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VP16 fusion induces the multiple-knockout phenotype of redundant transcriptional repressors partly by Med25-independent mechanisms in *Arabidopsis*

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ABSTRACT

Biological functions of only some plant transcriptional repressors are known owing to the lack of knockout lines or unclear phenotypes because of redundancy. Here we show that strong viral activation domain VP16 fusion to the transcriptional repressor FLOWERING LOCUS C reversed its function and caused a stronger phenotype than that of the multiple-knockout line of redundant genes, suggesting the potential of this technique to identify transcription factor function that cannot be detected in a single-knockout line. Loss-of-function of transcriptional coactivator Mediator25 did not affect VP16 activity despite their in vivo interaction, suggesting the existence of other key mechanism(s) in plants.

Structured summary of protein interactions:
VP16 and **Med25** physically interact by bimolecular fluorescence complementation (View interaction)
VP16 physically interacts with **Med25** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

Most single loss-of-function mutants in *Arabidopsis* do not show any detectable phenotype because of gene redundancy [2], which makes gene function analysis difficult. CRES-T (Chimeric REpressor gene Silencing Technology) is one technique that solves such problems and is now widely used in basic and applied research [16,26,27]. CRES-T is a powerful plant gene silencing system, which converts a transcriptional activator into a strong repressor by fusion with a plant-specific small repression peptide SRDX, which contains the plant-specific repression motif xLxLxL named EAR motif. CRES-T causes a strong, dominant phenotype, which can often be observed only by multiple-knockout mutations of redundant genes. However, this technique is ineffective for native transcriptional repressors to cause thier knockout phenotypes, and progress in transcriptional repressor functional analyses has been hampered by gene redundancy. A promising technique besides CRES-T is conversion of transcriptional repressors to activators by fusion with the strong transactivation domain of VP16 (hereafter called "VP16"), which has been isolated from the herpes simplex virus [41]. Several reports have shown that fusion with VP16 is capable of turning a repressive transcription regulator into an activator, which causes a similar or stronger phenotype compared to those of knockout *Arabidopsis* plants [21,12,6,40,10]. However, data is lacking which compares the phenotype and target gene expression in multiple-knockout lines and the VP16-fusion line to show how much the VP16 fusion system is capable of inducing a strong phenotype, which can only be seen in multiple-knockout lines, such as in the CRES-T system. In addition, Ohta et al. [32] reported that VP16 transactivation activity is strongly inhibited by fusion with the tobacco ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 3 (ERF3) fragment containing a repression motif. This result suggests that there are cases in which VP16 fusion to the transcriptional repressor fails to convert the function. Furthermore, the molecular mechanism of VP16 transcriptional activation in plants is unknown. Therefore, it is important to evaluate the phenotype and target gene expression in a VP16 fusion line and multiple-knockout lines, and show how functional conversion by VP16 fusion occurs in plants.

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Mediator, first identified in *Saccharomyces cerevisiae* [18,8], is the central coactivator of transcription in eukaryotes ranging from yeast to humans [37]. Mediator forms a large protein complex that binds to the C-terminal domain of the RNA polymerase II holoenzyme, acting as a bridge between this enzyme and transcription factors. Studies show that this complex influences almost all transcription stages [3]. Mediator25 (Med25), a metazoan-specific Mediator subunit, has been identified as a specific VP16 target in mammalian cells and is critical for VP16 transactivation activity [28,42]. The Med25 N-terminus is required for Mediator protein complex formation, and the C-terminal activator-interacting domain (ACID) is essential for interaction with VP16. The *Arabidopsis* Mediator complex components were identified by Bäckström et al. in 2007. Interestingly, 21 of the components are relatively conserved in other organisms, but another six are *Arabidopsis* specific. PHYTOCHROME AND FLOWERING TIME1 (PFT1, [4]) was identified as Med25 in *Arabidopsis* based on the conserved N-terminus. ACID of Med25 is less conserved between metazoans and plants compared to the conservation among plants, suggesting a plant-specific function or machinery. ACID of *Arabidopsis* Med25 interacts with many transcription factors in the yeast two-hybrid system [35,5], suggesting its importance in transcriptional regulation in plants. However, whether plant Med25 also interacts with VP16 and whether it is required for VP16 transactivation activity is unknown.

One of the most studied *Arabidopsis* transcriptional repressors is FLOWERING LOCUS C (FLC), a MADS-box transcription factor [24,39,7,13]. FLC is one of the main negative flowering regulators that suppress the transition from the vegetative to the reproductive phase. FLC forms dimers and functions with other redundant MADS-box proteins to suppress flowering by repressing the transcriptions of floral activators, such as *FLOWERING LOCUS T* (*FT*), a gene encoding florigen, and *SUPPRESSOR OF OVEREXPRESSION OF CO* (*SOC1*), another MADS-box transcription factor [25,14,22,23,9,11,38]). *FLC* knockout causes early flowering, and multiple knockout mutants of *FLC* and its redundant gene *SHORT VEGETATIVE PHASE* (*SVP*) show a stronger early-flowering phenotype under short-day conditions. Although *FLC* expression regulation has been analyzed in detail [7,13], the molecular mechanism by which FLC represses the expression of these target genes is undefined. FLC has a typical plant-specific repression motif xLxLxL (ELMLKL) in its C-terminus [26]; however, whether this motif is required for its transcriptional repression activity is unknown.

In this study, we clearly demonstrate that VP16 fusion to FLC reversed its transcriptional repressor activity, leading to a stronger early-flowering phenotype than that of a single FLC loss-of-function line and an *flc svp* double loss-of-function line. Our results suggest the efficacy of VP16 fusion for transcriptional repressor studies such as those using the CRES-T system. We also suggest the involvement of Med25 and other unidentified mechanisms in VP16-mediated transcriptional activation machinery in plants.

