The Plant Zinc Finger Protein ZPT2–2 Has a Unique Mode of DNA Interaction*

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ZPT2–2 is a DNA-binding protein of petunia that contains two canonical TFIIIA-type zinc finger motifs separated by a long linker. We previously reported that ZPT2–2 bound to two separate AGT core sites, with each zinc finger making contact with each core site. Here we present our further characterization of ZPT2–2 by using selected and amplified binding sequence imprinting and surface plasmon resonance analyses; together, these assays revealed some unusual features of the interaction between ZPT2–2 and DNA. These experiments allowed us to conclude that 1) the optimal binding sequence for the N-terminal zinc finger is AGC(T), and that of the C-terminal one is CAGT(2); 2) multiple arrangements of the two core sites accommodate binding; and 3) the spacing between the two core sites affects the binding affinity. In light of these observations, we propose a new model for the DNA-ZPT2–2 interaction. Further, consistent with this model, a high affinity binding site for ZPT2–2 was found in the promoter region of the ZPT2–2 gene. This site may serve as a cis-element for the autoregulation of ZPT2–2 gene expression.

The TFIIIA (Cys₂/His₃)-type zinc finger proteins, first discovered in transcription factor IIIA of Xenopus (1), represent an important class of eukaryotic transcription factors. To date, numerous genes have been found that encode this type of zinc finger motif, and many of their products have been implicated in various regulatory roles. The TFIIIA-type zinc finger tetrahedrally coordinates a zinc atom to form a compact structure that interacts with the major groove of DNA in a sequence-specific manner. Generally, in animals, multiple zinc finger motifs are present as tandem arrays that are separated by a conserved short sequence known as an HC link (2). These cluster-type zinc finger proteins interact with contiguous sets of triplet sequences, with each zinc finger making contact with a triplet. In Sp1, Krox20, Zif268, and GAGA, specific amino acid residues in the a-helical region of the DNA binding surface have been shown to interact with specific nucleotides in target sequences (3–6).

The EPF family is a subfamily of TFIIIA-type zinc finger proteins of plants. Members of the EPF family have been implicated in floral organ-specific (7–9) and stress-responsive (10) transcriptional regulation and other regulatory processes (11). The proteins of this family are characterized by the long (19–65 amino acids) linkers of various lengths that separate the zinc fingers. Moreover, the zinc finger motif itself contains a highly conserved sequence, QALGGH (12, 13), which is located within the region that corresponds to the DNA-binding surface of TFIIIA-type zinc finger proteins of animals (3–6).

Our previous DNA binding studies showed that ZPT2–2, with a linker of 44 amino acids between the two fingers, interacted with two tandemly repeated AGT core sites, which were separated by 10 bp, at a dissociation constant (Kd) of 120 nM (14). The binding affinity was sensitive to the spacing between the two core sites. Another protein, ZPT2–1, with a linker of 61 amino acids, showed a different preference for the spacing in target DNA. In light of these observations and considering the diverse lengths of the linker region among the members of this protein family, we proposed that EPF proteins recognize their cognate DNAs not only by the sequences of the core sites but also by the spacing between the core sites (13, 14).

The binding affinity of ZPT2–2 to the tandemly repeated two AGT sequences is, however, rather weak (Kd = 120 nM) compared with those reported for other transcription factors and their cognate binding sequences (Kd = 0.1–1.0 nM). This weak affinity could be because of the suboptimal affinities of the respective fingers to the AGT core sequences. These speculations led us to perform SAAB imprinting assays to identify optimal binding sequences of ZPT2–2 from random sequences. We then quantitatively characterized selected sequences by using surface plasmon resonance (SPR) analysis. These experiments have revealed optimal binding sequences for the respective zinc fingers of ZPT2–2 and corroborated our previous conclusion regarding the specificity for the spacing between the two core sites. Moreover, our current results revealed some new features in the protein-DNA interaction of ZPT2–2. These observations allowed us to propose a revised model for the interaction between DNA and ZPT2–2, which is unique among eukaryotic DNA-binding proteins.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids—**To construct the plasmids for the production of recombinant proteins in Escherichia coli, we used the polymerase chain reaction (PCR) to amplify DNA sequences encoding the N-terminal zinc finger of ZPT2–2 (F1), the C-terminal one (F2), or both by PCR to amplify DNA sequences encoding the N-terminal and C-terminal zinc fingers of ZPT2–2.

