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Supplemental Information

High-Resolution Crystal Structure of Arabidopsis FLOWERING LOCUS T Illuminates Its Phospholipid-Binding Site in Flowering

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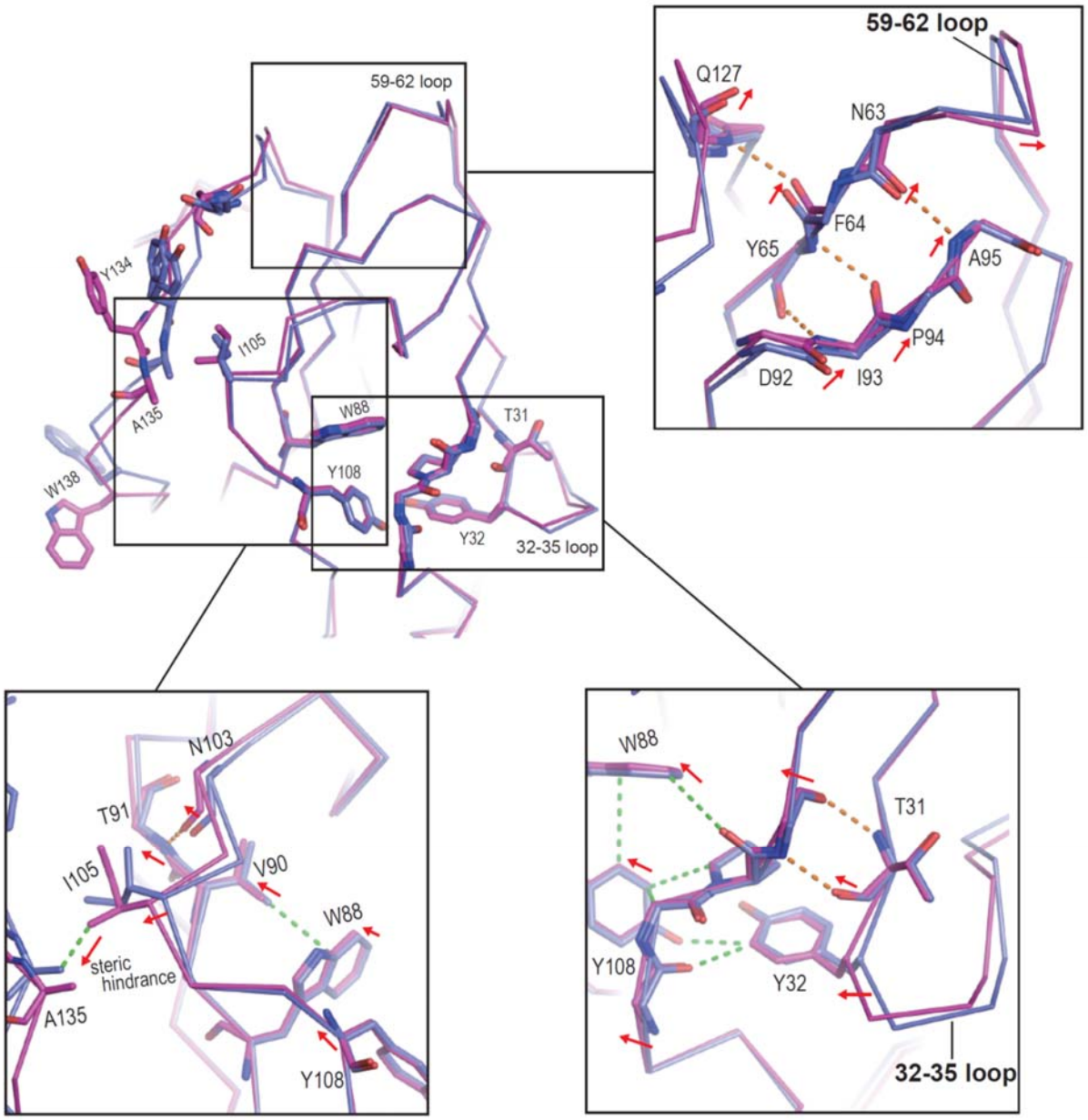

Figure S1 Details of structural correlation between the segment B conformation and 14-3-3 binding loops (shown in Fig. 1E), Related to Figure 1. Insets show close-up views of the square regions. Hydrogen bonds and van der Waals contacts are represented by dashed red and green lines, respectively. Red arrows depict the displacement of the indicated residues upon the small rotation of the 32-35 loop.