2. Materials and methods

2.1. Plant materials and growth conditions

All *Arabidopsis thaliana* plants used in this study were Columbia ecotype. *flc* (*flc-101*) and *svp* (*svp-32*) were obtained from the *Arabidopsis* Biological Resource Center. *flc svp* was generated by crossing. Plants were grown at 22 °C under long-day (16-h light and 8-h dark) or short-day conditions (8-h light and 16-h dark). Plants were grown on Murashige and Skoog medium containing 0.8% agar, 30 mg/l hygromycin, and 250 mg/l vancomycin. To observe flowering, plants were grown on medium and transferred to soil 14–16 days after they started growing.

2.2. Vector construction

Information on the vectors used in this study is summarized in Supplementary Table 1. The *FLC* or *Med25* coding region, without a stop codon, was amplified by polymerase chain reaction (PCR) from an *Arabidopsis* cDNA library fusing Gateway attB1 and attB2 sequences (Life Technologies, Carlsbad, CA, USA) at the 5′ and 3′ ends, respectively, to prepare plasmids for constitutive expression of *FLC*, *FLC-VP16*, *FLC-SRDX*, or *FLAG-Med25*. Each fragment was cloned into pDONR207 (Life Technologies) and introduced into the binary vectors pDEST_35S_3fstop_BCKH [33], pDEST_35S_VP16_HSP_GWB5, pDEST_35S_SRDX_HSP_GWB5 (described below), or pGWB11 [30], respectively, using Gateway LR Clonase II (Life Technologies). The 35S promoter-omega-attR1-ccdB-attR2-VP16 or 35S promoter-omega-attR1-ccdB-attR2-SRDX fragments from pDEST35SVP16HSP [34] or pDEST_35S_SRDX_HSP [33], respectively, were digested with *Hin*d III and cloned into the *Hin*d III site of R4pGWB5_SRDX_HSP [33] after removing the attR4-ccdB-attR2-SRDX fragment to construct pDEST_35S_VP16_HSP_GWB5 and 35S_SRDX_HSP_GWB5.

DNA fragments of green fluorescent protein (GFP) or *β-glucuronidase* (GUS) without a stop codon were amplified by PCR and introduced into pDEST_35S_VP16_HSP_GWB5 (as described above) to construct 35S:GFP-VP16 and 35S:GUS-VP16. The pDEST_35S_VP16_HSP_GWB5 vector, without the LR reaction, was used to generate vector control transgenic plants for flowering and quantitative reverse-transcription PCR (qRT-PCR) analysis. The *FT* or *SOC1* 3 kb promoter regions were amplified by PCR to add the Gateway attB1 [33] and attB2 sequences cloned into

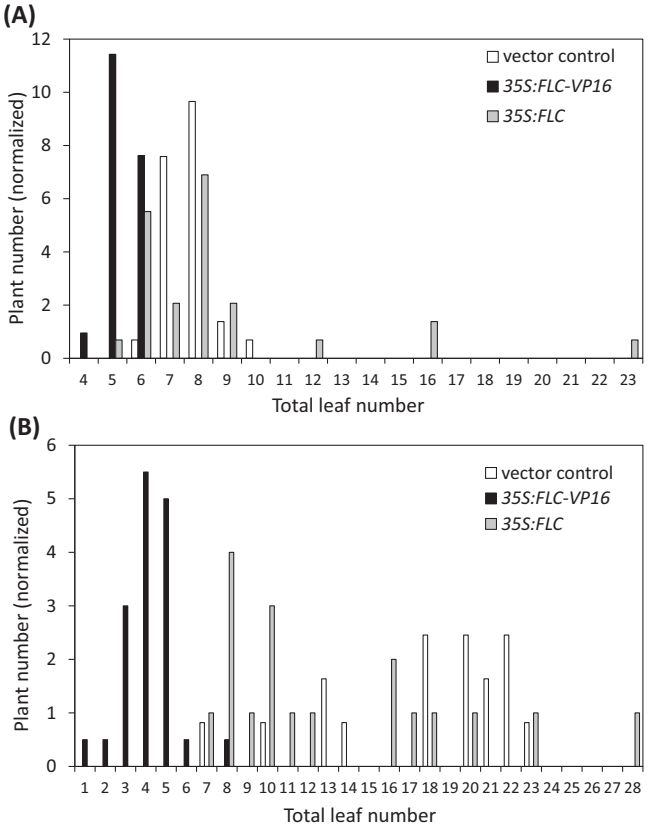

Fig. 1. *35S:FLC-VP16* plants flower early under long- and short-day conditions. Flowering time distribution in *35S:FLC*, *35S:FLC-VP16*, and vector control plants under long- (A) and short-day conditions (B). Total leaf numbers at the bolting stage were counted, and plant numbers were normalized to adjust to that of the vector control. $n = 18–35$.

pDONRG-P4P1R using the BP reaction (Life Technologies) and then transferred into the R4L1pDEST_GLHSP vector by the LR reaction for reporter plasmid construction. The HSP terminator fragment fused with the *Xba* I/*Bam*H I sites was amplified by PCR and cloned into the *Xba* I/*Bam*H I sites of pGL4.10 (Promega Inc., Madison, WI, USA) to construct R4L1pDEST_GLHSP. The plasmid was digested with *Sfi* I and ligated with the attR4-ccdB-attL1 fragment fused with *Sfi* I sites, which were amplified from R4L1pDEST_GUS_BCKK [34] by PCR. The primers used for PCR are shown in Supplementary Table 2.

To construct binary vectors for the BiFC assay, the *Xba*I-*Sac*I fragment from nYFP/pUGW0 [31], nYFP/pUGW2, or cYFP/pUGW2 (Ito and Uemura, unpublished) was introduced into the *Xba*I-*Sac*I site of pDEST_35S_SRDX_HSP_GWB5 (described above), and then the DNA fragment of *FLC*, *FLC-VP16*, *VP16*, *GUS*, or *Med25* in pDONR207 was introduced into the constructs using the LR reaction. nYFP/pUGW0, nYFP/pUGW2 and cYFP/pUGW2 vectors were produced by the same strategy as reported by Hino et al. [15].

