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A New Family of Zinc Finger Proteins in Petunia: Structure, DNA Sequence Recognition, and Floral Organ–Specific Expression

Hiroshi Takatsuji,[a,1] Norihisa Nakamura,[b] and Yukihisa Katsumoto[b]

[a] Laboratory of Developmental Biology, National Institute of Agrobiological Resources, 2-1-2 Kannondai, Tsukuba, Ibaraki, 305 Japan

[b] Suntory Research Center, 1-1-1 Wakayamadai, Shimamoto-cho, Mishima, Osaka, 305 Japan

We have previously cloned a gene for a zinc finger protein (EPF1) that is expressed specifically in petals and interacts with the promoter region of the 5-enolpyruvylshikimate-3-phosphate synthase gene in petunia. In an attempt to isolate genes encoding additional factors that interact with this promoter, we cloned four novel genes encoding zinc finger proteins (EPF2-5a, EPF2-5b, EPF2-4, and EPF2-7). Sequence analyses revealed that overall similarity between the EPF1 and the EPF2 protein family, except in the zinc finger motifs and the basic amino acid cluster, was very low, suggesting that the two groups belong to different subfamilies. DNA binding specificities of EPF1, EPF2-5, and EPF2-4 were very similar, as expected from the conserved zinc finger motifs. However, EPF2-7 showed no binding to the probes tested in spite of having the conserved motifs. DNA binding studies using a series of spacing mutant probes suggested a binding mechanism in which the EPF proteins recognize spacings in target DNA. RNA gel blot analyses and histochemical analyses with a promoter and β-glucuronidase fusion revealed that expression of the EPF2-5 gene (*EPF2-5*) was petal and stamen specific. Expression of the EPF2-7 gene (*EPF2-7*) was sepal and petal specific and localized in vascular tissues. The preferential expression in two adjacent floral organs raises the possibility that these genes are downstream transcription factors of floral homeotic genes.

INTRODUCTION

5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS) is an enzyme that catalyzes an essential step in the shikimate pathway leading to aromatic amino acids and then to phenylpropanoid compounds. In petunia, expression of the gene encoding EPSPS (*EPSPS*) is petal specific, and the expression level increases dramatically during flower opening (Gasser et al., 1988). Interested in the regulation of gene expression at late stages of flower development, we have studied the transcription mechanism of this gene. Previously, we have reported on deletion analysis of the promoter region of *EPSPS* that confers the specific expression pattern (Benfey et al., 1990), characterization of a nuclear factor that interacts with specific sequences in the promoter region, and isolation of the gene encoding a DNA binding protein (EPF1) that interacts with one of the sequences (Takatsuji et al., 1992). EPF1 was found to be different from the nuclear factor that was originally detected in the nuclear extract. However, its parallel expression patterns to those of *EPSPS* in tissue and developmental stage specificity in petals and seedlings suggested strongly that EPF1 is a tissue-specific transcriptional activator of *EPSPS*.

EPF1 is a zinc finger protein with two canonical Cys_2/His_2-type zinc finger motifs. Most of the Cys_2/His_2-type zinc finger proteins reported from animals and yeast have clustered zinc finger motifs separated by orderly short spacers. Much work has been done on the protein–DNA interaction of the clustered Cys_2/His_2-type zinc finger proteins. Crystallographic (Pavletich and Pabo, 1991) and nuclear magnetic resonance studies (Omichinski et al., 1993) have revealed DNA–protein interactions between a single zinc finger motif and its target DNA sequence. The amino acids responsible for base recognition have been pointed out by statistical sequence analysis (Jacobs, 1992). By domain swapping studies, it was shown that each zinc finger in the clustered zinc finger motifs spans a unit of three bases in the target sequence (Nardelli et al., 1991). In contrast to the clustered motifs, the two zinc finger motifs of EPF1 are separated by a long spacer. This unusual structure of EPF1 may provide a system to study the contribution of each zinc finger motif to DNA recognition and how the zinc finger proteins with separated zinc finger motifs recognize their target sequences.

Molecular mechanisms controlling floral organ formation are rapidly being elucidated (Coen and Meyerowitz, 1991). Several genes that control the establishment of floral organ

[1] To whom correspondence should be addressed.

identities have been isolated from Antirrhinum (Sommer et al., 1990; Trobner et al., 1992; Bradley et al., 1993), Arabidopsis (Yanofsky et al., 1990; Jack et al., 1992), and petunia (Angenent et al., 1992; Kush et al., 1993; Tsuchimoto et al., 1993). Gene products of these homeotic genes are putative transcription factors as deduced from their structural features. Gene expression leading to the floral organ formation is probably controlled by a complicated network of transcriptional regulation that consists of a few levels of transcriptional hierarchy. Therefore, many of the target genes of the homeotic genes themselves are likely to be transcription factors that regulate downstream events, thereby playing more specialized roles in flower development. Many of Cys_2/His_2-type zinc finger proteins, such as Krüppel (Rosenberg et al., 1986) and Hunchback (Tautz et al., 1987) of Drosophila, have been implicated in transcriptional control of development. In plants, this type of DNA binding protein, including EPF1, may represent a class of transcription factors that control organ development at a level downstream of the homeotic genes in the transcriptional hierarchy.

Our previous studies suggested the presence of additional factors that interact with the *EPSPS* promoter. In this study, we describe the isolation and characterization of four genes encoding novel zinc finger proteins that share common functional domains with EPF1 but belong to a different subfamily from EPF1. DNA binding studies revealed that their gene products as well as EPF1 recognize spacings in target DNA. We propose a mechanism of DNA recognition by separated zinc fingers. Expression patterns of some of the genes were specific in two adjacent floral organs. Based on the expression patterns, possible roles of the *EPF* genes in flower development in connection with floral homeotic genes are discussed.

RESULTS

Isolation of *EPF2* Family Genes

Our previous studies suggested the presence of other factors that interact with the same sequence as the binding sequence of EPF1 in the *EPSPS* promoter (Takatsuji et al., 1992). In an attempt to isolate the genes for such factors, we screened a new petal-specific cDNA expression library using a tetramer of the binding sequence (EP1: TGATTTTGACAGTGTCACCTT) as a probe. Several clones were obtained that showed binding activity to the probe and only weak binding to a tetramer of EP1m (TGATTTTCAGAGTGTCACCTT) containing two base substitutions (data not shown). In our previous study, the binding activity of EPF1 was sensitive to zinc-chelating treatment, whereas that of the nuclear factor was insensitive to the treatment. To distinguish the two types of clones, the isolated clones were tested for sensitivity of their binding activities to treatment with EDTA (Hanas et al., 1983). Incubation of the clones on nylon filters in a probe solution containing 10 mM EDTA showed that none of the clones were active under these conditions (data not shown). These results suggested that the new clones were not for the nuclear factor and that the properties of their DNA binding activity were similar to those of EPF1.

