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Differential Expression of Two MADS Box Genes in Wild-Type and Mutant Petunia Flowers

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We isolated and characterized two flower-specific genes from petunia. The protein products of these genes, designated floral binding protein 1 (FBP1) and 2 (FBP2), are putative transcription factors with the MADS box DNA binding domain. RNA gel blot analysis showed that the *fbp1* gene is exclusively expressed in petals and stamen of petunia flowers. In contrast, the FBP1 protein was only detectable in petals and not in stamens, suggesting post-transcriptional regulation of the *fbp1* gene in these tissues. The *fbp2* gene is expressed in petals, stamen, carpels, and at a very low level in sepals but not in vegetative tissues. We analyzed the spatial expression of these *fbp* genes in floral organs of two homeotic flower mutants. In the *blind* mutant, whose flower limbs are transformed into antheroid structures on top of normal tubes, identical expression levels of both genes were observed in the antheroid structures as in normal anthers. In the homeotic mutant *green petals*, the petals are replaced by sepaloid organs in which the expression of *fbp1* is strongly reduced but not completely abolished. Our results suggest a regulation of the *fbp1* gene expression by the *green petals* (*gp*) gene. Expression of the *fbp2* gene was not affected in the *green petals* mutant. In contrast to the proposed models describing floral morphogenesis, our data indicated that homeotic genes can be functional in one whorl only.

INTRODUCTION

With the identification and isolation of a number of floral homeotic genes, the interest in the vital process of the reproductive organ formation in higher plants rapidly increases. Several recent studies have been focused on the development of flowers (for reviews, see Coen, 1991; Gasser, 1991). Despite the increased interest in flower morphogenesis, a broad and detailed understanding of this developmental process is still limited due to its complexity.

A crucial step in the flowering process is the transformation of the flower primordium into primordia of the four types of floral organs. This switch to determinacy is controlled by homeotic genes and constitutes an important step toward the formation of floral organs with distinct reproductive functions, forms, and spatial arrangements (Bernier, 1988).

A rapidly increasing number of homeotic genes have been isolated and analyzed for their spatial and temporal expression. The flower homeotic genes *agamous* (*ag*; Yanofsky et al., 1990), *apetala3* (*ap3*; Jack et al., 1992), and the *agamous*-like (*agl*) gene family (Ma et al., 1991) have been cloned from Arabidopsis. Two homeotic genes, *deficiens* (*defA*) and *floricaula* (*flo*), have been isolated from snapdragon (Coen et al., 1990; Sommer et al., 1990). Recently, Pnueli et al. (1991) reported the isolation and characterization of a homeotic gene family expressed in tomato flowers. Sequence analysis has revealed that most of the proteins encoded by these homeotic genes contain a region with a striking homology to the putative DNA binding domains of transcription factors from humans (SRF; Norman et al., 1988) and yeast (MCM1; Passmore et al., 1988). The conserved motif of this region is called the MADS box (for *M*CM1, *A*G, *D*EFA, and *S*RF; Schwarz-Sommer et al., 1990). The observation that the majority of the floral homeotic genes characterized so far encode transcription factors suggests that the complex process of flower morphogenesis requires the action of many regulatory proteins controlling the proper formation of floral organs.

Several models, which are based on floral homeotic mutants of Arabidopsis and snapdragon, have been proposed for the functions and interactions of floral homeotic genes (Schwarz-Sommer et al., 1990; Coen, 1991; Coen and Meyerowitz, 1991; Lord, 1991). The model for Arabidopsis, which is basically similar to the snapdragon model, postulates the action of three classes of genes (A, B, and C), each affecting the identities of two adjacent floral whorls. Gene A determines the initiation and differentiation of sepals (whorl 1), whereas expression of genes A and B leads to the formation of petals (whorl 2). If both B and C are expressed, stamens (whorl 3) are formed and expression of gene C only results in the development of carpels (whorl 4). Finally, experiments with Arabidopsis genes *ag* (class C gene) and *apetala2* (class A gene) have demonstrated that genes A and C regulate each other negatively (Drews et al., 1991). Expression of a gene C stops the cyclic

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process of flower morphogenesis and prevents the reinitiation of sepal formation by gene A.

In plants, especially for Arabidopsis and snapdragon, many homeotic floral mutants are known and they can serve as useful tools in the study of genes directing the fates of the organ primordia. For petunia, two homeotic flower morphology mutants, *green petals* (recessive for the gene *gp*) and *blind* (recessive for the gene *bl*), have been partially characterized (Gerats et al., 1988; van Tunen et al., 1990; Gerats, 1991). Flowers of the *blind* mutant have limbs replaced by antheroid structures on top of the tube (Wiering et al., 1979; Vallade et al., 1987). In the second homeotic mutant, *green petals*, the petals are converted into sepals, leaving the other floral whorls unaffected (Wiering et al., 1979). Recently, we have indicated that some characteristics of the homeotic genes *gp* and *bl* do not seem to fit into the models describing floral morphogenesis (van Tunen and Angenent, 1991). In contradiction to these models, only one whorl is affected by mutations in these homeotic genes. To gain further understanding into the action of homeotic genes in petunia, we have isolated two genes with the MADS box motif. In this study, we describe the molecular cloning and characterization of these genes encoding proteins designated floral binding protein 1 (FBP1) and 2 (FBP2). The sequence of both full-length cDNA clones and the genomic structure of *fbp1* are presented. In addition, the expression patterns of these *fbp* genes in petunia wild-type plants and the homeotic mutants *blind* and *green petals* are investigated. Our results suggest that *fbp1* is a potential B-type homeotic gene, which is post-transcriptionally regulated. Furthermore, we discuss the relationship between the *fbp1* gene and the homeotic genes *gp* and *bl* and the position of these genes within the existing models describing floral morphogenesis.