We then quantitatively characterized selected sequences by using surface plasmon resonance (SPR) analysis. These experiments have revealed optimal binding sequences for the respective zinc fingers of ZPT2–2 and corroborated our previous conclusion regarding the specificity for the spacing between the two core sites. Moreover, our current results revealed some new features in the protein-DNA interaction of ZPT2–2. These observations allowed us to propose a revised model for the interaction between DNA and ZPT2–2, which is unique among eukaryotic DNA-binding proteins.

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1 The abbreviations used are: bp, base pair(s); SPR, surface plasmon resonance; PCR, polymerase chain reaction; TEV, tobacco etch virus.
Expression and Purification of Truncated Forms of ZPT2—E. coli BL2(TEV)-ZPT2 was grown at 37 °C in LB medium with ampicillin (0.1 mg/ml). After bacterial growth reached an A600 of 0.3–0.6, the culture was supplied with isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM and allowed to grow for an additional 3 h. The cells were harvested, resuspended in Buffer A (20 mM Tris-HCl, pH 8.0, 0.01 mM ZnSO4, 2 mM dithiothreitol, and 0.02% Tween 20), and sonicated with a Sonifier 250 (Branson, Danbury, CT). The resulting cell lysate was centrifuged at 5000 rpm for 15 min, and the supernatant was loaded onto an amylose resin affinity column (New England BioLabs, Beverly, MA) in-frame with the coding sequence of the maltose-binding protein to yield pMAL-(TEV)-ZPT2-F1, -F2, and -F12.

Amplification of truncated forms of ZPT2—E. coli BL2(TEV)-ZPT2 was grown at 37 °C in LB medium with ampicillin (0.1 mg/ml). After bacterial growth reached an A600 of 0.3–0.6, the culture was supplied with isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.5 mM and allowed to grow for an additional 3 h. The cells were harvested, resuspended in Buffer A (20 mM Tris-HCl, pH 8.0, 0.01 mM ZnSO4, 2 mM dithiothreitol, and 0.02% Tween 20), and sonicated with a Sonifier 250 (Branson, Danbury, CT). The resulting cell lysate was centrifuged at 5000 rpm for 15 min, and the supernatant was loaded onto an amylose resin affinity column (New England BioLabs, Beverly, MA) in-frame with the coding sequence of the maltose-binding protein to yield pMAL-(TEV)-ZPT2-F1, -F2, and -F12.

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Fig. 1. Production of truncated forms of ZPT2—proteins. A schematic representation of full-length ZPT2 and its truncated forms. Closed box, basic amino acid cluster (B-box); shaded box, Ser-Thr-rich region; hatched box, zinc finger motif. Amino acids are numbered in regard to those of the full-length protein. B, SDS-polyacrylamide electrophoresis of purified ZPT2—proteins. Lane 1, molecular mass markers; lane 2, P12; lane 3, P2; lane 4, P12.

These DNAs were applied to the surface of a sensor chip for a contact time of 5 min, resulting in the capture of 400–1000 response units of the oligonucleotides. Each binding cycle was performed by injecting ZPT2—proteins into a constant flow (10 μl/min) of HBZP buffer (25 mM HEPES-KOH, pH 7.6, 150 mM KCl, 0.01 mM ZnCl2, and 0.02% Tween 20) across the surface via a sample loop. The sensor chip then was washed with HBZP buffer for an additional 500–1000 s to measure dissociation rates. All reactions were carried out at 25 °C. Data were collected at 2 Hz and analyzed by using the BIAEvaluation program 2.1 (BIAcore AB). We assumed that the reaction between immobilized DNA and ZPT2—proteins follows the first-order kinetics model in the following equation,

\[ \text{ZPT2} + \text{DNA} \rightleftharpoons \text{ZPT2}—\text{DNA} \]  

where \( K_\text{on} \) is the apparent association rate constant, and \( k_\text{off} \) is the dissociation rate constant.

