The SUPERMAN protein is an active repressor whose carboxy-terminal repression domain is required for the development of normal flowers

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Abstract SUPERMAN was identified as a putative regulator of transcription that acts in floral development, but its function remains to be clarified. We demonstrate here that SUPERMAN is an active repressor whose repression domain is located in the carboxy-terminal region. Ectopic expression of SUPERMAN that lacked the repression domain resulted in a phenotype similar to that of *superman* mutants, demonstrating that the repression activity of SUPERMAN is essential for the development of normal flowers. Constitutive expression of *SUPERMAN* resulted in a severe dwarfism but did not affect cell size, indicating that SUPERMAN might regulate genes that are involved in cell division.

Key words: Active repressor; Floral development; Cell proliferation; *SUPERMAN*; *Arabidopsis*

1. Introduction

Transcriptional repressors play central roles in development and in the regulation of cell proliferation in eukaryotes. Repressors can be divided into two broad categories, passive and active, and active repressors, unlike passive repressors, include an independent repression domain [1]. Numerous active repressors have been reported in yeast, mammals, and *Drosophila* [1], but only a few transcription factors have been identified as active repressors in plants [2]. We reported recently that the class II ERF transcription factors and a number of TFIIIA-type zinc-finger transcription factors are active repressors in plants and that the repression domain of each of these factors is located in the carboxy-terminal region that contains a conserved ERF-associated amphiphilic repression (EAR) motif (L/FDLNL/FXP), which is essential for the activity of each repressors [2]. However, biological functions of these active repressors are still unknown.

In this study, we found a sequence similar to an EAR motif in the SUPERMAN (SUP) protein, a putative regulator of transcription involved in floral development [3]. We demonstrated that SUP is an active repressor and its repression activity is essential for the development of normal flowers. We identified that the repression domain of SUP is located in the carboxy-terminal region, as is an EAR-like motif. Ectopic expression of SUP that lacked the carboxy-terminal repression domain resulted in a phenotype similar to that of *superman* (*sup*) mutants [4,5], with flowers having extra stamens, for example, demonstrating that the repression activity of SUP is essential for the development of normal flowers. On the other hand, constitutive expression of SUP resulted in a severe dwarfism but did not affect cell size. Based on these results, possible function for SUP in floral meristems was discussed.

2. Materials and methods

2.1. Transient expression

Analysis of transient expression in *Arabidopsis* leaves after particle bombardment was described previously [2]. In co-transfection assays, we used 1.6 μg of reporter and 1.2 μg of effector constructs for each bombardment. Luciferase (LUC) assays were performed with the Dual-Luciferase™ Reporter Assay System and a luminescence reader (TD-20/20; Promega, Madison, WI, USA). To normalize values after each transfection, 0.4 μg of plasmid, pPTRL, which included LUC gene from *Renilla* under the control of the 35S promoter of cauliflower mosaic virus, was used as an internal control.

2.2. Cloning and transformation

The SUP coding region was amplified by PCR with the *Arabidopsis* genomic TAC clone # K14B15 provided from the Kazusa DNA Research Institute (Kisarazu, Japan) as template. The DNA coding of full-length *SUP* and of 1/174 region of *SUP* (SUPΔRD) were fused downstream of the 35S promoter of cauliflower mosaic virus and cloned into the *Hin*dIII–*Sac*I sites of the transformation vector pBIG-HYG [6] to create p*35S::SUP* and p*35S::SUPΔRD*. These constructs were used to transform *Agrobacterium tumefaciens* strain GV3101 and were introduced wild-type *Arabidopsis* plants (Col-O) by vacuum infiltration [7]. Transgenic plants were selected on hygromycin-containing medium.

3. Results and discussion

One member of the plant family of TFIIIA-type zinc-finger transcription factors [8], SUP of *Arabidopsis*, is involved in the maintenance of the stamen/carpel whorl boundary [3–5]. By database search analysis, we found that SUP contains an amphiphilic amino-acid sequence similar to the EAR motif in its carboxy-terminal region (Fig. 1), as do three other TFIIIA-type zinc-finger proteins in *Arabidopsis*, namely, ZAT12 [9], ZAT7 [9] and ZFP7 [10] (Fig. 1).