RESULTS

Isolation and Sequence of *fbp1* and *fbp2* cDNA

Our strategy to isolate floral morphogenesis genes from petunia was based on the use of a conserved sequence found in DNA binding domains of transcription factors of plant, fungal, and human origin (MADS box; Yanofsky et al., 1990). A set of degenerated oligonucleotides deduced from the decapeptide sequence KRRNGLFKKA present within the MADS box was synthesized. These oligonucleotides and oligo(dT) were used for an amplification of MADS box cDNA clones synthesized from floral mRNA by polymerase chain reaction (PCR). Subsequently, the resulting PCR products were used to screen a λgt11-based, petal-specific cDNA library. Initially, one cDNA clone, designated *fbp1*, was isolated and analyzed in more detail. Rescreening of the cDNA library with *fbp1* cDNA as probe resulted in the isolation of an additional MADS box cDNA clone, designated as *fbp2*. The complete nucleotide sequences of *fbp1* (780 bp) and *fbp2* (960 bp) were determined and are depicted in Figure 1. The sequence of the *fbp1* cDNA (Figure 1A) clone contains one large open reading frame with a calculated coding capacity of 24.6 kD, as well as 5′ and 3′ nontranslated regions. A small poly(A) tail was found at the 3′ terminus of *fbp1*. The *fbp2* cDNA (Figure 1B) encodes for

A

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                PRIMER 1
GGAAAATATGGGGAGAGGAAAGATAGAGATAAAAAGAATAGAAAACTCAAGCAACAGACA 60
      M  G  R  G  K  I  E  I  K  R  I  E  N  S  S  N  R  Q
AGTAACTTACTCAAAAAGAAGAAATGGGATCTTGAAAAAAGCTAAGGAAATTAGTGTTCT 120
  V  T  Y  S  K  R  R  N  G  I  L  K  K  A  K  E  I  S  V  L
TTGTGATGCTCGTGTTTCTGTTATCATTTTTGCTAGCTCTGGCAAGATGCATGAGTTCTC 180
  C  D  A  R  V  S  V  I  I  F  A  S  S  G  K  M  H  E  F  S
TTCTACTTCGTTGGTTGATATTTTGGATCAATATCACAAGCTTACTGGTAGAAGATTGTT 240
  S  T  S  L  V  D  I  L  D  Q  Y  H  K  L  T  G  R  R  L  L
GGATGCTAAGCATGAGAACTTGGACAATGAAATCAACAAAGTCAAGAAAGACAATGACAA 300
  D  A  K  H  E  N  L  D  N  E  I  N  K  V  K  K  D  N  D  N
CATGCAAATTGAACTCAGGCACTTGAAGGGTGAAGATATCACATCTTTGAACCATAGAGA 360
  M  Q  I  E  L  R  H  L  K  G  E  D  I  T  S  L  N  H  R  E
GCTCATGATATTGGAAGATGCCCTTGAAAATGGACTCACTAGTATTCGTAACAAACAGAA 420
  L  M  I  L  E  D  A  L  E  N  G  L  T  S  I  R  N  K  Q  N
TGAGGTTCTGAGGATGATGAGGAAAAAGACTCAAAGTATGGAGGAGGAGCAAGACCAACT 480
  E  V  L  R  M  M  R  K  K  T  Q  S  M  E  E  E  Q  D  Q  L
TAATTGCCAATTGCGCCAACTTGAGATAGCAACCATGAATAGGAATATGGGAGAAATTGG 540
  N  C  Q  L  R  Q  L  E  I  A  T  M  N  R  N  M  G  E  I  G
CGAAGTGTTTCAGCAGAGGGAGAATCATGACTACCAAAACCATATGCCTTTTGCCTTCCG 600
  E  V  F  Q  Q  R  E  N  H  D  Y  Q  N  H  M  P  F  A  F  R
AGTACAACCAATGCAGCCAAATTTGCAGGAGAGGTTGTAAAAAAAGACCTTGATCTACTT 660
  V  Q  P  M  Q  P  N  L  Q  E  R  L  *
GGTGACGACCTTTTAATATTGTCTTGTTTGTATTTTGTGCTATCAAAAAAACTTGGTGTG 720
TATTATCAAGACTCGTGTACCTTATCGTTTAAGTGACATTATCTATCTATAAGACTAAAA 780
                PRIMER 2
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B

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GTGAGTTTCAGTTTTCTTAGCAAGAAAAAAAAAATATGGGAAGAGGTAGAGTTGAGCTTA  60
                                      M  G  R  G  R  V  E  L
AGAGAATAGAGAACAAAATCAATAGACAAGTTACCTTTGCTAAGAGAAGAAATGGACTAT 120
K  R  I  E  N  K  I  N  R  Q  V  T  F  A  K  R  R  N  G  L
TGAAAAAAGCTTATGAACTTTCTGTTCTTTGTGATGCTGAAGTTGCTCTTATTATTTTCT 180
L  K  K  A  Y  E  L  S  V  L  C  D  A  E  V  A  L  I  I  F
CTAATAGGGGAAAATTGTACGAGTTTTGCAGTAGCTCTAGCATGCTCAAGACCTTAGAGA 240
S  N  R  G  K  L  Y  E  F  C  S  S  S  S  M  L  K  T  L  E
GGTACCAGAAGTGTAACTATGGAGCACCAGAGACTAATATATCCACACGAGAAGCACTGG 300
R  Y  Q  K  C  N  Y  G  A  P  E  T  N  I  S  T  R  E  A  L
AAATAAGCAGCCAACAAGAGTACTTGAAGCTTAAAGCACGTTACGAAGCATTACAGCGAT 360
E  I  S  S  Q  Q  E  Y  L  K  L  K  A  R  Y  E  A  L  Q  R
CACAGAGGAATCTTCTTGGTGAAGATCTTGGCCCTTTGAACAGCAAAGAACTTGAATCAC 420
S  Q  R  N  L  L  G  E  D  L  G  P  L  N  S  K  E  L  E  S
TTGAAAGGCAGCTTGATATGTCACTGAAACAAATCAGATCAACTCGGACTCAGCTGATGT 480
L  E  R  Q  L  D  M  S  L  K  Q  I  R  S  T  R  T  Q  L  M
TGGATCAACTTCAAGATCTTCAGAGAAAGGAACATGCATTAAACGAAGCAAACAGAACCT 540
L  D  Q  L  Q  D  L  Q  R  K  E  H  A  L  N  E  A  N  R  T
TGAAACAAAGGTTGATGGAAGGAAGCACACTAAATCTGCAGTGCAGCAAAATGCACAAGA 600
L  K  Q  R  L  M  E  G  S  T  L  N  L  Q  Q  C  S  K  M  H
TGTGGGCTACGGCAGACAAGCAACTCAAACTCAGGGAGATGGCTTCTTTCATCCTTTGGA 660
K  M  W  A  T  A  D  K  Q  L  K  L  R  E  M  A  S  F  I  L
ATGTGAACCCACTTTACAAATTGGGTATCAAAATGATCCAATTACAGTAGGAGGAGCAGG 720
W  N  V  P  L  Y  K  L  G  I  K  M  I  Q  L  Q  *
GCCAAGTGTGAATAACTACATGGCTGGCTGGTTGCCTTGAAAGCTCATTCTGATAAAGTA 780
TATGCTCAATGCTTTTAATTTCCTATCATAAAAAATTGTCCTAATTTCTGTATTTTGTTT 840
TGACTAATACTTTTAATTCTGGACTAATTAATTGGGGCCCATAAGGAGGCCATTGTTGTA 900
GTAATCATAACTAGATTCTTGAGGCTTATCAGTGAGTCTTTCTTCATTCTTTGGATCGAA 960
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Figure 1. Nucleotide Sequences of *fbp1* and *fbp2* cDNA Clones.

