Zinc Release from the CH2C6 Zinc Finger Domain of FILAMENTOUS FLOWER Protein from Arabidopsis thaliana Induces Self-assembly*

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The FILAMENTOUS FLOWER gene from Arabidopsis thaliana is a member of a gene family whose role is to specify abaxial cell fate in lateral organs. Analysis of the amino-terminal region of the FILAMENTOUS FLOWER protein suggests that seven cysteine residues at positions 14, 26, 30, 33, 54, 56, and 57, and two histidine residues at positions 18 and 24 contribute to a putative zinc finger motif. Cys-X2-His-X2-His-X-Cys-X2-Cys-X2-Cys-X2-Cys-X2. Zinc determination experiments revealed that the FILAMENTOUS FLOWER protein binds two zinc ions per molecule. Chemical modification was required to release one zinc ion, whereas the other was released spontaneously or more rapidly in the presence of metallochromic indicator. The loss of a zinc ion and the subsequent structural change of the zinc finger domain were correlated with the multimerization of the FILAMENTOUS FLOWER protein. A cysteine residue at position 56 in the FILAMENTOUS FLOWER protein potentially interferes with zinc ligation within the zinc finger and causes this zinc release. In support of this, substitution of the Cys by alanine suppressed both the zinc release and the multimerization of the FILAMENTOUS FLOWER protein. Deletion analysis showed that the region between positions 45 and 107 functions in the intermolecular contacts between FILAMENTOUS FLOWER proteins. This region corresponds to the carboxyl-terminal half of the zinc finger domain and the following hydrophobic region containing two putative α-helices. Our results suggest that the FILAMENTOUS FLOWER protein forms a range of different conformers. This attribute may lead to a greater degree of functional flexibility that is central to its role as an abaxial cell fate regulator.

In higher plants, lateral organs produced from the flanks of the apical meristem exhibit defined adaxial-abaxial polarity. Recent molecular genetic analysis has identified a number of factors involved in establishing adaxial-abaxial organ polarity (1). These investigations suggest that normal juxtaposition of these factors involved in establishing adaxial-abaxial organ polarity (1). These investigations suggest that normal juxtaposition of these factors is necessary for normal organ growth and may feed-back to support the maintenance of the apical meristem (2, 3).

The filamentous flower (fil) mutant was isolated from Arabidopsis thaliana as one of the mutants that have defects in flower development and morphogenesis (4). The fil mutant forms two types of flowers. One is a flower with an aberrant number and arrangement of organs, and the other is not a shape of flower but a filament. It has been thought that the FILAMENTOUS FLOWER (FIL) gene has a role in the formation and development of floral meristems and determination of the numbers of floral organs (5, 6). Recently, it is proposed that the FIL gene is a member of a gene family whose role appears to be pivotal in specifying the abaxial cell fate of lateral organs (3). In addition to FIL, this gene family consists of six members, including the flower specific genes CRABS CLAW (CRC) (7), INNER NO OUTER (INO) (8), and YABBY2 (YAB2) (YAB73 (YAB3), and YABBY5 (YAB5) (3). YAB2, YAB3, and FIL are expressed abaxially in all developing lateral organs and are proposed to redundantly promote the abaxial fate of all lateral organs in the plant (2, 3). The putative proteins encoded by this gene family share amino-terminal zinc finger and carboxyl-terminal HMG-box like domains (2, 3). The presence of these domains suggests that these genes may function as transcriptional regulators. Consistent with this, the FIL protein has been shown to contain a zinc ion and be localized to the nucleus (2).

Recent studies have indicated that, in addition to DNA binding, zinc fingers can work in protein-protein interactions supporting the formation of homo- and heterodimers as well as protein self-association (9). This suggests the aspects that the protein function of FIL and other members of this gene family may be mediated through protein-protein interactions. Several sequence motifs have been observed to accompany the zinc finger domain when they act in protein-protein interactions. These motifs include the Kruppel-associated box (10), the poxprotein interaction motifs that may be mediated through protein-protein interactions (10). Nuclear bodies containing large assemblies of GATA family proteins are also capable of protein-protein interactions (13). Among many zinc finger families, the LIM domain contains double zinc finger structures that mediate specific contacts between proteins participating in the formation of a multiprotein complex (15). The LIM motif displays the consensus amino acid sequence Cys-X2-Cys-X16-23His-X2-Cys-X2-Cys-X2-Cys-X16-23-Cys-X2-Cys (where X is any amino acid (16)), and spectroscopic studies of some LIM domains have revealed that they

