The FILAMENTOUS FLOWER protein has a zinc finger domain, hydrophobic region, proline-rich region, and a HMG box-like domain. We have reported that zinc release at the zinc finger is probably facilitated by the non-canonical cysteine residue at position 56, and that EDTA causes the structural change and enhances the self-assembly of the protein (Kanaya, E., Watanabe, K., Nakajima, N., Okada, K., and Shimura, Y. (2001) J. Biol. Chem. 276, 7383–7390). To investigate this aspect further we examined the DNA binding function of the FILAMENTOUS FLOWER protein. Gel retardation experiments showed that the FILAMENTOUS FLOWER protein binds to DNA without sequence specificity. Deletion analyses suggested that the zinc finger domain and the hydrophobic region are not required but the proline-rich region and the HMG box-like domain are indispensable for the DNA binding by the FILAMENTOUS FLOWER protein. The DNA binding by the protein consisting of the zinc finger domain and the rest of the regions was reduced with the addition of EDTA. This result probably suggests that the zinc release, the structural change probably occurring in the zinc finger domain, the intermolecular interaction, and the self-assembly of the protein are related to the dissociation of the FILAMENTOUS FLOWER protein from DNA.

In higher plants, all lateral organs exhibit abaxial-adaxial asymmetries, and common genetic mechanism is likely to work to specify organ polarity (1–6). The FILAMENTOUS FLOWER (FIL) gene is thought to be a member of a gene family whose role appears to be pivotal in specifying the abaxial cell fate of lateral organs (1–3). In this gene family, FIL, YABBY2(YAB2), and YABBY3(YAB3) are expressed abaxially in all developing lateral organs (1–3). The protein encoded by the FIL gene is thought to be a member of a gene family whose role appears to be pivotal in specifying the abaxial cell fate of lateral organs (1–3). The protein encoded by FIL consists of 229 amino acid residues, and has the amino-terminal zinc finger domain between positions 10 and 60, the hydrophobic region between positions 60 and 100, the proline-rich region between positions 127 and 145, and the carboxyl-terminal HMG box-like domain between positions 145 and 180 as shown in Fig. 1a. The presence of these domains, in particular the HMG box-like domain, suggests that the FIL gene may function as a transcriptional regulator, and the FIL protein is likely to work as a DNA-binding protein (1).

The HMG box is a conserved domain of about 80 amino acid residues, which mediates DNA binding by the HMG box protein (7). The solution structure of the HMG domain consists of a global fold of three α-helices (helix I, helix II, and helix III) stabilized in an L-shaped configuration (8, 9). The HMG proteins are divided into two classes. The HMG box proteins in the first class are transcription factors and bind to DNA in a sequence-specific manner, such as the human sex-determining factor SRY (10), the lymphoid enhancer binding factor LEF1 (11, 12), and the T-cell factor TCF-1 (13). The HMG box proteins in the second class are more abundant and bind to DNA in a non-sequence-specific manner. Although their biological functions are currently not clear, they are thought to participate in DNA recombination and repair, activation and repression of transcription, and nucleosome assembly and disassembly. Examples include HMG1 and 2 (HMG1/2) proteins from higher eukaryotes (14, 15), NHP6A and NHP6B from Saccharomyces cerevisiae (16), and HU from Escherichia coli (17). The HMG1/2 proteins strongly distort DNA upon binding, and stabilize bent and supercoiled DNA. NHP6A induces a large bend of DNA upon binding. HU exists in the cell as homo- and heterodimers and seems to participate to wrap the oriC sequence (18).

The amino acid sequence of the HMG box-like domain between positions 145 and 180 of the FIL protein which contains the putative helix 3 and helix 4 (Fig. 1b), shows a homology to those of the regions containing helix I and helix II of both the HMG domain of human mitochondrial transcription factor 1 and mouse testis-specific HMG (1). However, homology was not found in the region corresponding to helix III. A loop structure is predicted for the region corresponding to helix III and a short helix (helix 5) follows it in the FIL protein (Fig. 1b). The structure of the HMG box-like domain of FIL is different from those of the typical HMG domain in the region around helix III. Murphy et al. (19) identified three specificity determinants to differentiate between the non-sequence specific and sequence-specific HMG domains. First all non-sequence-specific HMG proteins have a serine residue at position 146 (Ser146) whereas all sequence-specific HMG proteins have an asparagine at the corresponding position. Second, non-sequence specific proteins have a hydrophobic residue at position 168, whereas sequence-specific proteins have a hydrophilic residue at the corresponding position. The presence of a Ser146 in the FIL protein agrees with FIL having a non-sequence specific HMG domain but the presence of His168 in FIL does not. The third determining domain is in a C-terminal hydrophobic core. But, the HMG box-like domain of the FIL protein has no corresponding region.

