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Original Paper

VP16 fusion efficiently reveals the function of transcriptional repressors in Arabidopsis

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Abstract Proper gene expression regulated by transcription factors is essential for plants to achieve proper growth and development. However, the biological functions of many transcription factors remain largely unknown. Furthermore, although there are transcription factors which possess a plant-specific repression domain(s), their biological functions and whether such transcription factors function as transcriptional repressors are unclear. Thus, aiming for searching clues to understand their functions, we generated transgenic plants in which a putative transcriptional repressor fused with a VP16 viral trans-activation domain was expressed constitutively. Several plants with strong morphological phenotypes such as leaf and flower development defects were isolated from those lines expressing potential transcriptional repressors with unknown functions, giving the clue to reveal the yet-to-be analyzed functions of each protein. Reversal of function of the well-known transcriptional and floral repressor SHORT VEGETATIVE PHASE by VP16 fusion was observed, exemplifying successful functional reversion by this system. Plants constitutively expressing VP16 fused WUSCHEL, which is known to function both as a transcriptional activator and repressor, showed both phenotypes reported in its overexpression and loss-of-function lines. Taken together, our data provide examples showing the efficacy of VP16 fusion to provide helpful information to uncover the unknown functions of potential transcriptional repressors. This technique could also be effective to produce "super plants" which obtained strong and useful traits for application by strongly activating genes which are usually silent.

Key words: Arabidopsis, transcription factor, VP16, repression domain.

Plants achieve proper growth and development in a fluctuating environment through the coordinated functions of transcriptional activators and repressors. Transcriptional activation and repression are believed to function in a balanced manner to achieve proper regulation of gene expression. Approximately 15% of Arabidopsis transcription factors possess a plant-specific transcriptional repression domain(s), suggesting their functions as transcriptional repressors (Mitsuda and Ohme-Takagi 2009). Understanding the biological and molecular functions of transcriptional repressors and unraveling their roles in transcriptional networks is of great importance for both basic and applied studies in plants. However, the biological and molecular functions of transcriptional repressors remain largely unknown. For example, it is unclear how many of them and which ones actually work as transcriptional repressors. Analyses of transcriptional repressor knockout lines are useful to understand their biological functions. However, such an approach is not always possible because of the lack of knockout lines. Furthermore, most single loss-of-function mutants do not show a detectable phenotype because of redundancy (Bolle et al. 2011). Thus, alternative techniques are required to overcome such shortages of knockout analyses. CRES-T (Chimeric REpressor gene Silencing Technology) is a powerful tool for analyzing the biological functions of transcriptional activators, which has been widely used in both basic and applied studies (Hiratsu et al. 2003; Mitsuda et al. 2011). In this technique, a transcriptional activator is converted to a dominant transcriptional repressor by fusing a plant-specific repression domain called SRDX, which results in a strong phenotype that can usually be observed by producing multiple knockout lines of redundant genes. In contrast, fusion of a strong viral transactivation domain VP16 is a candidate technique to reverse or dominantly repress the function of a transcriptional repressor (Triezenberg et al. 1988).

Abbreviations: AG, AGAMOUS; CaMV, cauliflower mosaic virus; CLV3, CLAVATA3; CRES-T, Chimeric REpressor gene Silencing Technology; ERF, ETHYLENE-RESPONSIVE FACTOR; ESR1, ENHANCER OF SHOOT REGENERATION 1; FT, FLOWERING LOCUS T; LEP, LEAFY PETIOLE; PCR, polymerase chain reaction; SOC1, SUPPRESSOR OF OVEREXPRESSOR OF CO 1; SPL, SQUAMOSA BINDING PROTEIN LIKE; SVP, SHORT VEGETATIVE PHASE; TPL, TOPLESS; TPR, TOPLESS-RELATED; TT1, TRANSPARENT TESTA; WUS, WUSCHEL.

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However, one critical problem with this technique is the lack of examples. Although VP16 fusion has been used in plant transcription factor studies, only a few reports used VP16 for the repressive regulator of transcription, in which the repressor was converted to an activator (Hanano and Goto 2011; Koo et al. 2010). Furthermore, one report showed that VP16 transactivation activity is strongly eliminated by the tobacco ETHYLENE-RESPONSIVE FACTOR3 (ERF3) fragment containing a repression domain as assessed by the GAL4DB transient assay system, suggesting that there are cases in which the VP16 fusion system is not sufficiently effective to reverse the transcriptional repressor function (Ohta et al. 2001). Analyzing more lines expressing VP16-fused potential transcriptional repressors is important to test the efficacy of this technique. If this technique is effective, it will be a powerful tool to reveal the biological functions of yet-to-be studied potential transcriptional repressors and to awake important and useful traits that have been latent because of the repressor function.

In this study, we investigated efficacy of VP16 fusion technique in the study of biological function of potential transcriptional repressors in plant. We randomly picked the potential transcription factors with a known repression domain(s) and searched for those that showed a visible morphological phenotype when fused with VP16 and constitutively expressed. Several lines with strong and unique phenotypes were successfully isolated, suggesting the potential of this system for functional analyses of transcriptional repressors whose biological functions remain unknown. This system could also be useful for applied studies aimed at producing plants which obtained useful traits.

