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Measuring specific interaction of transcription factor ZmDREB1A with its DNA responsive element at the molecular level

Yaxin Jiang, Feng Qin[1], Yiqin Li[1], Xiaohong Fang* and Chunli Bai

Institute of Chemistry, Chinese Academy of Sciences, Beijing 100080, China and [1]Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China

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ABSTRACT

Specific interactions between transcription factors and DNA responsive elements are of fundamental importance in understanding how genetic regulatory proteins control gene transcription. Here we have developed a new method of using atomic force microscopy (AFM) to quantitatively study the single molecular specific interaction between ZmDREB1A, a transcription factor from maize, and its DNA responsive element, *d*ehydration-*r*esponsive *e*lement (DRE) with core sequence A/GCCGAC. It was found that ZmDREB1A bound to both DRE ACCGAC and GCCGAC efficiently. The single molecular interaction forces of ZmDREB1A with DRE A/GCCGAC were determined to be 101 ± 5 and 108 ± 3 pN, respectively. The point mutation of ZmDREB1A in its DNA-binding domain or single base substitution of the DRE core sequence greatly reduced the binding affinity, demonstrating the high sensitivity of the AFM measurements. AFM is expected to be a simple, quick, sensitive and reliable method that offers valuable information for the characterization of transcription factors and the identification of their potential DNA responsive elements in functional genomics research.

INTRODUCTION

The recognition and binding of a transcription factor to its DNA responsive element (promoter or enhancer) is the basis for the initiation and regulation of gene expression in all cells (1,2). As a transcription factor controls the expression of a set of genes containing the same responsive element and acts as a switch of regulatory cascades, the manipulation of transcription factors is more effective and promising for genetic improvement of plants or animals than that of other downstream functional genes (3). With the development of molecular biology, more and more transcription factors and their corresponding responsive elements in the promoter regions of different kinds of genes have been identified and characterized. The understanding of the specific interactions between transcription factors and DNA promoters are of fundamental importance in understanding how genetic regulatory proteins affect gene transcription. Gel electrophoresis-based assays have been commonly used in biology to directly study the binding of a transcription factor to a DNA element (4). However, it is not easy to obtain accurate quantification and the DNA sequence preference of the binding due to high experimental variations. Efforts have recently been made to develop rapid and quantitative DNA-binding assays (5). We report in this paper the development of a new technique using atomic force microscopy (AFM), to study their interactions quantitatively at the single molecule level.

Dehydration-responsive element (DRE) binding factors (DREBs), the transcription factors involved in the regulation of plant tolerance to drought, low temperature and high salinity, have recently received attention in plant molecular biology (6–8). The manipulation of one of the DREBs in transgenic *Arabidopsis,* named DREB1A, has given insights into genetic improvement of agriculturally important crops' tolerance of environmental stress through the DREB regulatory system (3). DREBs belong to the AP2/EREBP transcription factor family, which are unique in plants. They bind to the DRE (A/GCCGAC) sequence in the promoter region of many stress tolerance-related genes. In the study of the molecular mechanism of DREBs in DNA-binding and transcription activation, many questions are still open. For example, DREBs share a highly conserved DNA-binding domain (ERF/AP2 domain consisting of 58 or 59 amino acids) with *e*thylene-*r*esponsive *e*lement (ERE) binding factors in the same AP2/EREBP transcription factor family, but they discern two different DNA sequences to control the expression of two different sets of genes (9–12). Recent reports have also suggested that in the family of DREBs, different proteins have different DNA sequence preferences to regulate varied target genes (13). To gain a better understanding of DREBs' function, we have recently cloned one of the DREB transcription factor genes from maize, named *ZmDREB1A* (GenBank accession

*To whom correspondence should be addressed. Tel: +86 10 62650024; Fax: +86 10 62650024; Email: xfang@iccas.ac.cn
Correspondence may also be addressed to Chunli Bai. Email: clbai@iccas.ac.cn

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

no. AF450481) (14). The results from yeast one-hybrid and gel mobility shift assay have shown that it binds to the DRE but not the ERE sequence. In order to study the affinity of *ZmDREB1A* to different DNA sequences, we have quantitatively measured the binding forces by AFM.