RESULTS

Identification of Optimal ZPT2—Binding Sequences—To reveal the optimal binding sequences for ZPT2—proteins, a truncated form of ZPT2—(P12) that included both the N-terminal (F1) and C-terminal (F2) zinc fingers was expressed in E. coli (Fig. 1A), purified to homogeneity (Fig. 1B), and subjected to SAAB imprinting assays. The P12 protein was incubated with various end-labeled 55-bp oligonucleotides, each of which included a 25-bp central stretch of randomized sequence and binding sites for PCR primers at both ends. The P12-bound oligonucleotides were separated from unbound oligonucleotides by using gel electrophoresis (Fig. 2A), eluted from the gel, amplified by PCR, and subjected to further rounds of SAAB imprinting procedures.

Enrichment of ZPT2—binding sequences was tested by determining a dissociation constant (\( k_\text{d} \)) for a pool of selected oligonucleotides. The \( k_\text{d} \) of the interaction between P12 and the oligonucleotides after five rounds of selection was similar to that after four rounds (\( K_\text{d} = 2.9 \text{ nM} \); Fig. 2, B and C), which indicated saturation of the selection. Hence, we amplified,
cloned, and sequenced the oligonucleotides from the fourth round of selection. A sequence of CATG was found in all clones (Fig. 2D), which suggests that this sequence is the binding site for one of the two zinc fingers. This result is consistent with our previous findings demonstrating that the core binding sites of ZPT2–2 are AGT (14). Further, this current result indicates that a cytosine just upstream of the AGT also is recognized during binding.

Our previous results led us to expect another AGT–10 bp upstream or downstream of the CAGT sequence (14). Unexpectedly, however, such a sequence was not found in any of the clones; instead, an AGC or AGG was found within the randomized region or primer binding sequences at various distances (upstream, downstream, or both) from the CAGT sequences. The sequences could be classified into the following three categories: type I, AGC(T) present at 10–12 bp downstream of the CAGT site (CAGT-10-12-AGCT); type II, AGG present at 10–12 bp downstream of the CAGT site (CAGT-10-12-AGG); and type III, AGC present at both 4–6 bp downstream and 7 or 8 bp upstream of the CAGT site. Separated by 7 or 8 bp from the CAGT site, an upstream AGC(T) was also present in most of the type I sequences. These results suggest that one of the two zinc fingers of ZPT2–2 recognizes the CAGT, and the other finger has affinity for AGC(T) or AGG. Binding affinities to the selected sequences as determined by gel shift assays are shown in Fig. 2D. The $K_d$ values for type I sequences (0.45–1.9 nM) were much lower than those for type II sequences (3.4–11 nM). This suggests that ZPT2–2 prefers AGC to AGG for its binding site.