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‡ The abbreviations used are: HMG, high mobility group; FIL, FILAMENTOUS FLOWER; YAB2, YABBY2; YAB3, YABBY3; AP1, APETALA1; EMSAs, electrophoretic mobility shift assays; FITC, 5'-fluorescein 5-isothiocyanate.

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The HMG box-like domain of the FIL protein is partially different from the typical HMG box domains.

Recently, we have reported that the non-canonical cysteine residue at position 56 (Cys56) in the FIL protein potentially interferes with a zinc ligating within the zinc finger and probably facilitates the zinc release, and that the addition of EDTA causes the structural change and enhances the multimerization of the FIL protein. The COOH-terminal half of the zinc finger domain and the following hydrophobic region participate with the region between positions 45 and 107 functioning in the intermolecular contacts between the FIL proteins (20).

To place these data within the framework of the potential transcriptional role of the FIL protein we examined the relation among the zinc finger domain, the hydrophobic region, the proline-rich region, and the HMG box-like domain in DNA binding using gel retardation. We found that the proline-rich region and the HMG box-like domain are required for the DNA binding by the FIL protein with the zinc finger domain. The addition of EDTA reduced the DNA binding by the FIL protein. The COOH-terminal half of the zinc finger domain and the following hydrophobic region participate with the region between positions 45 and 107 functioning in the intermolecular contacts between the FIL proteins (20).

Construction of the Expression Vector—Construction of the vector for the expression of the FIL protein FIL-(6–229) was performed as described previously (1, 20). Construction of the truncated FIL genes for the expression of the seven FIL proteins, FIL-(6–229), FIL-(120–229), FIL-(143–229), FIL-(6–203), FIL-(6–183), FIL-(6–165), and FIL-(120–183) was done using PCR with synthetic DNA oligomers as primers and using KOD DNA polymerase for the polymerase chain reaction were from Toyobo Co., Ltd. Isopropyl-β-D-thiogalactopyranoside was from Wako Pure Chemical Industries, Ltd. DNA oligomers were synthesized by Sawady Technology Co., Ltd. Other chemicals were of reagent grade.

Experimental Procedures

Cells and Plasmids—Plasmids pET28a, pET29b and E. coli BL21(DE3) were from Novagen, Inc. Competent cells of E. coli JM109 (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, Δ(lac-proAB)/ FtraD36, proAB+, lacF′, lacZAM15) for plasmid construction were from Takara Shuzo Co., Ltd. Cells were grown in Luria-Bertani medium (21) containing 50 µg/ml kanamycin.

Materials—Restriction and DNA-modifying enzymes were from Takara Shuzo Co., Ltd. The DNA ligation kit (ligation high) and KOD DNA polymerase for the polymerase chain reaction were from Toyobo Co., Ltd. Isopropyl-β-D-thiogalactopyranoside was from Wako Pure Chemical Industries, Ltd. DNA oligomers were synthesized by Sawady Technology Co., Ltd. Other chemicals were of reagent grade.