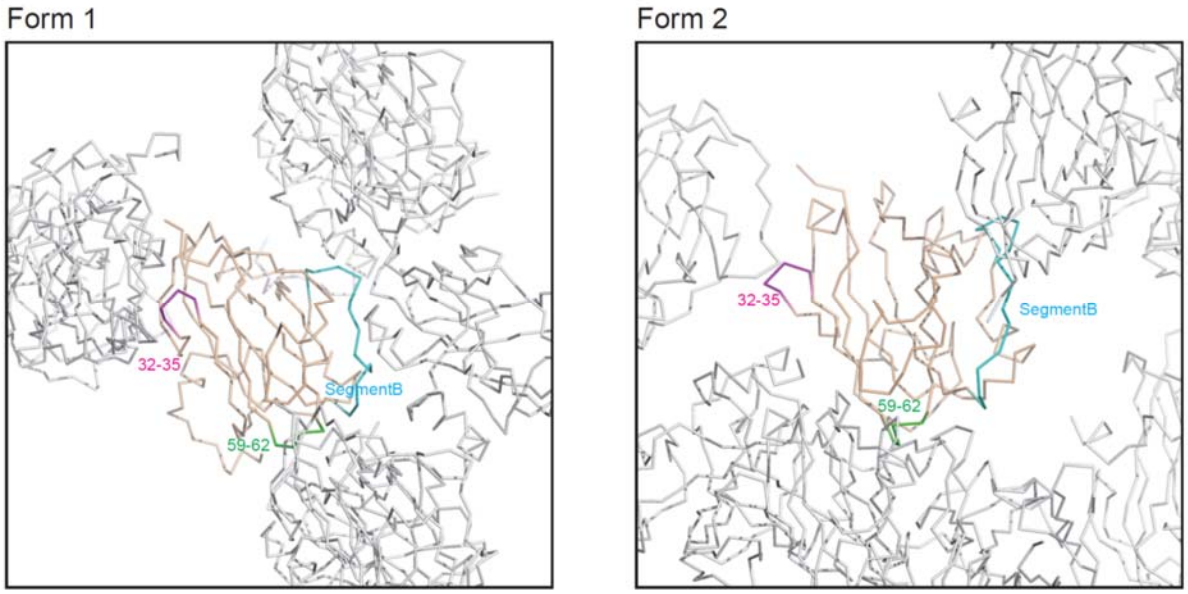

Figure S2 Comparison of crystal packings of Form 1 (left) and Form 2 (right), where symmetric molecules are shown in gray, Related to Figure 1. Segment B, 32-35 and 59-62 loops of the asymmetric molecule are colored in cyan, magenta and green, respectively.