(A) Nucleotide and deduced amino acid sequence of the *fbp1* cDNA. The conserved amino acids used for the synthesis of the degenerated PCR primers are underlined. The intron/exon junctions are denoted by arrowheads. The *fbp1* cDNA sequences present in primers 1 and 2, which were used for amplification of the coding region by PCR, are indicated by lines.

(B) Nucleotide and deduced amino acid sequence of the *fbp2* cDNA.

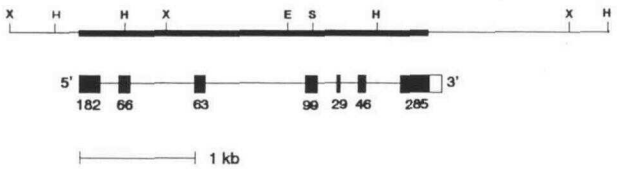


Figure 2. Genomic Structure and Restriction Map of the *fbp1* Gene.

The boxes indicate exons (sizes in nucleotides are given), and the lines between them represent introns. The untranslated region at the 3′ end of the cDNA is indicated by an open box. Restriction enzyme sites are as follows: EcoRI, E; HindIII, H; SstI, S; XbaI, X.

a putative peptide with a molecular mass of 26.3 kD and exhibits a long track of 10 adenine residues in its nontranslated 5′ end. The N termini of both putative FBP proteins contain the complete MADS box domain (see also Figure 4B below).

Genomic Organization of the *fbp1* Gene

The complete *fbp1* cDNA insert was used to isolate the *fbp1* gene from a genomic library of petunia line R27. Hybridization under high-stringency conditions revealed four positive clones, which were further purified and subjected to PCR analysis using primers 1 and 2 (Figure 1A). PCR fragments of 2.8 kb were obtained from two λ phages, which demonstrated the presence of the complete coding sequence in these genomic inserts. The nucleotide sequence of the *fbp1* gene in one of these clones as well as ±1 kb of upstream sequence were determined, resulting in the genomic structure shown in Figure 2.

To determine the number of MADS box genes and *fbp* genes present in the genome of petunia, DNA gel blot analysis was performed. Figures 3A and 3B show genomic DNA digested with various restriction enzymes hybridizing with a 5′ and 3′ probe of *fbp1* cDNA. Hybridization with the 5′ terminal probe containing the MADS box (Figure 3A) revealed a large number of bands, indicating that the petunia genome contains a high number of genes encoding MADS box proteins. The result of the hybridization with the *fbp1*-specific 3′-terminal probe (Figure 3B) is in agreement with the genomic structure of the single *fbp1* gene depicted in Figure 2. Hybridization with a *fbp2*-specific probe demonstrated that the *fbp2* cDNA corresponds to a single gene in the genome of petunia (results not shown).

fbp Genes Encode Putative Transcription Factors

Comparison of the FBP protein sequences and various floral homeotic gene products revealed two domains of relatively high homology. Figure 4A shows schematically both domains and their position in the proteins. The alignment of the MADS box domains of petunia FBP1 and FBP2, the tomato protein TM5 (Pnueli et al., 1991), the Arabidopsis proteins AGL2 and AG (Yanofsky et al., 1990; Ma et al., 1991), and the snapdragon gene product encoded by *defA* (Sommer et al., 1990) is shown in Figure 4B. In the MADS box, approximately 70% of the amino acid residues are identical between FBP1 and the other MADS box domains depicted in Figure 4B. Alignment of the MADS box domains of FBP1 and the protein of the snapdragon gene *globosa* (*glo*) revealed only four mismatches (Z. Schwarz-Sommer, personal communication). No differences in amino acid sequence were found in the MADS box domains of FBP2 and AGL2, and only one mismatch with the sequence of TM5. Furthermore, both petunia FBP proteins have, as all DNA binding domains of the MADS proteins, a conserved potential phosphorylation site (RXX[S/T]; Cohen, 1988; line above the sequence in Figure 4B). It has been demonstrated for several transcription factors that phosphorylation is essential for DNA binding (Prywes et al., 1988; Mylin et al., 1989).

In addition to the MADS box, the FBP proteins share a second domain with striking sequence similarities with TM5, AGL2, AG, and DEFA proteins (Figure 4C). This domain has been designated the K box, because of its similarity to the coiled-coil domain of human type II keratin (Tyner et al., 1985). In particular, the leucine residues in this domain are very conserved or are replaced by other hydrophobic amino acids. It has been

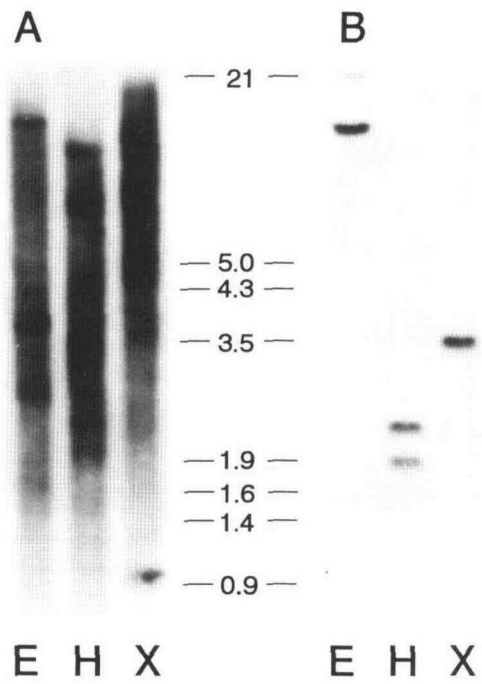


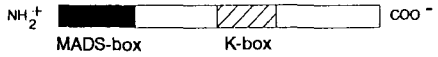
Figure 3. DNA Gel Blot Analysis of Petunia R27 Genomic DNA.

(A) The blot was probed with the 5′ terminal part (nucleotides 1 to 220) of *fbp1* cDNA.

(B) The blot was probed with the 3′ terminal part (nucleotides 221 to 780) of *fbp1* cDNA.

The lengths of marker fragments are given in kilobases. DNA was digested with EcoRI (E), HindIII (H), and XbaI (X), respectively.

