here is likely the stable core for the GluTR dimer.

The FLU^{TPR}-GluTR^{DD} Interaction—The FLU^{TPR}-GluTR^{DD} complex structure reveals how FLU recognizes GluTR without significant conformational changes. When the structures of FLU^{TPR} in its free and GluTR^{DD}-bound forms are superimposed (Fig. 6A), the root mean square deviation of backbone Ca atoms is only 0.79 Å. Rigidity is a common property for repeat proteins including TPR proteins (23). Interestingly, detailed analysis of the FLU^{TPR}-GluTR^{DD} structure demonstrates that the TPR2 of FLU^{TPR} does not participate in direct protein-protein interaction (Fig. 6B). This differs from the usual concave binding pocket formed by TPR proteins (24). Only TPR1 and TPR3 are involved in GluTR binding, and the FLU^{TPR}-GluTR^{DD} interactions include five salt bridges: Arg²¹⁵-Glu⁴⁶², Glu²⁸⁴-Arg⁴⁸⁵, Asp²⁹¹-Arg⁴⁵⁰, Glu²⁹⁵-Lys⁴⁴⁷, and Asp³⁰⁷-Arg⁵⁰⁰, and a hydrogen bond between Tyr³⁰⁹ and Arg⁴⁵⁰ (Fig. 6B). In a yeast two-hybrid assay, deletion of the C-terminal 43 residues of GluTR (Cys⁵⁰¹-Lys⁵⁴³) abolished interaction with

FLU (7). The FLU-GluTR interaction, as represented by the FLU^{TPR}-GluTR^{DD} structure, does not require this C-terminal region, which is instead involved in GluTR dimerization. Deletion of this region would disrupt the GluTR dimer, suggesting that a 2:2 complex is the functional unit for FLU-mediated GluTR regulation.

FLU^{TPR} Down-regulates GluTR Activity—GluTR is the rate-limiting enzyme for ALA formation (6). The inhibitory role of FLU on GluTR activity has been suggested physiologically in the *flu* mutant (1, 7). To experimentally address the effect of FLU on GluTR activity, we measured ALA synthesis by an *in vitro* coupled enzyme assay (Fig. 7A). The rate of ALA formation decreased significantly in the presence of FLU^{TPR} when compared with the rate in the absence of FLU^{TPR}. FLU^{TPR} also repressed ALA formation when GluTR-binding protein (GluBP), a positive regulator of GluTR, was added to the assay. These results show that FLU and GluBP have antagonizing effects on GluTR activity and function independently from each other. Indeed, FLU exclusively binds to the C-terminal dimeric domain, whereas GluBP binds to the N-terminal catalytic domain of GluTR (9). Notably, the surface area of GluTR

