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Review

Zinc-finger transcription factors in plants*

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Abstract. Several classes of zinc-finger motifs are present in transcription factors and function as parts of DNA-binding and protein-protein interaction domains. Most of the known classes of zinc-finger motifs earlier identified in other eucaryotes have also been found in a number of (putative) transcription factors in plants. In addition, some novel classes of zinc fingers have been identified in plants. Many of these proteins have been implicated in the regulation of important biological processes that are unique to plants, such as flower development, light-regulated morphogenesis and pathogen responses. Thus, plants seem to have adopted preexisting prototype zinc-finger motifs as well as generated new zinc-finger domains to adapt them to various regulatory processes. Detailed analyses of TFIIIA-type plant zinc-finger proteins revealed unique manners of interactions with target DNA sequences, i.e. recognition of spacing, suggesting that plants have developed unique mechanisms even when proto-type functional motifs were adopted. In this review, attempts were made to summarize the current knowledge of (putative) zinc-finger transcription factors according to a structure-based classification, in view of their involvement in specific regulatory processes and interaction with target DNA sequences.

Key words. Zinc finger; transcription factor; target sequence recognition; plant; gene regulation.

Introduction

Modulation of transcriptional activity is fundamental to the regulation of gene expression associated with most biological phenomena and is largely mediated through proteins that interact directly or indirectly with specific DNA sequences (*cis* elements) in the promoter region of genes. To date, a vast amount of information about eucaryotic transcription factors has become available, including their biological roles, interaction with target DNA sequences and other regulatory proteins, and three-dimensional structures. In general, transcription factors have modular structures composed of a few functional domains for binding to target DNAs, for interaction with other proteins including other transcription factors and components of basic transcriptional machinery, and for other functions.

The biology of plants is in many aspects common to that of other organisms; there are, however, a number of biological processes that are unique to plants, e.g. photosynthesis, nitrogen fixation, the reproductive process, development and responses to environmental signals. In the past decade, hundreds of transcription factors have been identified in plants that are involved in the regulation of many biological processes. Their protein structures suggest that plants have in some cases adopted preexisting prototype functional motifs and modified them for specific regulatory processes. Indeed, most of the functional motifs present in eucaryotic

* A systematic nomenclature zinc-finger proteins in plants is currently being organized by Dr. B. W. Tague at Wake Forest University, USA. Therefore, most of the proteins and genes described in this review might be renamed according to new rules.

transcription factors have their counterparts in the plant kingdom. In the other cases, plants seem to have evolved new classes of functional motifs that are not present in other organisms (at least in reference to current knowledge). The modified and new functional motifs are considered to have coevolved with regulatory processes that are unique to plants.

The term 'zinc finger' represents the sequence motifs in which cysteines and/or histidines coordinate a zinc atom(s) to form local peptide structures that are required for their specific functions. The zinc-finger motifs, which are classified based on the arrangement of the zinc-binding amino acids, are present in a number of transcription factors and play critical roles in interactions with other molecules. Some classes of zinc-finger motifs (e.g. TFIIIA- and GATA types) are, in most cases, parts of DNA-binding domains of transcription factors and have been shown to be directly involved in the recognition of specific DNA sequences. Other classes (e.g. LIM- and RING-finger types) are mostly implicated in protein-protein interactions. Most of the eucaryotic zinc-finger motifs have also been found in plants. In addition, some novel motifs (e.g. WRKY and Dof motifs) have been identified in plants.

Many of the (putative) zinc-finger transcription factors have been implicated in important biological processes. Mutations in some of the genes coding for zinc-finger proteins have been found to cause profound developmental aberrations or defective responses to environmental cues. Other zinc-finger proteins are known to play a regulatory role by interacting with the *cis* elements of specific target genes. Detailed analyses of the interaction of TFIIIA-type proteins with the respective target DNA sequences revealed some features that seem to be unique for this type of protein family in plants. In this review, attempts have been made to summarize the current knowledge regarding (putative) zinc-finger-containing transcription factors according to a structure-based classification with an emphasis on the TFIIIA class that has been the area of interest of our group.

TFIIIA type

EPF family of petunia

In a TFIIIA-type zinc finger [1], two cysteines and two histidines in a conserved sequence motif ($CX_{2-4}FX_5LX_2HX_{3-5}H$) tetrahedrally coordinate a zinc atom to form a compact structure that interacts with the major groove of DNA in a sequence-specific manner [2]. Generally, in animals, multiple zinc-finger motifs are present as tandem arrays separated by conserved short sequences (seven amino acids) known as HC links.

The first TFIIIA-type zinc-finger protein in plants (ZPT2-1, renamed from EPF1) was identified from petunia during the study of the regulatory mechanism of petal-specific expression of the gene for enolpyruvyl shikimate-3-phosphate synthase (EPSPS), an enzyme in the shikimate pathway leading to the flower pigments, anthocyanins [3]. The petal-specific expression of the *EPSPS* gene is regulated by its 5′-upstream region [4, 5]. The gene for ZPT2-1 was cloned by the southwestern method using the binding sequence within the regulatory region as a probe. The expression of the *ZPT2-1* gene is also petal-specific and coincides with that of the *EPSPS* gene in cell-type specificity and timing; hence, ZPT2-1 is considered a potential candidate for the organ- and stage-specific activator of the *EPSPS* gene.

ZPT2-1 contains two canonical TFIIIA-type zinc-finger motifs ($CX_2CX_3FX_5LX_2HX_3H$). It is characterized by a long spacer between the two zinc fingers (61 amino acids between the second histidine of the N-terminal finger and the first cysteine of the C-terminal finger, fig. 1), in contrast to clustered-type protein animals. Another structural feature of ZPT2-1 is that both the zinc-finger motifs contain a sequence, QALGGH, in the putative DNA-contacting surfaces. This sequence is quite highly conserved in many TFIIIA-type zinc-finger proteins in plants as described below. The long and variant length of spacers between zinc fingers is also a common feature among these proteins.

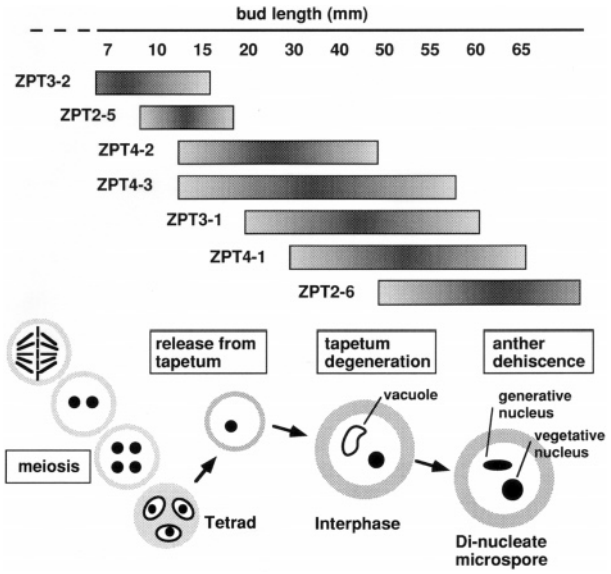

Figure 1. Temporal patterns of the expression of the EPF genes in anthers and filaments. Developmental stages are standardized by reference to bud lengths. The bars in the upper part indicate temporal patterns of the expression of respective EPF genes, with gradients in the density in the bars representing relative expression levels. Developmental stages of anthers and pollen corresponding to respective bud lengths are shown in the lower part.