A



B

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                    1
FBP1    1  MGRGKIEIKRIENSSNRQVTYSKRRNGILKKAKEISVLCDARVSVIIFASSGKMHE
FBP2    1  ****RV*L*****KI*****FA*****L****Y*L******E*AL***SNR**LY*
TM5     1  ****RV*L****GKI*****FA*****L****Y*L******E*AL***SNR**LY*
AGL2    1  ****RV*L*****KI*****FA*****L****Y*L******E*AL***SNR**LY*
AG     50 S***********TT*****FC*****L****Y*L******E*AL*V*S*R*RLY*
DEFA    1  *A****Q******QT***********LF***H*L*V****K**I*MIS*TQ*L**
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C

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FBP1    104     RHLKGEDITSLNHRELMILEDALENGLTSIRNKQNEVL
FBP2    111     *N*L***LGP**SK**ES**RQ*DMS*KQ**STRTQLM
TM5     111     *N*L***LGP**SK**ES**RQ*DMS*KQ**STRTQLM
AGL2    124     *N*L***LGP**SK**EQ**RQ*DGS*KQV*SIKTQYM
AG      157     *Q*M**T*G*MSPK**RN**GR**RSI*R**S*K**L*
DEFA    106     *QRM**SLND*GYEQIVN*IEDMD*S*KL**ERKYK*I
```

D

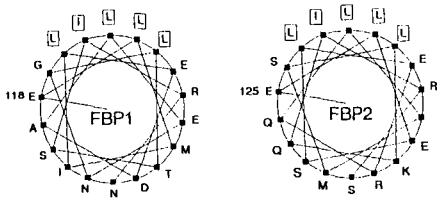

Figure 4. Comparison of Deduced FBP1 and FBP2 Protein Structures and Alignment of Conserved Domains of TM5, AGL2, AG, and DEFA Proteins.

(A) The MADS box domain is represented by a solid box, and the K box domain by a dashed box.

(B) Alignment of the MADS boxes of FBP1, FBP2, TM5, AGL2, AG, and DEFA. The conserved phosphorylation site (1) is indicated by a line above the sequence. Identical amino acid residues are represented by asterisks.

(C) Alignment of the putative dimerization domains of FBP1, FBP2, TM5, AGL2, AG, and DEFA.

(D) Helical wheel models of 18 amino acids of FBP1 (positions 118 to 135) and FBP2 (positions 125 to 142) representing five turns of the coiled structure. The leucine and isoleucine residues forming the hydrophobic face of the helix are boxed.

proposed that these leucines and other nonpolar residues exist in an α-helical conformation and might form a hydrophobic face similar to that in leucine zipper proteins (Landschulz et al., 1988). Figure 4D shows helical wheel models of FBP1 (starting at Glu-118) and FBP2 (starting at Glu-125), based on an ideal α-helix containing slightly more than 3.5 residues per turn. In both FBP proteins, four leucines and one isoleucine are located at one site of the coiled coil structure. This region is a functionally important domain of the snapdragon DEFA protein. Deletion of a single amino acid causes a mutant phenotype of the flower (Schwarz-Sommer et al., 1992). For proteins with a leucine zipper motif (i.e., FOS and JUN), it has been demonstrated that these leucines are essential for dimer formation (Kouzarides and Ziff, 1988). Similarly, the hydrophobic motif of repeated leucine residues within the coiled coil structure of the FBP proteins might facilitate intrahelical interaction with other FBP molecules or transcription factors.

Another feature of certain types of transcription factors is found in the region between the putative dimerization domain and the C terminus of FBP1. This domain, comprising the C-terminal 60 amino acids of the protein, is glutamine rich (18%). This is considered to be characteristic for transcriptional activating domains that interact with general transcription factors such as TATA-binding TFIID (Mitchell and Tjian, 1989). Similar glutamine-rich regions have been found in a number of transcription activators from Drosophila, yeast, and mammals (Mitchell and Tjian, 1989). Also, the C-terminal part of the proteins encoded by the Arabidopsis *agl* gene family is glutamine rich (Ma et al., 1991), whereas such regions are not present in FBP2 and the other plant MADS box proteins.

Expression of the *fbp* Genes in Wild-Type Petunia

To study the pattern of *fbp1* and *fbp2* gene expression in wild-type petunia, RNA was isolated from leaves, roots, and the different floral organs. Floral tissue was collected from flower buds of different developmental stages. Figure 5B shows the result of a hybridization of these RNAs with the 3′ *fbp1*-specific probe. This RNA gel blot reveals that *fbp1* is exclusively expressed in petals and stamen and not in sepals and carpels or in vegetative tissues. In tubes and limbs, together forming the petunia petal, similar *fbp1* mRNA levels were observed (Figure 5B, whorls 2a and 2b). Furthermore, *fbp1* is continuously expressed during flower development: *fbp1* mRNA was detectable in young flowerbuds (as small as 5 mm) as well as in mature flowers (results not shown). Recently, Weiss et al. (1992) demonstrated by run-on transcription that the presence of *fbp1* mRNA in petals of different developmental stages is probably the result of de novo transcription. Similar persistent expression throughout flower development was also observed for the snapdragon gene *defA* (Sommer et al., 1990).

Transcripts of *fbp2* were only observed in petals, stamen, carpels, and at a very low level in sepals, as is shown in Figure 5C. This expression pattern matches the spatial expression of the tomato MADS box gene TM5, which exhibits extensive sequence homology with *fbp2* (Pnueli et al., 1991).

To examine the presence of FBP1 protein in the different floral organs, we raised a polyclonal antiserum against the FBP1 protein. The complete *fbp1* cDNA was expressed in *Escherichia coli* using a pET11 expression vector (Rosenberg et al., 1987). For the insertion of the cDNA into the right reading frame, the sequence upstream of the translation initiation codon was modified into a NcoI restriction site by PCR. Comparison of the crude protein extracts of *E. coli* cells containing or lacking the *fbp1* cDNA revealed an additional protein band with a molecular mass of approximately 28 kD, as is shown in Figure 6A. This protein was easily enriched by sonication of the lysed cells and collecting the pellet after centrifugation (Figure 6A, lanes 3 and 4). The majority of the FBP1 protein