To examine whether SUP functions as an active repressor,

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Abbreviations: EAR, ERF-associated amphiphilic repression; SUP, SUPERMAN; GAL4DB, yeast GAL4 DNA-binding domain; LUC, luciferase; SUPRD, 175/204 repression domain of SUPERMAN; SUPΔRD, 1/174 region of SUPERMAN; *AP3*, *APETALA3*

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 EAR motif                                  L/FDLNL/FxP
*SUPERMAN (175/204) NDEIISLELEIGLINESEQDLDLELRLGFA
 ZAT7      (124/168) PETTTVTALKKFSSGKRVACLDLDLDSMESLVNWKLELGRTISWS
*ZAT12     (121/162) LPEPTVTTLKKSSSGKRVACLDLSLGMVDNLNLKLELGRTVY
 ZFP7      (165/209) RQVVEEAEAPVVVVASTESGLDLNSVAANGGVDNNSSKPDLTLRL
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Fig. 1. Alignment of amino acids in the carboxy-terminal regions of TFIIIA-type zinc-finger proteins that contain an EAR-motif-like sequence. Numbers in parentheses indicate the positions of amino acid sequences. Reverse type indicates amphiphilic regions homologous to the EAR motif. Asterisks indicate peptides whose repression activities were analyzed in transient expression assays.

we performed a transient expression assay with an effector plasmid in which the coding region of SUP or a truncated version was fused to that of the yeast GAL4 DNA-binding domain (GAL4DB) under control of 35S promoter of cauliflower mosaic virus [SUP(D)]. We bombarded *Arabidopsis* leaves with the effector plasmid together with a plasmid in which a reporter gene for LUC was under the control of 35S promoter with five copies of the GAL4-binding site inserted immediately upstream of the TATA box (35S-GAL4-LUC) (Fig. 2A). We then examined whether the effector could repress expression of the reporter gene for LUC. As shown in Fig. 2B, the level of expression of the reporter gene was reduced by 75% when the effector that contained full-length SUP (1/204) was co-expressed with the reporter plasmid, while truncated SUP from which the carboxy-terminal region that included the EAR-motif-like sequence has been deleted (SUPΔRD) did not affect the expression of the reporter gene. When the effector plasmid encoded only the carboxy-terminal region of SUP (156/204 or 175/204), the level of expression of the reporter gene was reduced by more than 97%. These results indicated that SUP was an active repressor and that its repression domain was located in the carboxy-terminal region, namely, the region from residue 175 to residue 204 that includes an EAR-motif-like sequence. We confirmed the repression activity of the carboxyl-terminal 175/204 region of SUP using another reporter gene, GAL4GCC-LUC [2,11], whose transcription is activated by the AtERF5 effector, which is a GCC-box-specific activator of transcription [11]. As shown in Fig. 3, the effector plasmid that encoded the 175/204 repression domain of SUP (SUPRD) fused to GAL4DB (GAL4DB–SUPRD) reduced the AtERF5-activated level of expression of the reporter gene by 85% (ratio of extents of induction = 2.6/17.5) and it also reduced the basal activity of the reporter gene by 57% (ratio of extents of induction = 0.43/1). These results indicated that repression by SUP was not specific for the 35S promoter and that SUP did not act by inhibiting specific transcriptional activators for specific promoters. The GAL4–SUPRD effector did not repress the expression of the reporter gene when the latter lacked a GAL4 binding site (data not shown). Thus, binding to DNA was required for repression, as in the case of the ERF3 repression domain of tobacco [2]. We also confirmed that the carboxy-terminal region of 42 amino acids of ZAT12, which contains an EAR-motif-like sequence (Fig. 1), functioned as a repressor when expressed as a fusion protein with GAL4DB (data not shown). Our results suggested that amphiphilic amino-acid sequences similar to an EAR motif in these plant zinc-finger proteins might be important for repression and might function in the same manner as the EAR motif.