2.3. RNA extraction and qRT-PCR

RNA extraction and qRT-PCR were performed as described previously [10]. The gene-specific primers used for qRT-PCR are shown in Supplementary Table 2.

2.4. Transient effector–reporter analysis

Protoplasts were isolated from *Arabidopsis* mesophyll cells and transiently transformed using the polyethylene glycol method according to Yoshida et al. [43]. Firefly luciferase driven by *FT* or *SOC1* 5′ upstream 3 kb sequence was employed as a reporter. Along with the reporter construct, *35S:FLC*, *35S:FLC* with an *SRDX* or *VP16* binary vector was introduced as an effector. The plasmid construction is described in the "Vector Construction" section. A modified *Renilla* luciferase gene (*hRLUC*; Promega) driven by the 35S promoter (phRLHSP; [29] was used to normalize the reporter activity as the internal reference.

2.5. Immunoprecipitation assay

Transient protein co-expression in *Nicotiana benthamiana* and co-immunoprecipitation were performed as described previously [20] with some modifications as follows. Dynabeads Protein A (Life Technologies) was used for immunoprecipitation. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed with e-PAGEL (Atto, Tokyo, Japan), and the proteins were transferred to a polyvinylidene difluoride membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). The blots were incubated with α-GFP antibody (1:2500; Abcam, Cambridge, UK) or α-FLAG-HRP (1:15,000; Sigma–Aldrich, St. Louis, MO, USA) and were detected using ECL prime (Life Technologies).

2.6. BiFC assay

Transient protein coexpression in *N. benthamiana* was performed as described previously [20]. Leaf epidermis was peeled and subjected to microscopic analysis using the BZ9000-Generation II Biorevo (Keyence, Osaka, Japan).

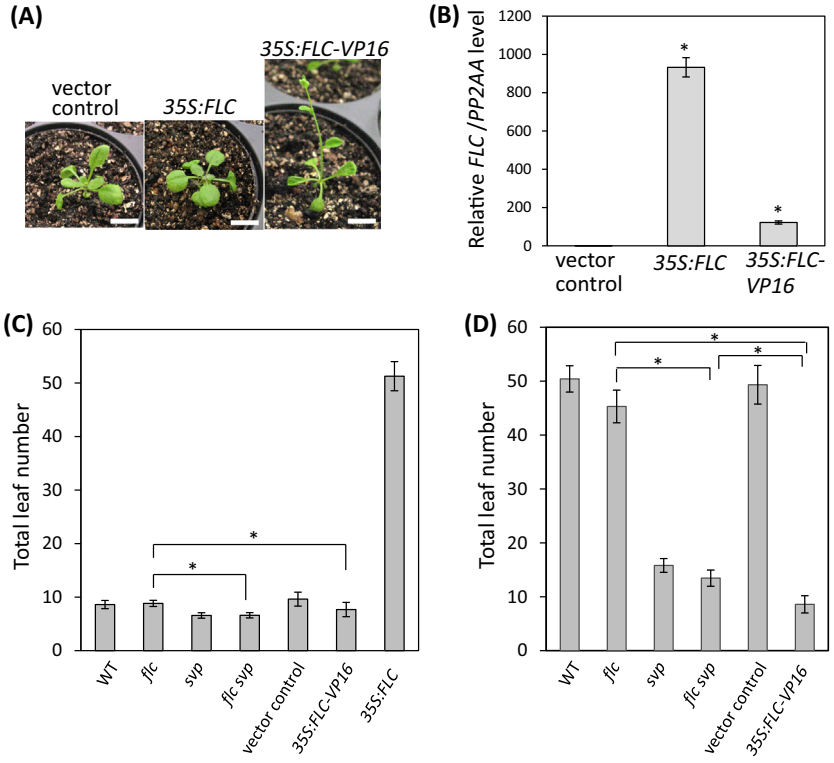

Fig. 2. Early-flowering phenotype of *35S:FLC-VP16* plants is similar to or even stronger than that of the *flc svp* double-knockout line. (A) Representative vector control, *35S:FLC*, and *35S:FLC-VP16* plants grown for 18 days under long-day condition. Bars = 1 cm. (B) Relative *FLC* transcript levels in representative vector control, *35S:FLC*, and *35S:FLC-VP16* plants. The plants were grown for 8 days under long-day conditions, and their whole seedlings were used. The value for the vector control was set to one, and relative values are shown for *35S:FLC* and *35S:FLC-VP16* plants. Mean values of three replicates are shown. Error bars represent standard deviations. Asterisks represent significant differences between the control and each value ($P < 0.05$). (C and D) Flowering time comparison of the vector control, *35S:FLC*, *35S:FLC-VP16*, WT, *flc*, *svp*, and *flc svp* under long- (C) and short-day (D) conditions. Mean values of total leaf numbers at bolting are shown. *35S:FLC* plants under short-day condition have not started to bolt at the time when they produced 60 leaves. Error bars represent standard deviation. Asterisks represent selected combinations with significant differences ($P < 0.05$).