produced was present in this pellet. A rabbit was immunized with FBP1 isolated from preparative SDS–polyacrylamide gels.

The immunoblot presented in Figure 6B revealed that the antiserum raised was able to recognize FBP1 specifically in protein extracts of *E. coli* cells containing the *fbp1* cDNA (lanes E− versus E+). Subsequently, the expression and subcellular location of the protein were determined in different floral organs of petunia line R27. Figure 6B shows that the polyclonal antiserum strongly reacts with two proteins, both accumulating in petals. One protein band comigrates with the FBP1 protein synthesized in *E. coli*, suggesting that it represents FBP1. The other protein has a molecular mass of approximately 45 kD, and its relation with the FBP1 protein is unknown. The accumulation of FBP1 in nuclear extracts is in agreement with the role of FBP1 as a transcriptional activator. Surprisingly, the protein was only observed in extracts of petals and not in stamens. This disagrees with the results obtained with the RNA gel blot analysis, where accumulation of *fbp1* mRNA was observed in both petals and stamens (Figure 5B). Analysis of the protein samples on gels stained with Coomassie Brilliant blue R250 did not show any protein breakdown in the various nuclear extracts (results not shown).

Expression of the *fbp* Genes in Homeotic Flower Mutants

The two homeotic flower morphology mutants *green petals* and *blind* possess highly modified whorl 2 organs (Wiering et al., 1979). In *green petals*, the petals are replaced by sepaloid organs, whereas the flowers of *blind* lack limbs and instead develop antheroid structures on top of the tube. Figure 5A shows the flowers of wild-type, *green petals*, and *blind* plants.

The expression pattern of the *fbp1* gene was analyzed in *green petals* and *blind* by an RNA gel blot experiment using the 3′-terminal part of *fbp1* cDNA as a probe. This hybridization experiment, depicted in Figure 5B, showed that the antheroid limbs of *blind* accumulated *fbp1* transcripts similarly to normal anthers. Furthermore, *fbp1* expression in tubes of *blind* was not affected by the conversion of limbs into antheroid structures. To investigate whether these modified limbs are petaloid or antheroid, an immunoblot analysis was performed. Because accumulation of the FBP1 protein is restricted to petals (Figure 6B), a discrimination between petaloid and antheroid tissue can be made at the level of FBP1 protein accumulation. Figure 5D shows again the discrepancy between mRNA and protein accumulation in stamens. As in wild-type petunia, FBP1 protein was absent in nuclear extracts of stamens as well as in extracts of the antheroid structures of the *blind* mutant. This demonstrates that the antheroid petals behave like normal anthers with respect to *fbp1* expression. These results show that the spatial expression of *fbp1* follows the changes in organ development caused by the mutation in the *bl* gene, as was also observed for the flavonoid genes encoding chalcone isomerase (van Tunen et al., 1990). Finally, an additional band with a lower mobility was observed in tubes of the *blind* mutant and wild type and not in the limbs, which might be the result of differential modification of FBP1 in tubes and limbs. The accumulation pattern of *fbp2* mRNA was not affected by the mutation in the *blind* mutant compared to those of wild-type flowers, as is shown in Figure 5C.

In the green petals (whorl 2) of the *green petals* mutant, the *fbp1* mRNA level was strongly reduced in comparison with the expression levels in *blind* and wild-type whorl 2 organs (Figure 5B). However, Figure 5B shows that the *fbp1* mRNA level in the green petals was not identical to whorl 1 organs. In the green petals, low amounts of *fbp1* transcript still accumulate. Similarly, Figure 5C shows that the expression of *fbp2* was not identical in whorl 1 and 2 of the *green petals* mutant. These observations demonstrate that, in spite of the morphological similarity of sepals and green petals, these organs are not identical. In contrast to the *fbp1* expression, the mRNA level of *fbp2* was not reduced in whorl 2 organs of the mutant (Figure 5C), suggesting that *fbp2* expression is not regulated by the *gp* gene.

The FBP1 protein accumulation in the green petals is in agreement with the mRNA levels depicted in Figure 5B: the protein was hardly detectable in extracts of green petals (Figure 5D; whorl 2).

DISCUSSION

In this study, we report the isolation and characterization of two flower-specific genes (*fbp1* and *fbp2*) from petunia. Both gene products are putative transcription factors sharing two domains with other floral homeotic genes of Arabidopsis, snapdragon, and tomato. The N-terminal MADS box domain—the putative DNA binding region—has been found in a number of organisms, including mammals and fungi. This suggests that the MADS box domain is evolutionarily conserved. The second conserved domain, the K box, is probably involved in formation of dimer molecules. In addition, the immunological localization of the FBP1 protein in a nuclear fraction is compatible with FBP1 acting as a transcription factor. Furthermore, FBP1 exhibits extensive sequence homology with the protein of the homeotic gene *globosa*. The overall amino acid sequence similarity between these two proteins is 86% (Z. Schwarz-Sommer, personal communication). The FBP2 protein has 93% of the amino acid sequence in common with the tomato TM5 product, suggesting that they are also cognate homologs (Pnueli et al., 1991). The highest degree of similarity (58%) of FBP2 with an Arabidopsis MADS box protein was observed with AGL2 (Ma et al., 1991). These similarities between flower-specific MADS box genes from unrelated plant species suggest that these genes are involved in general mechanisms regulating the development of the flower.