1 The abbreviations used are: FIL, FILAMENTOUS FLOWER; HMG, high mobility group; LB, Luria-Bertani; PCR, polymerase chain reaction; GST, glutathione S-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside; PAR, 4,4′-pyridylidyloxazoloresorcinol; FMPS, p-hydroxymercurophenyl sulfonate; PAGE, polyacrylamide gel electrophoresis; DOC, sodium deoxycholate; TCEP-HCl, tris(2-carboxyethyl)phosphine hydrochloride; CD, circular dichroism; HRP, horseradish peroxidase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
Cells and Plasmids—Plasmid pET28a and Escherichia coli BL21 (DE3) (Novagen, Inc., Madison, WI) were transformed with DNA solutions from Takara Shuzo Co., Ltd. Competent cells of E. coli JM109 (recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lacI(qproAB) F’traD36, proAB-, lacIq, ZamaD15) for plasmid construction were from Takara Shuzo Co., Ltd. Cells were grown in Luria-Bertani (LB) medium (21) containing 50 mg/ml kanamycin or 100 mg/ml ampicillin.

Materials—Restriction and DNA-modifying enzymes were from Takara Shuzo Co., Ltd. The DNA ligation kit (ligation high) and KOD containing 50 mg/liter kanamycin or 100 mg/liter ampicillin. We investigated the FIL protein focusing on the zinc finger domain. The FIL protein acts as a transcriptional regulator in the plant, but the molecular basis of its function has yet to be demonstrated. We discuss the possibility that structural variability of the FIL protein due to the changing conformation of the zinc finger domain may be central to its molecular and biological action.

EXPERIMENTAL PROCEDURES

Transformation of the FIL gene—The FIL gene has been isolated as described previously (2). The gene was amplified by PCR using the synthetic DNA oligonucleotides P1 (5′-GGGCGGCTCCGCGCTCCGCTCCGTCACGCTG-3′) and P2 (5′-TTATTCGCTGGAAAGTAGTACTACCAACGG-3′) as primers. Recognition sites of restriction endonucleases NcoI and XhoI are shown by the two underlined areas, respectively. Thirty cycles of PCR were performed using a Tri-Thermoblock (Biometra) apparatus with KOD DNA polymerase and using the procedures recommended by the supplier. The DNA product was digested with NcoI and XhoI, and purified by agarose gel fragment of plasmid pET28a to generate plasmid pETFil, in which the FIL gene, encoding Fil-(6–229) with a histidine tag at a carboxyl terminus, is under the control of bacteriophage T7 transcription and translation signals. Mutation of Cys5′ to Ala was generated by overlap PCR. Two initial PCR were performed with primers P1 and P3 (5′-GGTGGTGAACACACCATCAGG-3′) and P4 and P5 (5′-CGATGTGAGTGTTGTGCACC-3′), where the substituted residues are underlined. The amplified DNA fragment of FIL genes in pETFil were confirmed by gel electrophoresis in agarose 1.5%, and sequenced using the ABI Prism 310 genetic analyzer from PerkinElmer Life Sciences. Truncation of the FIL gene was performed via PCR with the synthetic DNA oligonucleotides carrying BamHI and EcoRI sites as primers, and using KOD DNA polymerase, according to the procedures recommended by the supplier.

The DNA product was digested with BamHI and EcoRI and ligated to the large BamHI-EcoRI fragment of plasmid pGEX-2T, thereby creating in-frame fusion with the glutathione S-transferase (GST) gene. The nucleotide sequences of the truncated FIL genes were confirmed as described above.

Overproduction of the FIL Protein—Expression of the FIL protein was induced in E. coli BL21(DE3) cells harboring the plasmid pETFil by the addition of IPTG in the presence of 10 μM zinc acetate. Cultivation of the E. coli transformants was carried out as described previously (2). Cells were harvested by centrifugation and subjected to the purification procedures described below. The production of the FIL protein in cells was examined by analyzing the whole-cell extract by sodium dodecyl sulfate-PAGE (22). The solubility of FIL in cells was examined as described previously (23).