AFM is a rapidly developing technique for probing affinity and recognition properties at the molecular level. It is advantageous in terms of high force resolution, high spatial resolution, low sample consumption as well as the capacity to provide quantitative intermolecular force information (15–17). As a direct force measuring method, it has been applied to the study of a variety of non-covalent specific interactions such as that of receptor/ligand (18–20), antibody/antigen and other protein/protein (21–26), carbohydrates (27), crown ether/ammonium (28), strands of DNA (29) and DNA aptamer/protein (30), etc. Although AFM been used in investigating structural–functional interactions between protein transcription factors and DNA by imaging the large protein–DNA complex (31,32), to the best of our knowledge, this is the first example of AFM measurement of the binding force between a transcription factor and its DNA promoter at the single molecular level. The successful application of AFM in transcription factor/DNA promoter interaction study would provide us a new approach to the study of functional genomics.

MATERIALS AND METHODS

Materials

All the DNA sequences were custom synthesized from SBS Genetech Co. Ltd. (Beijing, China). These include the DRE element sequence (ACCGAC), 5′-NH_2-GATATACTACCGACATGAGTTC-3′, and its complementary ssDNA, 3′-CTATATGATGGCTGTACTCAAG-5′; the DRE element sequence (GCCGAC), 5′-NH_2-GATATACTGCCGACATGAGTTC-3′, and its complementary ssDNA, 3′-CTATATGACGGCTGTACTCAAG-5′; the ERE element (GCCGCC), 5′-NH_2-CGCAGACATAGCCGCCATTT-3′, and its complementary ssDNA, 3′-GCGTCTGTATCGGCGGTAAA-5′; the mutant DRE element sequence (ACCGAG), 5′-NH_2-GATATACTACCGAGATGAGTTC-3′, and its complementary ssDNA, 3′-CTATATGATGGCTCTACTCAAG-5′ (the element sequences are underlined). 3-Amino-propyltriethoxysilane (APTES) and toluene (99.99%, HPLC grade) were obtained from ACRO (USA). Milli-Q purified water (18.2 MΩ) was used for all the sample preparations.

ZmDREB1A protein and its point mutation

The amino acid sequence of the wild-type ZmDREB1A protein was deduced from the cDNA sequence of its gene (AF450481). For the mutated protein (named V14A), the 14th valine (GTG) in ZmDREB1A was singly replaced by alanine (GCG). PCR strategy was used to introduce the point mutation into the wild-type sequence by mutant primer pairs which were V14A-Forward, 5′-GGTGGGCGTGCGAGGTGCGCGTCCCGG-3′, and V14A-Reverse, 5′-CCGGGACGCGCACCTCGCACGCCCACC-3′. The reverse mutant primer was paired with ZmDREB1A full-length forward primer, 5′-AAAGAATTCATGGACACGGCCGGCCTC-3′, to generate a smaller fragment of ZmDREB1A. The forward mutant primer was paired with ZmDREB1A full-length revere primer, 5′-AAAGCGGCCGCCTAGTAGTAGCTCCAGAGCG-3′, to produce a larger fragment of ZmDREB1A. This pair of fragments was used as the second PCR templates to generate the full-length and mutant ZmDREB1A sequences. The wild-type and mutated DNA sequences were cloned into pBluescriptSK vector with EcoRI and NotI restriction enzyme sites, respectively.

GST fusion ZmDREB1A proteins and gel mobility shift assays

The wild-type and mutant of 479 bp fragments of ZmDREB1A, containing the DNA-binding domain, were prepared by the primer pair, 5′-TGACGAATTCGCTTCCTCACCAC-3′ (forward) and 5′-TGACGTCGACGCACGTGCTCAAG-3′ (reverse), respectively. Each of these fragments was cloned into the EcoRI–SalI sites of the pGEX-4T-1 vector and transformed into *Escherichia coli* to produce the GST-fusion proteins (33). The GST fusion proteins for ZmDREB1A and its mutant were purified using Glutathione Sepharose 4B column (Amersham Pharmacia Biotech, USA) according to the manufacturer's instructions. Gel mobility shift assays were conducted as described previously (34). A 75 bp fragment from the promoter region of *Arabidopsis RD29A* gene (34) and a 90 bp fragment from the promoter region of *Arabidopsis HOOKLESS1* gene (35) were used as DRE and ERE probes, respectively, in the gel mobility shift experiments.

