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Letter to the Editor

Structural Analysis of FRIGIDA Flowering-Time Regulator

Dear Editor,

Flowering is a major developmental switch in the life cycle of flowering plants. FRIGIDA (FRI) activates *FLOWERING LOCUS C (FLC)* transcription, a major repressor of this switch regulating flowering time (Johanson et al., 2000). FRI is highly conserved and many natural variations in FRI are found to affect flowering time. However, little is known about the molecular characteristics of FRI.

To reveal the molecular features of FRI, we determined the crystal structure of the highly conserved core domain (106–394 amino acids [aa]) of grape (*Vitis vinifera*) FRI ($_{Vv}$FRI) (Supplemental Figures 1, 2, and Supplemental Table 1). The $_{Vv}$FRI core domain is composed of 14 α-helices (H) and four one-turn α-helices (sH) (Figure 1A). The 14 α-helices are linked by loops or the short one-turn α-helices, and stack on each other in clockwise manner to form an extended helical bundle structure with no homology with other known structures. In this configuration, the H7 is buried by H5, H6, H8, H9, and H10. Any mutation in H7 that changes the hydrophobicity probably causes a deleterious effect on the structure, which is likely to make FRI non-functional.

As no biochemical function of FRI is known, we decided to map a potentially functional surface based on the structural information and sequence conservation (Figure 1B) revealing that the surface composed of H2, H4, and H6, which are highly conserved, might be functionally important. Furthermore, an electrostatic surface presentation of the $_{Vv}$FRI structure shows that a highly positively charged surface coincides with the conserved surface (Figure 1B). Therefore, to examine if the surface identified is important for FRI function *in vivo*, we generated several mutants of *Arabidopsis* FRI ($_{At}$FRI) based on the $_{Vv}$FRI structure that disrupt positive charges on the surface. These mutants include $_{At}$FRI$_{mut1}$ (K155A/K159A/K195A in $_{At}$FRI equivalent to R124A/K128A/R164A in $_{Vv}$FRI), $_{At}$FRI$_{mut2}$ (K155A/R158A/K159A/K195A in $_{At}$FRI equivalent to R124A/R127A/K128A/R164A in $_{Vv}$FRI), and $_{At}$FRI$_{mut3}$ (K155A/K159A/K195A/K254A in $_{At}$FRI equivalent to R124A/K128/R164A/K218A in $_{Vv}$FRI). We then introduced these mutant genes, wild-type (wt) $_{At}$*FRI* ($_{At}$*FRI*$_{wt}$) and wt $_{Vv}$*FRI* ($_{Vv}$*FRI*$_{wt}$), into *Arabidopsis thaliana* Columbia-0 (Col-0) accession, and measured their effects on flowering using the first generation (T1) transgenic plants (Figure 1C). As Col-0 accession has no functional *FRI* allele (Johanson et al., 2000), we observed that introduction of $_{At}$*FRI*$_{wt}$ caused the transgenic plants with delayed flowering. Among the three mutant genes, $_{At}$*FRI*$_{mut1}$ also resulted in delayed flowering at a comparable level to $_{At}$*FRI*$_{wt}$, suggesting that $_{At}$*FRI*$_{mut1}$ is still functional. However, $_{At}$*FRI*$_{mut2}$ did not cause delayed flowering (49 of 50 T1 plants flowered like Col-0), indicating that this quadruple *FRI*-mutant gene is not functional. In addition, two thirds of the $_{At}$*FRI*$_{mut3}$ T1 plants did not show delayed flowering, suggesting that the function of $_{At}$*FRI*$_{mut3}$ is partially disrupted. It should be noted that the expression levels of these mutant proteins are comparable with that of the wild-type indicating that the mutation does not affect the protein expression levels (Figure 1E and 1F). These data imply that the conserved surface identified based on the structure is functionally important.

We also tested if $_{Vv}$*FRI*$_{wt}$ is able to phenocopy $_{At}$*FRI*$_{wt}$ (Figure 1C). However, we were not able to observe a flowering-delay effect from $_{Vv}$*FRI*$_{wt}$ in Col-0 in spite of the high degree of sequence conservation between $_{At}$FRI and $_{Vv}$FRI. It has been reported that the N- and C-terminal regions of $_{At}$FRI are functionally important (Risk et al., 2010). As the N-terminal and C-terminal regions outside of the highly conserved FRI core domain are not well conserved between $_{At}$FRI and $_{Vv}$FRI, it is possible that the difference in the N- and C-terminal region contributes to the inability of $_{Vv}$FRI to complement $_{At}$FRI. Alternatively, the subtle difference in amino acid sequence between these two proteins in the FRI core domain might be critical for FRI function in *Arabidopsis* cells, such as for forming a functional complex with other proteins.