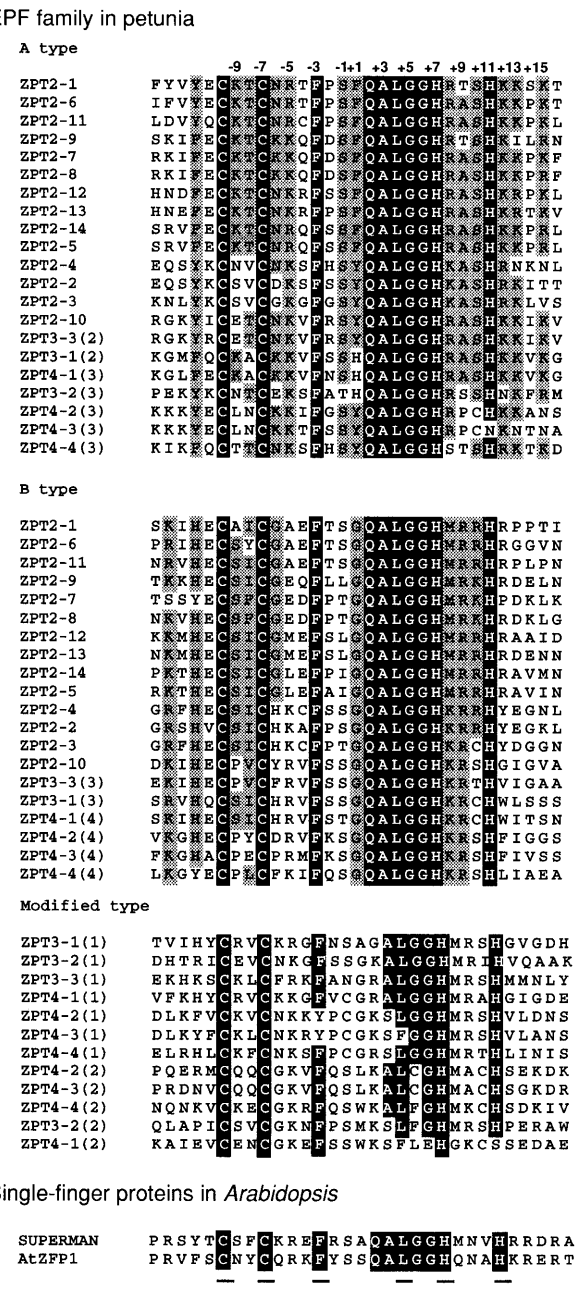

Figure 2. Zinc-finger sequences of the EPF family, SUPERMAN and AtZFP1 Zinc-finger sequences of the petunia EPF family are shown at the top. A- and B-type zinc fingers are aligned separately. Modified type represents the zinc fingers in which some amino acids in the QALGGH sequence were replaced. Consensus amino acids of EPF-type zinc fingers are indicated by solid boxes, and the amino acids characteristic for the A- and B-type zinc fingers are shaded. Zinc-finger sequences of two single-fingered proteins, SUPERMAN [8] and AtZFP1 [23], are also shown at the bottom. Positions of amino acids are numbered at the top of sequences relative to the first putative helical residue (+1). Numbers in parentheses after some protein names represent the positions of zinc fingers in the respective protein, with the most N-terminal one being number 1. The six amino acids characteristic for TFIIIA-type zinc fingers are indicated by bars at the bottom of sequences.

In petunia, more than 30 proteins have been found to contain zinc fingers containing the QALGGH sequence (figs 2 and 3). These proteins will be referred to hereafter as the EPF family. Among the EPF family, seven members are predominantly expressed in anthers, with five of them being specifically expressed in anthers. The expression patterns of these genes led us to speculate about the involvement of these putative transcription factors in the development of anther and pollen [6]. All these genes encode EPF-type zinc-finger proteins with very diverse structures in terms of the numbers of zinc fingers (two, three or four) and the lengths of spacers between zinc fingers (ZPT3-2, 2-5, 4-2, 4-3, 3-1, 4-1, 2-6 in figs 2 and 3). Detailed analyses of their expression patterns revealed that the seven genes are expressed sequentially during the course of anther development from the meiosis stage through the dehiscence of the anther as summarized in figure 1. Within the anther, the transcripts were found predominantly in pollen. The sequential expression pattern of the seven transcription proteins is reminiscent of the regulatory cascade of transcription factors that controls the embryogenesis of *Drosophila* [7]. In a fertilized *Drosophila* egg, transcription factors encoded by maternally provided messenger RNAs (mRNAs) form gradients along the anteroposterior or the dorsoventral axis. These transcription factors trigger the onset of regulatory cascades consisting of several classes of transcription factors, and the regulatory cascades proceed in a position-dependent manner according to the initial gradients of maternal mRNAs. A series of developmental events, including segmentation of the embryo and specification of the identity of segments, follow the sequential activation (or repression in some cases) of downstream transcription factors by the upstream ones. Likewise, development of anthers and pollen from undifferentiated cells of stamen primordia might also be regulated by a regulatory cascade involving several transcription factors. And the EPF proteins could very well form the backbone of the molecular basis involved in the development of this male organ. This hypothesis, however, is solely based on the expression patterns and has to be examined by reverse genetical and biochemical methods.

SUPERMAN

Among TFIIIA-type zinc-finger proteins in plants, SUPERMAN protein in *Arabidopsis* is the best characterized in terms of its genetic function in flower development [8]. Mutations in the *SUPERMAN* gene cause extra stamens in place of normal development of carpels [9]. SUPERMAN protein contains only one TFIIIA-type zinc finger that is similar to those of the EPF family in petunia in terms of the presence of the QALGGH sequence (fig. 2).

Genetic control of the specification of floral-organ identity has been extensively studied during the last decade.

A genetic model (the ABC model) explains the specification of organ identity as follows [10–12]. In a typical flower, four kinds of floral organs (sepal, petal, stamen and carpel) arise in four concentric whorls. The identity of each organ is specified by a unique combination of the activity of either one or two of the three classes of genes, each of which is expressed in two adjacent whorls, class A genes being expressed in the first (outermost) and the second whorls, class B genes in the second and the third whorls, and a class C gene in the third and the fourth whorls. Hence, sepals are specified by class A genes only, petals by class A and B genes, stamens by class B and C genes, and carpels by class C genes only. All these genes encode putative transcription factors called MADS-box proteins [10–12], with the exception of *APETALA2* [13], one of the A class genes. Before its cloning, the presumed action of the *SUPERMAN* gene was to suppress the expression of class B genes in the fourth whorl because the ectopic expression of class B genes in this whorl, in combination with class C genes, should lead to the generation of stamens, consistent with the mutant phenotype. After the cloning of the *SUPERMAN* gene, however, its expression was found to be under the control of B class genes. It was just the opposite of what was originally presumed and was against the initial model, which assumed that SUPERMAN controlled the expression of B class genes [8]. Histological localization of *SUPERMAN* transcripts revealed that they were present in the subdomain of the third whorl adjacent to the fourth whorl [8]. A revised model based on expression analyses in wild-type and mutant plants proposes that the function of SUPERMAN is to repress cell division in the third whorl by defining a boundary between the third and the fourth whorl cells [8, 14]. According to this model, the generation of extra stamens in the place of carpels in the *SUPERMAN* mutant is due to the loss of the boundary between the third and the fourth whorls resulting in excessive cell division in the third whorl primordia that suppresses proliferation of cells in the fourth whorl.