Sequence analysis of the clones revealed that they were derived from two very homologous genes (*EPF2-5a* and *EPF2-5b*). Nearly an equal number of clones for the two genes were represented in the clones, suggesting that the two genes are expressed in similar levels in the petal. By homology screening using a partial cDNA fragment of *EPF2-5a* as a probe, full-length cDNA clones of both genes were isolated from the same cDNA library. Protein coding regions were deduced from the comparison with DNA and protein sequences of EPF1 (Takatsuji et al., 1992) and the presence of an in-frame stop codon six triplets upstream from the predicted initiation codon (data not shown). A genomic clone of *EPF2-5a* was isolated from a genomic DNA library of petunia. Sequence analysis revealed a putative TATA box 45 bp upstream from the 5′ end of the full-length cDNA and 140 bp upstream from the predicted initiation codon, further supporting the predicted coding region. In the 5′ upstream region of *EPF2-5a*, two sequences (CCATAAAGGA and CCTAAACAAGG) that are similar to the consensus binding sequence of MADS-box proteins were found (data not shown).

In addition to the genomic clones of *EPF2-5a*, some clones showing weak hybridization were isolated during screening of the genomic library. Sequence analysis revealed that these clones represent two genes (*EPF2-4* and *EPF2-7*) that share considerable homology with *EPF2-5*. Protein coding regions of the genes were predicted from the sequence comparison with *EPF2-5*. The predicted protein coding regions were further supported by the presence of an in-frame stop codon 24 triplets and 31 triplets upstream from the predicted initiation codons of *EPF2-4* and *EPF2-7*, respectively, with no methionine between the predicted initiation codons and the upstream stop codons. Neither of these genes contained introns.

Genomic DNA gel blot analyses showed that each *EPF2* gene is present in one or a few copies in the petunia genome. As shown in Figure 1, the *EPF2-5* probe revealed two strong bands and a few weaker bands. The two strong bands were found to correspond to *EPF2-5a* and *EPF2-5b* by restriction mapping of their genomic clones (data not shown). The weaker bands were not a result of *EPF2-4* or *EPF2-7* subfamilies because the positions of the bands do not correspond to those revealed by the *EPF2-4* and *EPF2-7* probes, suggesting the presence of another subfamily that was not obtained in this screening. The *EPF2-4* probe revealed two bands in XbaI digest, suggesting that there are two members in this subfamily. The *EPF2-7* probe revealed only one strong band. These results were obtained by hybridization and washing in high stringency; therefore, some more related genes might be detected in the petunia genome in a lower stringency condition.

Structures of EPF2 Proteins

Predicted protein sequences encoded by the *EPF2* genes and the *EPF1* gene are shown in Figure 2. EPF 2-5a and EPF2-5b

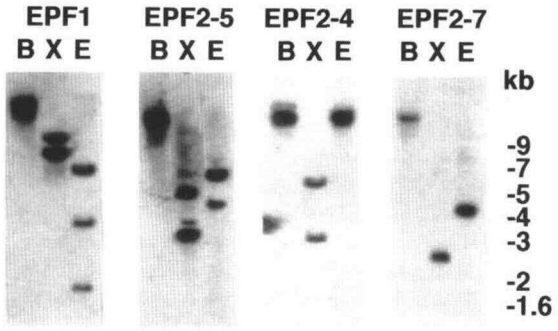

Figure 1. Genomic DNA Gel Blot Analyses of the *EPF* Genes.

Genomic DNA of petunia (10 μg each) was digested with restriction enzymes and run through a 0.8% agarose gel. The gel was blotted to a nylon membrane and hybridized with ^{32}P-labeled partial cDNA fragments of *EPF1*, *EPF2-5b*, *EPF2-4*, and *EPF2-7*, respectively. The hybridization was conducted at 65°C in 1 M NaCl, 10% SDS, and 50% dextran sulfate, and then the membrane was washed at 65°C in 2 × SSC and 1% SDS. B, BamHI; X, XbaI; E, EcoRI. Molecular length markers are indicated at right in kilobases.

are highly homologous, sharing 91% identical amino acids. EPF2-4 and EPF2-7 have moderate similarity to EPF2-5 (53 and 41% identity to EPF2-5a, respectively). In contrast, EPF1 has very low similarity to the EPF2 family, that is, 26% identity of amino acids to EPF2-5a, 18% to EPF2-4, and 22% to EPF2-7. Significant similarities between EPF1 and the EPF2 family were found only in the (putative) functional domains, that is, the two zinc finger motifs and the clusters of basic amino acids near the N termini (B-box). A phylogenic tree based on the protein sequences shows that *EPF1* is distantly related to the *EPF2* family (Figure 3). Therefore, we consider that *EPF2* and *EPF1* can be assigned to the same gene family in view of the conserved functional domains and to different subfamilies on the basis of the low similarity in the protein sequences.

As represented schematically in Figure 4B, each EPF protein has two zinc finger motifs with various spacings between the motifs. As described previously for EPF1 (Takatsuji et al., 1992), the distances between the motifs are unusually long as compared to the clustered motifs in many zinc finger proteins reported from animals and yeast, which are separated from each other by well-conserved H-C links consisting of seven amino acids. Amino acid sequences in the first and the second zinc finger motifs are aligned in Figure 4A. These motifs are canonical Cys_2/His_2-type as characterized by six conserved amino acids (Cys-X_2-Cys-X_3-Phe-X_5-Leu-X_2-His-X_3-His). This structure is a typical one in the DNA binding domains of many well-characterized transcription factors, such as Krüppel and Hunchback, Drosophila transcription factors of the gap class (Rosenberg et al., 1986; Tautz et al., 1987), and TFIIIA, a Xenopus transcription factor (Hanas et al., 1983). It should be noted that six consecutive amino acids (QALGGH motif), which were common in the two fingers in EPF1, are also conserved in both fingers of all the EPF2 proteins. These regions, which form the right face of the finger structure, have been demonstrated to interact directly with DNA by crystallographic analysis (Pavletich and Pabo, 1991) and nuclear magnetic resonance studies (Omichinski et al., 1993). Also, two of the three positions, which were pointed out to be the determinants of DNA binding specificity by statistical sequence analysis (Jacobs, 1992), are contained in this motif (QALGGH). From the conserved amino acid sequences in the DNA recognition region of the zinc finger motifs, similar DNA binding specificities were predicted for the EPF proteins. As described below, this was found to be partially the case because DNA binding specificities of EPF1, EPF2-5, and EPF2-4 were very similar, whereas that of EPF2-7 was different.