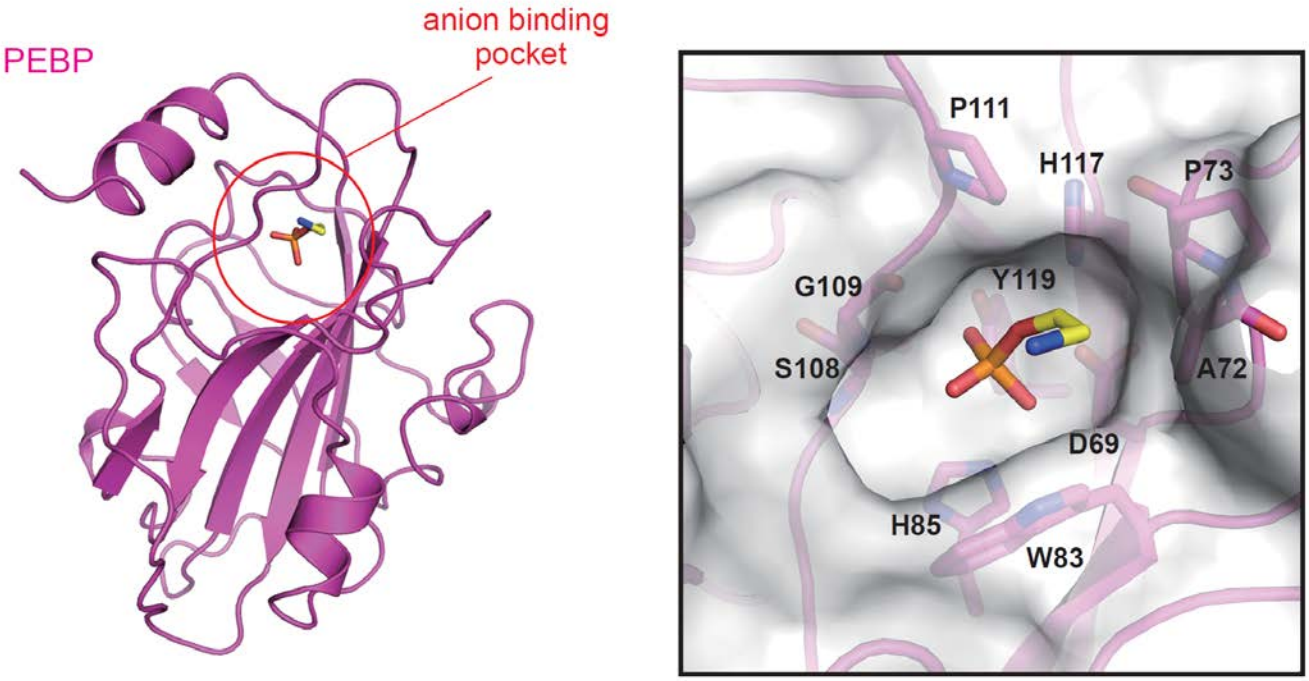

Figure S3 Structure of phosphorylethanolamine (PEtn)-bound PEBP, Related to Figure 2. *Left:* Ribbon diagram of crystal structure of PEtn-bound PEBP (PDB ID: 1B7A). The bound PEtn is shown in a yellow stick model. The anion binding site is indicated by a red circle. *Right*: Close-up view of the anion binding site.

Table S1 List of oligonucleotide primers used in this study, Related to Figures 1, 3, 4 and 5

Name	Sequences (5' to 3')
FTm-F	GAGGGAAGGATTTCACATATGTCTATAAATATAAGA
FTm-R	ACCTGCAGGGAATTCGGATCCCTAACTCTCTCCCTC
JL393	AGAGACCCTCTTATAGTAAGCGCAGTTGTTGGAGCCGTTCTTGATCCGTTTAATAGATC
JL394	TCCAAGTCCTAGCAACCCTCACCTCGCAGAATATCTCCATTGGTTGGTGACTG
JL395	GCACCAGGGTGGCGCCAGAACTTCGCCACTCGCGCGTTTGCTGAGATCTACAATCTCGG
JL396	TGTTGGAGACGTTCTTGATCCGTTTGCTAGATCAATCACTCTAAAGGTTAC
JL397	AGGGTGGCGCCAGAACTTCAACACTGCCGAGTTTGCTGAGATCTACAATCTCG
JL398	TGGAGACGTTCTTGATCCGTTTAATGCATCAATCACTCTAAAGGTTACTTATG
JL399	CTAAAGGTTACTTATGGCCAAAGAGCGGTGACTGCTGGCTTGGATCTAAGGCCTTCTCAG
JL439	CCGCTCGAGATGTCTATAAATATAAG
JL440	CGTCTAGACGGCTAAAGTCTTCTTCCT
KK63	GAACAACCTTTGGCAATGAGATTGTGTCTTACGAAAATCCAAGTCCCACTGCAGG
KK64	GCAGTTTTCTACAATTCTCAGAGGGAGAGTTAGGGTACCCCGGGTCGACCTGCAGCCAAG
KK175	ACTGCATGCTCTATAAATATAAGAGACCCTCTTATAGTAAGCAG
KK176	GTCGGTACCCTAAAGTCTTCTTCCTCCGCAGCCAC

Transparent Methods

Cloning of mutagenized FT constructs

The protein coding sequence of *FT* was amplified with Arabidopsis leaf cDNA using primers KK175 and KK176, digested with *Sph*I and *Kpn*I, and ligated into the corresponding cloning site of pQE30 (Qiagen) to obtain pYN1041. Next, site-directed mutagenesis was performed to pYN1041 with primers KK63 and KK64 to replace Cys107 and Cys164 with Ser, remove Cys170, and the last 7 amino acid residues at C-terminus as previously reported (Ahn et al., 2006). Using the resulting plasmid (pKK512) as a template, we next amplified FT without the last 24 bp before the stop codon by the primers FTm-F and FTm-R and cloned into *Nde*I/*BamH*I sites of pMAL-c5X (pJL116), which expresses FT fused N-terminally to maltose-binding protein (MBP) in *E. coli*. The substitution of respective amino acid residues with Ala involved PCR-based site-directed mutagenesis to pJL116 (Sawano and Miyawaki, 2000): R13A, D17A and R83A with the primers JL393 and JL394 to obtain pMAL-c5X-FTm1 (pJL124); N143A and E146A with the primer JL395 to obtain pMAL-c5X-FTm2 (pJL125); N23A and R145A with the primers JL396 and JL397 to obtain pMAL-c5X-FTm3 (pJL127); and R24A, E36A and N39A with the primers JL398 and JL399 to obtain pMAL-c5X-FTm4 (pJL126).