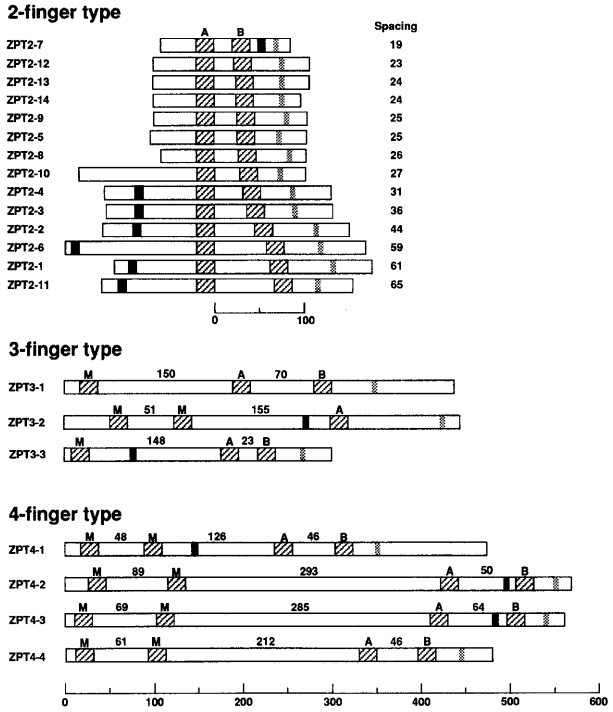

Figure 3. Schematic structures of petunia EPF proteins. Shown are schematic protein structures of all EPF-type zinc-finger proteins identified in petunia. Zinc fingers are shown by hatched boxes, hydrophobic regions including the DLNL sequence by shaded boxes and basic regions by closed boxes. Numbers of amino acids in the spacers (between the second histidine of N-terminal zinc fingers and the first cysteine in the C-terminal zinc fingers) are indicated on the right of the schematic structures for two-fingered proteins or on each spacer region for three- and four-fingered proteins, respectively. The two-fingered proteins were arranged with the first zinc finger at a basal position to emphasize the diversity in lengths of spacers. Types of zinc fingers are indicated by A (A-type), B (B-type) or M (modified type).

SUPERMAN protein contains only one TFIIIA-type zinc finger. We found that one finger of ZPT2-2, which is a two-fingered protein, makes contact with only three or four bases, and the affinity of DNA binding by one finger was very weak [15]. Therefore, one finger is by itself insufficient for recognition of specific target DNA. However, another protein, the GAGA factor [16] of *Drosophila*, which contains only one TFIIIA-type zinc finger like SUPERMAN, utilizes the N-terminal extension of the zinc-finger motif to interact with the target DNA [17, 18]. The SUPERMAN protein also contains two overlapping leucine-zipper-like motifs in the C-terminal region, which might serve as the site for protein-protein interactions, as has been proposed for a heterodimerization domain of *Saccharomyces* MATa1 [19]. In this regard, possible dimerization of SUPERMAN proteins or their association with other DNA-binding proteins might be the mechanism that enhances the specificity and affinity of DNA binding.

Other TFIIIA-type zinc-finger proteins

Several other proteins containing TFIIIA-type zinc fingers have been reported from a number of plant species. WZF1 was identified as a protein which specifically interacts with the promoter region of histone H3 and H4 genes in wheat [20]. STZ in *Arabidopsis* complements the salt-sensitive phenotype of a yeast mutant which is deficient in the phosphoprotein phosphatase calcineurin [21]. The expression of the *STZ* gene increases with salt treatment in plants. These observations suggest the role of STZ in the regulatory processes

associated with salt tolerance in plants. In *Arabidopsis*, altogether 12 different proteins of this class have been reported [22]. These proteins have either two or three TFIIIA-type zinc fingers, including the QALGGH sequence. Eight proteins containing single EPF-like zinc-finger motifs have been identified in *Arabidopsis* (AtZFPs) [23]. The AtZFPs are similar to SUPERMAN in that they have only one zinc-finger motif and a C-terminal leucine-rich region. The genes for AtZFPs are expressed predominantly in vegetative tissues [23, 24].

It should be noted that all the proteins mentioned above contain the QALGGH sequence in zinc-finger motifs. Interestingly, this conserved sequence motif has not been reported from organisms other than plants, suggesting that this type of zinc-finger protein, which forms a major class of transcription factors in plants, might be involved in controlling the processes that are unique to plants. The only TFIIIA-type zinc-finger protein without the QALGGH sequence is PCP1 in potato [25]. The PCP1 gene enabled a yeast strain deficient in secreted invertase to grow on sucrose as the sole carbon source by suppressing the sucrose uptake deficiency.

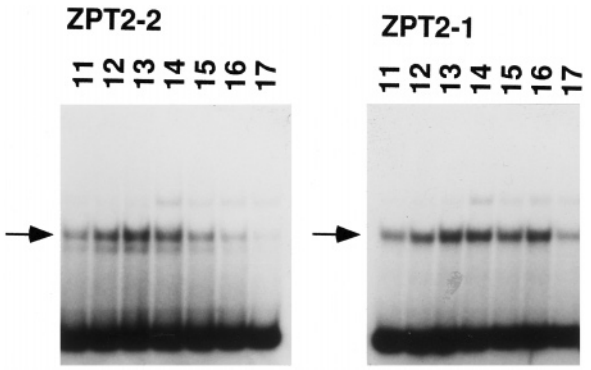

Figure 4. Differential recognition of the spacing in the target DNAs by EPF proteins. Recombinant ZPT2-1 and ZPT2 proteins were tested for the binding to the radiolabelled probe DNAs containing two tandem AGT core sites that were separated by the spacing of various lengths. Numbers on the panels indicate the spacing between two core sites in the probe DNAs. The sequences of the probes are TTGACAGTGTCA$(N)_{0-6}$TGACAGTGTCA, in which N's are alternate G and C (core sites are underscored).