The B-boxes are also conserved in all the EPF proteins (Figure 2). The highly conserved sequences, in spite of the diversity in the entire sequences between EPF2 and EPF1 families, suggest that these regions are functionally conserved domains. As pointed out previously for EPF1, the abundance of basic amino acid sequences makes these candidates for nuclear targeting signals.

EPF1 has a serine-and-threonine–rich sequence, a putative activation domain by analogy to some other transcription factors (Theill et al., 1989). The serine-and-threonine–rich sequences were also found in EPF2-5 and EPF2-4, although there is no apparent similarity in the primary sequences. Therefore, EPF2-4 and EPF2-5 may also be transcriptional activators. In contrast, this characteristic sequence was not found in EPF2-7. If these sequences serve as transcriptional activation domains, EPF2-7, which lacks this domain, could be a transcriptional repressor.

DNA Binding Specificities of the EPF Proteins

To study DNA binding specificities of the EPF proteins, they were produced by coupled transcription and translation using an in vitro transcription system with T3 or T7 RNA polymerase and the rabbit reticulocyte in vitro translation system. The gene products were tested for DNA binding activity to various mutant probes containing base substitutions as shown in Figure 5. In a preliminary experiment, a tetramer of EP1S (13 bp) that lacks flanking sequences outside the inverted repeat in EP1 (21 bp) was found to show several-fold higher binding (data not shown) because of the optimal spacing between each sequence unit, as described below in detail. Therefore, EP1S, instead of the EP1, was used as a base sequence for the mutation analysis of the binding sequence. The tetramer of the EP1S (Figure 5, probe A) showed strong binding to EPF1, EPF2-5, and EPF2-4. When two base mutations were introduced into a half site of the inverted repeat (Figure 5, probe B), the binding activities were severely reduced. The binding was completely abolished by the mutations in both half sites of the inverted repeat (Figure 5, probe C). These results suggested that minimal sequence requirement for binding may be a half site of the inverted repeat. The three EPF proteins showed very weak binding activities to probe D (Figure 5), in

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EPF1        MEF------- ---------- -SEDSIDHTL VF-KGKRSKR PRQLSPDIYS  31
EPF2-5a     MALEALKSPT AATPTLPPRY EDQVDMSNLD SWVKGKRSKR PR-I------  43
EPF2-5b     MALEALKSPT AATPSLPPRY EDHVDMNNLD SWVKGKRSKR PR-I------  43
EPF2-4      MTLETLKSSS TPKTSKPTIP LPPKPINDAI DIHKRKRSKR PR-I------  43
EPF2-7      MALEALNSPT TTTP--PSFQ FENNGLKYLE SWTKGKRSKR QRSM------  42

EPF1        SSTTSTTQIS SSSSREEDED MANCLILLAQ SG--QSHK-- --QKFSSRKF  75
EPF2-5a     ---------- -ETPPSEEEY LALCLIMLAR SGNGTTPSSI PGSTDTTTIS  82
EPF2-5b     ---------- -ETPPSEEEY LALCLIMLAR SGNGTTPGS- TDTTITTTIS  81
EPF2-4      ---------- -ETPPSEKEF LALCLIMLAR SG-GKNPTT- TPTTITNEPL  80
EPF2-7      ---------- -ERQCTEEEY LALCLIMLAR SD-GSVNNS- --RSLPPPPL  77

EPF1        TETATSTGK- AGFY-----V YECKTCNRTF PSFQALGGHR TSHKKSKTIA 119
EPF2-5a     KEPEKKNRDV APVYQETEQS YKCSVCDKSF SSYQALGGHR ASHRKITTIA 132
EPF2-5b     KEPEKKNREL TPVHQETEQS YKCSVCDKSF SSYQALGGHK ASHRKITTIA 131
EPF2-4      QVQEPINKPL QVQEPINEQS YKCNVCNKSF HSYQALGGHK ASHRN-KNLS 129
EPF2-7      PPSVPVTSQI NATLLEQKNL YKCSVCGKGF GSYQALGGHK ASHRKLVSMG 127

EPF1        AEKTSTLEDH HQQQERVAQE EGEFIKIIPS ISTQIINKGN NMQSNFNSKS 169
EPF2-5a     T--TALLDDN NNNPTTSNST NGNVVNNIST LN-------- --------PSG 165
EPF2-5b     T--TALLDDN NNNPTTSNST SGNVVNNISA LN-------- --------PSG 164
EPF2-4      T--TT-VSYD DTNPSTSNS- ---------- LN-------- --------PSG 150
EPF2-7      G--DEQSTTS TTTNVTGTSS --------AN VN-------- -------GNG 152

EPF1        KIHECAICGA EFTSGQALGG HMRRHRPPTI TANITNTKVT LSTTIDDTSN 219
EPF2-5a     RSHVCSICHK AFPSGQALGG HKRRHYEGKL GGNNNNN-HR DGGGHSGSVV 214
EPF2-5b     RSHVCSICHK AFPTGQALGG HKRRHYEGKL GGNNNN--HR DGGGHSGSVV 212
EPF2-4      RFHECSICHK CFSSGQALGG HKRRHYEGNL GGGVS----- -----RGDTV 190
EPF2-7      RTHECSICHK CFPTGQALGG HKRCHYDGGN GNGNGS---- ----VSVGVT 194

EPF1        YTSESSHDYD EIKEKPRIIL SLDLNLPAPP EDDHHSDNTK FDFSGNKQCL 269
EPF2-5a     TTSDGGASTH TLRDFDLNML PPSPELQLGL SIDCGLKSQQ VPIEQEVESP 264
EPF2-5b     TTSDGGASTH TLRDFDLNML PPSPELQLGL SIDCDLKSQ- IPIEQEVESP 261
EPF2-4      ISSEGGGSAV IRRDFDLN-L PPSPELTLGM SVDCERKSQ- LSGEQEVESP 238
EPF2-7      SSEGVGSTIS HHRDFDLN-I PALPEFWPGF GSGEDEVE-- -SPHPAKKSR 240

EPF1        VFSAAALVDC HY     281
EPF2-5a     MPLKKPRLLF SMD    277
EPF2-5b     MPLKKPRLLF SMD    274
EPF2-4      MPTKKPRLAF RIDGN  253
EPF2-7      LSL-PPKLEL FKGLM  254
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Figure 2. Primary Sequence Comparison of the EPF Proteins.