Regulation and Differential Expression of *fbp1*

We have shown by RNA gel blot analysis that the *fbp1* gene is exclusively expressed in petals and stamens of wild-type petunia. However, the FBP1 protein was only detectable in

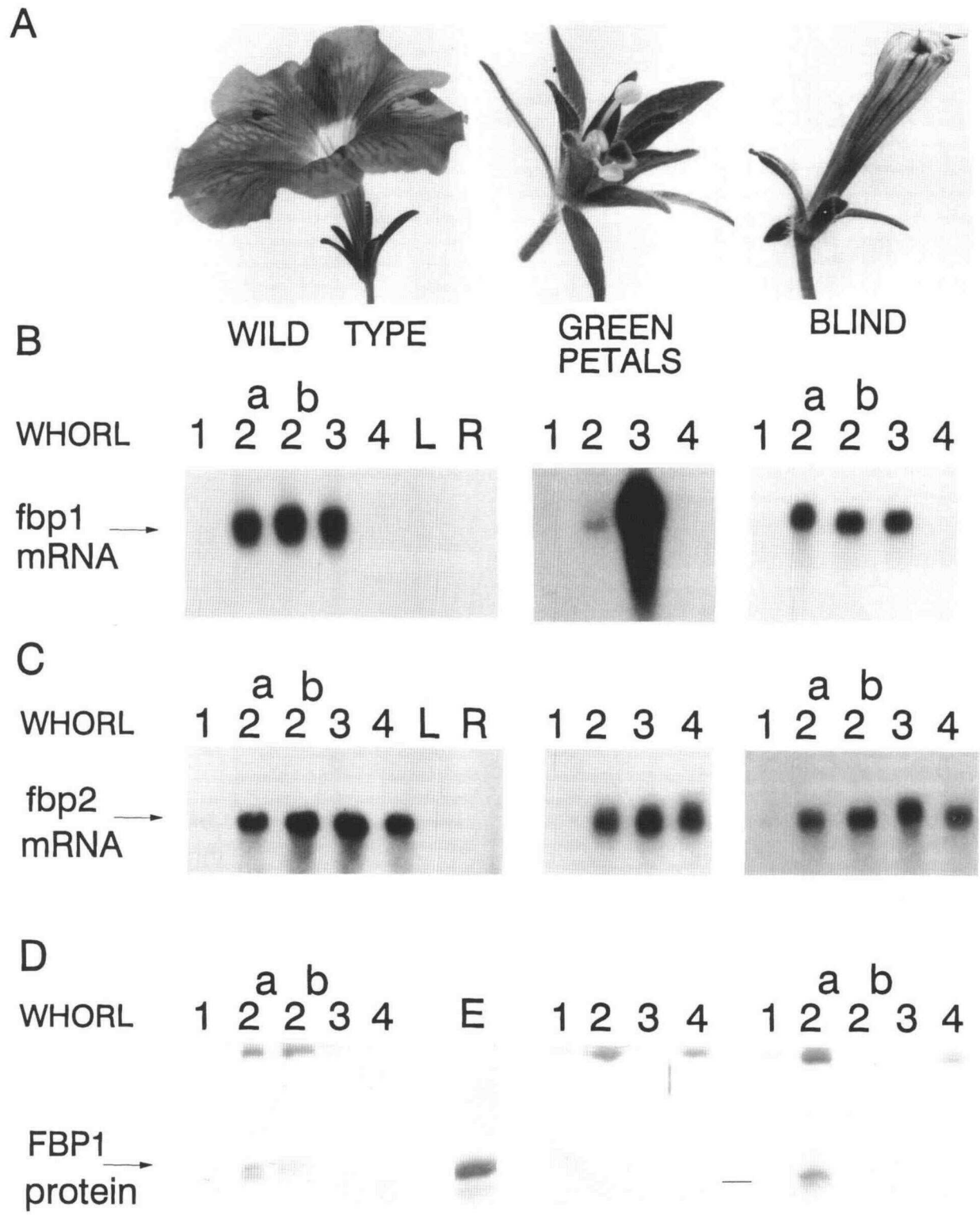

Figure 5. Differential Expression of *fbp1* and *fbp2* and Protein Accumulation in Floral Organs of Homeotic Mutants *green petals* and *blind* and Wild-Type Petunia.

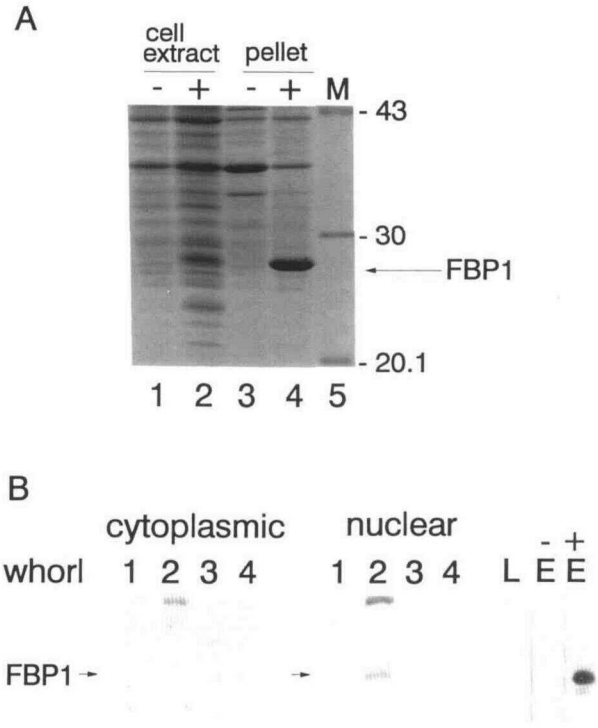

Figure 6. FBP1 Protein Accumulation in *E. coli* and Petunia.

(A) SDS–polyacrylamide gel (12.5%) with protein extracts of *E. coli* cells containing (lanes 2 and 4; +) or lacking (lanes 1 and 3; −) the *fbp1* cDNA. Lanes 1 and 2 were loaded with crude cell extracts, and lanes 3 and 4 represent proteins present in the pellet fraction after sonication of the cells. The molecular masses (kD) of marker proteins (lane 5; M) are given. The gel was stained with Coomassie blue.

(B) Immunoblot analysis of proteins reacting with FBP1 antiserum. Proteins were isolated from floral organs (sepals, lanes 1; petals, lanes 2; stamens, lanes 3; carpels, lanes 4; leaves, lane L) and *E. coli* cells with (lane E+) or without (lane E−) the *fbp1* cDNA. Cells from the floral organs were fractionated in a cytoplasmic and nuclear fraction, and identical amounts (100 μg) of protein were loaded onto the gel. The position of the FBP1 protein is indicated with arrows.

nuclear extracts of petals but not in those of stamens. This discrepancy can be explained by a post-transcriptional control of *fbp1* gene expression. This regulation might be directed by organ-specific factors that control translation efficiency in a differential way. Also, the turnover of the protein can be influenced by interaction with organ-specific proteins, i.e., by the formation of heterodimer transcription factors. Similar translational control in plants has been observed for a number of genes as a response to environmental or developmental signals (Berry et al., 1990; Franken et al., 1991). In our immunoblot analysis, we have used protein extracts from flowerbuds of different developmental stages (flowerbuds from 0.5 cm to mature size) in which the separate floral organs can already be distinguished. We cannot draw any conclusions on the role of *fbp1* during the formation of stamen primordia and the early stages of stamen development. It might also be possible that the FBP1 protein is functional in only a limited number of cell types of the stamen. This possibility will be investigated in the near future by immunolocalization of the FBP1 protein.

Three classes of homeotic genes—A, B, and C—are considered to direct the identity of the organs. They are active in three overlapping regions, each comprising two adjacent whorls (for a review, see Coen and Meyerowitz, 1991). Class B genes are expressed in petals and stamens, and a mutation in these genes results in phenotypic effects in whorls 2 and 3. For instance, the homeotic Arabidopsis mutant *Pistillata* (*pi*) and snapdragon mutant *Globosa* (*glo*) show sepaloid structures in whorl 2 and the stamens are replaced by carpels (Hill and Lord, 1989; Schwarz-Sommer et al., 1990). The differential expression of *fbp1* mRNA suggests that this gene can be assigned as a class B homeotic gene. Furthermore, β-glucuronidase (GUS) expression data revealed that the *fbp1* promoter is already active at the moment petal and stamen development is initiated (G. C. Angenent and A. J. van Tunen, unpublished data). These results and the homology between *fbp1* and the homeotic gene *glo* strongly indicate that *fbp1* is a B-type homeotic gene involved in flower morphogenesis.

As a class B gene, *fbp1* seems to be unique, because protein accumulation was not detectable in whorl 3 organs. Class B homeotic genes from Arabidopsis and snapdragon that are functional in one whorl only have not been reported yet.

Figure 5. (continued).

(A) Phenotype of wild-type R27 (left), *green petals* (middle), and *blind* (right) flowers.

(B) RNA gel blot analysis of the *fbp1* expression in various plant organs of wild-type petunia (R27) and in floral organs of *green petals* and *blind*. Total RNA was isolated from sepals (whorl 1), green petals (whorl 2; *green petals*), tubes (whorl 2a; wild type and *blind*), limbs (whorl 2b; wild type), antheroid limbs (whorl 2b; *blind*), stamens (whorl 3), carpels (whorl 4), leaves (L), and roots (R). The blot was probed with labeled cDNA corresponding to nucleotides 220 to 780 of the *fbp1* cDNA sequence. The blot with *green petal* RNAs was exposed five times as long as the other blots. The position of *fbp1* mRNA is indicated with an arrow.

(C) Similar blot as is shown in **(B)** probed with labeled cDNA corresponding to nucleotides 330 to 960 of the *fbp2* cDNA sequence. The position of *fbp2* mRNA is indicated with an arrow.

(D) Immunoblot analysis of FBP1 accumulation in wild-type, *green petals*, and *blind* flowers. Proteins (nuclear fraction) were isolated from the same tissue as was used for the RNA gel blot analysis in **(B)**. Lane E shows the control of FBP1 protein synthesized in *E. coli*. The position of FBP1 is indicated with an arrow.

Relation between *fbp1* and Petunia Flower Morphogenesis Mutants