Interaction of EPF proteins with target DNA sequences

Recognition of spacing in target DNA sequence. Interaction with target DNA sequences has been best characterized in three members of EPF family in petunia. One of the unique structural features of the EPF family in petunia and related proteins in other plant species is the diversity in the lengths of spacers between zinc fingers. DNA-binding studies using the two-fingered proteins (ZPT2-1 and ZPT2-2) produced in *Escherichia coli* revealed some unique interactions with target DNA, which seems to be based upon the separation of the zinc fingers [15, 26]. Cluster-type zinc-finger proteins, typical in animals, are known to bind to contiguous sets of triplets in target DNA, with each finger making contact with one triplet. By contrast, the two proteins of the EPF family were both found to bind to two tandem but separated core sites (AGT), with each finger making contact with one core site, as revealed by hydroxyl radical footprint and competition experiments [15]. However, the two proteins, which have spacers of different lengths between the two fingers, differed in their preference for the spacing between the two core sites [15]. ZPT2-2, whose spacer is 44 amino acids in length, shows rather high specificity for a spacing of 13 bp, whereas ZPT2-1, with a spacer of 61 amino acids, tolerated a spacing of 13 to 16 bp (fig. 4). The specificity of the spacing was weaker when the sequence between the two core sites was AT-rich rather than GC-rich, presumably due to the difference in the flexibility in the structure of DNA. Thus, it was suggested that the members of the EPF family distinguish their target sequences not only by the sequences of core sites but also by the spacing between the core sites. This proposed mechanism for target-sequence recognition is reminiscent of that for the POU domain family in animals [27].

Supporting this mechanism of target recognition, other members of the EPF family have spacers of varying lengths as summarized in figure 3 [28]. A total of 21 proteins have been characterized, which can be categorized into three subclasses based on number of zinc fingers, i.e. two, three and four. Fourteen of these proteins are of the two-fingered type. Interestingly, the lengths of spacer regions between the two zinc fingers are highly variable among the members of the family, ranging from 19 amino acids in ZPT2-7 to 65 amino acids in ZPT2-11. Given the different preferences for spacing of ZPT2-1 and ZPT2-2 in target sequences as mentioned above (fig. 4), one can presume that these two-fingered proteins with very diverse lengths of spacers have rather variable preference for the spacings in the target DNA sequence. The spacing in the target DNA is presumed to be roughly proportional to the respective lengths of the spacers. It would require an almost fully stretched conformation if the protein with a spacer as short as 19 amino acids, for example, bound to two core motifs separated by 13 bp.

These speculations can be extended for three- and four-fingered types. In these subclasses, most zinc fingers appear to be paired; relatively shorter spacers (23 to 89 amino acids) are present between two fingers (fig. 3). This notion is supported by the presence of two types of

zinc-finger sequences near the C-termini of the three- and four-fingered proteins (except in ZPT3-2) with the same relative positions as in two-fingered proteins: type A finger is always located N-terminal to type B finger (figs 2 and 3). Two N-terminal fingers in four-fingered proteins (denoted type M for modified type) also appear to be paired. The paired zinc fingers can presumably act as a minimal DNA-binding domain, considering the observation that two fingers are sufficient for high-affinity DNA binding ($K_d \approx 10^{-7}$ M) [15]. Rather long spacers (126 to 293 amino acids) separate the paired fingers from other single or paired fingers. As observed in DNA-binding experiments with ZPT2-1 and ZPT2-2 (fig. 4), the longer spacer was more tolerant of different spacing in target DNA, which suggests that the binding domain becomes more flexible as the length of the spacer increases. Therefore, the longer spacers in the three- and four-fingered proteins, perhaps, allow relatively independent behaviour of the two putative minimal DNA-binding domains located at the ends.

These DNA-binding studies and comparative sequence analyses suggesting a general mechanism of target sequence recognition of the EPF family can be summarized as follows: (i) Two fingers separated by relatively short spacers behave as a minimal DNA-binding domain; (ii) different lengths of spacers might confer different target sequence specificity to each member of the EPF family even with relatively similar core sequences; (iii) the minimal DNA-binding units separated by long spacers in the three- and four-fingered proteins behave relatively independent of each other. These hypotheses, however, remain to be experimentally verified by determining the target sequence of each protein and structural analyses of protein-DNA complexes.

Base-determinant positions. In some TFIIIA-type zinc-finger proteins in animals, such as Sp1, Krox20 and Zif268 [2, 29, 30], three positions in the α-helical region (CXXCXXXFXX^{a}XXX^{b}LXX^{c}HXXXH) have been shown to participate in major interaction with nucleotides in target sequences. Each amino acid in these positions recognizes one nucleotide in a triplet of the target sequence, as demonstrated by mutational, x-ray crystallographic and nuclear magnetic resonance (NMR) studies [2, 29–32]. This rule of three base-determinant positions seems to hold for many zinc fingers according to the statistical sequence analyses of zinc fingers by Jacobs [33]. The amino acids that occupy three of the base-determinant positions are highly variable among different proteins [33], which in turn means that the target sequences of respective zinc fingers are very diverse.

The sequence QALGGH is highly conserved in zinc-finger motifs among the petunia EPF family, *Arabidopsis* SUPERMAN and many other TFIIIA-type zinc-finger proteins in plants as mentioned above. This sequence is located within the region that corresponds to the DNA-recognition surface (within the α-helical region) in animal proteins [2, 29, 30]. Each amino acid in this sequence has been found to be essential for the DNA-binding activity by site-directed mutagenesis [28]. The substitution of any of the A, L, G, G or H residues at positions 3, 4, 5, 6 and 7, respectively, in the first finger of ZPT2-2 completely abolished the binding (see fig. 2 for the numbering of positions). When the Q at the position 2 was mutated, the binding was greatly reduced. Consistent with these observations, a G-to-D mutation at position 5 in the zinc finger of SUPERMAN protein leads to loss of function in terms of the phenotype in mutant plants [8].

Within the QALGGH sequence, the alanine at position 3 and the glycine at position 6 correspond to the two highly variable base-determinant positions b and c, respectively, in animal TFIIIA-type zinc-finger proteins. However, the high conservation of this sequence among several members of plant EPF-type zinc-finger proteins (more than 30 proteins in petunia) argues against the possibility of these positions being base-determinant, because the occurrence of the same amino acids in base-determinant positions in different proteins would greatly limit the diversity of target sequences that ought to be highly diverse. Furthermore, the glycine at position 6, which has no side chain, is unlikely to make contact with DNA. If these two positions are involved in interaction with the target DNA, differential recognition of the spacing in target sequences alone will not be sufficient to explain the diversity of target DNAs. Another possible mechanism could be that respective EPF proteins interact with other DNA-binding proteins to achieve specificity for target sequences. Hydrophobic regions at the C-terminus of all the EPF proteins in petunia (fig. 3) [6, 28] and other TFIIIA-type zinc-finger proteins in plants [8, 23, 34] might be the site for such protein-protein interactions. Even if these mechanisms account for the diversity of target sequences, it still seems quite possible that the EPF proteins use different positions as base determinants from those of animal proteins.