Construction of the Expression Vector—Construction of the vector for the expression of the FIL protein FIL-(6–229) was performed as described previously (1, 20). Construction of the truncated FIL genes for the expression of the seven FIL proteins, FIL-(6–229), FIL-(120–229), FIL-(143–229), FIL-(6–203), FIL-(6–183), FIL-(6–165), and FIL-(120–183) was done using PCR with synthetic DNA oligomers as primers and using KOD DNA polymerase according the procedures recommended by the supplier. As the primers have recognition sites of restriction endonucleases NcoI and XhoI, the PCR products were digested with NcoI and XhoI, and ligated to the large NcoI-XhoI fragment of pET28a to generate plasmid pETFIL-(6–229), pETFIL-(120–229), pETFIL-(143–229), pETFIL-(6–203), pETFIL-(6–183), pETFIL-(6–165), and pETFIL-(120–183). The sequence of each FIL gene in the plasmid was confirmed by cycle sequencing using ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kits and ABI PRISM™ 310 Genetic Analyzer from Perkin-Elmer Life Sciences. Overexpression and purification of the FIL proteins were performed as described previously (1, 20).

The APETALA1 (AP1) gene was cloned from an Arabidopsis thaliana cDNA library by PCR using the synthetic DNA oligomers with recognition site of restriction endonuclease NdeI or HindIII, respectively. The PCR was performed as described above. The DNA product was digested with NdeI and HindIII and ligated to the large NdeI-HindIII fragment of pET29b. The sequence of the AP1 gene was confirmed as described above. Overproduction of the AP1 protein and the purification
of the AP1 protein using a HiTrap chelating column with Ni\textsuperscript{2+} ions were performed according to the procedure described previously (22). Concentrations of the FIL proteins and the API protein were determined according to the procedure described previously (22).

**Electrophoretic Mobility Shift Assays (EMSA)**—EMSA were performed with synthetic double-stranded oligonucleotide probes listed in Table I. 5’-Fluorescein 5-isothiocyanate (FITC)-labeled double-stranded random oligonucleotide pool of 76-mer in Table I was generated by polymerization in one direction using 5’-FITC-labeled

\( \text{ACGCGTTGCGAGCTCTCAGATCAG}^{-3} \)

as a primer, 5’-ACTCGAGGAAATTGGAATCTCCGCGATCAAC-GAGGTTACGCCGAGCTCTCAGCTCAG-3’ as a template, and KOD DNA polymerase from Toyobo Co. at 74°C for 15 min. Double stranded oligonucleotides with different lengths and sequences summarized in Table I were synthesized by Sawady Technology Co., Ltd. A 20-μl reaction for EMSA containing Buffer A (20 mM Tris-HCl, pH 7.7, 10% glycerol, 1 mM dithiothreitol, 0.1–0.5 μg of poly(dI-dC)-poly(dI-dC), 0.4% sodium deoxycholate, and 0.04 mM NaSCN), 2.5 pmol of FITC-labeled or 0.2 pmol of \(^{32}\)P-labeled double-stranded oligonucleotide probe, and 0.1–30 μg of the FIL protein were incubated at 20°C for 30 min or 15 h. The solution of the mixture was electrophoresed in a 6% polyacrylamide gel (60:1 acrylamide: bisacrylamide) at room temperature. Fluorescence of FITC was detected by FMIBIO II Multi-View from TAKARA. The amount of \(^{32}\)P-labeled double-stranded oligonucleotide probe in the gel was quantified from the autoradiogram using a laser-excited image analyzer (BAS5000, Fuji Film, Tokyo, Japan).

**Stoichiometry of the FIL Protein-DNA Complex**—The 15-amino acid Stag peptide was fused to the NH\(_2\)-terminal end of the FIL protein using the expression vector pET28a from Novagen. The Stag fused FIL protein was purified as described above, and the gel mobility shift of the Stag-FIL protein-DNA (the 51-bp DNA in Table I) complex was identical to that of the FIL-(6-229)DNA complex. The band of the Stag-FIL protein-DNA complex was excised from the gel, and the complex was eluted from the gel in the buffer containing 20 mM Tris-HCl, pH 8.0, 0.1 mM NaSCN, 1 mM dithiothreitol, 10% glycerol, 1.0% sodium deoxycholate by shaking at 20°C for 15 h. The amount of the Stag-FIL protein in the complex was quantified by Stag Rapid Assay kit from Novagen. The molar amount of DNA was determined by comparison to several known amounts of the DNA fragment electrophoresed on the same gel.