3. Results

3.1. Constitutive expression of VP16-fused FLC causes early flowering

To evaluate the efficacy of VP16 fusion to reverse functions of transcriptional repressors, we selected FLC as a representative, well-studied transcriptional repressor. We produced transgenic *Arabidopsis* plants constitutively expressing *FLC* fused with *VP16* at the C-terminus (hereafter called *35S:FLC-VP16*) and compared the plants with a vector control and *FLC*-overexpressing (*35S:FLC*) transgenic plants. We found that all *35S:FLC-VP16* T1 plants flowered early under both long- and short-day conditions compared with the mean value of control plants; a total of 7.69 and 19.18 leaves at bolting, respectively (Fig. 1). In contrast, late-flowering

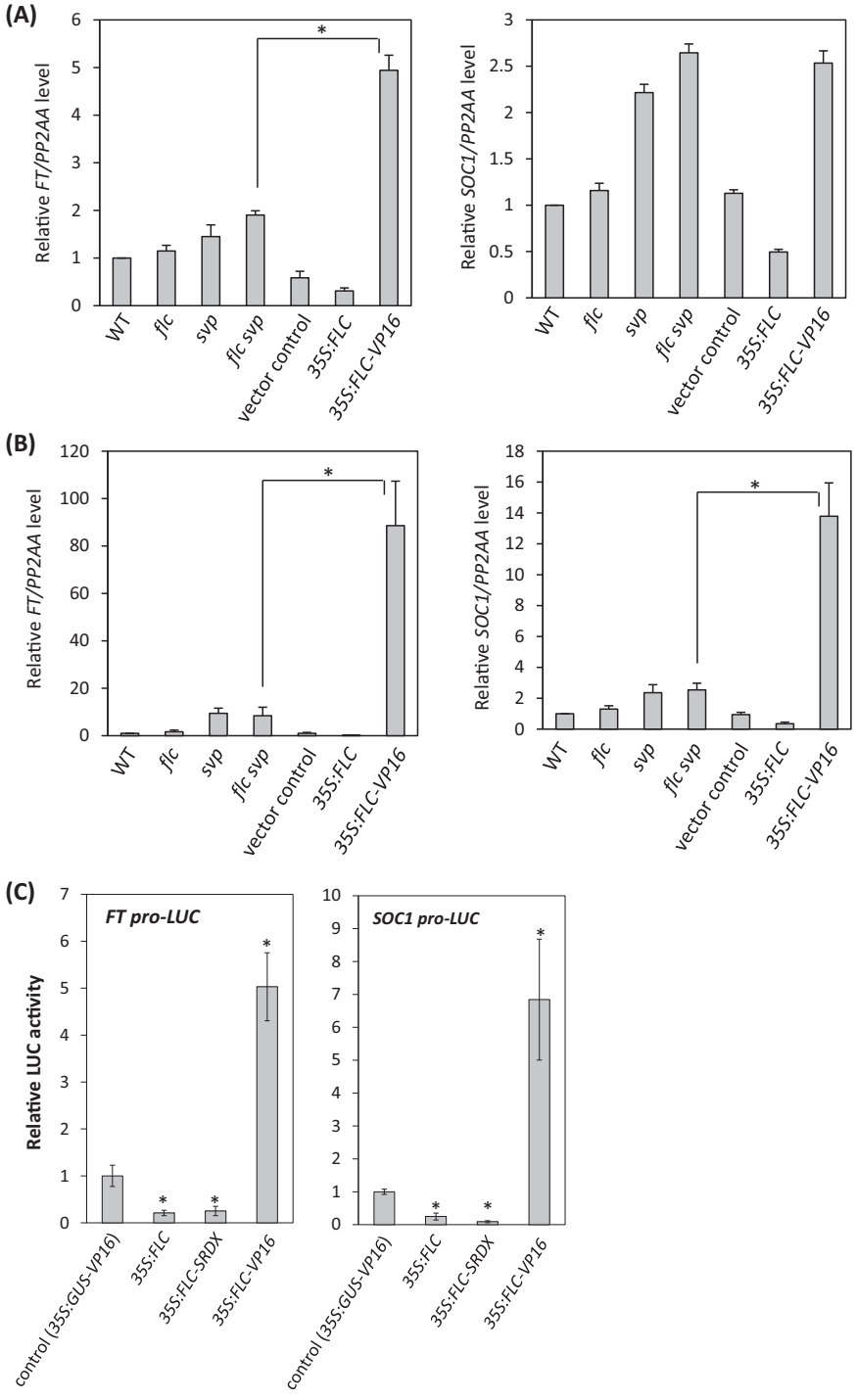

Fig. 3. *FT* and *SOC1* transcription was upregulated by*35S:FLC-VP16*. *FT* and *SOC1* transcript levels were analyzed by quantitative reverse transcription polymerase chain reaction using whole seedlings grown under long- (A) or short-day (B) conditions for 5 or 14 days, respectively, and harvested at ZT16 (ZT, hours after the light period started) or ZT14, respectively, at the time at which the diurnal *FT* transcript level oscillation showed its maximum level in the wild-type. Mean values of three replicates are shown. Error bars represent standard deviation. Asterisks represent selected combinations with significant differences ($P < 0.05$). (C) Reporter plasmids (*FTpro:LUC* or *SOC1pro:LUC*) and effector plasmids (*35S:FLC*, *35S:FLC-SRDX*, *35S:FLC-VP16*, or *35S:GUS-VP16* as a control) were introduced into *Arabidopsis* mesophyll protoplasts, and luciferase activity was quantified. Asterisks represent significant differences between the control and each value ($P < 0.05$).