Naturally occurring variations in *FRI* result in varying flowering times (Le Corre et al., 2002; Gazzani et al., 2003; Shindo et al., 2005; Kuittinen et al., 2008; Mendez-Vigo et al., 2011). However, there has been no explanation of these non-functional alleles at the molecular level. To gain insights into the molecular and structural basis of non-synonymous mutations resulting in non-functional *FRI* alleles, we analyzed naturally occurring non-synonymous mutations along with the FRI structure based on a previous study. The study by Dean's group examined *FRI* allelic variations in relation to flowering time and a haplotype tree was generated from the association between non-synonymous mutations and the loss-of-function early-flowering phenotypes together with the expression levels of *FLC* (Shindo et al., 2005). Among the 192 accessions examined, three accessions (Wil-2, Wa-1, and Tottarp-2) share a single non-synonymous mutation of Leu294Phe in $_{At}$FRI protein, which corresponds to Leu258 in $_{Vv}$FRI. These three accessions show early-flowering phenotypes with relatively low expression levels of *FLC* indicating that FRIs in these accessions are not functional. To understand the molecular basis of the non-functional FRI caused by this mutation, we mapped Leu258 residue on the structure (Figure 1D). The corresponding residue Leu258 in $_{Vv}$FRI is located on H8 and is tightly surrounded by several hydrophobic residues such as Leu220, Leu226, Leu237, and Leu238 (Figure 1D). Therefore, the mutation of Leu to Phe in FRI of Wil-2, Wa-1, and Tottarp-2 accessions would cause steric hindrance with other hydrophobic residues leading to a non-functional allele. Three accessions collected from Sweden

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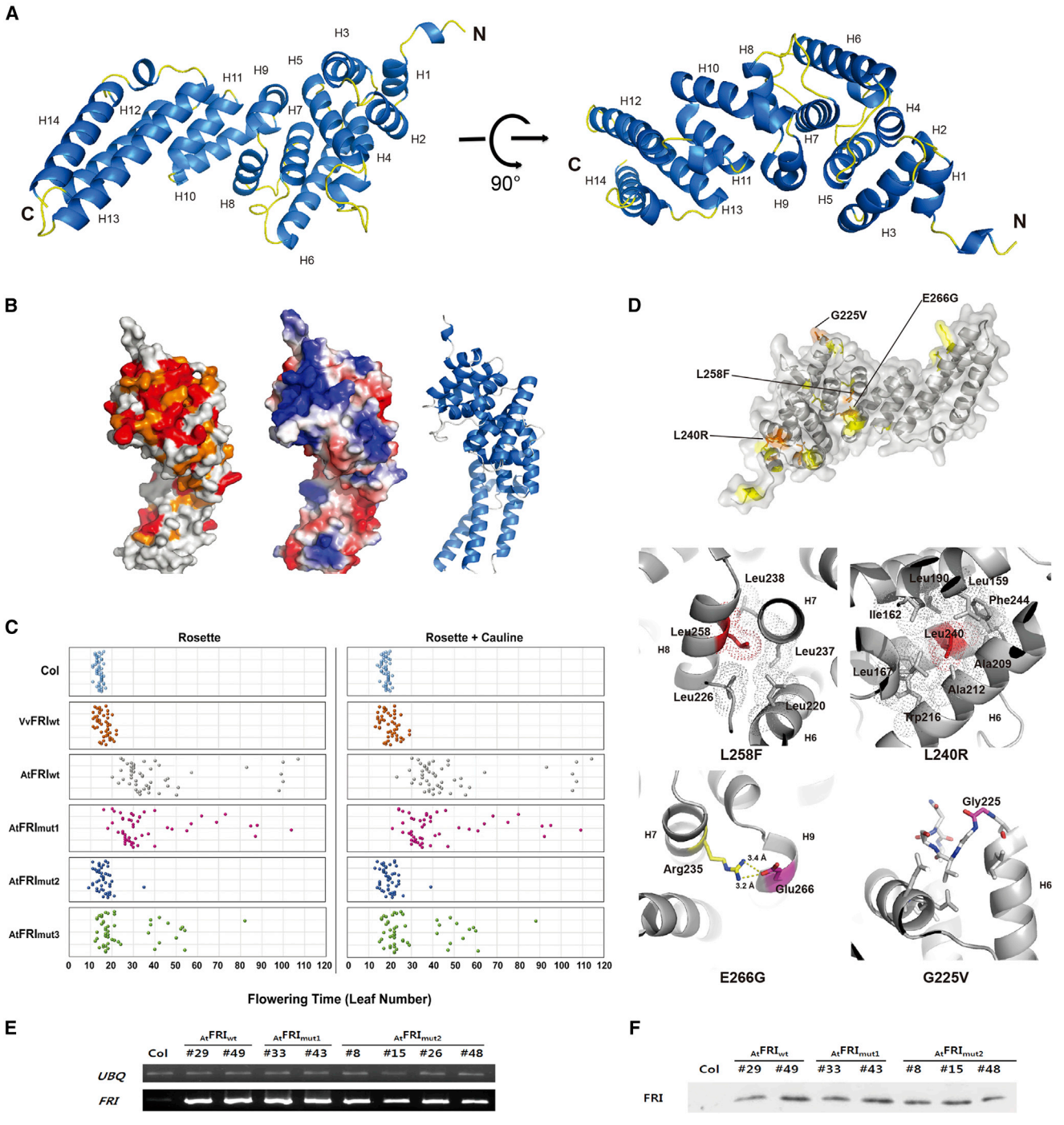