**RESULTS**

**Non-sequence-specific DNA Binding by the FIL Protein**—The FIL protein (FIL6-229) was mixed with a 5’-FITC-labeled random double-stranded oligonucleotide pool of 76-bp, 5’ FITC-labeled ACGCGTTGCGAGCTCTCAGATCAG-3’ (Table I) which is the Smal digest of the random 76 bp DNA (data not shown). The length of DNA became shorter, the binding efficiency was decreased. The FIL protein hardly bound to the 20-bp DNA.

**Stoichiometry of the FIL Protein-DNA Complex**—To determine the stoichiometry of the FIL protein-DNA complex,
Stag:FIL fusion protein was constructed, and was mixed with the 51-bp DNA in Table I. The FIL-DNA complex was isolated from the gel, and the protein and DNA were quantified as described under “Experimental Procedures.” Quantification of three individual FIL-DNA complexes gave average molar ratios of 2.3 ± 0.1. This result suggests that two FIL protein molecules bind to one 51-bp DNA.

**DNA Binding by the Truncated FIL Proteins**—The FIL protein has four regions, that is, the amino-terminal zinc finger domain between positions 10 and 60, the hydrophobic region between positions 60 and 100, the proline-rich region between positions 127 and 145, and the carboxyl-terminal HMG box-like domain between positions 145 and 180 as shown in Fig. 1a. To determine the region which is necessary for the DNA binding, eight different truncations of the FIL protein were constructed, that is, FIL-(6–229), FIL-(65–229), FIL-(120–229), FIL-(143–229), FIL-(6–203), FIL-(6–183), FIL-(6–165), and FIL-(120–183), as shown in Fig. 1c. These proteins were expressed in E. coli and purified as described previously (1, 20). The zinc finger domain was removed in FIL-(65–229). Both the zinc finger domain and the following hydrophobic region were removed in FIL-(120–229). The zinc finger domain, the hydrophobic region, and the proline-rich region were removed in FIL-(143–229). The C-terminal α-helix (helix 5 in Fig. 1b) predicted by the secondary structure prediction was removed in FIL-(6–203). The region between positions 184 and 229 and that between positions 166 and 229 were removed in FIL-(6–183) and FIL-(6–165), respectively. As shown in Fig. 4, among the eight truncated FIL proteins, FIL-(6–229), FIL-(65–229), FIL-(120–229), FIL-(6–203), FIL-(6–183), and FIL-(120–183) bound to the 52-bp DNA, while the two FIL proteins FIL-(143–229) and FIL-(6–165) did not. These results suggest that the region between positions 120 and 143 containing the proline-rich region and the HMG box-like domain between positions 145 and 180 are required for the DNA binding by the FIL protein, while the zinc finger domain, the following hydrophobic region, and the C-terminal region between positions 184 and 229 are not indispensable for the DNA binding by the FIL protein (Fig. 1c).

**Effect of EDTA on the DNA Binding by the FIL Protein**—The FIL protein has seven cysteine residues at positions 14, 26, 30, 33, 54, 56, and 57 in the zinc finger domain, and among them, it is thought that four cysteine residues at positions 30, 33, 54, and 57 are in the typical canonical positions for zinc ligation. We have reported that the cysteine residue at non-canonical position 56 (Cys<sup>56</sup>) probably facilitates the zinc release from the zinc finger domain of the FIL protein, and the loss of the zinc ion and the subsequent structural change probably occurring in the zinc finger domain are correlated with the self-assembly of the FIL protein, which are enhanced by the addition of EDTA (20). To investigate whether the changes in FIL protein structure influence DNA binding, FIL protein-DNA complex formation was assessed in the presence of EDTA.
EDTA (12.5, 25, 50, or 100 mM) was added to the solution containing the FIL protein (FIL-(6–229), FIL-(65–229), or C56A) and the 52-bp DNA. C56A is a mutant fil protein in which Cys56 is replaced by Ala allowing a means to investigate the role of the non-canonical Cys56 in the zinc release, structural change, and self-assembly of the FIL protein (20). EMSAs were performed to investigate the effect of EDTA on the formation of each FIL-DNA complex (Fig. 5). The proportion of the FIL-(6–229)-DNA complex fraction decreased from 62 to 30% as the concentration of EDTA was increased (Fig. 5, a and b). The decrease of the proportion of the FIL-(65–229)-DNA complex fraction under the same conditions was significantly less (from 75 to 67%; Fig. 5, a and b), and that of the C56A-DNA complex fraction from 74 to 66% (Fig. 5, a and b). The results (Fig. 5) suggest that the zinc release, structural change of the zinc finger domain, and self-assembly of the protein cause FIL-(6–229) to dissociate from DNA.