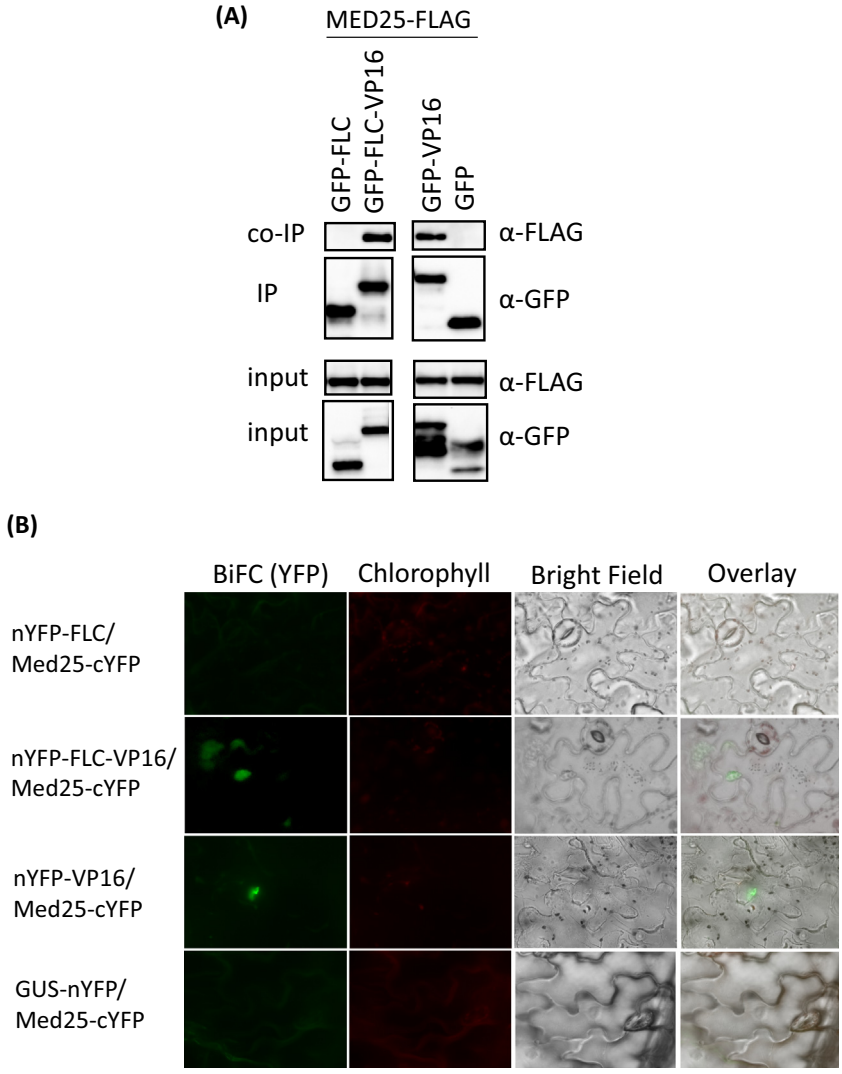

Fig. 4. VP16 associates with *Arabidopsis* Med25 *in vivo*. (A) FLAG-tagged Med25 (FLAG-Med25) was co-expressed with GFP-FLC, GFP-FLC-VP16, GFP-VP16, or GFP in *N. benthamiana* leaves, and the protein complexes including GFP-tagged proteins were immunoprecipitated using an α-GFP antibody. Inset, immunoprecipitated (IP) and co-immunoprecipitated (co-IP) samples were detected by α-GFP or α-FLAG antibodies. Representative data from three biological replicates are shown. (B) BiFC assay using the *N. benthamiana* protein expression system. nYFP-fused FLC, FLC-VP16, VP16, and GUS (negative control) were coexpressed with cYFP-fused Med25 BiFC in *N. benthamiana* leaves, and their peeled epidermis was subjected to microscopic analysis.

plants were observed among the *35S:FLC* T1 plants under both long- and short-day conditions. The flowering phenotype of the *35S:FLC* T1 plants varied between individual plants, showing late, normal, and early flowering, which was comparable with a previous report [36]. We isolated homozygous transgenic lines with high FLC transgene expression levels (Fig. 2A and B) and compared their flowering time with that of *flc* and *svp* single- and *flc svp* double-knockout lines under long- and short-day conditions (Fig. 2C and D). SVP is reported to be a redundant floral repressor of FLC and the *flc svp* double loss-of-function line shows a stronger early-flowering phenotype under short-day conditions [9]. As expected, *35S:FLC* and *35S:FLC-VP16* showed late and early flowering, respectively, compared with control plants under long- and short-day conditions. The early-flowering phenotype was more distinct under the short-day condition in which long-day *Arabidopsis* wild-type (WT) plants flowered late; *flc* and *svp* flowered earlier than WT, and *flc svp* flowered much earlier, as mentioned earlier. *35S:FLC-VP16* flowered earlier than single- and double-knockout lines. These data indicate that the VP16 fusion system worked strongly and caused a strong phenotype as in a multiple loss-of-function line with redundant genes.

3.2. FLC transcriptional repressor activity was converted to activator activity by VP16 fusion

To identify the cause for the strong early-flowering phenotype of *35S:FLC-VP16* plants, we analyzed the expression levels of major repression targets of FLC and SVP, *FT* and *SOC1* (Fig. 3A and B). As expected, *FT* and *SOC1* transcript levels in the late-flowering *35S:FLC* were lower than those in control plants, but they were significantly upregulated in the *35S:FLC-VP16* plants. Under short-day conditions, in which the photoperiod pathway-dependent induction of *FT* and *SOC1* expression does not occur in WT *Arabidopsis*, *FT* and *SOC1* transcript levels were low in the vector control and

35S:FLC, but significant upregulation was observed in *35S:FLC-VP16* plants (Fig. 3B). The upregulation of *FT* and *SOC1* in *35S:FLC-VP16* was stronger than that of *flc*, *svp*, and *flc svp*, suggesting a stronger effect on target gene expression by VP16 fusion to FLC.

To test whether this upregulation was caused by reversion of FLC transcriptional repressor activity due to VP16 fusion, we performed a transient assay using the *Arabidopsis* mesophyll cell protoplast system (Fig. 3C). The activities of luciferase (LUC) fused with the *FT* (*FTpro:LUC*) or *SOC1* promoter (*SOC1pro:LUC*) were significantly lower when *35S:FLC* was used as the effector compared with that when the negative control effector plasmid *35S:GUS-VP16* was used. In contrast, *35S:FLC-VP16* significantly induced *FTpro:LUC* and *SOC1pro:LUC* activities. These results show that FLC transcriptional repressor activity was effectively converted to activator activity by fusion with VP16, and this could be the cause of the multiple-knockout-like phenotype of *35S:FLC-VP16*.