Expression and purification of recombinant protein

For protein expression, *E. coli* strains C41 were cultured with Terrific Broth (TB) medium containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. The expression of recombinant proteins was induced by adding 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD_{600} about 0.5. Protein purification was conducted according to the manufacturer's instructions for the pMAL-c5x expression system (New England Biolabs). The purified proteins were concentrated with ultracentrifugation filters (Amicon, Merck-Millipore). The concentration of proteins was determined by using the BCA protein assay kit (Pierce, Thermo Scientific). For crystallization, MBP-tag was cleaved off with Factor Xa, and the proteins were applied onto a size exclusion column (Superdex75 10/300 GL) equilibrated with buffer containing 20 mM HEPES pH 7.5 and 150 mM NaCl.

Crystallization of FT in the presence of phospholipids

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; Cat. No. 850375) or 1,2-dipropionyl-*sn*-glycero-3-phosphocholine (Cat. No. 850302, both Avanti Polar Lipids, Alabaster, AL) was suspended in MilliQ water and the suspension was sonicated for a few minutes. Crystallization samples were prepared by mixing purified FT (5~10 mg/ml in 20 mM HEPES pH 7.5 and 150 mM NaCl) with 1~1.6 mM phospholipid suspension. Crystals of FT in the presence of phospholipid were grown in 4 different forms by the sitting drop vapor diffusion method at 20˚C: Condition 1: 9.9% (v/v) polyethylene glycol (PEG) 1500, 2.64% (v/v) 2-methyl-2,4-pentanediol (MPD), and 0.1 M Tris-HCl, pH 8.5; condition 2: 9.9% (v/v) isopropanol 4.95% (w/v) PEG 3350 and 0.2 M ammonium citrate/citric acid, pH 7.5; condition 3: 0.33% (v/v) PEG 4000 and 0.33 M ammonium citrate/citric acid, pH 5.5; condition 4: 2 M NaCl, 20% (w/v) PEG 3350, 0.1 M $MgCl_2$ and 0.1 M imidazole HCl, pH 6.5. In each condition, drops were made by mixing 0.2-1.0 µL protein solution with an equal volume of reservoir solution and equilibrated against 80 µL reservoir solution for 1 week.

Before data collection, reservoir solution with 25% (v/v) ethylene glycol was added into the drops several times, then crystals were flash-cooled in a nitrogen stream at 100 K.

Data collection and structure determination

The X-ray diffraction data were collected on the BL1A beamline at the Photon factory and the BL44XU beamline at SPring-8. Diffraction images were processed with the HKL2000 package (Otwinowski and Minor, 1997) or XDS (Kabsch, 2010). The FT structures at 1.0~1.5 Å resolution were determined by the molecular replacement method with Molrep (Vagin and Teplyakov, 1997) by using the previously determined FT structure (PDB ID: 1WKP) as a search model. The structures were refined with several cycles of manual model-building with COOT (Emsley and Cowtan, 2004) and refinement with PHENIX (Adams et al., 2010), and were validated with MolProbity (Chen et al., 2010). Structural figures were prepared by using PyMOL (http://www. pymol.org).

Molecular docking of FT-phospholipid interaction

Molecular docking of DOPC into FT was performed using Autodock Vina (Trott and Olson, 2010). Docking models were first calculated by removing all bound water molecules and using

whole areas of the FT molecule, which predicted 4 possible binding sites. Docking models were further optimized by limiting the search area to each predicted binding site.

Liposome preparation and liposome co-precipitation assay

To make 10 mM liposome, 120 μg DOPC dissolved in chloroform was dried under an N_2 stream and under a vacuum pump overnight. The dried lipid film was rehydrated in 300 μL HK Buffer (50 mM Hepes-KOH, pH 7.2, 120 mM KOAc) at 50 °C for 1 h, followed by 5 cycles of freezing and thawing. The liposome was prepared by using Mini Extruder (Avanti Polar Lipids, Alabaster, AL) with 400-nm size-exclusion membranes. For protein-liposome precipitation assay, 1 mM DOPC liposome was incubated at 500 rpm with 2 μg purified protein in HKM buffer (HK buffer containing 1 mM $MgCl_2$) at 30°C for 30 min (Lu and Benning, 2009; Cabrera et al., 2010). After incubation, liposomes were precipitated by centrifugation at 20,000*g* for 15 min at 4°C to separate the precipitate from supernatant. After 2 washes with HK buffer, the pellet was denatured with 2x sample buffer at 80°C for 3 min for analyzing the amount of liposome-bound protein by quantitative immunoblotting. Protein bands corresponding to MBP-FT were detected with anti-MBP monoclonal antibody (New England Biolabs, 1:5,000 dilution) and the ECL Plex goat-anti-mouse IgG-Cy3 secondary antibody (1:1,250 dilution). Fluorescent signals were quantitatively detected by using LAS 4000 (GE Healthcare Life Sciences).