The DNA binding ability of the five truncated FIL proteins and the mutant fil protein C56A were compared in both the absence and presence of EDTA by estimating the concentration of protein required to shift 50% of the input DNA into slower mobility complex upon polyacrylamide gel electrophoresis (Table II). Images of the gels obtained in the gel shift assays using the six FIL proteins in both the absence and presence of EDTA are shown in Fig. 6. The proportion of the protein-DNA complex fraction was increased as the concentration of the protein was increased as shown in Fig. 6, a–f. These increases in the proportion of the FIL-(6–229)-DNA and FIL-(6–203)-DNA fractions were reduced when EDTA was present in the reaction mixture as shown in Fig. 6, a and b. While, the effect of EDTA seems to be small in the rest of four FIL-DNA complexes as shown in Fig. 6, c–f.

Typical reciprocal plots are shown in Fig. 7 to illustrate the affinities of the 52-bp DNA for the six FIL proteins in both the absence and presence of EDTA. In the plots for FIL-(6–229) and FIL-(6–203), differences were observed between the value of the × intercept in the absence of EDTA and that in the presence of EDTA as shown in Fig. 7, a and b. While, the differences between these values were small in the plots for FIL-(65–229), FIL-(120–229), and C56A as shown in Fig. 7, d–f. The measurements were repeated at least three times for each protein, and the values summarized in Table II represent the averages. When the apparent KD values in the presence of EDTA were compared with those in the absence of EDTA, FIL-(6–229) displayed a 1.6-fold reduction, FIL-(6–203) displa-

![Fig. 6. Comparison of the DNA binding ability of the six FIL proteins in the absence of EDTA and that in the presence of EDTA. A 20-μl reaction for EMSA containing buffer A (20 mM Tris-Cl, pH 7.7, 10% glycerol, 1 mM dithiothreitol, 0.1 μg of poly(dI-dC)-poly(dI-dC) (0.008 μM for the average length of 951), 0.4% sodium deoxycholate, and 0.04 mM NaSCN), [32P]labeled 52-bp DNA (Table I) (0.01 μM) and each FIL protein in the absence or presence of EDTA were incubated at 20 °C for 15 h. The concentration of FIL-(6–229) (a), FIL-(6–203) (b), FIL-(6–183) (c), FIL-(65–229) (d), FIL-(120–229) (e), or C56A (f) was increased as indicated in the figures. The solution of the mixture was electrophoresed as described under “Experimental Procedures.” Part of the radioactive material remained at the top of some lanes (b and d).](image-url)
slower mobility complexes. Affinities (\(K_{D}/H_{9262} \)) \((5.0/H_{9262} \text{ (7.9–8.9)})\) suggests that 1.6-fold less DNA binding by FIL-(6–203) compared with FIL-(65–229) (Table II), suggesting that the decrease of the zinc release in the C56A protein by the substitution of the cysteine residue at position 56 to alanine, suppressed the reduction in DNA binding caused by loss of the zinc ion and the self-assembly of the protein caused by EDTA.

As summarized in Table II, the apparent \(K_{D} \) value for FIL-(6–203) \((4.4 \mu M)\) is smallest and similar to that for FIL-(65–229) \((5.0 \mu M)\). While, the apparent \(K_{D} \) value for FIL-(6–229) \((7.9 \mu M)\) suggests 1.6-fold less DNA binding compared with FIL-(65–229). The apparent \(K_{D} \) value for FIL-(6–203) \((13.0 \mu M)\) suggests 1.6-fold less DNA binding by FIL-(6–203) compared with FIL-(6–229) \((7.9 \mu M)\). These results suggest that the zinc finger domain seems to reduce the DNA binding ability of the HMG box-like domain of the FIL protein, while, the COOH-terminal region between positions 203 and 229 containing putative helix 5 (Fig. 1b) is not indispensable but contains an important enhancer of DNA binding by the FIL protein.