Zinc and Sulphydryl Group Determination—Zinc release experiments were performed as described previously (24). The protein solution was dialyzed against a buffer containing 10 mM Tris–HCl, pH 8.0, 250 μM TCEP, 0.6% DOC, and 10% glycerol to remove β-mercaptoethanol. The purity of the FIL protein was analyzed by SDS-PAGE.

Circular Dichroism—CD spectra (200–260 nm) were measured on a J-720 automatic spectropolarimeter (Japan Spectroscopic Co., Ltd.). Spectra were obtained using solutions containing the FIL protein at 0.3 mg/ml in 10 mM Tris–HCl, pH 8.5, containing 0.1 mM dithiothreitol. The EDTA-treated samples were prepared by incubating the FIL protein with 10 mM EDTA for 1 h at 20 °C, followed by the removal of excess EDTA with a 20-h dialysis. The mean residue ellipticity [θ], expressed in units of deg cm2/mole dm−1, was calculated by using an average amino acid molecular weight of 110. The helical content of the protein was calculated by the method of Wu et al. (26).

Secondary Structure Prediction and Homology Search—The secondary structure of FIL was predicted using a neural network system that was offered as a service on the Predict Protein Server on the Web. The protein sequence of FIL was analyzed by the BLASTP (29), and the sequence alignment was performed by ClustalW program was provided by Rost and Sander (28).

RESULTS

Overproduction and Purification—Expression of the FIL gene in the plasmid pETFil was induced in E. coli by the addition of IPTG as described previously (2). The cultivation of the transformants was performed at 30 °C to obtain the FIL protein in soluble form. Five amino acid residues at the amino terminus of FIL were removed to avoid the heterogeneity of the expressed protein. FIL-(6–229) was efficiently and selectively trapped by the chelating column when a histidine tag was attached to the carboxyl terminus. Elution with buffer A (20 mM Tris–HCl, pH 8.0, 0.1 mM NaSCN, 5 mM β-mercaptoethanol,
0.6% DOC, and 10% glycerol) containing 100 mM imidazole yielded 2.0 mg of protein product containing FIL from 1 liter of culture, which was identified as a single band using SDS-PAGE analysis (data not shown).

Self-assembly and Dissociation of the FIL Protein—When the FIL protein purification was performed in the absence of solubilizers and dissociating agents, the FIL protein formed a precipitate. This phenomenon prevented both the characterization and further increase in the concentration of the FIL protein. Changing pH and the addition of glycerol, Triton X-100, octyl-glycoside, CHAPS, and NaCl failed to prevent the formation of this precipitate, but the addition of 0.55M arginine and 0.44 M sucrose did (31). Therefore, gel filtration of the FIL protein using Superose 12 was performed at pH 5.5 in the absence of EDTA were monitored for 6 days by comparing the areas of the peaks corresponding to the multimer and the monomer in the elution profile. c, EDTA (10 mM) was added to the solution containing the wild-type FIL protein. It was applied immediately (thin line) or after standing at 4 °C for 6 days (thick line) to the same column. d, the relative amounts of the FIL multimer (●) and the FIL monomer (○) in the wild-type FIL solution kept at 4 °C in the presence of 10 mM EDTA were monitored for 6 days as described above.

Among several dissociating agents examined to dissociate the multimer of the FIL protein, DOC (32) and sodium thiocyanate (33) were most effective. Therefore, gel filtration was performed at pH 8.0 in the presence of 0.6% DOC and 0.1 M sodium thiocyanate. The elution profile showed two peaks that corresponded to the multimer eluted with the void volume (0.9 ml), and the FIL monomer protein eluted with a retention volume of 1.25 ml (Fig. 1a). Sedimentation equilibrium experiments were carried out to determine the molecular mass of this protein. Concentration versus radial distance profiles were obtained at three different rotor speeds (data not shown), and a nonlinear least squares analysis revealed that the data fit a simple single species model exhibiting a slight tendency for aggregation and a molecular mass of 27,100 ± 240 Da. This is very close to the theoretical mass of 26,305. When the FIL solution was applied to the column for gel filtration, immediately after recovery from the chelating column, the elution profile showed that the multimer and monomer forms of the FIL protein constituted 27 and 73% of the FIL protein, respectively (Fig. 1b).