Figure 1. Structural Analysis of FRIGIDA Flowering Time Regulator.

(A) The ribbon representations of the crystal structure of $_{Vv}$FRI in two different views (90° rotated). α helices are colored in blue and loops in yellow.

(B) Conserved amino acids are marked on the surface presentation of FRI. Invariant residues are colored in red and highly conserved residues are in orange (left). In the electrostatic surface representation, the positive surface is shown in blue, the negative surface in red, and the non-charged surface in white (middle). The ribbon representation in the same orientation (right).

(C) Indicated *FRI* genes were transformed into *Arabidopsis thaliana* Col-0 plants, and the flowering times of 50 T1 plants for each construct were analyzed by counting the number of rosette and cauline leaves formed before bolting. $_{Vv}$FRI$_{wt}$, wt $_{Vv}$FRI; $_{At}$FRI$_{wt}$, wt $_{At}$FRI; $_{At}$FRI$_{mut1}$, *Arabidopsis* K155A/K159A/K195A FRI mutant; $_{At}$FRI$_{mut2}$, *Arabidopsis* K155A/R158A/K159A/K195A FRI mutant; $_{At}$FRI$_{mut3}$, *Arabidopsis* K155A/K159A/K195A/K254A FRI mutant.

(D) Allelic variations are mapped on the structure of $_{Vv}$FRI based on a previous study (Shindo et al., 2005). Leu258 $_{Vv}$FRI equivalent to Leu294 of $_{At}$FRI and Leu240 of $_{Vv}$FRI equivalent to Leu276 of $_{At}$FRI are surrounded by hydrophobic residues. Glu266 of $_{Vv}$FRI equivalent to Glu302 of $_{At}$FRI makes a salt bridge of Arg235. Gly225 of $_{Vv}$FRI equivalent to Gly261 of $_{At}$FRI is located at the loop connecting H6 and H7.

(E and F) RT–PCR and western blot show that the expression levels of $_{At}$FRI$_{wt}$, $_{At}$FRI$_{mut1}$, and $_{At}$FRI$_{mut2}$ are all comparable.

(Bä-1-2, Bä-4-1, and Bä-5-1) contain Leu276Arg and Glu302Gly mutations in $_{At}$FRI protein with early-flowering phenotypes as well as low *FLC* expression suggesting that FRI with these mutations is not functional. Leu240 of $_{Vv}$FRI corresponding to Leu276 of $_{At}$FRI, which is located on H7, is also surrounded by hydrophobic residues (Ile162, Leu159, Leu167, Leu190, Ala209, Ala212, Trp216, and Phe244) (Figure 1D). Having a large positively charged Arg at this position would disrupt the hydrophobic core making FRI non-functional. In addition, these accessions also contain Glu302Gly mutation. In the $_{Vv}$FRI structure, Glu266 (Glu302 in $_{At}$FRI) interacts with an absolutely conserved Arg235, and Glu302Gly mutation would disrupt this salt bridge between Arg235 and Glu302. Another single accession (Kz-1) from Sweden flowers early, and has Gly261Val missense mutation with low *FLC* expression. Gly225 (Gly261 in $_{At}$FRI) is located at the loop connecting H6 and H7. The loop where Gly225 is located is sharply turned back into a cleft between H6 and H7, and Gly225 serves as a pivot for this configuration. Therefore, mutating Gly to Val would greatly disrupt this loop structure, which might lead to a non-functional FRI (Figure 1D). Overall, our data provide structural bases for non-functional *FRI* alleles that are found in natural habitats.

In summary, this study provides the first glimpse into the structure of FRI, a key flowering-time regulator, and establishes a structural basis to understand flowering-time regulation.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

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AUTHOR CONTRIBUTIONS

K.H., Y.-S.N, and J.-J.S. coined the ideas and designed the research, and K.H., J.E.O., and J.P. performed experiments. All authors analyzed the data, and K.H., J.E.O., Y.-S.N, and J.-J.S. wrote the manuscript.

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