**Wrapping of DNA by the FIL Protein**—The synergistic inflorescence phenotype of the fil ap1 double mutated plant as compared with the wild-type suggested that the FIL and AP1 proteins might interact in vivo to promote formation of floral meristems (23). We investigated a possible DNA binding interaction between FIL and AP1 proteins using DNA which has a target sequence of the AP1 protein, CArG box. We expressed the AP1 protein in E. coli, and purified as described under “Experimental Procedures.” The AP1 protein was mixed with the 52-bp DNA having CArG motif, 5′ −CCATTGGAG−3′ (Table I), and the mixture was analyzed by EMSA. As shown in Fig. 8, the AP1 protein bound to the 52-bp DNA (Fig. 8, lane 2). When 4 or 8 \(\mu g \) of the FIL protein was added to the solution of the mixture of the AP1 protein and DNA, the band corresponding to the AP1 protein-DNA complex disappeared, and another band corresponding to the FIL protein-DNA complex appeared (Fig. 8, lanes 3 and 4). When the FIL protein and the 52-bp DNA were mixed first and the AP1 protein was added to the solution of the mixture last, neither the band corresponding to the FIL protein-DNA complex disappeared nor the band corresponding to the AP1-DNA complex appeared (Fig. 8, lanes 5–7). These results suggest that the FIL protein can compete and displace the AP1 protein binding to DNA, whereas the AP1...
substitutions should reduce the binding ability of the FIL protein. But, these substitutions actually enhanced DNA binding by the FIL protein (data not shown). It seems likely that some of the basic amino acid residues in the proline-rich region may interact directly with DNA to support the HMG box-like domain-DNA interaction as is the case with the NH2-terminal basic amino acid segment of the NHP6A protein (27, 28). It might possibly predict that a state in which two FIL protein molecules bind to the DNA is required to stabilize the FIL protein-DNA complex. It is reported that interferon regulatory factor-2 cooperatively binds to DNA, in which the first binding causes more than 6-fold enhancement of the second binding. This cooperative binding is not induced by the contact between the two adjacent interferon regulatory factor-2 molecules, but induced by the conformational change of the DNA upon binding (29). Another cooperative DNA binding is also observed in the structure of 9-cis-retinoic acid receptor-DNA complex. Retinoic acid receptor cannot bind to DNA as a monomer, but can bind to DNA as a dimer. A weak interaction between the two protein molecules was observed in the structure of retinoic acid receptor-DNA complex (30). Similar cooperative DNA binding might be expected in the FIL protein-DNA complex.

The binding ability of FIL-(120–229), which has neither the zinc finger domain nor the hydrophobic region (Fig. 1c) was highest among the five deletion mutants summarized in Table II. Also, the binding ability of FIL-(65–229), which has no zinc finger domain (Fig. 1c), was similar to that of FIL-(120–229) (Table II). In contrast, FIL-(6–165) which consists of the zinc finger domain and the hydrophobic region (Fig. 1c) has no DNA binding ability, as shown in Fig. 4. These results suggest that the zinc finger domain and the hydrophobic region (Fig. 1) are not necessary for the DNA binding by the FIL protein.

The apparent $K_D$ values of FIL-(6–203) and FIL-(6–183) were increased as compared with that of FIL-(6–229) (Table II), suggesting that the region between positions 183 and 229 is not indispensable but contributes to the DNA binding by the FIL protein. Helix 5 is predicted in the region between positions 183 and 229 by the secondary structure prediction, as shown in Fig. 1b. It might be expected that the region containing the putative helix 5 might interact with DNA to support the DNA binding by the HMG box-like domain. Since the HMG box proteins in the second class have been observed to strongly distort DNA upon binding, ligase-mediated circularization assay was performed using 123-bp DNA according to the procedures described by Stros (31) and Pil et al. (32) to investigate the ability of the FIL protein to bend DNA. The formation of the circular 123-bp DNA was not observed, suggesting that the FIL protein has no ability to bend DNA.