Materials and methods

Plant materials and growth conditions

All plants used in this study were *Arabidopsis thaliana* Columbia. Plants were grown at 22°C under long-day conditions (16 h light and 8 h dark) or continuous light conditions. Transgenic plants were grown on Murashige and Skoog medium containing 0.8% agar, 30 mg/l Hygromycin and 250 mg/l Vancomycin and transferred to soil 14–16 days after they started growing.

Vector construction

To prepare plasmids for the constitutive expression of *VP16* fused transcription factors, the coding region of each transcription factor without a stop codon was amplified by polymerase chain reaction (PCR) from an *Arabidopsis* cDNA library, fusing Gateway attB1 and attB2 sequences (Life technologies, USA) at the 5′ and 3′ ends, respectively. The fragment was cloned into pDONR207 (Life technologies), and introduced into pDEST_35S_VP16_HSP_GWB5 (described below) by Gateway LR clonase II (Life technologies). For the pDEST_35S_VP16_HSP_GWB5 construction, the 35S promoter-omega-attR1-ccdB-attR2-VP16 fragment from pDEST35SVP16HSP (Oshima et al. 2013) was digested by *Hin*dIII and cloned into the *Hin*dIII site of R4pGWB5_SRDX_HSP (Oshima et al. 2011) after the removal of the attR4-ccdB-attR2-SRDX fragment. To prepare the plasmid for the constitutive expression of *SVP* without *VP16* fusion, the same entry clone (*SVP* in pDONR207) was used for the LR reaction for introducing the *SVP* fragment into the pDEST_35S_3fstop_BCKH (Oshima et al. 2011). Primers used for the vector constructions are listed in Supplementary Table 1. The pDEST_35S_VP16_HSP_GWB5 vector without LR reaction was used to generate vector control transgenic plants

RNA extraction and qRT-PCR

Total RNA was extracted using an RNeasy Plant Mini kit (Qiagen, Germany). Genomic DNA was removed by DNase digestion, following the manufacturer's instructions. One microgram of total RNA was subjected to first-strand cDNA synthesis using a PrimeScript RT reagent kit (Takara Bio, Japan). Quantitative RT-PCR was performed by the SYBR green method using an ABI7300 real-time PCR system (Life technologies). The gene-specific primers used for qRT-PCR are shown in Supplementary Table 1.

Results and discussion

Repression of flowering by the transcriptional repressor SHORT VEGETATIVE PHASE (SVP) was reversed by VP16 fusion

We analyzed the phenotype of transgenic Arabidopsis plants overexpressing*VP16*-fused *SVP* driven by the Cauliflower mosaic virus (CaMV) 35S promoter (*35S:SVP-VP16*) as a pilot experiment before starting the analyses of potential transcriptional repressors with unknown function. SVP is a floral repressor that represses flowering by repressing transcription of floral activator genes (Fujiwara et al. 2008; Hartmann et al. 2000; Jang et al. 2009; Lee and Yao 2007; Li et al. 2008). *SVP* encodes a MADS box-type transcription factor with two plant-specific repression domains, one in the N terminus and another in the C terminus (Supplementary Table 2, Hartmann et al. 2000; Mitsuda and Ohme-Takagi 2009). *SVP* knockout mutants show an early flowering phenotype, and the phenotype is strengthened by the combination with the knockout mutant of another MADS box-type transcription factor *FLOWERING LOCUS C* (Fujiwara et al. 2008; Hartmann et al. 2000; Li et al. 2008). *SVP* overexpression results in a late flowering phenotype under the long-day condition, and the phenotype is enhanced under the continuous light condition (Fujiwara et al. 2008; Li et al. 2008). We could obtain the plants constitutively expressing *SVP* (*35S:SVP*) showing late flowering phenotype under the continuous light condition, as reported previously (Figure 1A,