Chemical modification of the AFM tips and substrates

The single crystal silicon wafers and AFM silicon nitride (Si_3N_4) tips (type: NP with a radius of 20–60 nm, from Veeco, Santa Barbara, CA,) were cleaned following the previously reported procedure (30). The cleaned wafers or tips were transferred to a 1.0% v/v APTES in toluene solution, incubated for 2 h at room temperature, and then rinsed thoroughly with toluene to remove any unbound silane. The silanized wafers or tips were activated by incubating in a 0.1% glutaraldehyde solution in phosphate-buffered saline (PBS) buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl, pH 7.3) for 0.5 h at room temperature and then rinsed with the buffer. For the protein immobilization, the activated tips (or wafers) were immersed into the protein (ZmDREB1A–GST or others) solution (2 μg/ml in PBS) and incubated at room temperature for 0.5 h. For the DNA element sequence modification, the activated wafers were immersed in the duplex DNA solution (1.0×10^{-7} M) for 10 h at 4°C. After rinsing with the buffer, the functionalized wafers and tips were stored in PBS at 4°C until use.

Force curve measurements

A Nano Scope IIIa AFM (Veeco, Santa Barbara, CA) was used to perform force measurements in a liquid cell filled with the freshly prepared PBS buffer. Force measurements were performed with the protein-functionalized tips and DNA-modified wafers. The spring constants of the tips, calibrated by the thermal fluctuation method (36), were in the range of 0.038–0.079 N/m. The loading rates of the force–distance measurements ranged from 7.3×10^4 to 7.9×10^4 pN/s. The force curves were recorded and analyzed using the Nanoscope 5.30b4 software (Veeco, Santa Barbara, CA). The single

molecular interaction forces between the tips and substrates were directly extracted from the force curves by measuring the force difference between the break point (the lowest point of the peak) and baseline. The most probable single molecular interaction force was determined by fitting a Gaussian to the histogram of the force distribution. The errors were estimated by calculating SD/$\sqrt{N}$, where SD is the width of the distribution and N is the number of unbinding events in the histogram (24).

RESULTS AND DISCUSSION

Specific interaction of ZmDREB1A with the DRE sequence

The GST-fused ZmDREB1A protein (ZmDREB1A–GST) and the 22 bp oligonucleotide containing the DRE core sequence (ACCGAC) from the promoter region of *Arabidopsis rd29a* gene (34,37), were covalently attached to the AFM tips and silicon substrates, respectively, via a glutaraldehyde spacer. This protocol has been reported to be well suited for the force measurement of antibody/antigen and protein/DNA aptamer (30,38). The density of the immobilized protein on the AFM tip, mainly determined from the AFM topographical images of the ZmDREB1A–GST-modified silicon substrates under the same condition, was controlled to be low enough to result in only one molecule on the tip (22,23,26). In our experiments, the estimated density of ZmDREB1A–GST was 180 to 280 molecules per square micrometers, corresponding to 0.5–0.8 ZmDREB1A–GST per tip end of 2950 nm^2 with the estimated tip radius of 40 nm (26). This allows the detection of molecular recognition between a single protein/DNA pair by AFM (22,23).

Figure 1a is the representative force–distance curve when the ZmDREB1A–GST-modified tip and the DRE ACCGAC sequence-modified substrate were brought in and out of contact in the PBS buffer. It revealed that a significant level of adhesion existed between ZmDREB1A–GST and DRE ACCGAC. The histogram of the force distribution obtained from about 300 force–distance cycles is shown in Figure 2a. A single maximum in the histogram demonstrated that single molecule forces were measured (21–26). The reliable mean value, 95 ± 4 pN, for the specific single molecular interaction force of ZmDREB1A–GST/ACCGAC was calculated by fitting a Gaussian distribution to the single peak. This value was independent of the histogram arrangement with different width. It was found that if the ZmDREB1A–GST concentration was increased during the tip immobilization, multiple force peaks showed up in the histogram of the force distribution, indicating multiple pairs of the complex formed. To further confirm the reliability of the experimental condition for the measurement of the single pairs of protein–DNA element, we performed force measurements with a higher protein density on the tip and used the Poisson statistical method to derive the single rupture force of ZmDREB1A–GST/ACCGAC (20,30). The calculated single molecular force value was very close to that from the direct single pairs measurement (supplementary material). In the reported AFM measurements, the single molecule interaction forces in different antigen–antibody and ligand–protein systems are usually in the range of 50–300 pN (17). The individual force of the transcription factor ZmDREB1A with its DRE element also fell within the range.