Interaction of the ZPT2–2 Zinc Fingers with DNA—To characterize the roles of the respective zinc fingers of ZPT2–2 in its interaction with DNA, recombinant proteins containing only a single zinc finger were prepared (PF1 and PF2; see Fig. 1) and examined for DNA binding activity. Because the gel shift assay was not sensitive enough to detect the weak binding activity of the single zinc finger, we instead used an SPR technique. PF1 showed weak binding to the AGC site ($K_d = 150 \pm 60$ nM; see Fig. 3A and Table I), and its binding to the CAGT site was undetectable. In comparison, PF2 bound to both the CAGT site ($K_d = 4.7 \pm 0.1$ nM) and the AGC site ($K_d = 78 \pm 20$ nM; see Fig. 3B and Table I). Therefore, F1 can bind only to the AGC site, whereas F2 can bind to both sites, and its affinity for the CAGT site is much higher than that for AGC. When a probe containing both the core sites (CAGT-10-AGC) was tested, the $K_d$ values were $120 \pm 40$ nM for PF1, $3.9 \pm 0.4$ nM for PF2, and $14 \pm 1$ nM for PF12 (see Fig. 3D and Table I). Therefore, the binding affinity of the two zinc fingers together is two to three orders of magnitude higher than that of either single finger. This indicates that the two sites in PF12 act cooperatively upon binding to DNA. Hydroxyl radical footprinting experiments using PF12 revealed footprints at the CATG and the AGCT sites in a probe containing the two core sites (D). Dosages of proteins are (A) 40 $\mu$M PF1, (B) 20 $\mu$M PF2, (C) 10 $\mu$M PF12 and (D) 2.5, 5.0, 10, 20, and 40 nM PF12.
formed by the interaction of the second zinc finger (F2) with the CAGT site only; P_{F12}−DNA is a stable complex in which both zinc fingers are bound. $K_d$ is an affinity constant for the first interaction of F1 to the AGC site; this constant can be approximated by the $K_d$ for the binding of P_{F1} to the AGC site (Table I). $K_d$ is an equilibrium constant for the second interaction of F2 to the CAGT site, and this interaction is dependent on the prior binding of F1 to the AGC site. $K_d$ is an affinity constant for the first interaction between F2 and the CATG site; this constant can be approximated by the $K_d$ of P_{F2} to the CAGT. $K_d$ is an equilibrium constant for the second interaction by F1 to the AGC site, and this interaction is dependent on the prior binding of F2 to the CAGT site. As described previously, the affinity of F2 for CAGT is more than 30-fold higher than that of F1 for AGC; this difference is because of both an increased association rate ($k_{on}$) and a decreased dissociation rate ($k_{off}$; Table I). Therefore, P_{F12}−DNA* represents a major intermediate, and the binding reaction can be approximated by the two equilibrium constants $K_1$ and $K_2$. In light of these considerations, we conclude that, upon the binding of ZPT2−2 to the CAGT-10-AGC sequence, F2 makes the first interaction by preferentially binding to the CAGT site; this action is followed by the binding of F1 to the AGC site. Although the interaction of F1 with the AGC site is relatively weak, it helps to stabilize the ZPT2−2−DNA complex when the F1-AGC interaction occurs in conjunction with the binding of F2 to the CAGT site.

**ZPT2−2 Can Recognize Various Arrangements of the Two Core Sites**—In some sequences that were selected by the SAAB imprinting procedure, an AGC(T) sequence is present upstream of the CAGT site in addition to the downstream AGC(T) (Fig. 2D). The sequence AGC(T) can be regarded as two overlapping AGCs on two different strands. To examine whether these upstream AGC(T) sequences serve as binding sites, a model probe that contains an AGC sequence 7 bp upstream of the CAGT site (AGC-7-CAGT) was tested for its binding to P_{F12}. The AGC-7-CAGT probe bound to P_{F12} at a $K_d$ of 44 ± 3 nM (Table I), which indicates that the upstream AGC(T) sequences serve as binding sites for ZPT2−2. Hydroxyl radical footprinting experiments revealed a footprint at this upstream AGC(T), along with that of the CATG site (Fig. 4). This indicates that ZPT2−2 indeed interacted with both binding sites in this configuration, and F1 interacted with the AGC(T) site.

In the sequences selected by the SAAB imprinting procedure, the nucleotide T frequently followed the AGC. We used model probes to examine the molecular basis underlying this sequence preference. P_{F12} showed stronger binding to CAGT-10-AGC than to CAGT-10-AGCG (Fig. 5). The same preference (AGC-7-CAGT > AGC-7-CAGT; see Fig. 5) was observed when the F1-binding site was located upstream of the F2-binding site. Therefore, P_{F12} prefers T at the position following

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**Table I**

Kinetic constants for the binding of truncated ZPT2−2 proteins as determined by using the SPR technique.

| Peptide | Core sequence | $k_{on}$ | $k_{off}$ | $K_d$ |
|---------|---------------|---------|---------|-------|
| F1      | CAGT          | $1.2 \times 10^3$ | $1.3 \times 10^1$ | $4.0 \times 10^{-4}$ |
|         | AGC           | $2.9 \times 10^3$ | $2.8 \times 10^1$ | $1.5 \times 10^{-4}$ |
| F2      | CAGT          | $4.1 \times 10^3$ | $4.2 \times 10^1$ | $7.0 \times 10^{-6}$ |
|         | AGC           | $3.0 \times 10^3$ | $3.1 \times 10^1$ | $4.0 \times 10^{-6}$ |
| F12     | CAGT          | $2.6 \times 10^3$ | $2.7 \times 10^1$ | $3.0 \times 10^{-6}$ |
|         | AGC           | $2.2 \times 10^3$ | $2.3 \times 10^1$ | $3.0 \times 10^{-6}$ |