In order to have some clue to the real base-determinant positions, domain swapping and site-directed mutagenesis experiments were carried out [35]. ZPT2-2 and ZPT2-3 have different sequence specificities; ZPT2-2 can bind to the sequence TTGACAGTGTCACTTGACAGTGTCAC (core sites are underlined), whereas ZPT2-3 cannot [26]. By domain-swapping experiments it was determined that the second finger is critical for the differential binding specificity of the two proteins [35]. When the C in ZPT2-3 at position +10 was replaced by R (as it is in ZPT2-2), it dramatically enhanced binding (fig. 5). The reverse replacement (R by C in ZPT2-2) reduced binding. These results

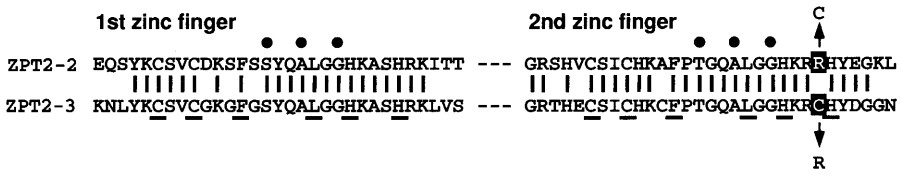

Figure 5. Zinc-finger sequences and possible base-determinant position of ZPT2-2 and ZPT2-3. Identical amino acids are linked by vertical lines. Six amino acids that are conserved in TFIIIA-type zinc-finger proteins are underlined. The amino acids that are responsible for the difference of DNA-binding specificity between ZPT2-2 and ZPT2-3 are highlighted, and the amino acid substitutions described in the text are shown. Three base-determinant positions for Sp1, Krox20 and Zif268 [2, 29, 30] are indicated by dots.

indicate that the difference in amino acids in this position is responsible for the difference in DNA-binding specificity of the two proteins, and hence this position is a strong candidate for one of the base-determinant positions. It should be noted that this position is apart from the QALGGH sequence and also different from the three base-determinant positions in animal proteins mentioned above [2, 29, 30]. However, it is within the putative α-helical region (fig. 5). Further attempts by site-directed mutagenesis, however, failed to reveal other base-recognition positions.

All the TFIIIA-type zinc fingers, as revealed by NMR [36–38] and x-ray crystallography [2, 39, 40], have basic structures composed of antiparallel, two-stranded β sheets and a short α helix. The α-helical region forms a DNA-contacting surface and includes the three base-determinant positions. This structure is primarily determined by two cysteines and two histidines which coordinate a zinc atom and two hydrophobic amino acids, usually F and L in positions 1 and 6, respectively, to form a hydrophobic core [41]. Do the EPF-type zinc fingers have similar structures? We are curious about this because the difference in the base-determinant positions as described above might be associated with a unique structure of the DNA-binding domain.

The WRKY family

A new type of Cys_2/His_2-type zinc finger (CX_4-$CX_{22-23}HX_1H$) unique to plants has been found within the conserved regions of the WRKY family of proteins (fig. 6). Three members of this protein family (WRKY1, 2 and 3) in parsley are implicated in the activation of *PR1* (pathogen-related 1) gene in response to fungal infection. These proteins bind specifically to three W-boxes, (T)TGAC(T), that are present in the promoter region of the *PR1* gene and are responsible for the activation of this gene in response to fungal elicitor Pep25, an oligopeptide. The elicitor rapidly induces the expression of two *WRKY* genes (*WRKY1* and *3*) in suspension-cultured cells of parsley. In contrast, the level of *WRKY2* mRNA declines rapidly with inverse kinetics with respect to *WRKY1* and *3*. The significance of the inverse kinetics is not well understood; however, the rapid changes in the *WRKY* mRNA levels in response to the defined signal molecule strongly suggest that the three WRKY proteins play key roles in a signal transduction pathway that leads from elicitor perception to PR gene activation.

Several other members of this protein family have been reported. ABF1 and ABF2 from wild oat bind specifically to the *cis* elements that are conserved in the promoters of *α-amylase 2* gene in wheat, barley and wild oat [42]. SPF1 from sweet potato specifically binds to the promoter sequences of *sporamin* and *β-amylase* genes [43]. By random binding site selection, ZAP1, an *Arabidopsis* protein, has been found to bind to a consensus sequence (CGTTGACCGAG), which includes a sequence similar to the W-boxes [44]. Sequence comparison of these proteins revealed that a conserved sequence (the WRKY domain) is extended to the N-terminal region of the zinc-finger motif, and there is a stretch of seven invariant amino acids (**WRKY**GQK) in this region (fig. 6). The WRKY proteins contain either one (WRKY3 and ABF2) or two (WRKY1, WRKY2, ABF1 and SPF1) WRKY domains. A requirement for a zinc atom for DNA-binding activity has been demonstrated for ZAP1 [44], ABF1 and ABF2 [42]. Furthermore, in vitro studies using truncated forms of ZAP1 have shown that the C-terminal finger is essential for DNA binding, whereas deletion of the N-terminal finger results in 2.5-fold reduced binding activity [44]. The high conservation in the basic N-terminal extension of zinc fingers might suggest that this region is also involved in DNA binding, as is the case in GAGA factor of *Drosophila*, which contains only one TFIIIA-type zinc finger [17, 18]. ZAP1 has been shown to act as a transcriptional activator, and its activation domain has been mapped adjacent to and possibly overlapping with the zinc-finger motif [44].

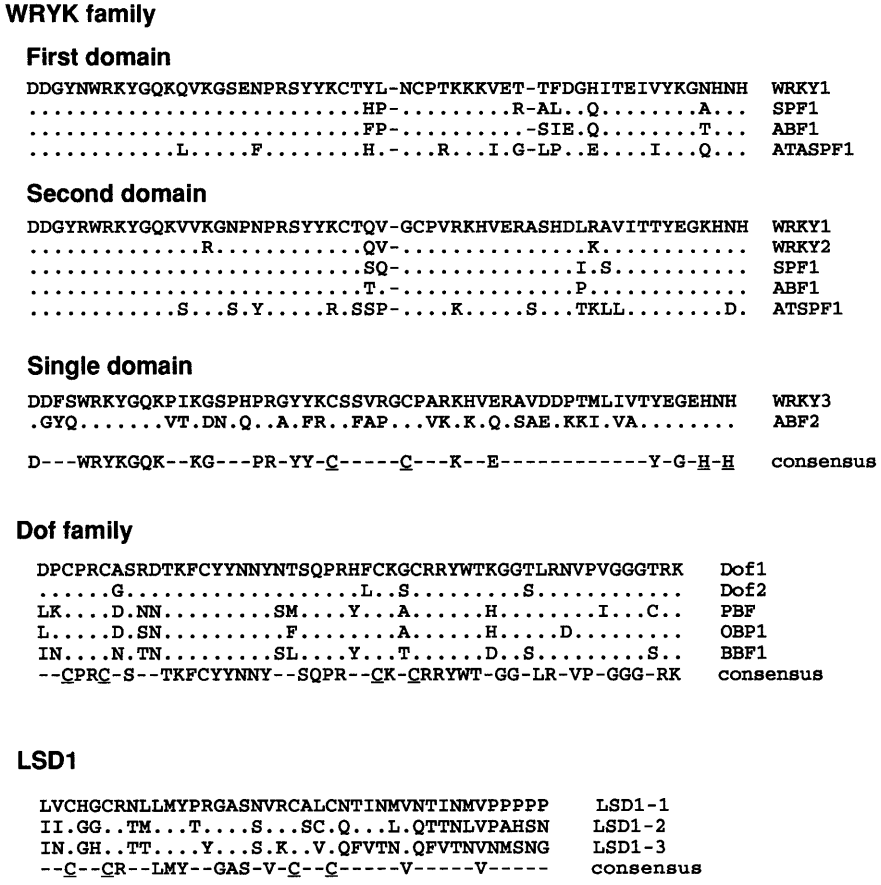

Figure 6. Novel classes of zinc-finger motifs found only in plants. Zinc-finger and surrounding sequences of the WRKY family, Dof1 family and three zinc-fingers in LSD1 are aligned separately. For the WRKY family, the first and second fingers in the double-fingered members and the fingers of single-fingered members are shown separately. The amino acids identical to those in the sequence shown at the top in respective classes are indicated by dots. Bars represents gaps. Putative zinc-binding amino acids are underscored. The sequence of the first finger in WRKY2 has not been reported yet. It should be noted that N- and C-terminal extensions of WRKY and Dof families, respectively, are highly conserved.