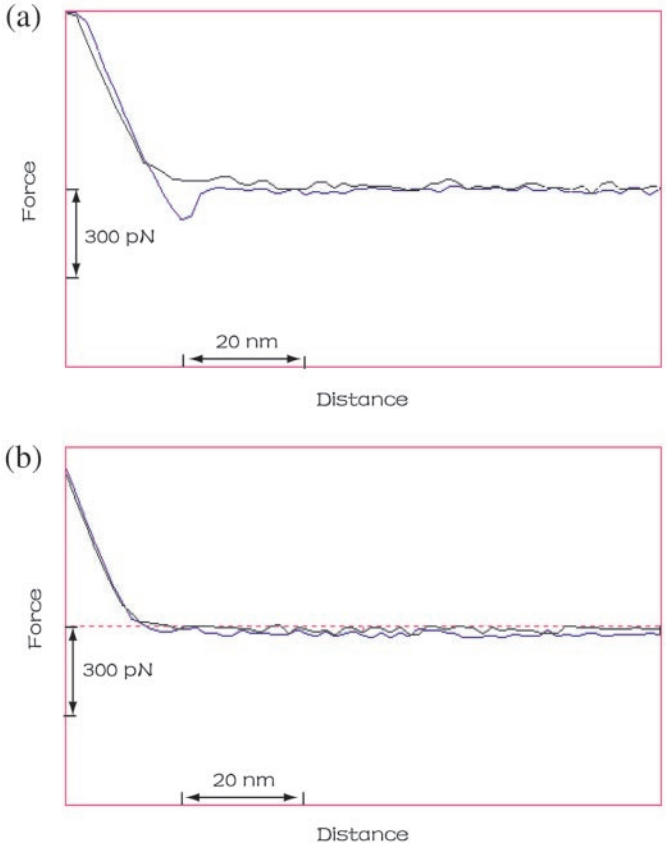

Figure 1. Typical force–distance curves using the ZmDREB1A–GST-modified AFM tip and DRE ACCGAC-modified substrates (**a**) or the GST-modified AFM tip and DRE ACCGAC-modified substrates (**b**) in the PBS buffer.

When the tip was changed to the one immobilized with GST alone, there was no obvious adhesion point in most of the force–distance curves (Figure 1b). Although we occasionally detected some unbinding events, the binding probability, which is defined as the ratio between the number of force curves showing adhesion events to the total number of curves, was much lower (about 0.3 with GST compared to 0.8 with ZmDREB1A–GST). Moreover, those rarely detected forces of GST/ACCGAC (shown in Figure 2b) were randomly distributed and did not follow a Gaussian profile, indicating those were non-specific interactions. Therefore, it was the ZmDREB1A protein but not GST that contributed completely to the specific interaction between ZmDREB1A–GST protein and the DRE sequence. It is noteworthy that the previously reported binding probabilities for single molecule protein–protein and ligand–protein interactions were in a wide range of 0.2–0.8 under different conditions (21–25). The binding probability of the ZmDREB1A–GST protein and the DRE sequence that we obtained was in the high range compared with those systems. This is probably because the density of the DNA element on the substrate in our experiment was relatively high and thus the possibility of binding of the single protein on the tip to the DNA element in each approaching retarding cycle was enhanced.