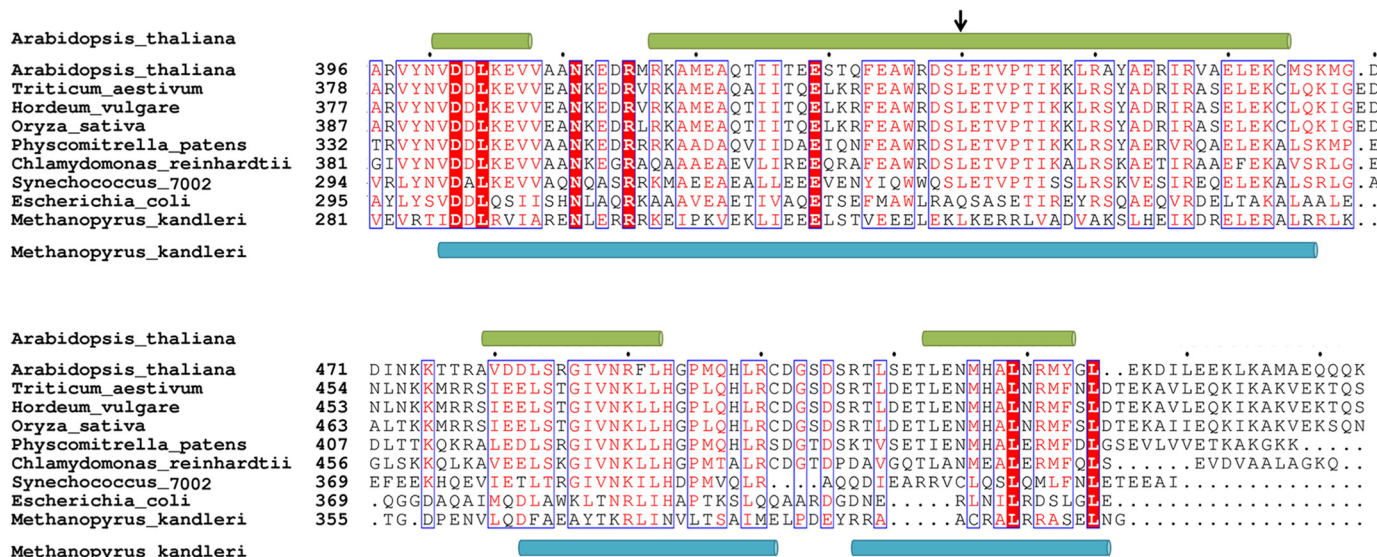


FIGURE 5. Multiple sequence alignment of the C-terminal regions of GluTRs. Identical amino acid residues are boxed in red, and similar residues are printed in red in a blue box. Dots indicate gaps introduced during alignment. The secondary structure elements, as observed in the GluTR structures from *Arabidopsis thaliana* and *Methanopyrus kandleri*, are shown on the top and the bottom of the aligned sequences, respectively. The N-terminal end of GluTR^{DD} in the FLU^{TPR}-GluTR^{DD} structure is indicated by an arrow.

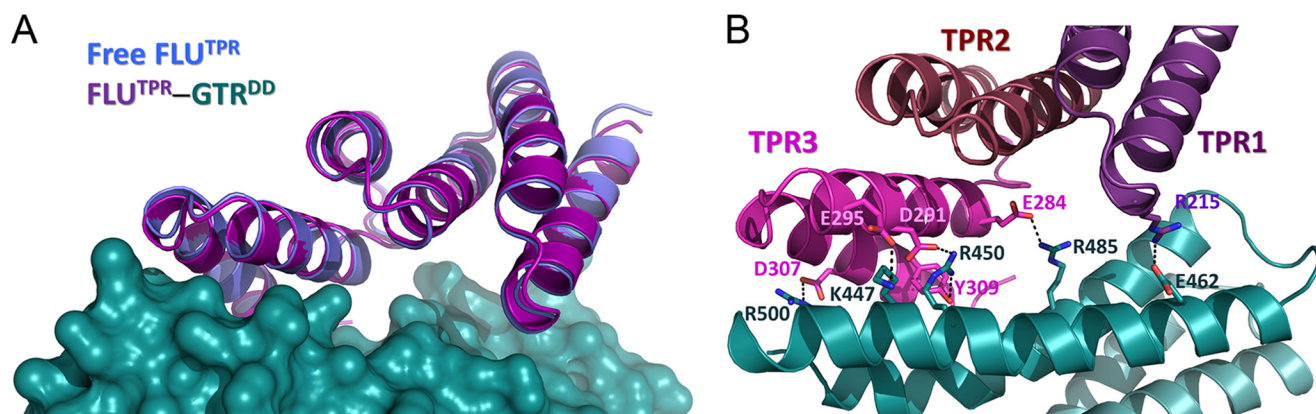


FIGURE 6. The FLU^{TPR}-GluTR^{DD} interaction. *A*, superimposition of FLU^{TPR} in its free and GluTR^{DD}-bound forms. GluTR^{DD} is in surface representation. *B*, detailed FLU^{TPR}-GluTR^{DD} interactions. The individual TPR motifs are color-coded. Side chains of residues forming intermolecular salt bridge and hydrogen bond are shown as sticks. Salt bridge and hydrogen bond are shown as dashed lines.