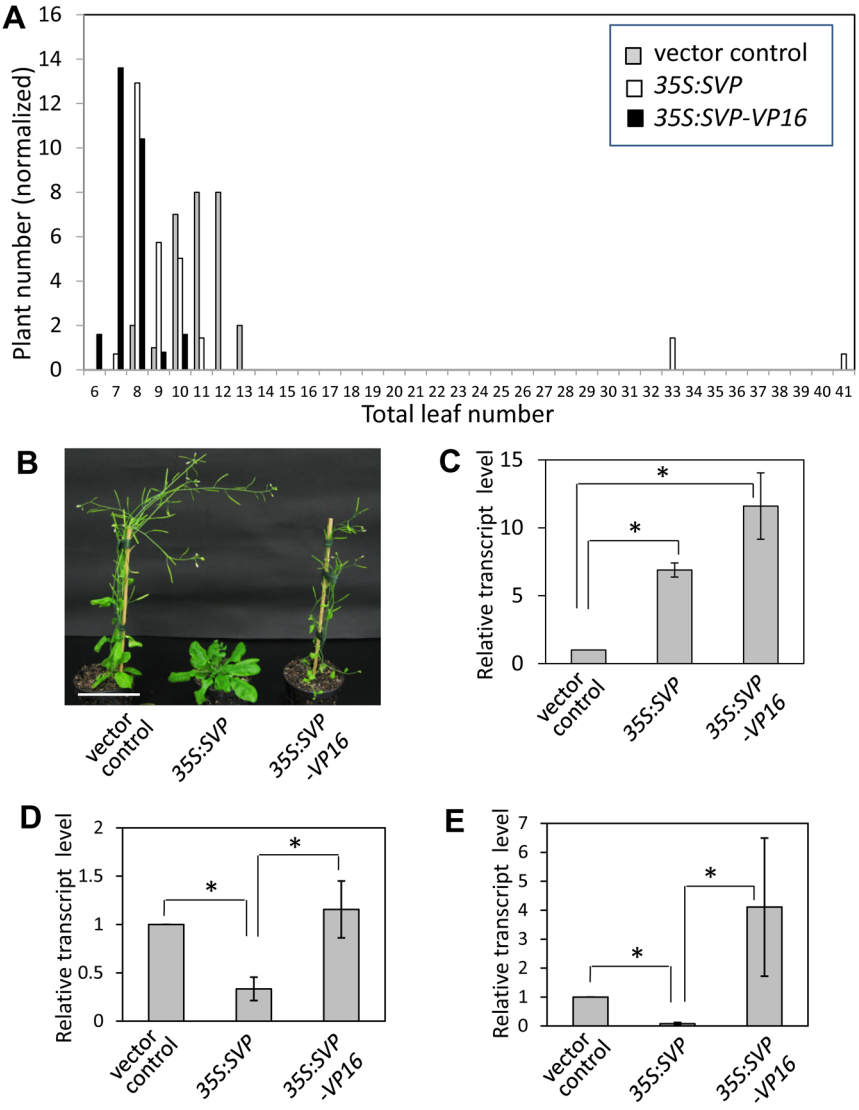

Figure 1. Reversal of SVP function as a floral repressor by VP16 fusion. (A) Distribution of flowering time of *35S:SVP*, *35S:SVP-VP16* and vector control T1 plants under the continuous light condition. Total leaf numbers at the bolting stage were counted. Plant numbers were normalized to adjust to that of the vector control. $n=28$–42. (B) Representative vector control, *35S:SVP*, *35S:SVP-VP16* T2 plants grown for 50 days. Bar=5 cm. (C) Relative *SVP* transcript levels in representative vector control, *35S:SVP*, *35S:SVP-VP16* plants. Plants were grown for 10 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:SVP* and *35S:SVP-VP16* plants. Mean values of three replicates are shown. Error bars represent the standard deviations. (D, E) Transcript levels of *FT* and *SOC1* were analyzed by qRT-PCR using the whole seedlings grown under the continuous light condition for 10 days. Mean values of three replicates are shown. Error bars represent the standard errors. Asterisks represent the combinations which showed significant differences (C–E, $p<0.05$).

Fujiwara et al. 2008). The flowering timing of T1 *35S:SVP* plants was segregated, as reported for that of *SVP* and other floral repressors encoding MADS box family transcription factors (Figure 1A, Cho et al. 2012; Ratcliffe et al. 2001). In total, 6, 17, and 19 plants showed later, normal, and earlier flowering, respectively, compared with the control plants transformed with an empty vector under the continuous light condition. In contrast, no T1 *35S:SVP-VP16* plants flowered late. In total, 32 and 3 plants showed earlier and normal flowering, respectively. We picked representative T2 lines of *35S:SVP-VP16*, *35S:SVP* and the control, which showed earlier, later and normal flowering phenotypes, respectively, and analyzed the *SVP* transcript levels (Figures 1B, C). *SVP* levels were approximately 7 and 12 times higher in the *35S:SVP* and *35S:SVP-VP16* plants, respectively, than those in the control plants. Transcript levels of major SVP target genes, *FLOWERING LOCUS T* (*FT*), which encodes the floral activator florigen, and *SUPRESSOR OF OVEREXPRESSOR OF CO 1* (*SOC1*), which encodes a MADS box transcription factor and also functions as a floral activator, were significantly downregulated in the *35S:SVP* plants but the repression was not observed in *35S:SVP-VP16* plants, compared with those in the vector control plants (Figures 1D, E, Fujiwara et al. 2008; Li et al. 2008). These data suggest that VP16 fusion

successfully repressed SVP function as a transcriptional repressor, resulting in the earlier flowering phenotype partly caused by cancellation of repression of floral activator genes including *SOC1* and *FT*. The finding that no T1 *35S:SVP-VP16* plants tested showed a late flowering phenotype indicates its strong flowering activation activity (Figure 1A). However, significant upregulation effects of SVP-VP16 on the *FT* and *SOC1* transcription were not detected in our condition when compared with the vector control plants, despite the clear flowering acceleration effects (Figure 1A, B, D, E). Since more than 5,000 genes are reported to be targeted by SVP (Gregis et al. 2013), transcriptions of many other genes must be changed in the *35S:SVP-VP16* plants. Analyzing the difference of the targets between SVP and SVP-VP16 will be helpful to further understand the VP16 fusion effect and the cause of the flowering acceleration.

