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# Expression of a kinase-dead form of CPK33 involved in florigen complex formation causes delayed flowering

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Regulation of flowering time is crucial for reproductive success of plants. FLOWERING LOCUS T (FT) protein is a central component of florigen and forms a ternary complex with 14-3-3 and FD, a basic leucine zipper transcription factor, in the shoot apex and promotes flowering. This complex formation requires phosphorylation of threonine residue at position 282 of FD. A calcium-dependent protein kinase CPK33 is responsible for the phosphorylation. However, possibly due to functional redundancy among calcium-dependent protein kinases, impact of the loss of CPK33 reported in the previous study was rather limited. Here, we report that expression of a kinase-dead form of CPK33 caused a clear delayed-flowering phenotype, supporting for an important role of CPK33 in florigen function through FD phosphorylation.

Regulation of timing to flower is crucial for the reproductive success of plants. Plants decide the appropriate time to flower by sensing environmental and internal cues and generate a long-distance signal called florigen. A central component of florigen is FLOWERING LOCUS T (FT). FT is transcribed and translated in the phloem companion cells, and moves to the shoot apical cells via phloem.[1-9] At the shoot apical meristem, FT forms a ternary complex with 14-3-3 and FD, a basic leucine zipper transcription factor.[1,2,10] Our recent work showed that the florigen complex formation is dependent on phosphorylation of threonine residue at position 282 (T282) of FD by calcium-dependent protein kinases (CDPK) including CPK33.[12] CPK33 was expressed in shoot apical meristem, directly interacted with FD, and phosphorylated T282. And, loss of CPK33 function caused delayed flowering and enhanced floral defect phenotype of *leafy* (*lfy*). These observations suggest that CPK33-mediated phosphorylation of FD T282 is an essential regulatory step in flowering time control and flower morphogenesis. However, possibly due to the functional redundancy among CDPK in *Arabidopsis*,[11] delay in flowering time of *cpk33* is much weaker than that observed in *fd*. Delayed flowering phenotype was observed only under certain conditions in which only limited induction of florigen occurs (SD/4LD/SD).[12] Therefore, clearer evidences in support for the impact of CPK33 on promotion of flowering are needed. Here, we report that expression of a kinase-dead form of CPK33 caused a clear delayed-flowering phenotype by interfering with the endogenous CDPK activity including CPK33.

To interfere with the endogenous CDPK activity against FD T282, we designed a dominant inhibition experiment by expression of a catalytically-dead form of CPK33 in the shoot apical meristem. We substituted a catalytic asparatic acid residue at position 197 (D197), crucial for phosphoryl group transfer, to asparagine residue ($CPK33^{D197N}$; hereafter termed as CPK33KD). A loss of the kinase activity of CPK33KD was confirmed by *in vitro* kinase assay with GST-tagged C4 peptide of FD as a substrate. The assay was based on the mobility shift by phos-tag SDS-PAGE as previously described.[12] Phosphorylation-dependent mobility shift of C4 peptide was detected for wild-type CPK33 in the presence of calcium, but not for CPK33KD (**Fig. 1A**). In addition, mobility shift of CPK33KD itself due to autophosphorylation was not observed (**Fig. 1A**). These results clearly indicate that CPK33KD lacks its kinase activity. While CPK33KD lacked its kinase activity, it could still interact with FD as wild-type CPK33 did (**Fig. 1B**). Based on these biochemical evidences, we expected that CPK33KD can interfere with the endogenous CDPK activity against FD T282. When expressed in shoot apical meristem under the control of FD promoter, 3FLAG-tagged CPK33KD caused delayed flowering phenotype under long day condition as expected (**Fig. 1D**). Strength of the late-flowering phenotype observed in 2 independent transgenic lines was correlated with the expression levels of 3FLAG-tagged CPK33KD examined by RT-PCR analysis (**Fig. 1C**). Taken our previous work.[12] and the dominant negative effect of CPK33KD in the present work together, we suggest that CPK33 is an important component of florigen complex formation via phosphorylation of FD T282. It is an important issue to demonstrate and reveal phosphorylation state of FD

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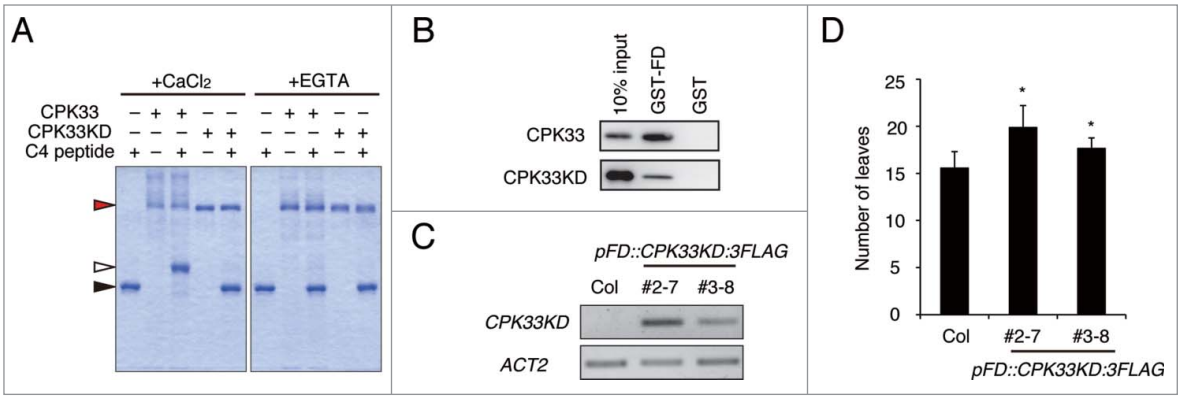


**Figure 1.** Dominant negative effect of CPK33KD. (**A**) Generation of a kinase-dead CPK33 (CPK33KD). Asp 197 was substituted to Asn by site direct mutagenesis. Loss of kinase activity of CPK33KD was confirmed. Phosphorylated and non-phosphorylated C4 peptides were separated by Phos-tag affinity SDS-PAGE. White and black arrowheads indicate phosphorylated and non-phosphorylated C4 peptide, respectively. Red arrowhead indicates CPK33 and/or CPK33KD. (**B**) *In vitro* pull down assay. Direct interaction between FD and CPK33KD was tested. CPK33 and CPK33KD were pulled-down with either GST-FD or GST in the presence of $Ca^{2+}$. Co-precipitated CPK33 and CPK33KD were detected with anti-His antibody. (**C**) RT-PCR analysis of *pFD::CPK33KD:3FLAG* transgenic plants. *CPK33KD* transgene was amplified with *CPK33KD:3FLAG* specific primers. *ACT2* was used as an internal control. (**D**) Dominant negative effect of CPK33KD on flowering time. Plants (n = 15-17) were grown under long-day (16h light/8h dark) condition. Flowering time was evaluated by the number of leaves at flowering (mean ± SD). Asterisks indicate statistically significant difference from Col (*: $P < 0.001$, Student's *t*-test).

T282 *in planta*. Spatiotemporal analysis of FD phosphorylation state using specific antibody that recognize phosphorylate FD T282 will provide us important information for understanding of the regulatory processes in floral transition in shoot apical meristem.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed


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