Both recessive mutants *blind* and *green petals* are affected in whorl 2 organs only, which makes it difficult to fit these mutants within the existing models for flower morphogenesis developed for Arabidopsis and snapdragon. So far, only the Arabidopsis homeotic mutant FLO10 is affected in a single whorl (Schultz et al., 1991). These authors suggest that the *flo10* gene prevents the expression of class B genes in whorl 4.

Recently, we have attempted to fit the *bl* gene into the existing models, including the critical aspect of timing of gene expression (van Tunen and Angenent, 1991). The *blind* phenotype might be the result of a mutation causing a shift in timing of gene A expression. Early abolishment of gene A expression leads to an inappropriate gene C expression resulting in anther development on top of the tubes. Occasionally, we have observed *blind* flowers with partially formed limbs or, on the other hand, anthers on top of incomplete tubes, suggesting that timing of gene expression during petal development is affected.

Our results with *fbp1* and previously reported data on flavonoid gene expression in *blind* (van Tunen et al., 1990) have demonstrated that the expression patterns of these genes follow the changes in tissue development. The antheroid structures on top of the tubes of *blind* flowers express these genes to the same extent as normal anthers. This demonstrates that the *Bl* gene is acting upstream of the *fbp1* gene and flavonoid genes in the hierarchy of flower development.

The second flower morphogenesis mutant, *green petals*, gives rise to flowers with sepaloid organs in whorl 2. These green petals behave like normal sepals with respect to the differential expression of the chalcone flavanone isomerase (*chiA*) gene (van Tunen et al., 1990). No *chiA* mRNA was observed in whorl 2 organs of *green petals.* In contrast, *fbp2* expression was not affected in the green petals of the mutant, demonstrating that *fbp2* expression is not regulated by the *gp* gene or influenced by the homeotic change to a sepaloid organ. These expression patterns suggest that *fbp2* is active in early stages of flower development. This is in contradiction to the tomato homolog TM5 gene, which is thought to be active in later stages of flower development during or after the formation of floral organs (Pnueli et al., 1991). The expression of *fbp1* in the green petals is dramatically reduced but not completely absent. This observation suggests that the transcription of *fbp1* is regulated by the *gp* gene in petals of wild-type flowers or that both genes are identical. An additional argument can be put forward to support the hypothesis that *fbp1* and *gp* are related. It is obvious that the mutation in *green petals* has no effect on the morphogenesis of the stamens, which is in agreement with the observation that the FBP1 protein is not present in the stamens. The possibility that *fbp1* is identical to the *gp* gene has been tested by genetic linkage experiments (data not shown). These experiments revealed that the *fbp1* gene did not co-segregate with the *green petals* phenotype, which demonstrates that these genes are not identical.

Our results have demonstrated that the mutation in *gp* has only an effect on *fbp1* transcription in petals and not in stamen. This suggests that the mutation in *gp* is in a regulatory sequence directing *fbp1* expression in petals or that *fbp1* transcription is not regulated by the *gp* gene in stamens of wild-type flowers. Mutations in regulatory sequences directing expression in an organ-specific manner can occur in petunia, as was reported for the *chiA* gene (van Tunen et al., 1991). A mutation within this gene results in an absence of *chiA* expression in anthers but not in petals. Also, a small deletion in the promoter region of the *chlorantha* allele of *deficiens* results in decreased gene expression specifically in the petals and stamens and not in the other organs (Schwarz-Sommer et al., 1990).

Additional experiments are necessary to further unravel the function of *fbp1* and *fbp2* in organ morphogenesis and the relation between *fbp1* and *gp*. Therefore, experiments to introduce *fbp1* and *fbp2* cDNA in an antisense or sense orientation into wild-type petunia will be performed in the near future.

METHODS

Plant Material

The *Petunia hybrida* varieties R27 (wild type), R51 (*blind*), and M64 (*green petals*) were grown under normal greenhouse conditions.

Polymerase Chain Reaction Analysis

Single-stranded cDNA was synthesized by priming with the oligonucleotide 5′-CCGGATCCTCTAGAGCGGCCGC$(T)_{17}$ from 10 μg of total RNA isolated from young corolla tissue (Maniatis et al., 1982). A second primer with the sequence 5′-GGGGTACCAA(G/A)CGI(C/A)(G/A)-I(A/C)(C/A)(T/C)GG(I/C)(T/C/A)T(I/C)(A/T)T(G/C/T)AA(G/A)AA(G/A)GC-3′ based on the conserved MADS box sequence KRRNGLFKKA was used for the initial polymerase chain reaction (PCR) analysis. PCR analysis was performed in 100 μL of PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$) containing 80 and 25 pmol of 5′ and 3′ primer, respectively, and 200 μM of each deoxynucleotide triphosphate. Amplification involved 30 cycles with a denaturing time of 20 sec at 94°C, cooling down in 90 sec to 37°C, an annealing time of 30 sec, and an extension time of 6 min at 60°C. Amplified cDNA was fractionated on 1% agarose gel, revealing one clear fragment of 0.8 kb. This band was isolated and subcloned into the M13mp19 vector using BamHI and KpnI restriction sites present in the 5′ and 3′ primers, respectively. This cDNA insert (*fbp1*) was used to screen the cDNA library for the isolation of a complete cDNA clone of *fbp1*. This clone was used to rescreen the cDNA library under low stringency conditions (60°C hybridization and wash with 2 × SSC [1 × SSC consists of 0.15 M sodium chloride and 0.015 M sodium citrate!]), and additional MADS box cDNA clones were isolated, including *fbp2.*