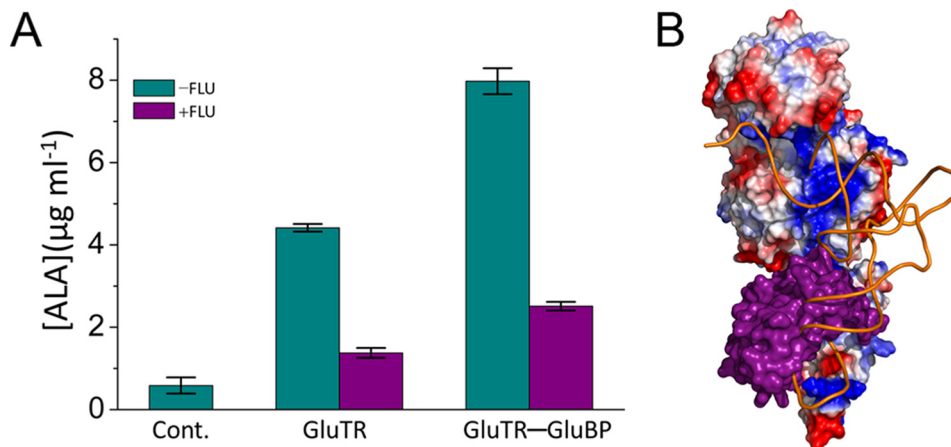


FIGURE 7. FLU^{TPR} inhibits GluTR activity. *A*, *in vitro* ALA synthesis assay. Data are presented as the mean ± S.E. of three independent experiments. *B*, predicted clash between tRNA and FLU^{TPR}. GluTR is represented as electrostatic potential surface, FLU^{TPR} is represented as purple surface, and tRNA is represented as an orange tube. Cont., control.

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that is shielded by FLU^{TPR} is mainly composed of positively charged residues (Fig. 6B). Therefore, it is likely that FLU may prevent the negatively charged tRNA from binding to GluTR due to stereo clash (Fig. 7B).

Discussion

The biosynthesis of tetrapyrroles is vital for plant growth and development (10, 11). Its biosynthetic pathway starts with a universal tetrapyrrole precursor, ALA, and branches into heme and chlorophyll pathways after the formation of protoporphyrin IX. The heme pathway requires only one further step, chelation with a ferrous iron, whereas the chlorophyll pathway needs several hallmark steps: chelation with a magnesium ion, formation of the fifth ring, light-dependent reduction of the fourth ring, and attachment of a phytol tail. FLU has been implicated in the formation of the universal precursor ALA and of PChlide, the product of the only light-dependent reaction in the chlorophyll pathway (1–5).

The non-canonical TPR motifs of FLU make them difficult to predict in previous studies (1, 3). The 1.45 Å FLU^{TPR} and 2.4 Å FLU^{TPR}-GluTR^{DD} structures reported here demonstrate how this three-repeat TPR domain recognizes the dimeric domain of the V-shaped GluTR dimer. The FLU-GluTR interaction, as represented by the FLU^{TPR}-GluTR^{DD} complex structure, indicates that the 2:2 complex is the functional unit for FLU-mediated GluTR regulation. In addition, the inhibitory effect of FLU on GluTR activity is confirmed by the *in vitro* ALA synthesis assay.