3.3. Med25 interacts with VP16-fused protein in vivo

Med25 has been identified as a VP16 interactor in metazoan studies and is essential for its transcriptional activation activity [28,42]. ACID of *Arabidopsis* Med25 does not show high similarity to that of metazoan Med25 [1], and it is unknown whether it interacts with VP16. To test whether plant Med25 also interacts with VP16 in vivo, we performed immunoprecipitation assays using the *N. benthamiana* system to co-express the tagged *Arabidopsis* proteins (Fig. 4A). FLAG-tagged Med25 (FLAG-Med25) was co-expressed with GFP-FLC, GFP-FLC-VP16, GFP-VP16, or GFP, and the protein complex including the GFP-tagged proteins was immunoprecipitated with an α-GFP antibody. FLAG-Med25 was co-immunoprecipitated with the VP16-fused proteins, GFP-FLC-VP16 and GFP-VP16, but not with GFP or GFP-FLC without VP16 fusion, indicating that VP16 directly or indirectly interacts with *Arabidopsis* Med25 in vivo. To further investigate the relationship between VP16 and Med25, we performed a BiFC analysis in *N. benthamiana* leaves (Fig. 4B). Specific fluorescence was detected only when the VP16-fused proteins and Med25 were co-expressed, suggesting a direct interaction between VP16 and Med25 in vivo.

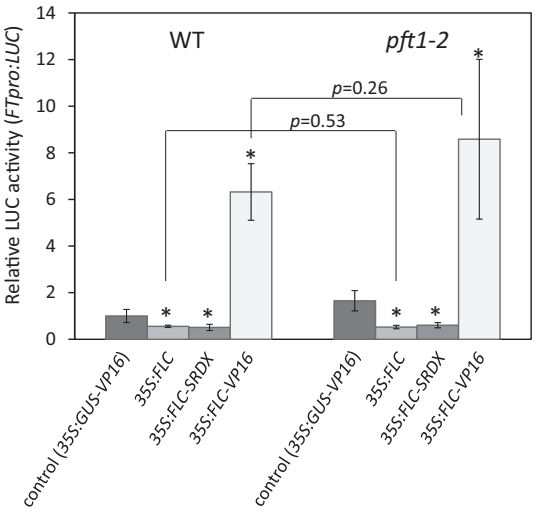

Fig. 5. *med25* single knockout does not affect VP16 activation activity. Reporter (*FTpro:LUC*) and effector plasmids (*35S:FLC*, *35S:FLC-SRDX*, *35S:FLC-VP16*, or *35S:GUS-VP16* as a control) were introduced into *Arabidopsis* mesophyll protoplasts, and luciferase activity was quantified. Asterisks represent significant differences between the control and each value ($P < 0.05$). No significant reporter activity differences between WT and *pft1–2* were detected for *35S:FLC* ($P = 0.53$) and *35S:FLC-VP16* ($P = 0.26$).

3.4. med25 single knockout does not affect VP16 activation activity

As we detected an interaction between VP16 and Med25, we performed a transient assay in WT and *med25* single-knockout line (*pft1–2*, [19] to test whether Med25 is essential for VP16 activation activity, as reported in metazoans (Fig. 5). No significant difference was observed in reporter activities between the WT and *pft1–2*, suggesting that Med25 is not an exclusive coactivator for VP16 in *Arabidopsis* and that other key machinery exists for transcriptional activation.

4. Discussion

In this study, we showed a clear example of the effectiveness of VP16 fusion to the transcriptional repressor to cause a strong phenotype similar to or even stronger than the multiple-knockout line of its redundant gene caused by transcriptional activation of direct target genes (Figs. 1–3). These results indicate that VP16 fusion is a useful technique to study the biological and molecular functions of transcriptional repressors in plants because it induced a strong phenotype that can only be seen when a multiple loss-of-function line of redundant genes is generated. This is similar to the CRES-T system, which has been widely used in plant transcriptional activator studies [16,27]. However, there is a report showing that VP16 fusion failed to reverse transcriptional repressor function [32]. Mutating the repression motif in addition to VP16 fusion could be a more definite technique to successfully revert transcriptional repressor function.

We found that *Arabidopsis* Med25 interacted with VP16 in vivo, as shown in metazoans, although plant and metazoan Med25 show low homology (Fig. 4, [1]. This result suggests that the transcriptional activation caused by the fusion of VP16 to transcription factors is achieved through the function of the Mediator complex, including Med25. *Arabidopsis* Med25 was originally isolated as a nuclear protein that acts downstream of phytochromeB to regulate *FT* expression [4]. Bäckström et al. [1] suggested that regulation of *FT* transcription by Med25 does not occur through the CONSTANS (CO)-dependent pathway, in which CO functions as a major *FT* activator in the photoperiod-dependent floral induction pathway. Later, Iñigo et al. [17] suggested that Med25 regulates flowering by regulating *FT* through both CO-dependent and -independent pathways, in which an unknown and putative transcription factor also plays a key role. Constitutive expression of *FLC-VP16* in *Arabidopsis* might mimic the active state of native *FT* and *SOC1* transcriptional activators through formation of an active mode Mediator complex caused by the VP16-Med25 association. However, to our surprise, single loss-of-function of *Med25* did not cause significant changes in transcriptional activation activity of VP16-fused proteins (Fig. 5), which was contrary to reports in animals. This finding suggests a plant-specific mechanism for the VP16-dependent activation machinery; there are other key regulators of transcriptional activation required for VP16-dependent transcriptional activation. Finding such other machinery could provide new insights into understanding the transcriptional regulatory mechanisms.

In summary, we report a clear example of the efficacy of VP16 fusion with a native transcriptional repressor that mimicked its multiple-knockout phenotype. This finding indicates the utility of the VP16 fusion technique for functional analysis of transcriptional repressors in plants. We also demonstrated the interaction between plant Med25 and VP16 *in vivo*, which could be involved

in the transactivation activities of VP16-fused transcription factors. However, no significant effect of Med25 loss-of-function to VP16 transactivation activity was observed, suggesting other unidentified key mechanisms. This line of findings provides important clues for investigating the yet-to-be determined molecular mechanisms of plant transcriptional regulation. Further functional analyses of Med25, VP16, and their interactors in plants are crucial to understand gene regulation mechanisms in plants and to develop new technologies to control gene expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.08.010.