Construction and phenotype observation of transgenic plants

Pro35S:FT ft-10: A 528-bp fragment of the *FT* coding sequence was amplified with the primers JL439/JL440, then cloned into the *Xho*I/*Xba*I sites of pYN2047 (Lin et al., 2015) to obtain *pENTR-Pro35S:FT* (pJL132). The substitution of amino acid residues with Ala was performed as described above with the same primers: R13A, D17A and R83A with the primers JL393 and JL394 to obtain *pENTR-Pro35S:FTm1* (pJL135); and N23A and R145A with the primers JL396 and JL397 to obtain *pENTR-Pro35S:FTm3* (pJL136). These constructs were then recombined into the pBGW destination vector by using LR Clonase (Invitrogen, Thermo Fisher Scientific, Watham, MA) (Karimi et al., 2005) to obtain *pBGW-Pro35S:FT* (pJL144), *pBGW-Pro35S:FTm1* (pJL145), and *pBGW-Pro35S:FTm3* (pJL139). These plasmids were transduced into *ft-10* by Agrobacterium-mediated transformation. Transformants were selected on soil by spraying 0.1% Basta solution. Resistant plants were genotyped to confirm transgenic constructs. At least 60

independent T1 transgenic lines for *Pro35S:FT ft-10* and *Pro35S:FTm1 ft-10*, and 26 lines for *Pro35S:FTm3 ft-10* were analyzed to obtain the distribution of flowering time.

qRT-PCR

Total RNA was isolated from 4 to 5 shoot apices of 7- or 14-day-old seedlings by use of TRIzol reagent (Thermo Fisher Scientific) including DNase treatment, and cDNA was synthesized by use of the SuperScriptIII First-Strand Synthesis kit (Invitrogen). For qRT-PCR, primer sets used for *SOC1*, *AP1*, and *PEX4* (control) were as previously described (Nakamura et al., 2014).

Immunoblotting

For immunoblot analysis, total protein was extracted from 8-day-old seedlings. Twenty seedlings were ground with 100 μL ice-cooled extraction buffer (2 mM K_2HPO_4/KH_2PO_4, pH 7.4, 1 mM EDTA, 1% Triton 100, 1 mM PMSF, 100x diluted protease inhibitor cocktail, 2% *β*-mercaptoethanol) and incubated on ice for 1 h. The protein extract was centrifuged at 4 ^oC for 1 h at 13,200 rpm. Protein concentration was determined by using the BCA protein assay kit (Pierce, Thermo Fisher Scientific). The supernatant was denatured with 2x sample buffer at 80^oC for 3 min. An amount of 300 μg protein was separated on 16% SDS-PAGE and blotted on PVDF membrane, which was blocked for 80 min with blocking solution containing 5% skim milk in TBS-T (0.1% Tween 20) buffer and probed with the primary anti-FT/TSF antibody (AS06 198, Agrisera, 1:1000 dilution) and horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG (Santa Cruz Biotechnology, 1:10,000) or anti-actin antibody (A0480, Sigma-Aldrich, 1:5,000) and HRP-conjugated secondary goat anti-mouse IgG antibody (Santa Cruz Biotechnology, 1:10,000) in blocking solution. Signals were detected with SuperSignal West Pico chemiluminescent substrate by using ImageQuant LAS 4000 (GE Healthcare Life Sciences).

Plant culture and phenotype observation

Arabidopsis thaliana (Columbia-0 ecotype) was used for *in planta* assays. Plants were grown under long-day conditions (16-h light/8-h dark) with light intensity 150 $\mu molm^{-2}s^{-1}$ and temperature 22 ^oC. Flowering time was measured by counting the number of rosette leaves when the inflorescence stem reached about 1 cm high (Koornneef et al., 1991).

Accession Number

AP1 (At1g69120), *FT* (At1g65480), *SOC1* (At2g45660)

Data Availability

Coordinates and structure factors have been deposited in the Protein Data Bank with accession codes 6IGG (condition 1), 6IGI (condition 2), 6IGH (condition 3) and 6IGJ (condition 4), respectively.