To examine the importance of repression by SUP, we characterized the function of the repression domain of SUP in vivo by expressing the SUPΔRD under the control of the 35S promoter (*35S::SUPΔRD*), in transgenic *Arabidopsis* plants. This region contained the C2H2-type zinc-finger region that is important for DNA-binding activity [8,12] but lacked the carboxy-terminal repression domain. If the function of endogenous SUP were to be titrated by SUPΔRD, which cannot act as a repressor, we would expect that repression by SUP would be abolished. Thus, plants would be expected to exhibit the same phenotype as those of plants with loss-of-function alleles of SUP as a result of a dominant-negative effect. The flowers of *sup* mutants have been well characterized. The boundary between the stamens and carpels is shifted toward the center of the floral meristem, with resultant formation of extra stamens, at the expense of carpels, and a defective gynoecium [4,5]. Wild-type *Arabidopsis* flowers have six stamens and two central carpels that fuse to form the female reproductive structure (Fig. 4A). Among the 60 independent *35S::SUPΔRD* transgenic plants that we obtained, six clearly resembled *sup* mutants in the T1 generation as follows. The most dramatic phenotypic alternation was evident in the fourth whorl of the flowers of *35S::SUPΔRD* transgenic plants, with extra stamens in the region normally occupied by carpels. We observed flowers with 7–12 stamens

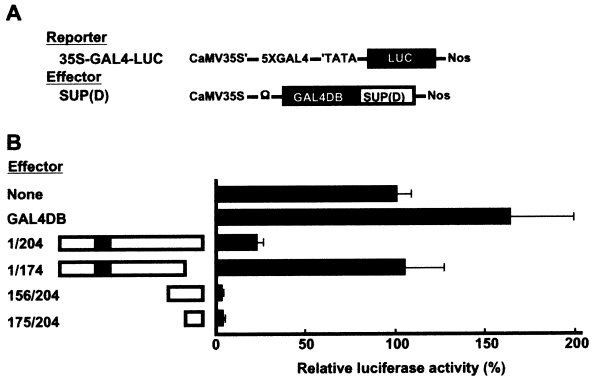

Fig. 2. Mapping of the repression domain of SUP. A: Schematic representation of the constructs used in bombardment experiments. The GAL4-responsive reporter, 35S-GAL4-LUC, contained the cauliflower mosaic virus (CaMV) 35S promoter (−800 to +8), in which five copies of the GAL4 binding site had been inserted in tandem at position −6, a translational enhancer sequence from tobacco mosaic virus (Ω), the firefly gene for LUC, and a nopaline synthase (Nos) terminator. Each effector construct contained a GAL4DB and part of coding region of SUP(D) under control of the CaMV 35S promoter. A translational enhancer sequence from tobacco mosaic virus (Ω) was located upstream of the site of initiation of translation. B: Relative LUC activities in *Arabidopsis* leaves that had been co-bombarded with reporter and effector plasmids. Transient expression was analyzed as described previously [2]. Diagrams of the deletion mutants of SUP are shown on the left. The indicated positions of SUP were fused to GAL4DB. Closed boxes indicate the C2H2-type zinc-finger domain. All LUC activities are expressed relative to values obtained with the reporter construct alone (with 'None' set arbitrarily at 100%). The values cited are averages, with standard deviations, of results from a minimum of three independent experiments.