The protein sequences of EPF2-5a and EPF2-5b were predicted from the cDNA and the genomic DNA sequences. Those of EPF2-4 and EPF2-7 were predicted from the genomic DNA sequences. The nucleotide and amino acid sequences were submitted to DDBJ, EMBL, and GenBank with the accession numbers D26083 (EPF2-5a), D26084 (EPF2-5b), D26085 (EPF2-4), and D26086 (EPF2-7). The sequence of EPF1 is shown in the top line for comparison. Identical amino acids as compared to EPF2-5a are shaded. The conserved B-box is indicated by a closed bar and the zinc finger motifs by open bars. Dashes were used to optimize alignment.

which two EP1S and two EP1Sdm sequences are arranged alternately in a tetramer. Taken together, three of the four EPF proteins showed similar DNA binding specificities, as expected from the structural similarity in the DNA binding domain.

The similar DNA binding specificities of EPF1, EPF2-5, and EPF2-4 suggest that these factors regulate the same set of target genes in different tissues. EPF2-7 showed no binding to any of the tested sequences, suggesting that it binds to different target sequences and controls a different set of target genes from those of EPF1, EPF2-5, and EPF2-4. This result was unexpected because the amino acid sequences in the two zinc finger motifs are very similar to those in the three other EPF proteins, especially the QALGGH motif in the DNA recognition region. Probably some differences in the sequences flanking the QALGGH motif also contribute to the binding sequence recognition. In contrast to nuclear receptor type–zinc finger proteins in which the amino acids responsible for DNA recognition have been determined by extensive mutational

analysis (Berg, 1989; Umesono and Evans, 1989), little work has been done on Cys_2/His_2-type zinc finger proteins. The EPF proteins, which have very similar zinc finger motifs but different target sequence specificities, will serve as a good system to find out determinant amino acids of the DNA sequence recognition.

EPF Proteins Recognize Spacing in Target DNA

The probe D has two EP1S and two EP1Sdm alternately arranged in the tetramer. Therefore, at least half of the binding activity was expected for this probe as compared to those with the probe A, which has four units of the EP1S, provided that a single unit of the EP1S serves as one binding site. However, the binding activities observed with the probe D were much lower than expected. One of the differences between the two probes is the spacing between each unit of EP1S, namely, 13 bp for the probe A and 26 bp for the probe D (distances between centers of each unit). On the other hand, the EPF proteins have two very similar zinc finger motifs that are unusually separated. Furthermore, disruption of the second zinc finger motif of EPF2-5 by site-directed mutagenesis of the first His to Asp severely reduced the binding activity (data not shown), indicating that the contribution of each zinc finger domain to the binding activity is not additive but synergistic.

These observations led us to speculate that optimal binding sequences for the EPF proteins consist of two units of the EP1S and that the spacing between the units may be a determinant for the binding affinity. To test this possibility, we made a series of spacing mutants by inserting spacers of various lengths between each unit of the EP1S in the tetramer and tested them for binding to the EPF proteins (Figure 6). The strongest activities were observed with the spacing of 13 and 15 bp for EPF1 and EPF2-5, respectively. With longer or shorter spacings than the optimal ones, the binding affinities became gradually reduced according to the difference from the optimal spacings. For EPF2-4, strongest activity was observed with the spacing of 13 bp and changes of the spacings reduced the binding more severely, indicating that its binding affinity is more sensitive to the spacing than EPF1 and EPF2-5. These results indicated that the binding affinities of the EPF proteins are greatly affected by the spacing between the units of the DNA motifs. A model for the mechanism of DNA recognition

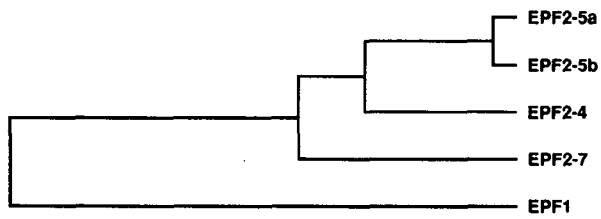

Figure 3. Phylogenic Analysis of the *EPF* Genes.

The dendrogram representing the relative similarities between the protein sequences was made with GeneWorks program (IntelliGenetics, Mountain View, CA). The length of the horizontal lines connecting one sequence to another is proportional to the estimated genetic distance between the sequences.

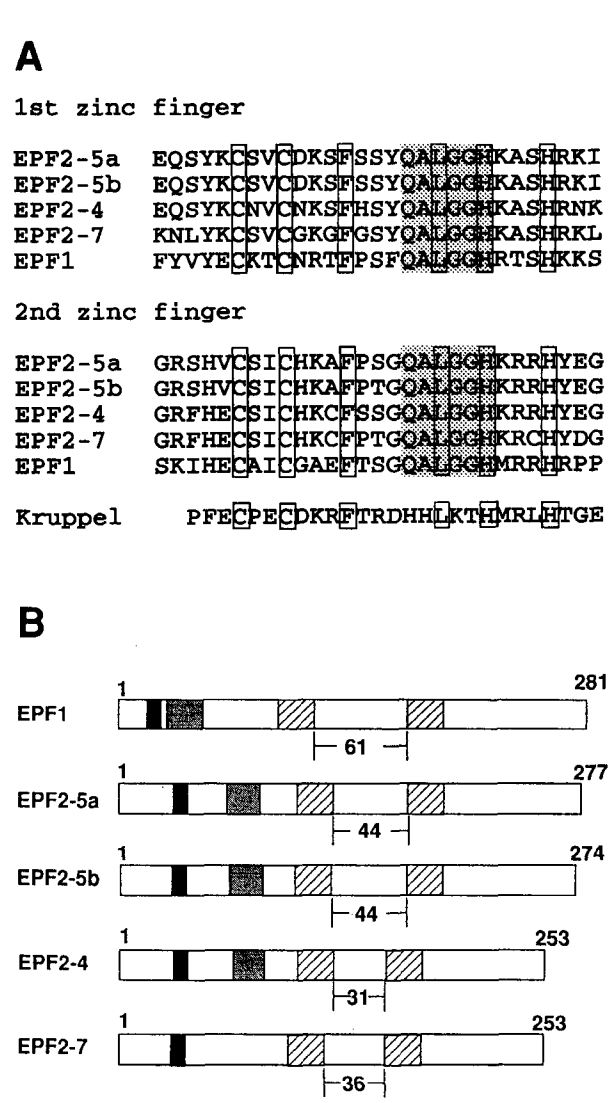

Figure 4. Functional Domains of the EPF Proteins.

(A) Amino acid sequence alignment of the first and the second zinc finger motifs, respectively. At the bottom, one of the zinc fingers of Drosophila Krüppel is shown for comparison. Six characteristic amino acids of the Cys_2/His_2-type zinc finger motif are boxed. Six consecutive amino acids (QALGGH motif) that are common in all eight zinc finger motifs are shaded.