GATA1-like

The GATA1 family forms one of the major families of the Cys_2/Cys_2-type zinc-finger transcription factors in eucaryotes. The DNA-binding domain consensus is $CX_2CX_{17}CX_2C$ and contains a zinc atom coordinated by the conserved four cysteines [45]. The original GATA1 was identified as a transcription factor required to promote the expression of globin genes in humans [46]. The gene for the first GATA1-type protein in plants (NTL1) was isolated from tobacco by polymerase chain reaction (PCR)-based methods [47] as a plant homologue of NIT2 [48], a well-characterized transcription factor in the fungus *Neurospora crassa*. NIT2 activates the expression of the genes for nitrogen-metabolic enzymes during nitrogen-limiting conditions. A single zinc finger of NTL1 is very similar to the finger of NIT2 (60% identity), as well as to fingers from several GATA1-type proteins in animals and yeast, although the spacing between the second and third cysteine is 18 instead of 17. Whether or not NTL1 is involved in the regulation of the nitrate assimilation pathway in plants has not been reported yet.

The CONSTANS protein that promotes flowering in *Arabidopsis* has two repeats of GATA1-like zinc fingers [49]. In *Arabidopsis*, the vegetative and reproductive (flowering) phases are clearly separated. The onset of flowering is normally promoted by long photoperiods. However, plants having mutations in the *CONSTANS* gene flower later than wild-type plants under these conditions. The two zinc fingers of the CONSTANS protein are in $CX_2CX_{16}CX_2C$ arrangement, and the zinc-finger sequences show low but significant similarity to those in the GATA1 family. The two zinc fingers are very similar to each other, with the most apparent conservation being in the C-terminal extension of each

finger that is rich in basic amino acids. The high conservation of the basic C-terminal region is again reminiscent of GATA1, in that this region is a basic domain required for DNA binding and is highly conserved [45, 50]. The importance of the zinc-finger region for its activity in the promotion of flowering is indicated by the fact that both of the two mutations in the *CONSTANS* gene affect the amino acids in the finger regions.

Another GATA1-like protein of *Arabidopsis*, STO [21], shares considerable similarity with CONSTANS protein in its zinc-finger sequence. Similar to the TFIIIA-type STZ mentioned above [21], the gene for STO complements the salt-sensitive phenotype of the yeast mutant deficient in the phosphoprotein calcineurin, which is implicated in the dephosphorylation of ion channels. In addition to reducing the growth of the mutant yeast in high-salt media, STO can partially compensate for the absence of calcineurin in all tested processes: recovery from the growth arrest induced by α factor and increased tolerance to Mn^{++}. Thus, STO might represent a component of the salt tolerance mechanism that is conserved in yeast and plants.

LSD1, an *Arabidopsis* protein, has three repeats of GATA1-like zinc fingers, defined by the sequence $CX_2CXRX_2LMYX_2GASXVXCX_2C$ (fig. 6) [51]. In plants, controlled induction of cell death occurs during the rapid, localized response to pathogens known as the hypersensitive response (HR) [52]. The HR is associated with most disease resistance reactions, though it is unclear whether HR is required to halt pathogen growth. The mutation in the *LSD1* (lesion simulating disease resistance 1) gene makes the plant hypersensitive to cell death initiation, and there is a failure in limiting the extent of cell death. Superoxide is a necessary and sufficient signal for cell death propagation; thus, LSD1 monitors a superoxide-dependent signal and negatively regulates the cell death pathway in plants. The zinc fingers of LSD1 protein share homology neither to CONSTANS nor to STO. The conserved zinc-finger domain of LSD1 is present in three additional *Arabidopsis* proteins, suggesting that LSD1 defines a new subfamily of GATA1-like proteins.

The Dof family

The Dof (DNA binding with one finger) family has been identified as sequence specific DNA-binding proteins that interact with the promoter sequences of several genes, whose expression is regulated tissue-specifically or in response to stress signals. The proteins of the Dof family are characterized by the presence of a conserved domain (Dof domain) including a zinc-finger-like motif, $CX_2CX_{21}CX_2C$, followed by a basic region (fig. 6) [53]. This motif is similar to the GATA1 motif; however, the Dof family is regarded as a distinct class because the spacing between the second and the third conserved cysteines is longer than those in the GATA family and because of their ability to bind to DNA with only one finger. Dof domain-like sequences are not present in the yeast genome, whose total sequence has been determined, nor have they been found in animals. Although it has been only a few years since this protein family was identified, several members have been implicated in the transcriptional regulation of various biological processes, including developmental and stress-induced gene expression.

The first member of this protein family, Dof1, was identified from maize first as the protein that specifically bound to an AAGG motif in the cauliflower mosaic virus 35S promoter [54] and then implicated in the light-regulated activation of the gene for C4-photoshynthetic phosphoenolpyruvate carboxylase (C4PEPC). Its single zinc finger has been shown to be essential for DNA-binding activity by site-directed mutagenesis and zinc-depletion experiments [55]. In a transient expression system in maize protoplasts, Dof1 acts as a transcriptional activator, with its C-terminal portion being an activation domain [56]. The activity was high in protoplasts from green leaves and very low in protoplasts from etiolated leaves, suggesting that the action of Dof1 was modulated by light. The light responsiveness appeared to be mediated by the modulation of DNA-binding activity. Another member of the protein family, Dof2 [55], was identified and found to inhibit the transcriptional activation by Dof1 [56]. This inhibition is considered to be mediated by direct interaction of the two proteins through their Dof domains [57]. Dof1 is expressed constitutively, while Dof2 is expressed in stem and root but not in leaves. Based on these observations, Yanagisawa proposed a model in which Dof1 alone regulates the light-dependent gene expression of the *C4PEPC* gene in leaves while, in stems and roots, Dof2 inhibits the action of Dof1 presumably via protein-protein interactions [56].

BBF1 from tobacco also has a Dof domain and binds specifically to the (A)CTTT(A) sequence within one of the essential *cis* elements (domain B) in the *rol B* gene (plant oncogene) promoter [58].