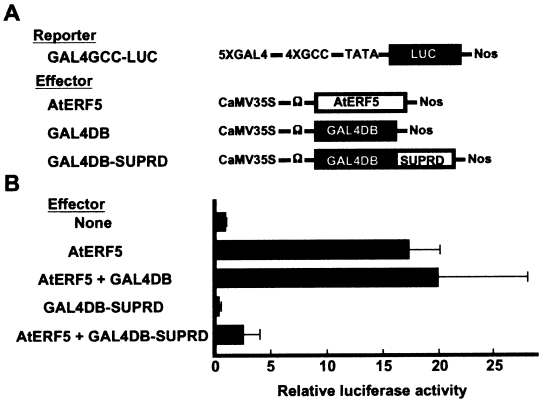

Fig. 3. Active repression of the expression of AtERF5-activated LUC activity by the repression domain of SUP (SUPRD). A: Diagram of the constructs used in co-bombardment experiments. The reporter construct, GAL4GCC-LUC, contained five copies of the GAL4 binding site, four copies of the GCC-box sequence (an AtERF5-binding site) in tandem, a minimal TATA region (starting at position −46) of the CaMV 35S promoter, the firefly gene for LUC, and a nopaline synthase (Nos) terminator. Each effector construct contained the coding sequence of AtERF5, a GAL4DB (shown as a closed box), and the SUPRD fused to GAL4DB (GAL4DB–SUPRD) as described in the legend to Fig. 1. Each construct was driven by the CaMV 35S promoter, and an omega sequence (Ω) from tobacco mosaic virus was located upstream of the site of initiation of translation. B: Relative LUC activities in *Arabidopsis* leaves that had been co-bombarded with reporter and GAL4-fusion effectors with or without the AtERF5 effector. Transient expression was examined as described previously [2]. The various effectors that were introduced into leaves with the reporter construct are shown on the left. All LUC activities are expressed relative to values obtained with the reporter construct alone (with 'None' set arbitrarily at 1). The values cited are averages, with standard deviations, of results from a minimum of three independent experiments.

on *35S::SUPΔRD* transgenic plants, and the carpel tissue that developed in the fourth whorl often failed to form a functional gynoecium (Fig. 4B,C). In addition to fertile stamens, the fourth whorl frequently developed mosaic organs that consisted of both stamen and carpel tissue (data not shown). We confirmed that the *sup*-like phenotype of transgenic plants was not due to disruption of the endogenous *SUP* gene during transformation by amplifying the endogenous *SUP* gene by PCR. Since the flowers of *35S::SUPΔRD* transgenic plants were very similar to flower on homozygous *sup* mutant plants [3,4], our results demonstrated that the repression domain of SUP is essential for SUP function in the regulation of the development of normal flowers.

The *sup* mutant phenotype is associated with the ectopic

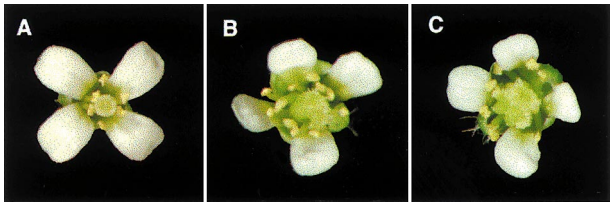

Fig. 4. Phenotype of flowers of *35S::SUPΔRD* transgenic plants. A: Wild-type (Col-O) flower, with the normal number of stamen (six) and a central gynoecium, which consisted of two fused carpels. B: Flower from a *35S::SUPΔRD* transgenic T1 plant with 11 stamens and two incompletely fused carpels. C: Flower from a *35S::SUPΔRD* transgenic T1 plant with seven stamens and three incompletely fused carpels.