Overexpression of VP16-fused WUSCHEL (WUS) caused multiple morphological defects

We generated another VP16 fusion line for the well-known transcription factor, WUS. WUS is a homeobox transcription factor essential for the maintenance of the stem cell pool (Laux et al. 1996). WUS has repression domains and is known to function both as a transcriptional activator and a repressor (Supplementary Table 2, Ikeda et al. 2009; Leibfried et al. 2005; Lenhard et al. 2001; Lohmann et al. 2001; Yadav et al. 2011; Yadav et al. 2013). WUS directly activates the expression of *CLAVATA3* (*CLV3*), and CLV3 in turn repress the *WUS* expression (Schoof et al. 2000; Yadav et al. 2011; Yadav et al. 2013). This feedback loop is important for the stem cell pool homeostasis in the shoot apical meristem. WUS also activates *AGAMOUS* (*AG*) expression which encodes a floral homeotic MADS box transcription factor, while AG represses the *WUS* expression (Ikeda et al. 2009; Lenhard et al. 2001; Lohmann et al. 2001). This feedback loop contributes to the proper floral meristem maintenance. On the other hand, WUS represses the transcription of *ARABIDOPSIS RESPONSE REGULATOR* genes and differentiation promoting transcription factors (Leibfried et al. 2005; Yadav et al. 2013). Therefore, we analyzed *35S:WUS-VP16* plants as an exceptional example as a transcription factor with repressive and activation activities. Among the 16 T1 *35S:WUS-VP16* plants, 8 showed severe morphological defects (Figure 2). *WUS* knockout lines were reported to produce bunch of leaves at different positions along the plant, and also to produce fused leaves (Hamada et al. 2000; Laux et al. 1996). Such phenotypes were observed in these *35S:WUS-VP16* plants, not in the wild-type plants (Figure 2A–D). However, loss of flower organs which has been reported in the *WUS* knockout plants was not observed in the *35S:WUS-VP16* plants (Kieffer et al. 2006; Laux et al. 1996). The floral organs of the *35S:WUS-VP16* plants were generally larger than those of the wild-type plants (Figure 2E). It suggests the possible enlargement of the shoot apical meristem size, which is opposite to the *WUS* knockout plants. In addition, *35S:WUS-VP16* showed strong multiple phenotypes reported for the plants overexpressing *WUS*, such as dark green and curly leaves, fasciation of influorescent stems, ectopic pedicel formation, formation of multiple cauline leaves on the top of the lateral influorescent stem, anthocyanin accumulation, and fused flowers (Figures 2B, F–J). Taken together, fusion of VP16 to WUS causes multiple morphological defects which are observed in loss-of-function or gain-of-function lines. This might reflect the bilateral function of WUS as transcriptional activator and repressor. Overexpression of VP16-fused WUS probably boosted the activation effect of WUS on the native activation targets. This example indicates that VP16 fusion does not always cause a knockout-like phenotype when the transcription factor functions as both a transcriptional activator and a repressor. One of the commonly reported phenotypes for plants ectopically expressing *WUS* is somatic embryo, adventitious shoot and floral bud formation on roots, stems or leaves (Gallois et al. 2004; Ikeda et al. 2009; Xu et al. 2005; Zuo et al. 2002). However, such phenotypes were not observed in any of the *35S:WUS-VP16* plants, implying that the *WUS* overexpression effect on the stem cell induction was attenuated by the VP16 fusion.

Although some transcription factors have functions both as a transcriptional activator and a repressor like WUS, the detailed mechanism of how they function differently is largely unknown. Furthermore, it is unknown how many transcription factors have such dual activities. Studies using VP16 and SRDX fusion will provide informative clues to understand the functions of such transcription factors.

Analysis of the VP16-fused potential transcriptional repressors with unknown function suggested their important roles in growth and development

Since we could confirm the efficacy of the VP16 fusion system for SVP and WUS as examples, we applied this technique to the randomly selected transcription factors with a repression domain(s) whose functions were unknown. Among them, we found several lines that showed strong visible phenotypes as described below (Figure 3).

Seven of the 16 T1 plants constitutively expressing VP16-fused *ERF084* (At1G80580) showed multiple phenotypes including dark green, curly, and rough surface leaves, dwarf, shorter petals and sepals, late flowering, and low fertility (Figure 3C). *ERF084* encodes a member of the group VIII (B-1) of the AP2/ERF family, which is a large transcription factor family