References

[1] Bäckström, S., Elfving, N., Nilsson, R., Wingsle, G. and Björklund, S. (2007) Purification of a plant mediator from *Arabidopsis thaliana* identifies PFT1 as the Med25 subunit. Mol. Cell 26, 717–729.
[2] Bolle, C., Schneider, A. and Leister, D. (2011) Perspectives on systematic analyses of gene function in *Arabidopsis thaliana*: new tools. Top. Trends Curr. Genomics 12, 1–14.
[3] Carlsten, J.O., Zhu, X. and Gustafsson, C.M. (2013) The multitalented Mediator complex. Trends Biochem. Sci. 38, 531–537.
[4] Cerdán, P.D. and Chory, J. (2003) Regulation of flowering time by light quality. Nature 423, 881–885.
[5] Çevik, V., Kidd, B.N., Zhang, P., Hill, C., Kiddle, S., Denby, K.J., Holub, E.B., Cahill, D.M., Manners, J.M., Schenk, P.M., Beynon, J. and Kazan, K. (2012) MEDIATOR25 acts as an integrative hub for the regulation of jasmonate-responsive gene expression in *Arabidopsis*. Plant Physiol. 160, 541–555.
[6] Chen, M.K., Hsu, W.H., Lee, P.F., Thiruvengadam, M., Chen, H.I. and Yang, C.H. (2011) The MADS box gene, FOREVER YOUNG FLOWER, acts as a repressor controlling floral organ senescence and abscission in *Arabidopsis*. Plant J. 68 (1), 168–185, http://dx.doi.org/10.1111/j.1365-313X.2011.04677.x. Epub.
[7] Crevillén, P. and Dean, C. (2011) Regulation of the floral repressor gene *FLC*: the complexity of transcription in a chromatin context. Curr. Opin. Plant Biol. 14, 38–44.
[8] Flanagan, P.M., Kelleher 3rd, R.J., Sayre, M.H., Tschochner, H. and Kornberg, R.D. (1991) A mediator required for activation of RNA polymerase II transcription *in vitro*. Nature 350, 436–438.
[9] Fujiwara, S., Oda, A., Yoshida, R., Niinuma, K., Miyata, K., Tomozoe, Y., Tajima, T., Nakagawa, M., Hayashi, K., Coupland, G. and Mizoguchi, T. (2008) Circadian clock proteins LHY and CCA1 regulate SVP protein accumulation to control flowering in *Arabidopsis*. Plant Cell 20, 2960–2971.
[10] Fujiwara, S., Kigoshi, K., Mitsuda, N., Suzuki, K. and Ohme-Takagi, M. (2014) VP16 fusion efficiently reveals the function of transcriptional repressors in *Arabidopsis*. Plant Biotechnol. 31, 123–132.
[11] Gu, X., Le, C., Wang, Y., Li, Z., Jiang, D., Wang, Y. and He, Y. (2013) *Arabidopsis* FLC clade members form flowering-repressor complexes coordinating responses to endogenous and environmental cues. Nat. Commun. 4, 1947.
[12] Hanano, S. and Goto, K. (2011) *Arabidopsis* TERMINAL FLOWER1 is involved in the regulation of flowering time and inflorescence development through transcriptional repression. Plant Cell 23, 3172–3184.
[13] He, Y. (2012) Chromatin regulation of flowering. Trends Plant Sci. 17, 556–562.
[14] Helliwell, C.A., Wood, C.C., Robertson, M., James Peacock, W. and Dennis, E.S. (2006) The *Arabidopsis* FLC protein interacts directly *in vivo* with *SOC1* and *FT* chromatin and is part of a high-molecular-weight protein complex. Plant J. 46, 183–192.
[15] Hino, T., Tanaka, Y., Kawamukai, M., Nishimura, K., Mano, S. and Nakagawa, T. (2011) Two Sec13p homologs, AtSec13A and AtSec13B, redundantly contribute to the formation of COPII transport vesicles in *Arabidopsis thaliana*. Biosci. Biotechnol. Biochem. 75, 1848–1852.
[16] Hiratsu, K., Matsui, K., Koyama, T. and Ohme-Takagi, M. (2003) Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis*. Plant J. 34, 733–739.
[17] Iñigo, S., Alvarez, M.J., Strasser, B., Califano, A. and Cerdán, P.D. (2012) PFT1, the MED25 subunit of the plant Mediator complex, promotes flowering through CONSTANS dependent and independent mechanisms in *Arabidopsis*. Plant J. 69, 601–612.
[18] Kelleher 3rd, R.J., Flanagan, P.M. and Kornberg, R.D. (1990) A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. Cell 61, 1209–1215.
[19] Kidd, B.N., Edgar, C.I., Kumar, K.K., Aitken, E.A., Schenk, P.M., Manners, J.M. and Kazan, K. (2009) The mediator complex subunit PFT1 is a key regulator of jasmonate-dependent defense in *Arabidopsis*. Plant Cell 21, 2237–2252.
[20] Kim, W.Y., Fujiwara, S., Suh, S.S., Kim, J., Kim, Y., Han, L., David, K., Putterill, J., Nam, H.G. and Somers, D.E. (2007) ZEITLUPE is a circadian photoreceptor stabilized by GIGANTEA in blue light. Nature 449, 356–360.
[21] Koo, S.C., Bracko, O., Park, M.S., Schwab, R., Chun, H.J., Park, K.M., Seo, J.S., Grbic, V., Balasubramanian, S., Schmid, M., Godard, F., Yun, D.J., Lee, S.Y., Cho, M.J., Weigel, D. and Kim, M.C. (2010) Control of lateral organ development and flowering time by the *Arabidopsis thaliana* MADS-box gene AGAMOUS-LIKE6. Plant J. 62, 807–816.