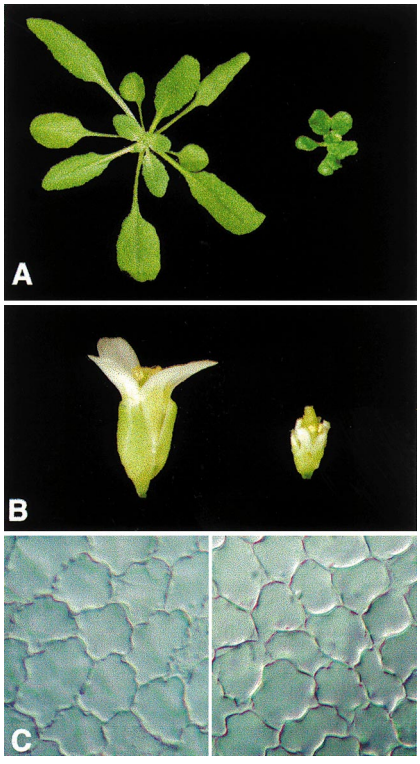

Fig. 5. Phenotype of *35S::SUP* transgenic plants. A: Wild-type plant (left) and *35S::SUP* plant (right). B: Flowers from a wild-type plant (left) and a *35S::SUP* plant (right). C: Epidermal cells (×300) from abaxial, distal portions of mature petals of wild-type (left) and *35S::SUP* (right) plants. Images were analyzed by using the NIH IMAGE program (http:/rsb/info.nih.gov/nih-image).

expression in the whorl 4 region of the floral homeotic gene *APETALA3* (*AP3*), which is required for development of the petal and stamen in whorls 2 and 3 [4,5]. It has been suggested that SUP acts as a transcriptional factor that represses expression of *AP3* in the fourth whorl of the flower [5]. However, a recent study provided evidence that the opposite is true since expression of *SUP* was found to be controlled by the floral homeotic genes *AP3*, *PISTILLATA* and *AGAMOUS* [13]. Furthermore, no SUP-responsive element was found in the promoter region of *AP3* [14]. Thus, it was suggested that SUP might repress cell division in the third-whorl primordia by defining the boundary between the third- and the fourth-whorl cells [13,15]. By contrast, it was reported that ectopic expression of the *SUP* gene in transgenic petunia and tobacco suppressed cell expansion but not cell division [16]. When full-length *SUP* was expressed from the 35S promoter (*35S::SUP*) in transgenic *Arabidopsis*, all of the T1 plants (19 lines) exhibited severe dwarfism (Fig. 5). In *35S::SUP* transgenic plants, all of the organs examined, such as leaves and floral organs, were much smaller than those of wild-type plants (Fig. 5A,B). We investigated the sizes and numbers of cells in mature petals of *35S::SUP* transgenic plants and of wild-type plants to determine whether the dwarf phenotype of *35S::SUP* transgenic plants was due to a reduction in cell number or to a reduction in cell size. We examined the distal portion of the petal epidermis because it contains diploid cells that are uniform in both size and shape [17]. As shown in Fig. 5C, there were no obvious major differences in terms of cell size and the number of cells per unit area between the trans-

genic plants and wild-type plants (wild-type/*35S::SUP* in cell size per unit area = 1.1). The minimal difference in cell size cannot explain the difference in petal between *35S::SUP* transgenic plants and wild-type plants (Fig. 5B). Thus, the dwarf phenotype of *35S::SUP* transgenic plants appeared to be associated with a decrease in cell number and not to a decrease in cell size. Since *35S::SUPΔRD* transgenic plants were of normal size, the putative decrease in cell number might have resulted from the repression activity of SUP.

Our results suggested that SUP might regulate cell proliferation negatively by repressing the transcription of genes that are involved in promotion of cell division, as proposed similarly by Sakai et al. [13]. For development of floral organs, the co-expression of the floral homeotic genes known as MADS-box genes in proper combination is sufficient [18], but spatial and temporal control of the expression of these genes is necessary for formation of a flower. SUP might act as a cadastral factor that determines the stamen/carpel boundary by restricting the excess proliferation of cells that express *AP3*, which develop into stamens. To clarify the mechanism of this phenomenon, efforts should be made to isolate the gene(s) whose expression is directly repressed by SUP.

We have described here the first evidence that an active repressor is involved in a developmental process in plants. Our results suggest, not unexpectedly, that repressors play important roles in plants. Many proteins containing an EAR motif [2] or an EAR-like motif have been identified but their biological functions remain unknown. Our findings provide some insight into the role of one such protein.

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