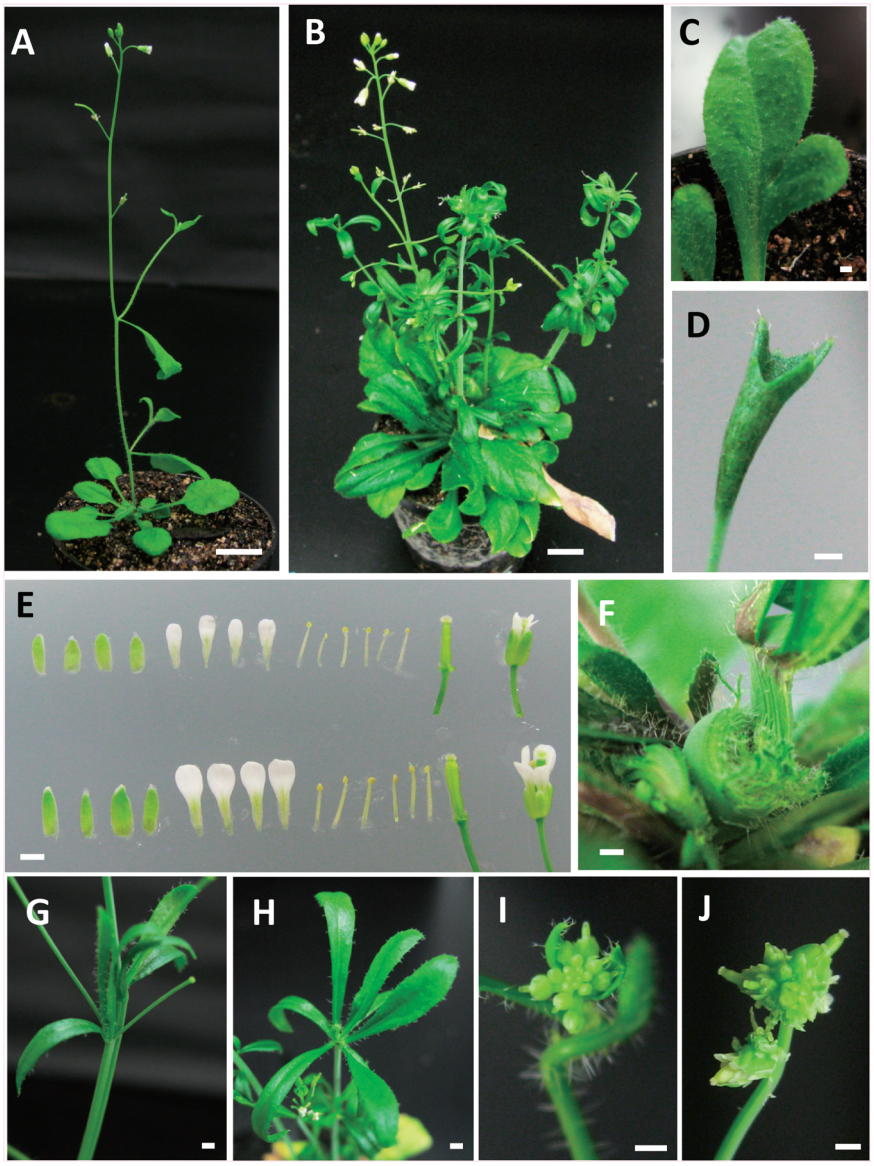

Figure 2. *35S:WUS-VP16* phenotypes. Four-week-old wild-type (A) and 10-week-old *35S:WUS-VP16* plants (B–D, F–J). (E) Flowers of wild-type (upper) and *35S:WUS-VP16* (lower). Bars represent 1 cm in A, B and 1 mm in C–J.

in plant (Nakano et al. 2006; Sakuma et al. 2002). Many ERF genes have been reported to have various functions involved in developmental and physiological processes in various plant species. However, the function of the ERF084 protein remains unknown. Interestingly, transcript level of *ERF084* is upregulated in the apex of *clv3* mutant (AtGenExpress, Schmid et al. 2005), suggesting its possible role in the shoot apical meristem. There are 15 members in the group VIII, including ENHANCER OF SHOOT REGENRATION1 (ESR1) and LEAFY PETIOLE (LEP). Overexpression of *ESR1* induces initiation of shoot regeneration in Arabidopsis (Banno et al. 2001; Kirch et al. 2003). The leaf phenotype of the activation-tagged *LEP* is similar to that of our *VP16*-fused *ERF084* overexpressor (van der Graaff et al. 2000). VP16-fused ERF084 might mimic relative transcription activators because LEP does not have a repression domain. Further studies using the knockout and overexpressor lines of *ERF084* and other related genes will lead to our understanding of regulatory mechanism of plant development.

Four of the 16 T1 *35S: SQUAMOSA BINDING PROTEIN LIKE 12* (SPL12) VP16 plants showed flower formation defects including fused terminal flower production (Figure 3D). The other 5 plants showed retarded growth and died after soil transplantation. The other 5 plants were dwarf and had late growth. These phenotypes suggested the importance of proper SPL12 function for plant growth and development. However, the biological function of SPL12 remains unknown. The SQUAMOSA BINDING PROTEIN domain containing superfamily members were originally identified in