A time course for the relative proportions of the FIL multimer and monomer forms were examined when the FIL protein with a total concentration of 2.0 mg/ml was kept in buffer A at pH 8.0 and 4 °C for 6 days. Fig. 1b shows the decrease of the monomer and the increase of the multimer forms of the FIL
The deduced amino acid sequence of the FIL protein was compared with those of the YAB2 and YAB3 proteins. Fig. 2 shows the alignment of the three sequences. The zinc finger domain of each protein is indicated by double underlining. The regions that form five putative α-helices in the FIL protein suggested by the secondary structure prediction and the corresponding regions conserved in the YAB2 and YAB3 proteins are indicated by rectangles.

Fig. 2. Sequence alignment of the FIL, YAB2, and YAB3 proteins. The alignment of the deduced amino acid sequences of the FIL, YAB2, and YAB3 proteins was performed by ClustalW (30). The zinc finger domains of the FIL, YAB2, and YAB3 proteins are shaded. The five conserved cysteine residues among the three sequences in the zinc finger domains are indicated by asterisks. All cysteine and histidine residues in the three zinc finger domains are underlined. The cysteine residue at position 56 in the zinc finger domain of FIL is indicated by an arrow. The region between positions 45 and 107 of the FIL protein, which mediates the FIL interaction, is indicated by double underlining. The regions that form five putative α-helices in the FIL protein are indicated by rectangles.

The FIL protein observed over the 6-day period. The shift from the FIL monomer to the multimer form occurred gradually over this time. After 6 days, the monomer had decreased to 67% from 73%, conversely, the multimer increased to 33% from 27% (Fig. 1b). After 30 days, the relative amount of the multimer was ~70% and that of the monomer was ~30% (data not shown). Lowering the pH of the buffer increased the multimerization of FIL, whereas dilution of the FIL solution diminished the multimerization (data not shown).

The addition of EDTA to the FIL solution markedly enhanced the multimerization of FIL protein (Fig. 1, c and d). When the relative decrease of the monomer and the increase of the multimer in the presence of EDTA at 4 °C was monitored over 6 days (Fig. 1d), the percentage of FIL monomer protein had decreased to 48% from 73% by the end of this period. Conversely, the percentage of FIL multimer protein had increased to 52% from 27% (Fig. 1d).

These results suggest that the formation of the FIL protein multimer could be related to a spontaneous release of a zinc ion from the FIL protein and that the addition of EDTA may enhance this process. We decided to examine the theoretical structure of the zinc finger domain of FIL to help investigate this possibility.

Comparison of the Amino Acid Sequences of the FIL, YAB2, and YAB3 Proteins—The FIL protein has the amino-terminal C-rich structure with a CX5HC3HX3CX2CXCXCC (CH2C6) motif between positions 14 and 57 and the carboxy-terminal HMG-box like domain for the potential DNA binding. The deduced amino acid sequence of the FIL protein was compared with those of the YAB2 and YAB3 proteins. Fig. 2 shows the alignment of the three sequences. The zinc finger domain of the FIL protein has seven cysteine residues at positions 14, 26, 30, 33, 54, and 57, and three histidine residues at positions 18, 29, and 56 (HCH3HC motif) (Fig. 2). The five cysteine residues at positions 26, 30, 33, 54, and 57 of the FIL protein are conserved in the three sequences (Fig. 2), and four cysteine residues at positions 14 and 56 of FIL are not conserved among the sequences of the three zinc finger domains shown in Fig. 2. Therefore, Cys56 was considered as a candidate residue for facilitating the observed zinc release from the FIL protein. To examine a possible role of the Cys56 in zinc ligation destabilization, alanine was substituted for cysteine at position 56 in the FIL protein.