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**Fig. 4. Hydroxyl radical footprinting of ZPT2−2-binding sites.** A DNA fragment containing ZPT2−2-binding sites was labeled with [ω−32P]dCTP at either terminus (BamHI or XbaI site) by using the Klenow fragment of DNA polymerase. The probes for top and bottom strands were incubated with (B) or without (F) truncated ZPT2−2 proteins (14), subjected to a hydroxyl radical reaction, and separated on a 15% polyacrylamide-urea sequencing gel with Maxam-Gilbert G sequencing reactions (G). Shown below are densitograms of the banding patterns.

In Scheme 1, P_{F12}(F1)-DNA* represents an intermediate complex formed by the interaction of the first zinc finger (F1) with the AGC site only; P_{F12}(F2)-DNA* is an intermediate complex

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AGC, regardless of which side of the F2-binding site (CATG) this F1-binding site may be located on.

When F1-binding sites (AGC) were placed on both sides of the F2-binding site (i.e., AGC-7-CAGT-10-AGC), the affinity of \( P_{F_{12}} \) for this probe was slightly higher than that for AGC-7-CAGT but lower than that for CAGT-10-AGCG. These results indicate that the upstream AGC acted in an inhibitory manner against the binding of F1 to the downstream AGC (Fig. 5).

The differences in the affinities of \( P_{F_{12}} \) toward these probes presenting various arrangements of the F1- and F2-binding sites were due primarily to differences in \( k_{on} \) values and not to changes in \( k_{off} \) values (Fig. 5). When \( P_{F_{12}} \) was tested for its binding to these probes, only very small differences were observed between all probes. These results indicate that \( K_d \) but not \( K'_{on} \) (Scheme 1) was influenced by differences in the arrangement and number of the binding sites.

**Effects of Spacing between the Core Sites**—The \( P_{F_{12}} \) binding sequences selected by the SAAB imprinting procedure (Fig. 2D) suggested that ZPT2-2 has a preference regarding the spacing between the F1- and F2-binding sites, in agreement with our previous observations (8, 14). To estimate quantitatively the effects of the spacing, SPR assays were completed by using probes with various spacings between the two binding sites.

When the F1-binding site was located downstream of the F2-binding site on the same strand, the highest affinity to \( P_{F_{12}} \) was observed for CAGT-10-AGC (\( K_d = 14 \pm 1 \) nM; see Fig. 6A). Increasing the spacing by 2 bp resulted in a 3.5-fold reduction in the binding affinity, and increasing it by 4 bp led to a 5.4-fold reduction. In comparison, decreasing the spacing by 2 bp reduced the binding affinity by 1.8-fold, and decreasing it by 4 bp reduced the binding affinity by 2.4-fold. The reduction in the affinity was due primarily to the increase in \( k_{off} \) rather than to the decrease in \( k_{on} \) (Fig. 6A).

When we tested \( P_{F_{12}} \) with the same set of probes, differences in the spacing barely affected the affinity (Fig. 6A), which indicates that the spacing does not influence \( K'_{on} \) (Scheme 1) during the interaction between ZPT2-2 and DNA. Therefore, the effect of spacing on the overall binding affinity of ZPT2-2 (\( K_{net} \)) is because of the differences in the \( K_d \). These results indicate that the effects of spacing become manifest only upon the second interaction, that is, upon the binding of F1 to the AGC site after the initial binding of F2 to the CAGT site.

We then assessed the effects of spacing for other arrangements of the core sites. In the AGC-n-CAGT arrangement, the binding affinity was again sensitive to the spacing, with the highest affinity being for AGC-5-CAGT (\( K_d = 25 \pm 3 \) nM; see Fig. 6B). The effects of spacing on the binding affinity were again due primarily to effects on \( k_{off} \) rather than on \( k_{on} \), and the binding of F2 was not affected by the spacing (\( K_{off} \) ranging from 20 to 40 \( \mu \)M; see Fig. 6A and B). Therefore, these results indicate that spacing affects \( K_{off} \) but not \( K'_{on} \).