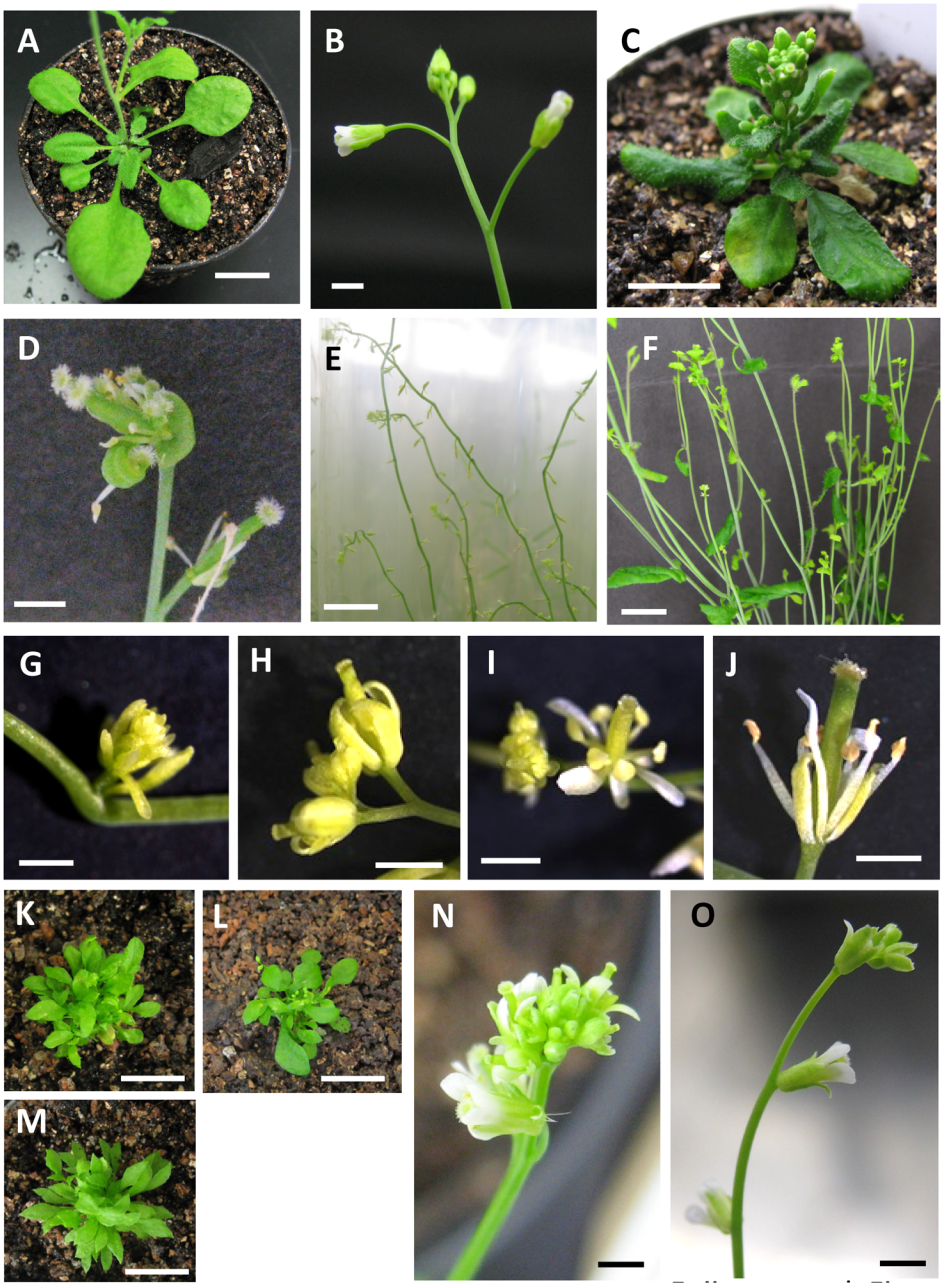

Figure 3. Phenotypes of VP16 fused overexpressors of potential transcriptional repressors with unknown function. Photos of wild-type plants (A, B), *35S:ERF-84-VP16* (C), *35S:SPL12-VP16* (D), *35S:At1g10455-VP16* (E–J), *35S:DUF313-VP16* (K), *35S:At5G60470-VP16* (L), *35S:At3G13840-VP16* (M) and *35S:WIP5-VP16* (N, O). Bars represent 1 cm (A, C, E, F, K–M) and 1 mm (B, G–J, N, O).

Antirrhinium majus, which binds to the promoter region of *SQUAMOSA* and regulates floral meristem identity (Klein et al. 1996). Seventeen *SPL* genes are present in Arabidopsis (Cardon et al. 1999; Xing et al. 2010). Based on sequence similarity and genomic structure, Arabidopsis *SPL* genes can be divided into two groups. SPL12 belongs to the same family with SPL1, −7, −14, and −16, which are constitutively expressed (Schmid et al. 2003; Xing et al. 2010). The group members except for SPL7 have one or two repression domains in their N terminus (Supplementary Table 2, Mitsuda and Ohme-Takagi 2009; Stone et al. 2005). Although the biological functions of members of this group are largely unknown, Stone et al. reported that SPL14 participates in plant development and sensitivity to the fungal toxin (2005). Further studies will provide instructive information to unravel the functions of members in this group. SPL1, which shares 69.4% identity with SPL12 at the amino acid level (Cardon et al. 1999), interacted with the transcriptional corepressor family proteins TOPLESS (TPL), TOPLESS-RELATED 2 (TPR2), TPR3, and TPR4 in a TPL family interactome study using yeast two hybrid system (Causier et al. 2012; Liu and Karmarkar 2008). Recent studies show that many transcriptional repressors