Substitution of Alanine for Cysteine at Position 56 Suppressed the Multimerization of the Protein and Zinc Release from the Zinc Finger Domain—To examine a possible role of the Cys56 in zinc ligation destabilization, alanine was substituted for cysteine at position 56. The solution of the C56A mutant protein was analyzed by gel filtration chromatography (Fig. 3a). Immediately after the elution from the Ni2+ resin, the approximate percentage of the fil C56A multimer was 10% compared with 80% for the fil C56A monomer and 10% for the fil C56A oligomer (Fig. 3b). This fil C56A oligomer was eluted with a retention volume of 1.09 ml, which was 0.16 ml smaller than that of the fil C56A monomer (1.25 ml) and 0.19 ml larger than that of the fil C56A multimer (0.9 ml) (Fig. 3a). The relative amount of the fil C56A multimer remained at 10% for 6 days (Fig. 3b). These results differed markedly from comparable experiments with the wild-type FIL protein (Fig. 1b), suggesting that the multimerization of the FIL protein is effectively suppressed by the substitution of Ala for Cys56. On the other hand, the monomer of fil C56A gradually decreased, with a commensurate, gradual increase in the concentration of the fil C56A oligomer over 6 days (Fig. 3b). The formation of an oligomer was not observed in the elution profile of the wild-type protein (Fig. 1a).

As was observed for wild-type FIL protein, the addition of EDTA increased the amount of fil C56A multimer but the effect
was less (Figs. 1c, 1d, 3c, and 3d). Moreover, it seems that the formation of the fil C56A oligomer was not influenced by EDTA (Fig. 3, b and d). This suggests that the fil C56A oligomer forms irrespective of whether there is zinc release from the zinc finger domain.

Structural Change of FIL Caused by the Addition of EDTA—Fig. 4 shows the far-UV CD spectra of the wild-type FIL and fil C56A proteins at pH 8.5 and 20 °C. Both the spectra (Fig. 4, a and b) are very similar to each other, and the helical content of the protein was 14.3% for the wild-type protein, and 14.9% for the C56A mutant. The addition of 10 mM EDTA to both the wild-type FIL and fil C56A mutant proteins produced CD spectral changes and a decrease in the helical content. (12.0% for the wild-type protein, and 13.1% for the fil C56A protein). Notably, the decrease in the helical content of the wild-type (2.3%) was larger than that of C56A (1.8%), indicating that EDTA affected a greater structural change on the wild-type FIL protein than on the fil C56A mutant protein. These results suggest that the addition of EDTA may interfere with zinc ligation by the zinc finger in the wild-type FIL protein causing zinc release. Under this proposal the loss of zinc then results in structural changes in the FIL protein that increases its tendency for self-association and multimerization. To investigate the potential role for zinc in this process we induced and monitored zinc loss from the wild-type FIL and fil C56A mutant proteins.

Release of Zinc Ions from FIL—The release of zinc ions from FIL was induced by chemical modification using PMPS and was monitored in the presence of the metallochromic indicator PAR (24). All zinc ions released from FIL were converted to the highly absorbant (PAR)\(_2\)Zn\(^{2+}\) complex (\(\Delta \varepsilon = 6.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}\) at 500 nm) (25), which was determined by measuring absorbance changes at 500 nm. As shown in Fig. 5a, the mixing of the wild-type or fil C56A mutant protein (2 \(\mu\)M) with PMPS (0.2 mM) in the presence of PAR (0.1 mM) resulted in the increase of absorbance at 500 nm for 10 min and reached plateau values of 0.25 (\(\Delta A_{500}\)). This value corresponds to the absorbance of 3.8 \(\mu\)M (PAR)\(_2\)Zn\(^{2+}\) complex solution and is equivalent to the release of 1.9 zinc ions per protein (the experimental value of 3.8 \(\mu\)M approximates the theoretical value...
of 4.0 μM for two zinc ions per protein assuming the oxidation of a few cysteine residues during the protein preparation. The mercaptide bond formation between PMPS and FIL cysteine residues was monitored at 250 nm in the absence of PAR. The absorbance change over the 20 min was 0.21 for the wild-type protein and 0.18 for the fil C56A mutant (Fig. 5b). These results are consistent with there being seven cysteine residues per FIL protein and six cysteine residues per fil C56A mutant protein as modified by PMPS (based on the assumption that 95% of sulfhydryl group was active in the cysteine modification).

The zinc release was observed from both the wild-type FIL or fil C56A mutant proteins in the presence of PAR alone without any cysteine modification (Fig. 5c). The absorbance change at 500 nm for 8 h was 0.13, indicating that almost one zinc ion was released per protein from both the wild-type and C56A mutant proteins during this period (Fig. 5c). These results suggest that, in the presence of PAR alone, only one of the two zinc ions in the FIL wild-type and fil C56A mutant protein can be released. At the same time, the rate of zinc release from the wild-type protein seemed to be about one order faster than that from the fil C56A protein (Fig. 5c). Thus the wild-type cysteine residue at position 56 appears to enhance the speed at which the zinc ion is released from the wild-type FIL protein.