[22] Lee, J.H., Yoo, S.J., Park, S.H., Hwang, I., Lee, J.S. and Ahn, J.H. (2007) Role of *SVP* in the control of flowering time by ambient temperature in *Arabidopsis*. Genes Dev. 21, 397–402.
[23] Li, D., Liu, C., Shen, L., Wu, Y., Chen, H., Robertson, M., Helliwell, C.A., Ito, T., Meyerowitz, E. and Yu, H. (2008) A repressor complex governs the integration of flowering signals in *Arabidopsis*. Dev. Cell 15, 110–120.
[24] Michaels, S.D. and Amasino, R.M. (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell 11, 949–956.
[25] Michaels, S.D., Himelblau, E., Kim, S.Y., Schomburg, F.M. and Amasino, R.M. (2005) Integration of flowering signals in winter-annual *Arabidopsis*. Plant Physiol. 137, 149–156.
[26] Mitsuda, N. and Ohme-Takagi, M. (2009) Functional analysis of transcription factors in *Arabidopsis*. Plant Cell Physiol. 50, 1232–1248.
[27] Mitsuda, N., Matsui, K., Ikeda, M., Nakata, M., Oshima, Y., Nagatoshi, Y. and Ohme-Takagi, M. (2011) CRES-T, an effective gene silencing system utilizing chimeric repressors. Methods Mol. Biol. 754, 87–105.
[28] Mittler, G., Stühler, T., Santolin, L., Uhlmann, T., Kremmer, E., Lottspeich, F., Berti, L. and Meisterernst, M. (2003) A novel docking site on Mediator is critical for activation by VP16 in mammalian cells. EMBO J. 22, 6494–6504.
[29] Nagaya, S., Kawamura, K., Shinmyo, A. and Kato, K. (2010) The HSP terminator of *Arabidopsis thaliana* increases gene expression in plant cells. Plant Cell Physiol. 51, 328–332.
[30] Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T. and Kimura, T. (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J. Biosci. Bioeng. 104, 34–41.
[31] Nakamura, H., Xue, Y.L., Miyakawa, T., Hou, F., Qin, H.M., Fukui, K., Shi, X., Ito, E., Ito, S., Park, S.H., Miyauchi, Y., Asano, A., Totsuka, N., Ueda, T., Tanokura, M. and Asami, T. (2013) Molecular mechanism of strigolactone perception by DWARF14. Nat. Commun. 4, 2613.
[32] Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H. and Ohme-Takagi, M. (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell 13, 1959–1968.
[33] Oshima, Y., Mitsuda, N., Nakata, M., Nakagawa, T., Nagaya, S., Kato, K. and Ohme-Takagi, M. (2011) Novel vector systems to accelerate functional analysis of transcription factors using chimeric repressor gene-silencing technology (CRES-T). Plant Biotechnol. 28, 201–210.
[34] Oshima, Y., Shikata, M., Koyama, T., Ohtsubo, N., Mitsuda, N. and Ohme-Takagi, M. (2013) MIXTA-like transcription factors and WAX INDUCER1/SHINE1 coordinately regulate cuticle development in *Arabidopsis* and *Torenia fournieri*. Plant Cell 25, 1609–1624.
[35] Ou, B., Yin, K.Q., Liu, S.N., Yang, Y., Gu, T., Wing Hui, J.M., Zhang, L., Miao, J., Kondou, Y., Matsui, M., Gu, H.Y. and Qu, L.J. (2011) A high-throughput screening system for *Arabidopsis* transcription factors and its application to Med25-dependent transcriptional regulation. Mol. Plant 4, 546–555.
[36] Ratcliffe, O.J., Nadzan, G.C., Reuber, T.L. and Riechmann, J.L. (2001) Regulation of flowering in *Arabidopsis* by an *FLC* homologue. Plant Physiol. 126, 122–132.
[37] Poss, Z.C., Ebmeier, C.C. and Taatjes, D.J. (2013) The Mediator complex and transcription regulation. Crit. Rev. Biochem. Mol. Biol. 48, 575–608.
[38] Searle, I., He, Y., Turck, F., Vincent, C., Fornara, F., Kröber, S., Amasino, R.A. and Coupland, G. (2006) The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in *Arabidopsis*. Genes Dev. 20, 898–912.
[39] Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J. and Dennis, E.S. (1999) The *FLF* MADS box gene: a repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. Plant Cell 11, 445–458.
[40] Shih, C.F., Hsu, W.H., Peng, Y.J. and Yang, C.H. (2014) The NAC-like gene ANTHER INDEHISCENCE FACTOR acts as a repressor that controls anther

dehiscence by regulating genes in the jasmonate biosynthesis pathway in *Arabidopsis*. J. Exp. Bot. 65, 621–639.
[41] Triezenberg, S.J., Kingsbury, R.C. and McKnight, S.L. (1988) Functional dissection of VP16, the *trans*-activator of herpes simplex virus immediate early gene expression. Genes Dev. 2, 718–729.
[42] Yang, F., DeBeaumont, R., Zhou, S. and Näär, A.M. (2004) The activator-recruited cofactor/Mediator coactivator subunit ARC92 is a functionally important target of the VP16 transcriptional activator. Proc. Natl. Acad. Sci. USA 101, 2339–2344.
[43] Yoshida, K., Sakamoto, S., Kawai, T., Kobayashi, Y., Sato, K., Ichinose, Y., Yaoi, K., Akiyoshi-Endo, M., Sato, H., Takamizo, T., Ohme-Takagi, M. and Mitsuda, N. (2013) Engineering the *Oryza sativa* cell wall with rice NAC transcription factors regulating secondary wall formation. Front. Plant Sci. 4, 383.