The results shown in Figs. 5–7 and summarized in Table II suggest that the DNA binding by the FIL protein is reduced by the addition of EDTA when the FIL protein has the zinc finger domain. We have reported that Cys$^{56}$ at non-canonical position 56 potentially interferes with zinc ligation within the zinc finger and probably facilitates this zinc release (20). EDTA was expected to enhance the zinc release by Cys$^{56}$, caused the structural change probably occurring in the zinc finger domain, and enhanced the multimerization of the FIL protein (20). It might be expected that the intermolecular interaction of the FIL proteins involving the structural change probably occurring in the zinc finger domain (20) might prevent the potential cooperative DNA binding by the FIL protein which is discussed above. The intermolecular interaction and multimerization of the FIL protein (20) is probably related to the dissociation of the FIL protein from DNA. Removal of the FIL protein from DNA might be required in some developmental processes to activate transcription in the abaxial cells. Multimerization of the FIL protein molecules would be required for the DNA binding by the FIL protein.
might also subsequently prevent the dissociated FIL protein from binding to DNA.

Regulation of transcriptional machinery may involve the reciprocal interactions among activators, repressors, accessory factors, and DNA sites. These factors may include not only site-specific DNA-binding proteins but also non-sequence-specific DNA-binding proteins. The FIL gene is expressed only at the abaxial side of primordia of leaves and floral organs but little is known about the in vivo function of the FIL protein. Two independently isolated fil mutants fil-1 and fil-2 have been analyzed morphologically and exhibit similar structural abnormalities in the inflorescence and floral meristem (23). Sequence analysis of the fil-1 molecular lesion reveals a new splice acceptor site in exon 5, resulting in the production of a short polypeptide of 165 amino acid residues with the major part of the HMG box-like domain and the following region between positions 154 and 229 being replaced by a new short polypeptide. On the other hand, the mutation in the fil-2 mutant protein is caused by a substitution of Tyr for Cys at position 30 and the fil-2 protein therefore lacks one of the canonical cysteine residues for zinc ligation. Thus despite the molecular disruptions being different from each other, the similar structural abnormalities of both mutated plants fil-1 and fil-2 may be explained if both mutant proteins cannot bind to DNA. In the case of fil-1 this may occur through the protein not having a positive affinity for the DNA (through loss of a major part of the HMG box-like domain), whereas for fil-2 alteration of the zinc finger may reduce affinity of the HMG box-like domain for DNA and increasing the propensity for fil-2 to multimerize.

The dramatically more severe inflorescence phenotype of the fil ap1 double mutated plant as compared with the fil and ap1 single mutants raised the possibility that the FIL protein and the AP1 protein interact in vivo contributes to the formation of floral meristems (23). The result shown in Fig. 8 suggests that the FIL protein displaces the AP1 protein which bound to DNA, while the AP1 protein is unable to bind to DNA, to which the FIL protein has already bound. It has been thought that the FIL protein is responsible for the normal development of leaves and floral organs by determining the abaxial character (1). Based on our results in vitro, it might be expected that the FIL protein might function in vivo to prevent the DNA binding by the site-specific DNA-binding proteins such as the AP1 protein through wrapping around the target site in the abaxial cells. It might be expected as follows. In the fil ap1 double mutated plant (23), the FIL protein cannot bind to DNA, or the AP1 protein also cannot bind to its naked target site CArG box in the abaxial side. Then, a set of factors that recognize the same target sequence of the CArG box as the AP1 protein and promote formation of the inflorescence might bind this naked target site. These might result in the conversion of flowers to inflorescences in the fil ap1 double mutants. Since classical surgical experiments have suggested that the establishment of abaxial-adaxial polarity requires a signal from the apical meristem and the abaxial cell fate is the default, the function of FIL in strongly binding to DNA may be consistent with this suggestion (33, 34).

In summary, the FIL protein was found to bind non-sequence specifically to DNA in EMSAs. The proline-rich region and the HMG box-like domain were required for the DNA binding by the FIL protein. DNA wrapping observed in vitro might be a function of the FIL protein in vivo. The addition of EDTA reduced the DNA binding by the FIL protein, suggesting that the zinc release, the structural change, and the self-assembly are likely to dissociate the FIL protein from DNA. Cys\textsuperscript{56} seems to be a key residue to start this dissociation.

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