PBF, another member of the Dof family in maize, has been implicated in the endosperm-specific expression of the genes for zein and other seed-storage proteins. In the activation of *zein* gene expression in seeds, a bZIP-type transcription factor, Opaque-2, has been known to play a critical role [59, 60]. However, Opaque-2 alone is not sufficient for the endosperm-specific expression of the *zein* gene. Another *cis* element, the prolamin box (or endosperm box), which is perfectly conserved in the promoter regions of seed storage protein genes in many cereal plants, has been shown to be essential for en-

dosperm-specific gene expression. Because the sequence of the prolamin-box (TGTAAAG) is similar to the Dof1-binding site, an endosperm-specific member of the Dof family was sought using a PCR-based method, and the gene for PBF (prolamin-box binding factor) was cloned [61]. PBF binds to the prolamin box specifically and also interacts with the Opaque-2 protein, whose target site lies 20 bp downstream of the prolamin box in the zein promoter.

The interaction of a Dof-type zinc-finger protein with a b-ZIP-type transcription factor has been found in another system as well. *Ocs* elements are present in the promoter regions of several pathogen-responsive genes and are known to mediate the response of gene expression from signals such as auxin, salicylic acid and hydrogen peroxide. Two b-ZIP-type transcription factors, OBF4 (*Ocs*-element binding factor 4) and OBF5, are known to interact with the *ocs* elements. In a screening for the protein(s) that interact with and modify the activity of OBFs, a Dof1 type protein OBP1 (OBF binding protein 1) was identified [62]. OBP1 enhances the binding of OBF4 and OBF5 to *ocs* elements in vitro. Subsequently, OBP1 protein was found to bind to the site next to the OBF-binding site (*ocs* element) in the promoter region of the stress-inducible *glutathione S-transferase 6* gene [63], the product of which plays critical roles in the detoxification of xenobiotics, thereby protecting the tissues against oxidative damage in both animals and plants [64].

Dof-type factors, thus, appear to act in association with b-ZIP-type factors in two different biological processes, i.e. the endosperm-specific expression of seed-storage protein genes (PBF) and the signal-induced expression of stress-responsive genes (OBF1). A common feature in the two systems is that the two classes of transcription factors bind to closely located target sites in the respective promoters and they interact with each other. Of interest are what the roles of the respective classes are and how the interaction of the two classes modulates promoter activity. The association of two classes of transcription factors is reminiscent of myb-like and myc-like factors that act in combination in various regulatory processes in plants [65]. Another interesting question is whether the association of bZIP- and Dof-type factors is a common theme of gene regulation in plants.

RING-finger type

The RING finger is a bipartite asymmetric motif, C-X_2-C-loop I-C-X-H-X_2-C-X_2-C-X_2-C-loop II-C-X-C (loops I and II are variable in length), which has been found in many regulatory proteins throughout the plant, animal, fungal, viral and protozoan kingdoms. This motif engages two zinc atoms [66] in such a way that the domain forms a peculiar structure: the first and third pairs of metal-binding residues bind one zinc atom, while the second and fourth pairs bind the other [67]. Many RING-finger-containing proteins have putative involvement in some aspects of transcriptional regulation [68, 69]. In these proteins, the RING-finger domain is considered more likely to mediate protein-protein interactions [70], but their direct involvement in DNA binding has not been ruled out. In plants, COP1 is the best-characterized regulatory protein containing the RING-finger motif.

Light has profound morphogenic effects on plant development. Seedlings grown in light show open and expanded cotyledons, short hypocotyls, chloroplast development and high expression levels of light-inducible genes (photomorphogenic phenotypes). In the dark, however, the seedlings exhibit long hypocotyls, closed and underdeveloped cotyledons with etioplasts instead of chloroplasts, and low expression levels of the light-inducible genes (skotomorphogenic phenotypes). *COP1* (*CONSTITUTIVE PHOTOMORPHOGENIC1*) is one of the several genetic loci of *Arabidopsis* that, when mutated, result in constitutive photomorphogenesis even in the dark. The mutant phenotypes and several lines of evidences from transgenic studies [71, 72] strongly suggest that COP1 acts as a light-inactivatable repressor of photomorphogenic development in light-specific signalling pathways. Recently, COP1 was also found to be involved in the regulation of the genes inducible by pathogen infection, hypoxia and developmental programmes [73]. Therefore, COP1 might act as a repressor of various kinds of gene expression that are targets for multiple signal transduction pathways and thus might play a general role as a nuclear regulatory protein.

In COP1 protein, there are three known structural motifs: a RING-finger domain near the N-terminus, a central coiled-coil region, and the WD40 repeats of the Gβ domain of trimeric G proteins. $TAF_{II}80$ [74], a TFIID subunit of *Drosophila*, shares significant similarity with COP1 [75]. This sequence similarity extends for the entire length of $TAF_{II}80$ and includes most of COP1, except for the RING-finger motif (21% identity and 44% similarity). More recently, a putative human counterpart of COP1 that shares much higher similarity than $TAF_{II}80$ was found in the expressed sequence tags (EST) of humans (X. W. Deng, personal communication).

The nucleocytoplasmic partitioning of COP1 is both light- and tissue-dependent [76]. COP1 is enriched in the nucleus in darkness, while exposure to light causes its depletion in the nuclei and a concurrent increase in the cytoplasm. This light-dependent nucleocytoplasmic partitioning was observed in hypocotyl cells. In roots,

COP1 is invariably nuclear localized, consistent with the established role of COP1 in suppressing chloroplast development in roots in both light and darkness [77]. These observations led to the conclusion that COP1 acts inside the nucleus and that light inactivation of COP1 involves a cell-type specific control of its nucleocytoplasmic partitioning. A cytoskeleton-associated protein, CIP1 (COP1-interactive protein), has been identified that interacts with COP1 via coiled-coil domains of both proteins [78]. It has been speculated that CIP1 is involved in the nucleocytoplasmic partitioning of COP1.

Besides COP1, two plant proteins that contain RING-finger-like motifs have been reported from *Arabidopsis* [79] and soybean [80]. The *Arabidopsis protein* (ATL2), which contains a RING-finger-like motif and a putative signal anchor sequence for membrane insertions, is toxic to yeast when overexpressed [79].

PHD-finger type

A new class of cysteine-rich sequence motifs, the PHD finger (Cys_4-His-Cys_3), is similar to the RING finger (Cys_3-His-Cys_4) in the arrangement of putative zinc-binding amino acids. The PHD finger (plant homeodomain finger) is so called because this sequence motif was originally noted in two plant proteins containing homeodomains, *Arabidopsis* HAT3.1 [81] and maize Zmhox1a [82]. Later, this motif was also found in two other homeobox-containing proteins, PRHAs from parsley and *Arabidopsis* [83]. Thus, PHD fingers in plant proteins reported so far have always been associated with homeodomains. All these proteins exhibit either sequence-specific or nonspecific DNA-binding activity. Zmhox1a specifically binds to three sites flanking the TATA box of the *Shrunken* gene [82]. A truncated polypeptide of Zmhox1a containing the homeodomain, but not the PHD finger, does not lose its specific DNA-binding capability; therefore, the PHD finger might not be involved in DNA binding. The PRHAs binds specifically to an 11-bp *cis* element responsible for the fungal elicitor-mediated expression of *pr2* (*pathogen-related 2*) gene [83]. In this case, however, sequence specificity was lost when the homeodomain without the PHD finger was tested. HAT3.1 is capable of interacting with DNA larger than 100 bp in a sequence-independent manner [81]. In summary, the involvement of PHD fingers in DNA binding is unclear. Interestingly, PHD fingers are often present in animal proteins that are implicated in chromatin-mediated transcriptional regulation, including Polycomb and trithorax group proteins [84]. In these types of proteins, the PHD finger is presumably the site of protein-protein interactions for combining multiple proteins in a large complex or for recognizing nuclear targets related to chromatin structure and chromatin regulation.