(B) Schematic representation of the EPF proteins. The B-boxes are shown by closed areas, Ser-Thr–rich regions by shaded boxes, and zinc finger motifs by hatched boxes. Numbers of amino acids between the last histidine of the first zinc finger and the first cysteine of the second zinc finger are indicated.

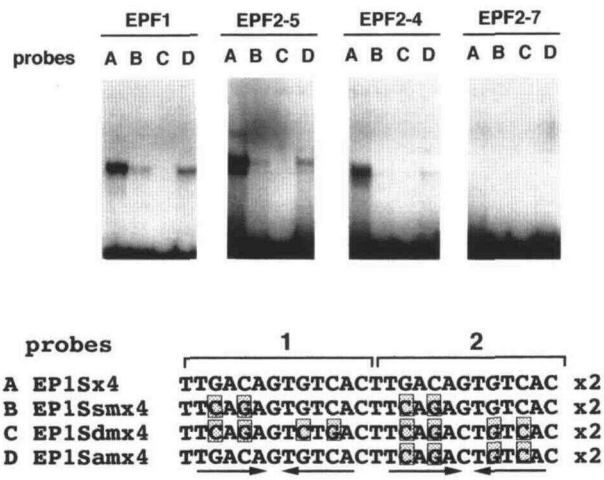

Figure 5. DNA Binding Specificities of the EPF Proteins.

The EPF proteins produced by coupled transcription of T7 or T3 promoters and translation in rabbit reticulocyte lysate were used for DNA binding assays. The probes tested were a tetramer of EP1S (probe A), a tetramer of EP1Ssm with two base mutations in a half site of the inverted repeat (probe B), a tetramer of EP1Sdm with two base mutations in both half sites of the inverted repeat (probe C), and a tetramer of alternate EP1S and EP1Sdm, EP1Sam (probe D). One-fiftieth of the translation product from 1 μg of the transcript was incubated with 10,000 cpm of ^{32}P-labeled probes in the gel shift assays. Regions 1 and 2 indicate the first and the second units of the binding sites, respectively. The notations ×4 and ×2 indicate that the sequences are tandemly repeated four times and twice, respectively. Shaded boxes indicate mutated nucleotides, and pairs of arrows in opposite orientations indicate inverted repeats.

by the EPF proteins based on these results is shown in Figure 7. The distances between the two fingers in the EPF proteins in the primary sequences are reflected in their tertiary configurations. When the distances between the two zinc finger domains in the tertiary structures match the spacings between the DNA motifs in the target DNA, the affinities of the protein–DNA interaction are strongest. The affinities become gradually weakened as a result of the strains either in the DNA or the protein resulting from the discrepancies between the distance in the proteins and the spacings in the DNA.

Floral Organ–Specific Expression Patterns of the *EPF2* Genes

Many of the Cys_2/His_2-type zinc finger proteins have been implicated in the transcriptional regulation of developmental control. To better understand the biological roles of the EPF2 proteins, their expression patterns were studied. In RNA gel blot analyses (Figure 8), expression of *EPF2-5* was found to be specific in the petal (limb and tube) and stamen, which are the second and the third whorls. The expression was stronger in the limb than in the tube, and the expression level in the limb increased during flower opening. Expression in other floral organs and vegetative tissues was very low or undetectable. These expression patterns are remarkably similar to those of *EPF1* and *EPSPS* (Takatsuji et al., 1992), the only difference being that *EPF2-5* is more strongly expressed in the stamen than the other two genes. *EPF2-7* was preferentially expressed in floral organs, and only very low expression was observed in vegetative tissues. In the floral organ, expression was highest

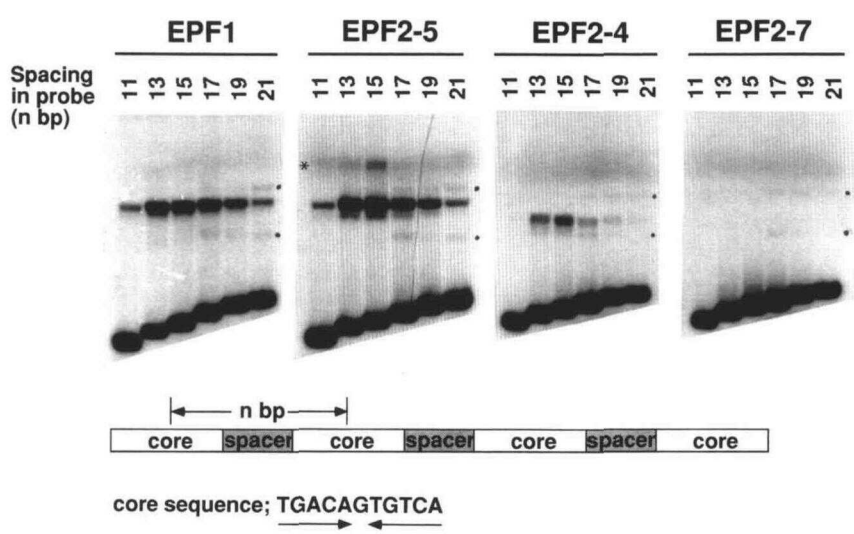

Figure 6. The EPF Proteins Recognize Spacing in the Target Sequences.

The EPF proteins produced in vitro were tested for binding activities to various spacing mutants in gel shift assays. The probes with the spacings from 15 to 21 bp were created by inserting spacers of TA × 2n (n = 1-4) between each unit of the EP1S (13 bp) in the tetramer. The probe with the spacing of 11 bp was created by deleting one base each from both ends of the EP1S (13 bp) in the tetramer. Bands with slower mobilities that appeared with EPF2-5 (*) are a result of binding by two protein molecules. Faint bands (•) are probably a result of DNA binding activities from the rabbit reticulocyte lysate that bind to AT repeats in the spacer sequence. Paired arrows in opposite orientations indicate the inverted repeat.

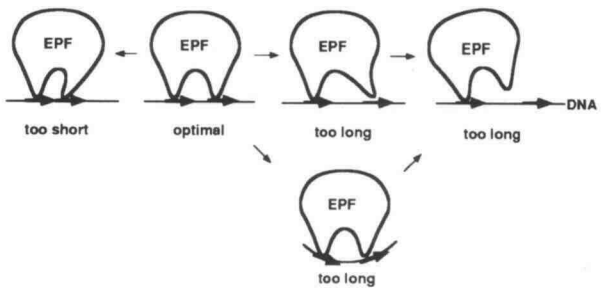

Figure 7. A Model for Spacing Recognition by the EPF Proteins.

Two tandemly repeated arrows on the DNA indicate core binding sequences. The zinc finger domains in the EPF are represented by two projections that contact with the DNA.

in the sepal and floral tube (the first and the second whorls) and lower in the limb and other floral organs. These expression patterns are reminiscent of floral homeotic genes that are expressed in two adjacent whorls of flowers, which will be discussed below. Expression of *EPF2-4* could not be detected in the tissues tested.