FLU has been identified as a negative regulator of chlorophyll biosynthesis over the past decades (1, 5). Furthermore, *Arabidopsis flu* mutant (or *tigrina d* of barley) has become a strategy to study the singlet oxygen-mediated signaling pathway in plants (25, 26). This strategy utilizes accumulation of the photosensitizer PChlide in the *flu* plants upon a dark/light shift. In the wild-type plants, PChlide does not accumulate because of FLU repression of GluTR. However, the link between PChlide accumulation and FLU-mediated GluTR inhibition remains unclear despite extensive research on PChlide-generated singlet oxygen responses (27). Based on an immunoprecipitation/mass spectrometry study, a model incorporating enzymes in the chlorophyll biosynthetic pathway was proposed in which magnesium protoporphyrin IX monomethyl ester cyclase and PChlide oxidoreductase form a complex with FLU. This FLU-containing complex would interact with GluTR upon accumulation of fluorescent PChlide, and thus inhibit ALA synthesis (14). Given the small size of FLU (27 kDa for mature protein) and its membrane-anchoring ability, to unravel its bridging role between membrane and GluTR regulation requires detailed information. The 2:2 FLU-GluTR complex model (Fig. 8) should provide a structural clue of how FLU acts as a bridge between GluTR and other chlorophyll synthesis enzymes. It is possible that the coiled-coil domain of FLU participates in binding of the other enzymes because the coiled coils are a protein motif involved in recognition between proteins (28).

Spatial regulation is a recently recognized strategy for GluTR (29). The way GluTR is anchored to the plastid membrane through FLU has been predicted (30). Based on the two states of FLU^{TPR} (Figs. 1A and Fig. 4) and the inhibitory effect of FLU^{TPR}

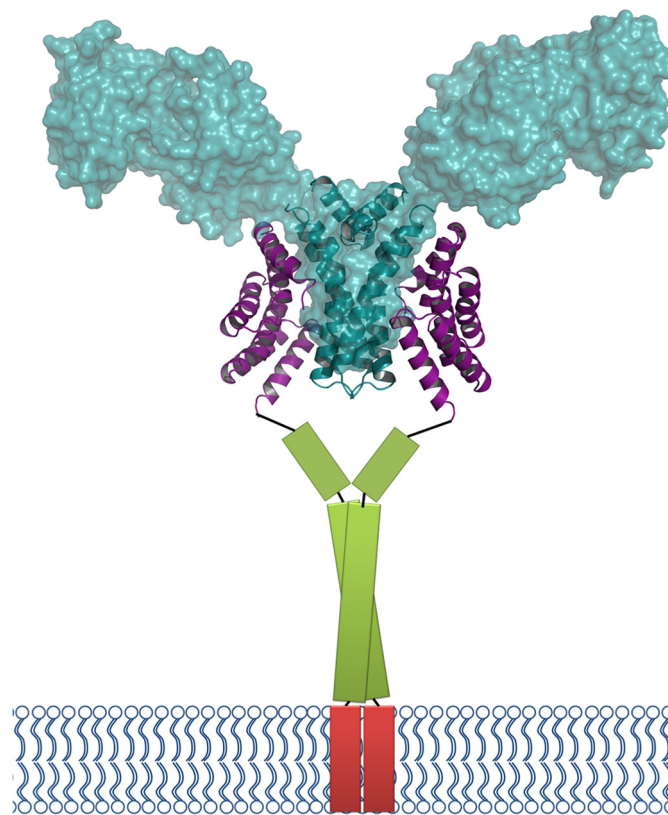


FIGURE 8. Model of the FLU-GluTR complex. The FLU^{TPR}-GluTR^{DD} complex in ribbon representation is overlaid with the transparent surface of GluTR (PDB code 4N7R, chains A and B). The predicted coiled-coil domain of FLU is shown as a *green column*, and the transmembrane domain is shown as a *red column*.

on GluTR activity (Fig. 7A), the working model proposed by Kauss *et al.* (14) is further developed here. In this scenario, the FLU-containing complex senses PChlide accumulation and switches FLU from a resting (Fig. 1A) to an active (Fig. 4) state. The active FLU pulls GluTR from the stroma to the proximity of plastid membrane, where binding of the substrate glutamyl-tRNA is inhibited.

Author Contributions—M. Z. and L. L. designed the research; M. Z., F. Z., Y. F., X. C., Y. C., and W. Z. performed experiments; M. Z., F. Z., and L. L. analyzed the data; H. D., R. L., and L. L. wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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