with a repression domain(s) repress target gene expression by interacting with TPL/TPR corepressors (Causier et al. 2012; Kagale and Rozwadowski 2011; Krogan et al. 2012; Liu and Karmarkar 2008; Shyu et al. 2012; Tao et al. 2013; Wang et al. 2013). SPL1 and SPL12 may also function as transcriptional repressors by interacting with TPL/TPR corepressor family members. It would be interesting to analyze whether they actually interact with TPL/TPR family proteins *in vivo* and whether the interaction is disturbed by VP16 fusion. Detailed mechanisms of how TPL/TPR corepressors contribute to transcriptional repression remain unknown. In fact, the mechanisms of how VP16-fused transcription factors function as transcriptional activators are also unknown. Molecular analyses of transcriptional repressors with and without VP16 fusion focusing on the relationships between TPL/TPR corepressors and other related proteins is the next project to unravel plant transcriptional regulation mechanisms.

All tested T1 *35S:At1g10455-VP16* plants showed defects in flower formation, and each plant had different types of defects, including low fertility, loss of pedicels (Figure 3E), flower-like organ formation only on the top of the influorecent stems (Figure 3F), loss of some floral organs (Figure 3G–J), over production of petals (Figure 3I) and formation of stamenoid petals (Figure 3J). At1g10455 is a relatively newly found gene that encodes a B3 transcription factor (Swaminathan et al. 2008). There are 118 genes in the plant-specific B3 superfamily, including the auxin response factor family proteins, whose studies are relatively advanced (Hayashi 2012). In contrast, At1g10455 belongs to the less studied REM family which is composed of 76 genes. Interestingly, among the 36 genes encoding predicted DNA-binding proteins that are positively regulated by the floral homeotic MADS transcription factor AG, 10 belong to the B3 family including 8 REM family genes (Gómez-Mena et al. 2005). The transcript level of At1g10455 in the flower is higher than that in other aerial vegetative organs (At-TAX database, Laubinger et al. 2008). This expression profile, the reported function of other REM family members, and the flower phenotype we detected suggest the important roles of At1g10455 gene and/or other REM genes in proper flower development.

At1G32030, encoding a domain of unknown function (DUF313), also belong to the REM family (Swaminathan et al. 2008). Five of the 16 T1 *35S:DUF313-VP16* plants showed lost or reduced apical dormancy (Figure 3K). Two died in soil and the others produced thin stems and many small and narrow leaves and flower buds with reduced internode length of the inflorescent stem. At1G32030 is expressed constitutively (eFP browser, Winter et al. 2007). These data suggest the importance of DUF313 for plant growth and development.

Our VP16-fused overexpressors might mimic the activator type B3 transcription factors and induce a strong phenotype. Analysis of genes affected by B3-VP16 overexpression and plants overexpressing other genes encoding the activator type B3 transcription factors might provide new insights into proper floral organ formation and other development and growth.

In addition, some T1 plants constitutively expressing VP16-fused *ID-domain 13* (At5G60470), encoding the INDETERMINATE1-like C2H2 transcription factor (Colasanti et al. 2006), and *SCARECROW-LIKE 29* (At3G13840), encoding a GRAS family transcription factor (Bolle 2004), showed phenotypes similar to those of the *35S:DUF313-VP16* plants (Figure 3L, M). Although the biological functions of these two genes are unknown, this phenotypic similarities suggest that they might function in close or the same pathways of plant growth regulation and development.

WIP5 and 5 other WIP proteins form a plant-specific subfamily of C2H2 zinc finger proteins in Arabidopsis (Appelhagen et al. 2010; Sagasser et al. 2002). This subfamily has zinc fingers in their highly conserved C-terminal region called the WIP domain. One member of the WIP family, TRANSPARENT TESTA 1 (TT1)/WIP1, is involved in seed coat development based on the yellow seed color of the knockout mutant and its specific expression in the developing ovule and young seeds (Sagasser et al. 2002). *TT1* overexpression causes multiple morphological phenotypes, including aberrant leaves, delayed flowering, repressed elongation of inflorescence internodes, smaller flowers, and flower development without pedicels. Among WIP members, TT1/WIP1, WIP4, and WIP5 have a repression domain (Supplementary Table 2, Mitsuda and Ohme-Takagi 2009), but whether they function as transcriptional repressors is unknown. We found that the *35S:WIP5-VP16* phenotype is partially similar to that of the *TT1* overexpressor, such as pedicle-less flowers, small flowers, aberrant leaf formation and repressed internode elongation (Figure 3N, O). None of the T2 seeds were yellow as in the *tt1* mutant seeds (data not shown). These observations suggest that either they are not transcriptional repressors or are repressors in which VP16 fusion is insufficient to reverse the transcriptional repressor activity. Because the *WIP5* expression pattern does not show clear organ or developmental stage specificity, TT1 and WIP5 do not appear to have the same function. These observations will be a clue to understand the biological and molecular functions of WIP family proteins.