Cell Type-Specific Expression Patterns of the *EPF2* Genes

To study cell type–specific expression patterns of each EPF2 gene, 5′ upstream regions of the genes including short N-terminal coding sequences were fused in-frame with β-glucuronidase (*GUS*) coding sequences in pBI101 vectors. These chimeric constructs were introduced into petunia, and then the distribution of GUS activities was determined in mature transgenic plants and seedlings of their F_1 plants.

A fluorometric determination of the GUS activity conferred by the *EPF2-5a* 5′ upstream region showed that the expression patterns in various tissues and floral organs were essentially consistent with those observed in the RNA gel blot analyses. A dramatic increase in the activity in the limb during flower opening was also observed. Histochemical staining of sections of mature flower showed that all cell types were stained in the limb of petals (Figure 9A), only upper epidermal cells were stained in the upper part of the floral tube (Figure 9B), and no expression was observed in the lower part of the tube (Figure 9C). These expression patterns were very similar to those of the *EPF1* promoter (Takatsuji et al., 1992). Strong expression was also observed in anthers of the mature flower (Figure 9D), in contrast to the *EPF1* promoter that showed only weak expression in this organ. In the seedlings, expression patterns similar to those of the *EPF1* promoter were observed (data not shown).

Consistent with the steady state level of the transcripts revealed by the RNA gel blot analyses, expression of *EPF2-7*::*GUS* was strong in the sepal and the floral tube, and the expression levels remained constant during flower opening. Histochemical staining revealed that the GUS activities were localized in vascular tissues in both floral tubes (Figure 9E) and sepals (Figures 9F and 9G). Higher magnification of the cross-section showed that the expression was primarily in phloem (Figure 9H). These expression patterns in the second whorl were in marked contrast with those of the *EPF2-5* and the *EPF1* promoters, that is, strong expression in the upper part and the absence of expression in the vascular tissue in the middle part by the promoters of *EPF1* and *EPF2-5* as opposed to stronger expression in the lower part and its localization in the vascular tissue by the promoter of *EPF2-7*. Weak expression was also observed in anthers and ovaries in mature flowers and in cotyledons of seedlings (data not shown). The expression of *EPF2-4*::*GUS* was not detected in any tissues tested.

DISCUSSION

A New Family of Zinc Finger Proteins

We have identified and characterized a new subfamily (*EPF2* family) of genes for zinc finger proteins of petunia. There seem to be at least nine genes in the *EPF* gene family, of which we have already cloned six, including a partial clone of another *EPF1* gene (H. Takatsuji, unpublished result). Similar to a family of basic leucine zipper proteins (Quatrano et al., 1992) that

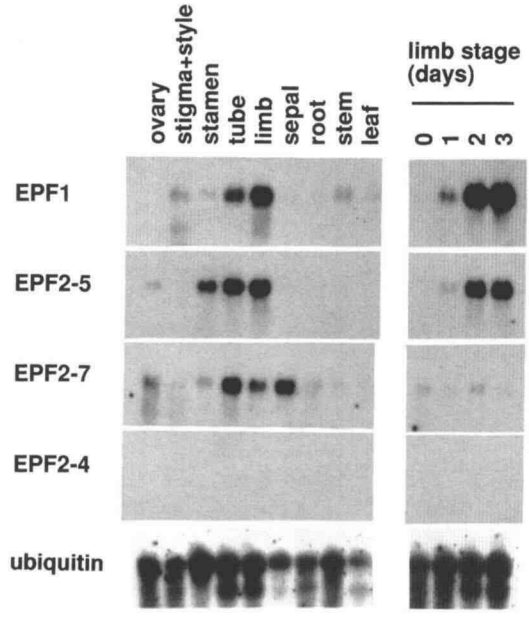

Figure 8. RNA Gel Blot Analyses of the *EPF2* Genes.

Total RNA (10 μg) from various organs and from limbs at various stages (0 to 3 days) of flower opening was analyzed as described in Methods. The stages of the flowers are as follows: 0, small bud; 1, limb unopen, tube elongated; 2, limb open, anther unopen; 3, anther open.