For the correct subcloning of *fbp1* cDNA into the *Escherichia coli* expression vector pET11d, the translation start site was modified into a NcoI site by PCR. The sequences of both 5′ and 3′ primers are 5′-GGGGATCCATGGGGAGAGGAAAGATAGAG-3′ and 5′-CCGGATCCTTAAACGATAAGGTACACGAGTC-3′, respectively. The underlined sequences correspond to the cDNA sequence shown in Figure 1A

(primers 1 and 2). The conditions for this PCR were denaturing at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 2 min.

Screening cDNA and Genomic Libraries

A λgt11 cDNA library made from poly(A)$^+$ RNA of young R27 petals (van Tunen et al., 1988) was screened. Plating and screening of the library using *E. coli* Y1090 cells as host were performed according to the Promega protocol. Probes were prepared by random oligonucleotide priming (Feinberg and Vogelstein, 1984); hybridization and washing of the Hybond-N membranes (Amersham International) were done at high stringency (65°C hybridization and final wash with 0.1 × SSC). The genomic library was a gift of L. A. Mur (Free University, Amsterdam) containing partial Sau3A fragments of R27 nuclear DNA in λ GEM12 (Promega). Approximately 150,000 plaques were screened with ^{32}P-labeled *fbp1* cDNA, and positive clones were isolated and purified. Subsequently, the inserts or part of the inserts were subcloned in pEMBL vectors (Boehringer Mannheim) and further analyzed by restriction enzyme digests, hybridization, and sequencing.

DNA Sequencing Analysis

The dideoxy chain termination method was used for sequencing of single-stranded DNA derived from pEMBL derivatives. Sequencing was done using the Sequenase kit (Promega) according to the manufacturer's protocol. All sequence data reported in this paper were derived from sequencing both DNA strands. If necessary, breakpoints were created by unidirectional digestion with exonuclease III according to the method of Henikoff (1984). The protein structure was predicted with Microgenie software. The GenBank accession numbers of the *fbp1* and *fbp2* cDNA sequences reported in this article are M91190 and M91666, respectively.

DNA and RNA Gel Blot Analyses

Total plant DNA was isolated from young petunia leaf material (Koes et al., 1986), and the isolation of total RNA from different plant organs was done according to Verwoerd et al. (1989). Flower buds of various developmental stages (from 5 mm to mature) were used as the source for RNA isolation. Ten micrograms of petunia DNA was digested with XbaI, HindIII, and EcoRI, electrophoresed, and blotted onto Hybond-N+ membranes. For RNA gel blot analysis, 10 μg of total mRNA was denatured by glyoxal/DMSO and separated on 1.2% agarose gels in 15 mM sodium phosphate buffer, pH 6.5. DNA and RNA gel blots were hybridized with labeled cDNA fragments from *fbp1* (nucleotides 1 to 240 for the 5′ end probe and nucleotides 241 to 780 for the 3′ end probe) or from *fbp2* (nucleotides 330 to 960). Hybridization was performed at 65°C in 1 M NaCl, 1% SDS, and 10% dextran sulphate and finally washed in 0.1 × SSC/0.5% SDS (65°C).

Immunoblot Analysis

Crude nuclear fractions were isolated using a modification of the method of Lawton et al. (1991). The floral organs were prepared from flower buds at developmental stages similar to those used for RNA gel blot analysis. About 1 g of tissue was homogenized in 5 mL of extraction buffer (50 mM MES, pH 5.8, 25 mM NaCl, 0.5% [v/v] Triton X-100, 5 mM $MgCl_2$, 30% glycerol, 5% sucrose; 10 mM 2-mercaptoethanol) supplemented with the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), chymostatin (2 μg/mL), and leupeptin (2 μg/mL). The homogenate was passed through Miracloth and centrifugated at 3000*g*. The pellet was gently resuspended in extraction buffer containing 80% [v/v] Percoll and centrifuged at 2700*g*. The pellet was washed with extraction buffer without Triton X-100. Identical amounts of nuclear protein (±100 μg) were electrophoresed on a 12.5% SDS–polyacrylamide gel (Laemmli, 1970). The proteins were subsequently transferred to nitrocellulose membranes (Schleicher and Schüll) and incubated with FBP1 antiserum (1:200 dilution). As a second antiserum, goat anti-rabbit IgG conjugated to alkaline phosphatase was used, and staining was carried out using standard conditions (Promega).

Bacterial Expression of FBP1 Protein

The complete *fbp1* cDNA clone was introduced in the bacterial expression vector pET11d (Rosenberg et al., 1987) using NcoI (5′ end) and BamHI (3′ end) sites. Both sites were generated by PCR. This construct was transformed into BI21, a host strain that contains an integrated copy of the isopropyl β-D-thiogalactopyranoside (IPTG)–inducible T7 RNA polymerase gene (Rosenberg et al., 1987). Cultures were grown in Luria broth to an OD of 0.3. Then IPTG was added to 0.4 mM, and incubation was continued for 3 hr. The bacteria were harvested and resuspended in 1/20 of the original culture volume of buffer containing 50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/mL lysozyme. The cells were sonicated three times for 15 sec at maximum amplitude with an MSE Soniprep 150. After centrifugation, the pellet was resuspended in loading buffer according to Laemmli (1970).

Immunization

A New Zealand white rabbit was immunized with 100 μg FBP1 protein purified by SDS–polyacrylamide gel. Booster injections (100 μg of protein) were given twice with an intervening period of 28 days, and the serum was collected after an additional 10 days.

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