The LIM family

The LIM domain is also a new functional motif and contains a cysteine-rich motif of CX_2-$CX_{17-19}HX_2CX_2CX_2CX_{16-20}CX_{2-3}C$. Based on association with other functional domains, LIM-domain-containing proteins are categorized into three subclasses [85]. One class of proteins contains a homeodomain (LIM-HD proteins). The second class has no homeodomain, but contains only LIM domains (LIM-only proteins). Another recently described protein has two LIM domains linked to a protein kinase domain. Many LIM-containing proteins have been implicated in the transcriptional regulation of cell differentiation and growth regulation. Some are associated with the cytoskeleton, while others are implicated in chromosome translocations. Recently, ample evidence has been accumulating suggesting that the LIM domain serves as the site for protein-protein interactions with itself (homodimerization) [86], with helix-loop-helix type transcription factors [87, 88] or with protein kinases [89].

So far, the only LIM-domain-containing protein reported from plants is PLIM-1 (renamed from SF3) in sunflower [90, 91]. PLIM-1 belongs to the LIM-only class and contains two LIM domains. The gene for PLIM-1 is expressed specifically in mature pollen grains, and PLIM-1 protein was initially regarded as a new type of transcription factor [90]. However, later immunological studies have revealed that PLIM-1 protein is not present in nuclei but is associated with actin cytoskeleton as a component of a protein complex (R. Baltz, personal communication). PLIM-1 is concentrated in patches in the cortical region of both ungerminated pollen grains and growing pollen tubes in its intracellular localization. Based on these observations, PLIM-1 is presumed to be involved in the formation and growth of pollen tubes by organizing the actin cytoskeleton at its adhesion sites with the plasma membrane and possibly also in the transduction of extracellular signals to the interior of the cell.

Other and uncategorized types

Tobacco 3AF1 can bind to an AT-rich sequence element in the light-responsive promoter of pea *rbcS-3A* promoter [92]. This protein contains two 100 amino-acids repeats, including zinc-finger-like sequences ($HX_{12}CX_2CX_{14}HX_{12}C$). This domain was shown to be involved in DNA binding because the activity was abolished by treatment with 1,10-phenanthroline, a metal chelator. At present, 3AF1 is the only protein that has this sequence motif.

Nuclear receptors form one of the major families of transcription factors in animals and insects. They have highly conserved DNA-binding domains, in which eight cysteines are arranged into two zinc-finger modules, with N-terminal fingers interacting with target DNAs and C-terminal fingers interacting with partner proteins for homo- or heterodimerization. In searching for their counterparts in plants, the gene for the ES43 protein was cloned from barley [93]. This protein contains only one zinc-finger-like sequence that shows some similarity to the first finger of nuclear receptors. The similarity does not seem to be high enough to conclusively support the relationship.

Conclusion

Plants have adopted most of the typical classes of zinc fingers as functional domains in (putative) transcription factors. In addition, new classes of motifs not found in other eucaryotes have been identified. The WRKY family is of the Cys_2/His_2 type but forms a distinct class from the TFIIIA type based on distinctive spacing between the zinc-chelating amino acids and the absence of two hydrophobic amino acids (F and L) conserved in the TFIIIA type. The Dof family (Cys_2/Cys_2 type) should also be classified as distinct because of its unique spacing between cysteines. LSD1 should be regarded as a distinct class from the GATA family, considering its low sequence similarity to other GATA1-like proteins and poor conservation in the C-terminal extension of each finger in LSD1, unlike those in other GATA1-like proteins. It is included in the section of GATA-like proteins in this review for the sake of convenience. When the ongoing genome projects reveal the total genome sequences of human and other eucaryotes, it will become evident whether the tentatively plant-specific classes are really meaningful or not. The proteins of the EPF family, in spite of adopting typical TFIIIA class zinc fingers as DNA-binding domains, appear to interact with target DNA in a unique manner with respect to the recognition of spacing in target DNAs as well as the base-determinant positions. Taken together, plants seem to have developed unique regulatory mechanisms to adapt to their life cycles either by generating new functional motifs or by modifying the usage of preexisting ones.

The vast amount of knowledge about zinc-finger proteins in animals and other eucaryotes has largely benefited their investigations in plants. In some cases, the information on transcriptional regulation in plants might have influenced investigations in other systems. For example, COP1 protein has a human counterpart of unknown function. Furthermore, COP9 and COP11 proteins, which act in the same signal transduction pathway as COP1, also have their counterparts in humans [75, 94]. These COP proteins and other components are suggested to play rather general roles in various signal transduction pathways [73]. Studies in plants might contribute to understanding molecular mechanisms in the final stages of signal transduction pathways common among eucaryotes.

Recent innovations and improved technology in plant sciences have greatly contributed to the identification of new transcription factors that are involved in many biological phenomena. Using genetic approaches (gene tagging and map-based cloning) several transcription factors have been identified that are involved in various important phenomena. In many cases, their actions at the molecular level (including target genes of respective factors) are poorly understood at present. On the other hand, a number of genes for transcription factors have been identified based on the interaction of their encoding proteins with other molecules (specific DNA and proteins) or due to their sequence homology with other known transcription factors. The relevance of the transcription factors identified by these approaches to particular biological phenomena is often ambiguous. In such cases, reverse-genetical analyses of their functions might be necessary, and transgenic technology has been successfully employed. A major improvement in transgenic technology in the past few years has been the development of conditional gene expression systems using chemical inducers such as tetracycline [95], steroid hormones [96, 97] and copper [98]. In addition, systems of gene disruption have been developed in some plant species, which include petunia [99], maize [100] and rice [101], exploiting endogenous transposons. In *Arabidopsis*, a collection of T-DNA tagged lines has been organized for the same purpose [102]. Homology-dependent gene disruption has also become a reality in *Arabidopsis* [103]. These systems will be appreciated even more when the ongoing genome projects in some model plants reveals numerous sequences of unidentified transcription factors. In vitro transcription systems [104–107] and microinjection technology [108] are also powerful tools for characterizing the molecular action of transcription factors. Furthermore, x-ray crystallography and NMR can provide us with valuable information on the interaction of transcription factors with target sequences and associating proteins. Employing these new technologies to good effect will increase our understanding of the molecular action of zinc-finger transcription factors of plants.

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