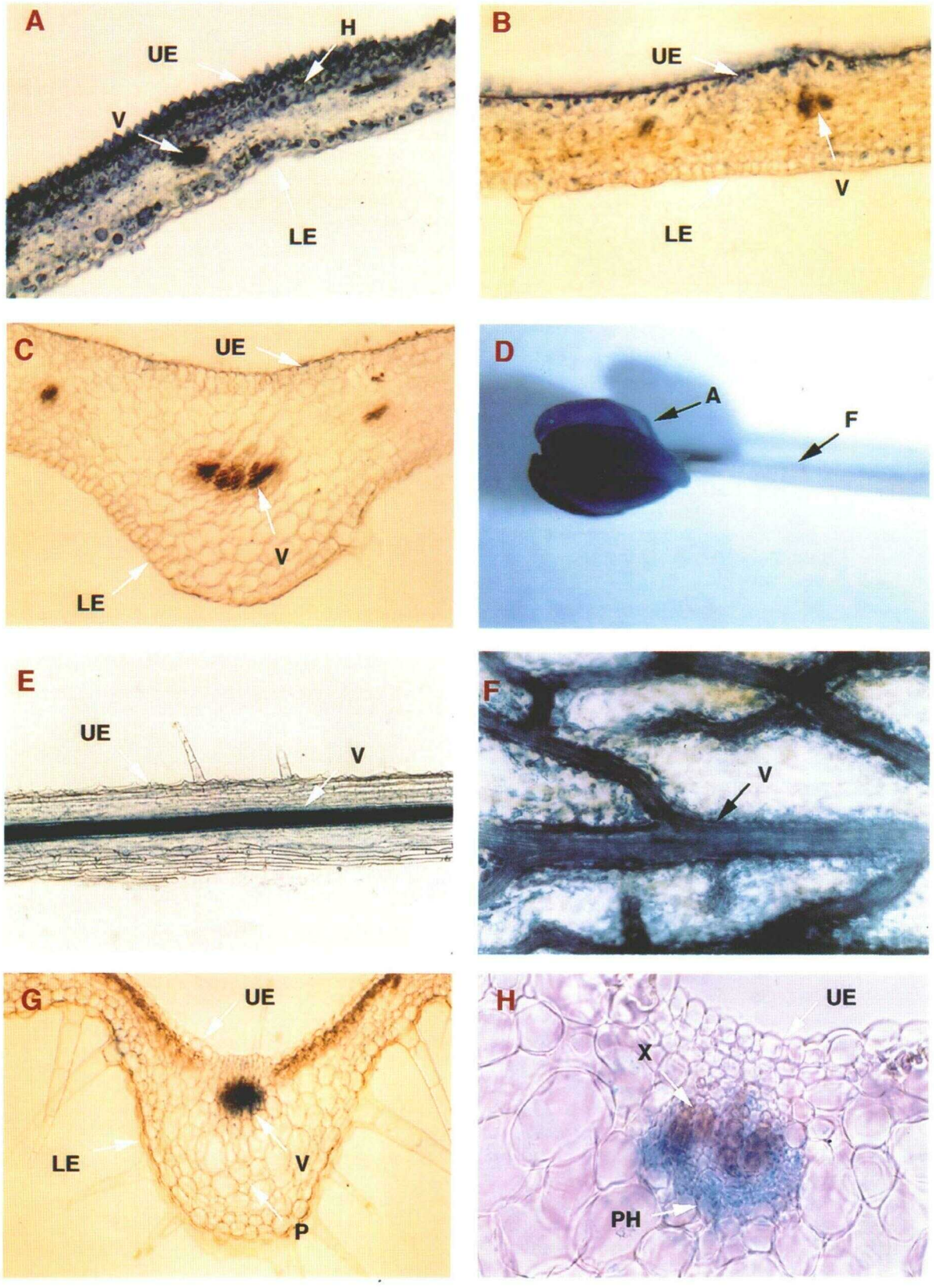

Figure 9. Histochemical Staining in Flower Sections of Transgenic Petunia Containing Promoter and *GUS* Translational Fusion Constructs.

The flower sections were prepared using a microslicer (Dohan, Kyoto, Japan). The histochemical staining was performed as described by Benfey and Chua (1989).

interact with various G-box–like promoter sequences and a family of MADS-box proteins (Sommer et al., 1990; Ma et al., 1991) that interact with consensus $CC(A/T)_6GG$ sequences, the EPF family represents a family of putative transcription factors that interact with consensus promoter sequences. EPF1 was the first zinc finger protein of the canonical Cys_2/His_2-type isolated from plants. Recently, cloning of the wheat zinc-finger protein 1 (WZF1) gene encoding a Cys_2/His_2-type zinc finger protein that interacts with a specific promoter sequence of histone genes was reported (Sakamoto et al., 1993). Interestingly, WZF1 also contains two separated zinc finger motifs with the QALGGH motifs in the same region and a basic amino acid cluster near its N terminus. These common structural features suggest that the WZF1 gene belongs to the same gene family as the *EPF* genes. Together with the new genes described in this study, all the genes of Cys_2/His_2-type zinc finger proteins so far reported from plants belong to the same gene family, in spite of distantly related plant species and without apparent relationship between their presumed target genes. This type of zinc finger protein with the two separated zinc finger motif and the QALGGH motif has not been found in animals, although more than 200 Cys_2/His_2-type zinc finger proteins have already been reported. Therefore, the characteristic structure represented by the EPF family might be specific for plants and typical for plant zinc finger proteins.

EPF Proteins Recognize Spacing in Target DNA

The EPF proteins have two widely separated zinc finger motifs with the common QALGGH motif in the DNA recognition region. This characteristic structure led to the discovery of the mechanism of DNA sequence recognition by this type of zinc finger protein. In our model, each zinc finger domain binds to each unit of a tandemly repeated DNA motif recognizing the spacing between the two DNA motifs. Binding affinity is highest when the distance between the zinc finger motifs matches the spacing in the target DNA, and the affinity decreases according to the degree of discrepancy between the distances. The optimal spacing of the EPF proteins (13 to 15 bp) is longer than the distance of one helical turn (10.5 bp) of double-stranded DNA; therefore, we imagine that the proteins contact the DNA not from one side but from both sides as if they fold the DNA by the two zinc finger motifs.

Most zinc finger proteins of animal and yeast have clustered zinc finger motifs separated by conserved H-C links consisting of seven amino acids (Rosenberg et al., 1986). In Sp1 and Krox-20, each one of the three clustered zinc fingers interacts with three bases in target DNA, which are tandemly repeated three times with no spacers (Nardelli et al., 1991). In this case, the spacings between the units of binding sequences are 3 bp, in contrast to 13 to 15 bp for EPF. Therefore, the distance between the zinc finger motifs in the primary sequence is reflected in the configuration of their tertiary structures and most likely determines the recognition of target DNA sequences. Probably, this is a general rule for zinc finger proteins. A few zinc finger proteins have been reported that have widely separated zinc finger motifs in the primary sequences, such as Suvar(3)7 (Reuter et al., 1990), teashirt (Fasano et al., 1991), and TRS-1 (Pays and Murphy, 1987). Binding sequences of these factors (many of which have not been identified) are probably split type, as are those of the EPF proteins.

Nuclear receptor–type zinc finger proteins (Cys_2/Cys_2-type) are known to recognize spacings in the target sequence, thereby distinguishing the cognate promoter sequences from those for other nuclear receptors with similar nucleotide sequences and different spacings (Naar et al., 1991; Umesono et al., 1991). In this case, the nuclear receptors bind to inverted repeats or direct repeats in the target sequence as dimers, with each monomer binding to a half site of the repeats. In contrast, EPF proteins bind to target DNA as monomers; therefore, the mechanism for spacing recognition is clearly different.

Floral Organ-Specific Expression of EPF Genes

A model has been proposed that explains genetic control of floral organ identities by the actions of homeotic genes (Coen and Meyerowitz, 1991). This model proposes three gene functions (*a*, *b*, and *c*) that act in two adjacent whorls, respectively, and the identity of each floral organ is established by the combination of their actions. In the first whorl, the sepals are established by the action of the *a* gene. Petals are established by the combination of *a* and *b* functions in the second whorl, the stamens are established by *b* and *c* functions in the third whorl, and the carpels are established by *c* function only in

Figure 9. (continued).

(A) to **(D)** The limb of the petal in **(A)**, the upper part of the floral tube in **(B)**, and the lower part of the floral tube and stamen in **(C)** and **(D)**, respectively, from the mature flower of transgenic petunia transformed with *EPF2-5::GUS*.
(E) and **(F)** Longitudinal sections of the floral tube in **(E)** and sepal in **(F)** from the mature flower of transgenic petunia transformed with *EPF2-7::GUS*.
(G) and **(H)** Cross-sections of the sepal in **(G)** and its higher magnification in **(H)** of transgenic petunia transformed with *EPF2-7::GUS*.
UE, upper epidermis; LE, lower epidermis; H, hypodermis; V, vascular tissue; P, parenchyma; A, anther; F, filament; PH, phloem; X, xylem.

the fourth whorl. Genes corresponding to the *b* and *c* gene functions have been isolated from some plants. Their gene products are regarded as putative transcription factors from their conserved putative DNA binding domains (MADS-box) (Sommer et al., 1990).