Spontaneous zinc release from the wild-type FIL protein (2 mg/ml in buffer A) in the absence of PAR and PMPS was also detected when the FIL protein was separated from the free zinc ions by dialysis. In this case the zinc release reached a plateau for 7 days at 4 °C (data not shown).

Determination of the Region Involved in FIL Self-association—The FIL protein contains two zinc ions, and one zinc ion is released more easily from the FIL protein than the other (Fig. 5). The loss of the zinc ion likely causes the structural change of the zinc finger domain illustrated in Fig. 4, which is linked to increased self-association by the FIL protein (Fig. 1). To determine the region that is involved in the self-association of FIL, (6–229) was mixed with glutathione-agarose beads coated with either GST, GST-FIL1–45, GST-FIL1–75, GST-FIL1–114, GST-FIL65–229, GST-FIL107–229, or GST-FIL1–229 (Fig. 6a). After extensive washing, the protein retained on the beads was subjected to electrophoresis (Fig. 6b) and Western blotting (Fig. 6c). As shown in Fig. 6c, the GST-FIL1–75, GST-FIL1–114, GST-FIL65–229, and GST-FIL1–229 fusions could retain FIL-(6–229). In contrast, the beads coated with GST, GST-FIL1–45, and GST-FIL107–229 could not retain it.
FIG. 6. Determination of the region that mediates the association of the FIL protein. a, diagrams of a GST protein and six GST-FIL fusion protein forms used for the analysis. Open rectangles indicate the GST proteins, and black rectangles indicate the truncated and full-length FIL proteins. The region indicated by gray shading (positions between 45 and 107) is that mediating the protein-protein interaction. b, SDS-PAGE. GST-FIL fusion proteins and GST revealed by Coomassie Blue staining after Western blotting are shown. c, Western blot. The bands of FIL(6–229), with histidine tag retained by Coomassie Blue staining after Western blotting are shown.

These results imply that the residues between positions 45 and 107 contribute to FIL self-association (Fig. 6a), and the residues between positions 45 and 60 and those between positions 60 and 100 are specifically involved. The former region corresponds to the carboxyl half of the zinc finger domain, and the latter corresponds to the adjacent hydrophobic region containing two putative α-helices.

Secondary Structure Prediction of FIL—The analysis to predict the secondary structure of the FIL protein suggested that residues at positions 74–80, 89–95, 152–157, 169–179, and 204–214 had the propensity to form α-helices (helices I, II, III, IV, and V in Fig. 2), whereas residues at positions 25–30, 34–40, and 45–54 in the zinc finger domain had the propensity to form β-sheet structures (data not shown). Of the five putative α-helices, helices I and II (Fig. 2) contain five leucine residues in the hydrophobic region adjacent to the zinc finger domain, whereas III, IV, and V in the HMG-box-like domain of FIL (Fig. 2) have no leucine residues. It is assumed that helices I and II are likely to participate in the association of proteins by way of the leucine-leucine interactions. These two helices (I and II) of FIL are not conserved in the sequences of the YAB2 and YAB3 proteins. Although, among the three helices in the carboxyl-terminal HMG-box-like domain of FIL, helices III and IV are conserved in the FIL, YAB2, and YAB3 proteins (Fig. 2).

DISCUSSION

Molecular genetic analysis indicates that FIL has an important role in specifying abaxial cell fate in the apical and floral meristems (2–6). Little is known, however, about the in vivo action of the FIL protein except that it is likely to be a transcriptional regulator. In this study we have investigated the role of zinc ions in the FIL zinc finger domain and the structural organization of the protein to understand the functional properties of the FIL protein.