In conclusion, we could show the efficacy of an analysis of VP16-fused putative transcriptional repressors. If VP16 fusion acts to reverse the function of transcriptional repressors as with SVP, we would expect a strong phenotype similar to its multiple knockout line phenotypes. It will be helpful to identify the biological

functions of transcriptional repressors that do not show any phenotype with single gene knockout. Because having a repression domain(s) does not always mean that the transcription factor functions as a repressor, there must be cases in which strong expression of the transcription factor with VP16 fusion enhances native transcriptional activation activity but does not reverse repressor activity. It would be also a powerful tool to find the potential function of these transcription factors. Phenotypic analyses of plants constitutively expressing the VP16 fused transcription factor provide the clue to understand the biological function of the transcription factor. In order to fully understand the function of the transcription factor, further studies such as phenotypic analyses of its loss-of-function, overexpression and CRES-T lines, analyses of transcriptome profiles in those lines, and molecular analyses of their transcriptional regulation activities are required.

Since VP16 fusion to the transcriptional repressor may cause strong expression of genes that are normally silent, it could cause a strong and/or unique phenotype that cannot be obtained by regular knockout or regular overexpression. We actually presented several examples in this study. In some cases, no visible phenotypic changes are observed, but examination of stress responses will be promising to identify novel regulator of stress tolerance. Therefore, this technique would be a powerful tool to isolate plants with strong useful phenotypes for applications such as higher yield of biomass, fuel, and material; strong stress tolerance; and useful morphology. Comprehensive production and analyses of transgenic plants expressing VP16-fused transcription factors, along with those of CRES-T and regular overexpressor and knockout lines could accelerate the overall understanding of transcription factors and transcriptional networks.

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Supplementary Table 1. Primers used in this study

Purpose	Gene Name	Primer sequence (5' to 3')
Entry clone preparation	*ERF-84*	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAAAACAGCTACACCGTTGATGGT
		GGGGACCACTTTGTACAAGAAAGCTGGGTTCTTCCTAGACAACAACCCTAAACTCAAATC
	SPL12	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAAGCTAGAATTGAAGGTGAAGTA
		GGGGACCACTTTGTACAAGAAAGCTGGGTTGCTTGTTCCATACTCAAGTAATTCCCACCT
	AT1G10455	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCACAAAATAAGAATCTCAATCTGG
		GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTCTTCTGTAATACGTTTTTCTGTGTCGA
	IDD13	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGACAAATCGGTTCTTTTGTGAA
		GGGGACCACTTTGTACAAGAAAGCTGGGTTTGGTTGCTTCCTGCCTACAATGGTTGCGGC
	SCL29	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTTGTTGGAAGAAACAGAACCACCAA
		GGGGACCACTTTGTACAAGAAAGCTGGGTTCTTCCACAATGAACAAAAGGAAACTGCCTC
	DUF313	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTACGTATTATAATGATCATCTCG
		GGGGACCACTTTGTACAAGAAAGCTGGGTTTGCCATGTCAGGAGGAGGAGGAAGA
	WIP5	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTAATCCAGCTTGTTCGAATCTCT
		GGGGACCACTTTGTACAAGAAAGCTGGGTTTTGCTCGATATCAGAAGCAGCATCATAGTA
	WUS	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGCCGCCACAGCATCAGCATCATCA
		GGGGACCACTTTGTACAAGAAAGCTGGGTTGTTCAGACGTAGCTCAAGAGAAGCGCAAGG
	SVP	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCGAGAGAAAAGATTCAGATC
		GGGGACCACTTTGTACAAGAAAGCTGGGTCACCACCATACGGTAAGCCGAGCCTA
Quantitative RT-PCR	*SVP*	TTCTGTAGCTCCAGCATGAAGGA
		AGATGGCTGATCAAGCTTCTCCAA
	FT	CTGCTACAACTGGAACAACCTTTG
		TTTGCCTGCCAAGCTGTCGAA
	SOC1	ATGAATTCGCCAGCTCCAATATGC
		TGGTGCTGACTCGATCCTTAGTA
	PP2AA3	GACCAAGTGAACCAGGTTATTGG
		TACTCTCCAGTGCCTGTCTTCA

Supplementary Table 2. Repression domains and their positions in the proteins analyzed in this study.

AGI code	common name (Alias)	short description	repression motif sequence	position of the repression motif (aa, position/full length)
AT1G80580	ERF-84	ethylene-responsive element-binding family protein	GLELDL	242/257
AT3G60030	SPL12	SPL12 (SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE 12); transcription factor	RLTLNL	95/928
AT1G10455	-	unknown protein	NLNLEL	5/153
AT5G60470	IDD13	zinc finger (C2H2 type) family protein	TLTLSL	21/450
AT3G13840	SCL29	GRAS family transcription factor	IDLDLPP	88/510
AT1G32030	-	Domain of unknown function (DUF313)	LDLNTIP	98/333
AT1G51220	WIP5	WIP domain protein 5	DLHLGL	104/337
AT2G17950	WUS1, PGA6, WUS	WUSCHEL 1, WUSCHEL, Homeodomain-like superfamily protein	TLPLFP	254/292
			SLELRL	286/292
AT2G22540	FAQ1, AGL22, SVP	SHORT VEGETATIVE PHASE, AGAMOUS-like 22, K-box region and MADS-box transcription factor family protein	SLELQL	83/240
			SLRLGL	231/240