The expression patterns of the *EPF* genes appear to be closely related to those of the floral homeotic genes. Expression of *EPF1* is much higher in the second whorl than in other tissues. This whorl corresponds to the overlapping area of *a* and *b* gene expression. *EPF2-5* is expressed in the second and third whorls, which correspond to the area of *b* gene expression. Expression of *EPF2-7* is much stronger in the first and the second whorls than the other two, corresponding to the area of *a* gene expression. The expression of the homeotic genes is generally highest soon after the emergence of the primordia of each floral organ and then declines during maturation of the flower. On the other hand, the expression of the *EPF* genes increases during the maturation of the flower. Although we have no direct evidence at the moment, the floral organ pattern of expression and the temporally sequential expression pattern between the homeotic genes and the *EPF* genes imply hierarchical relationships between the two classes of genes, that is, each *EPF* gene is a target gene of the gene products of corresponding homeotic genes. The presence of putative binding sites of MADS-box protein in the 5′ upstream region of *EPF2-5a* and also in *EPF1* (CCAATTAAGG) supports this possibility. The delay of the expressions of the *EPF* genes from those of the homeotic genes may be a result of positive autoregulation of their own promoters by their gene products as proposed for *DEFICIENS A* and *GLOBOSA* (Sommer et al., 1990; Trobner et al., 1992). The presence of EP1-like sequences in the promoter region of *EPF1* and *EPF2-5* (data not shown) is in favor of the autoregulation. However, the binding affinities of the EPF proteins to these sequences are supposed to be weaker than the optimum, because the sequences do not appear to be tandemly repeated.

In petunia, the *green petal* mutant shows a homeotic conversion of petal into sepal (de Vlaming et al., 1984). The *green petal* mutation was caused by a chromosomal deletion of the *pMADS1* gene, which was isolated (Kush et al., 1993) and shown to be a petunia counterpart of *DEFICIENS A*, one of the well-characterized *b* genes of Antirrhinum (van der Krol et al., 1993). This gene is expressed strongly in the second whorl, in which *EPF1* and *EPF2-5* are expressed, and weakly in the third whorl, in which *EPF2-5* is expressed. The correlation of the expression patterns between the *pMADS1* gene and the *EPF* genes suggests that the transcription of the *EPF* genes may be positively regulated by the *pMADS1* gene product.

The homeotic genes control identities of the floral organs in which they are expressed. Probably they control many downstream transcription factors that play more specialized roles in floral organ formation. If the EPF proteins are such downstream transcription factors, disturbing the expression of these genes might result in visible or biochemically detectable changes in the phenotypes of floral organs.

METHODS

Isolation of cDNA and Genomic Clones of Zinc Finger Protein *EPF2* Genes

To construct a cDNA expression library, total RNA was prepared from mature petal of *Petunia hybrida* (cv Titan Parade) according to Fromm et al. (1985), and poly(A) RNA was selected by Oligotex-dT30 (Roche, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized by avian myeloma virus reverse transcriptase using random primers (Amersham International) as primers. A cDNA expression library was constructed in λ gt11 using adapters at the termini of the cDNA (Wu et al., 1987). The cDNA library was screened for sequence-specific DNA binding activity by the method of Singh et al. (1989) using a tetramer of the EP1 sequence as a probe and a tetramer of the EP1m as a negative control probe. Using partial cDNA clones as probes, full-length cDNA clones were isolated by a hybridization screening of the same library. A genomic library of petunia was constructed as described previously (Takatsuji et al., 1992). The library was screened using a DNA fragment containing the entire cDNA of *EPF2-5b* as a probe.

In Vitro Translation of EPF Proteins and Gel Shift Assays

Full-length coding sequences of the *EPF* genes in the pBluescript SK– vector were transcribed from the T7 or T3 promoter by T7 or T3 RNA polymerases (Stratagene), respectively. The transcripts were translated in a rabbit reticulocyte in vitro translation system (Promega) with ^{35}S-labeled methionine. After checking the sizes and amounts of the protein products on a 12% SDS–polyacrylamide gel, the products were assayed for DNA binding activities by a gel shift assay as described previously (Takatsuji et al., 1992).

RNA Gel Blot Analysis

Total RNA was extracted as described previously (Takatsuji et al., 1992). The RNA was separated on a 1% agarose gel containing 6% formaldehyde, transferred to a nylon membrane filter, and UV cross-linked. The filter was sequentially hybridized with ^{32}P-labeled cDNA fragments by stripping and rehybridization. The probes used were an EcoRI-XhoI fragment (1 kb) of *EPF2-5*, a HindIII-XbaI fragment (0.6 kb) of *EPF1*, an XbaI-EcoRV fragment (0.6 kb) of *EPF2-4*, an AatII-EcoRI fragment (1.6 kb) of *EPF2-7*, and a SacI-KpnI fragment (0.2 kb) of the bean ubiquitin gene. The filter was hybridized at 60°C in 1 M NaCl, 10% SDS, and 50% dextran sulfate and then washed at 60°C in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.15 M sodium citrate) and 1% SDS for *EPF2-5*, *EPF1*, *EPF2-4*, and *EPF2-5* probes. For the ubiquitin probe, the hybridization and washing were conducted at 55°C in the same solutions.

Promoter Analyses by Chimeric Constructs with the β-Glucuronidase Gene

Translational fusions of promoter regions of *EPF2* genes and β-glucuronidase (*GUS*) reporter genes were constructed. For *EPF2-5a*::*GUS*, the SalI-HindIII (filled-in) fragment (1.9 kb) containing the

upstream sequence and the first 15 bp of the coding sequence was inserted into the SmaI-SalI sites of pBI101.3 (Clonetech Laboratories, Palo Alto, CA) in frame to the coding sequence of the *GUS* gene. For *EPF2-4*::*GUS*, the HindIII-XmaI (2.4 kb) fragment containing the upstream sequence and the first 108 bp of the coding sequence was inserted into the SmaI-HindIII sites of pBI101.3. For *EPF2-7*::*GUS*, the StuI-EcoRI fragment (1.7 kb) containing the upstream region and the first 22 bp of the coding sequence was subcloned into the EcoRI-SmaI sites of pBluescript SK−, and then the BamHI-EcoRI fragment containing the region was excised from the pBluescript SK− vector and reinserted into the BamHI-EcoRI sites of pBI101.2. These constructs were transformed into petunia (cv Mitchell, diploid) as described by Benfey and Chua (1989).

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