It is proposed that FIL is a member of the gene family including CRC (7), INO (8), YAB2, YAB3, and YAB5 (3). Among them, FIL, YAB2, and YAB3 are thought to be highly correlated with each other to specify the abaxial cell fate. The putative proteins encoded by these genes have a zinc finger domain toward the amino terminus and the HMG-box-like domain toward the carboxyl terminus. The YAB2 protein has a CHC3HC zinc finger motif, and the YAB3 protein has a HCHC3HC motif (Fig. 2) based on the putative amino acid sequences reported by Siegfried et al. (3). It has been predicted that the YAB2 and YAB3 proteins have a Cys5-Cys5-zinc-finger domain and bind a zinc ion per protein. On the other hand, our results show that the CH2C6 zinc finger motif of FIL binds two zinc ions per protein. It is possible that the YAB2 and YAB3 proteins bind two metal ions? It seems to be difficult for the zinc finger domain of YAB2 to bind two zinc ions. However, the two-metal binding by the YAB3 protein might be possible, if the seven potential zinc coordinating residues (His18, Cys26, His29, Cys30, Cys33, Cys54, and Cys57) of the YAB3 protein are arranged around two zinc ions as reported on the crystal structure of the RAG1 dimerization domain, in which two zinc ions share one cysteine residue in the binding site (34). It might be expected that the FIL, YAB2, and YAB3 proteins share in the in vivo function of the different zinc finger structures.

The arrangement of the cysteine and histidine residues of the zinc finger domain of FIL, particularly in the carboxyl-terminal half of the zinc finger domain, has some similarities to that of the LIM finger domain. Homology search using BLASTP (29) exhibited the 33% identity between positions 26–67 of FIL and the positions 583–662 of LimA from Dictyostelium discoideum, although the position and the number of histidine residues in the amino-terminal-half of the zinc finger domain of FIL are inconsistent with that of the LIM finger domain. It was confirmed that the fusion protein GST-FIL1–45 binds a zinc ion (data not shown). This result strongly suggests that the amino-terminal half of the zinc finger domain has an ability to bind a zinc ion. According to the arrangement of the LIM finger motif (16), it might be assumed that Cys14, His18, His29, and Cys30 of FIL are the zinc coordinating residues of what could be classified as site 1, and Cys33, Cys54, and Cys57 are those of site 2 in the zinc finger domain of the FIL protein (Fig. 2).

Our results strongly suggest that the cysteine residue at position 56 contributes to the weakening of the ligation of one of the zinc ions in the zinc finger domain of FIL. Significantly, extra cysteine residues like Cys56 of FIL are observed in other zinc finger domains such as the penultimate cysteine residue of the RING finger domain of COP1 (35) and the second cysteine residue of the first RING finger domain of PRT1 from Arabidopsis (36). Based on our results, it seems possible that these extra cysteine residues may disturb the zinc ligation by the zinc-coordinating residues at the canonical positions.

The slow but spontaneous release of the zinc ion from the zinc finger domain of FIL results in a structural change to the FIL protein. Presumably, this change in FIL structure exposes the adhesive surface of the FIL protein and allows for the formation of FIL multimers (Fig. 1, a and c). Although there is
no evidence that the formation of the fil C56A protein oligomers shown in Fig. 3 (a and c) are related to the loss of the zinc ion, their formation may reflect a similar but reduced tendency for self-association observed for the wild-type FIL protein. Zinc release and the theoretical exposure of the adhesive surface that results may be partially suppressed in the fil C56A mutant, preventing multimer formation but allowing oligomer formation (Fig. 3b).

It seems that the structure of the zinc finger domain of FIL is inherently unstable. This conformational flexibility may allow the rapid dissociation of FIL protein from the transcriptional machinery. Alternatively, the multimerization of the FIL protein that occurs after loss of a zinc ion in vitro, may (if it occurs in vivo) increase the potency of FIL as a transcription factor. This is supported by preliminary results indicating that FIL can also self-associate in the DNA-bound form (data not shown). There are also some examples of proteins that increase their local concentration and efficacy by self-association. The Ikaros protein forms a cluster in the association with transcription factors containing five of the nine (Fig. 2). Many transcription factors contain their local concentration and efficacy by self-association. The FIL can also self-associate in the DNA-bound form (data not shown).

In summary, the zinc finger domain of FIL was found to bind two zinc ions per protein. One of the two zinc ions is released spontaneously and is followed by the self-assembly of the FIL protein. The cysteine residue at position 56 is one factor that promotes rapid zinc release from the FIL protein. The hydrophobic leucine-rich region between positions 45 and 107 mediates the self-association of the FIL protein.

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