**A High Affinity ZPT2-2-binding Site Is Present in the Promoter of the ZPT2-2 Gene**—In light of the DNA binding specificity of ZPT2-2, we searched for ZPT2-2-binding sites in the upstream region of the ZPT2-2 gene. This action revealed a sequence, \( \text{CAGTCGTAAGCCAAAGCT} \) (PROM; core sites underlined), 320 bp upstream from a transcription initiation site of ZPT2-2. The PROM sequence contains optimal binding sites for the two zinc fingers with optimal spacing. An SPR assay...
revealed that \( P_{12} \) bound to PROM with high affinity \( (K_d = 9.5 \pm 1.2 \text{ nM; see Fig. 5}) \). This result strongly suggests that this sequence is a cognate binding site for ZPT2–2 and presumably serves as a cis-element for the autoregulation of the ZPT2–2 gene itself.

**DISCUSSION**

**Proposed Model for DNA Interaction by ZPT2–2**—In light of our present and previous characterization of the DNA interaction, we here propose a model for the DNA binding of ZPT2–2. ZPT2–2 can interact with two types of DNA sequences, which have different arrangements of the two core sites. The interaction of the F2 zinc finger with the CAGT site primarily initiates the binding. Then the F1 region interacts with an AGC(T) site that is located several nucleotides either upstream or downstream from the CAGT site, thereby stabilizing the complex. The equilibrium between the intermediate and final complexes greatly depends on the spacing between the CAGT and AGC(T) sites; this spacing determines the overall binding affinity. The unique manner in which ZPT2–2 interacts with DNA is due largely to the flexibility of the long linker region between the two zinc fingers.

Most transcription factors, including cluster-type zinc finger proteins, interact with continuous DNA sequences. However, some DNA-binding proteins, such as those belonging to the Oct-1 (17, 18) and ZnCy6 (19–21) families, are known to recognize separate DNA-sequence motifs, with each of two DNA-binding domains making contact with a different motif. These DNA binding proteins have very strict specificities for the spacing between core sequences, to the degree that even a 1-bp deviation in spacing drastically reduces the DNA binding affinity of Oct-1 (18) and PUT3 (a ZnCy6 protein; see Ref. 21).

Compared with those of Oct-1 and PUT3, the specificity of ZPT2–2 for spacing is moderate. In addition, ZPT2–2 will bind to two completely different arrangements of the two DNA-binding sites. Thus, the mechanism by which ZPT2–2 recognizes these noncontiguous binding sites differs from that of Oct-1 and PUT3.

**Biological Significance of the Unique Mode of DNA Interaction of ZPT2–2**—Is there any biological advantage to interacting with target DNA sequences in two different arrangements? At present, we have no answer to this question. However, it would be interesting if, depending on various conditions, intact ZPT2–2 could change its conformation between the conformations corresponding to the two arrangements of the two core sites. Because of steric hindrance, the intact ZPT2–2 may not be able to change its conformation as freely as does its truncated form. Even so, the conformation of intact ZPT2–2 may change depending on its state of protein modification or interaction with other regulatory proteins. Thus, ZPT2–2 could switch from one set of target genes to another in response to environmental or intracellular stimuli.

In the promoter region of the ZPT2–2 gene, we found a putative ZPT2–2-interacting site that is perfectly in accord with the results of our current *in vitro* study. Many transcription factors are known to regulate the transcription of their own genes through interacting with their promoter DNA sequences (autoregulation). Considering the ubiquity of autoregulation of transcription factors, together with the high affinity interaction of ZPT2–2 with DNA, the sequence we identified most likely serves as a *bona fide* cis-element for ZPT2–2 *in vivo*. Therefore, knowledge of the DNA binding specificity of ZPT2–2 *in vitro* led us to identifying an *in vivo* ZPT2–2 interaction site.

We expect that this information will facilitate our in *vivo* functional characterization of ZPT2–2 in the stress response in plants.

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