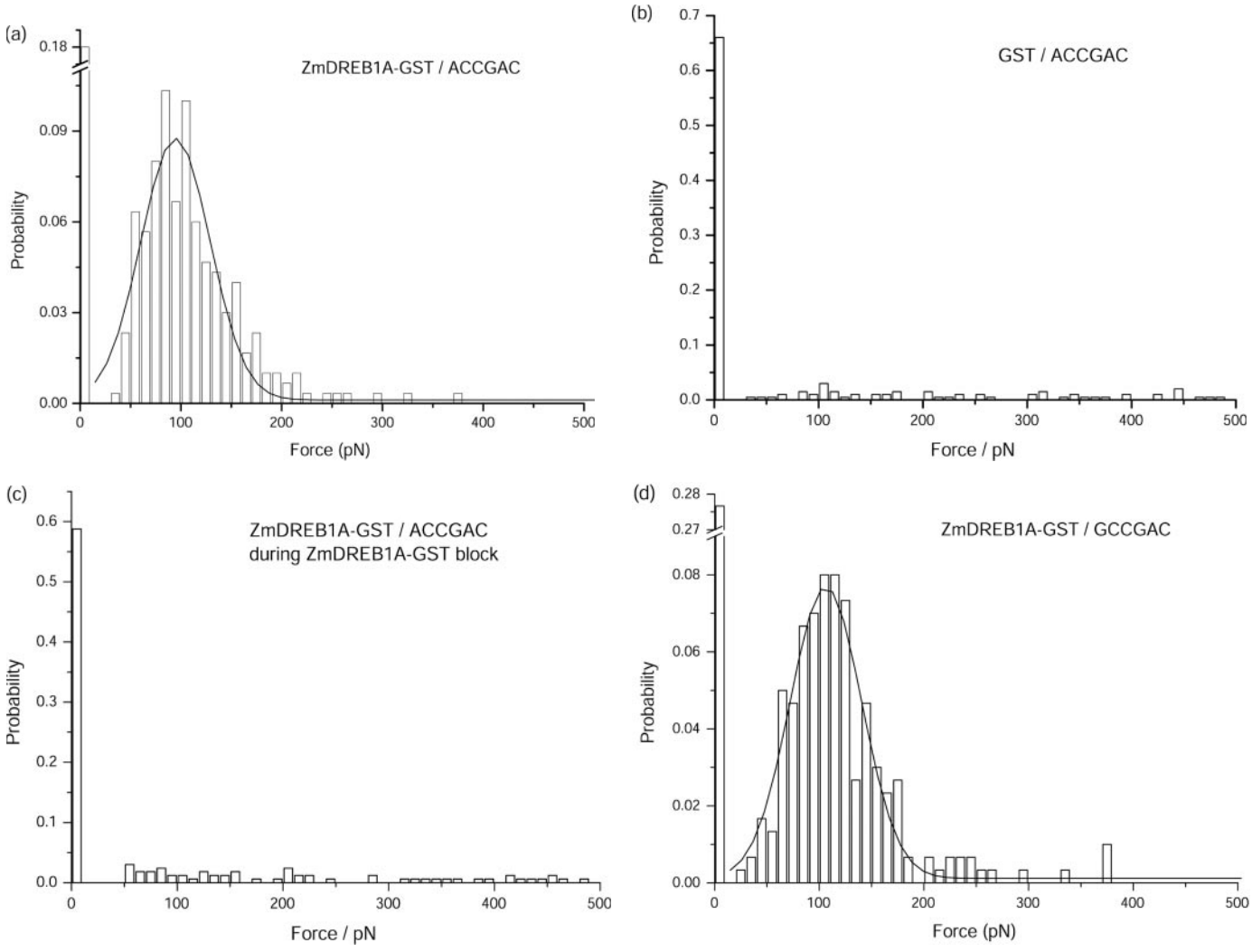

Figure 2. Histograms of the adhesion forces of ZmDREB1A–GST/ACCGAC (**a**), GST/ACCGAC (**b**) in the PBS buffer and ZmDREB1A–GST/ACCGAC after a free ZmDREB1A–GST solution was injected into the fluid cell (**c**), and ZmDREB1A–GST/GCCGAC in the PBS buffer (**d**). □ open bar, experimental data; solid line, theoretical Gaussian distribution curve.

Several other control experiments have been conducted including the blocking experiments to confirm the specific interaction force measured by AFM. The force distribution from the blocking experiments showed that if a solution of free ZmDREB1A–GST protein (2.0 μg/ml) or free DRE sequence (1.0×10^{-7} M) was injected into the fluid cell, the binding between ZmDREB1A–GST-modified tip and the DRE sequence-modified substrate decreased greatly, and the binding probability decreased to about 0.4 as well (shown in Figure 2c). Since both the binding force value and the binding probability can be obtained, AFM is expected to provide more information on the nature of molecular interaction than the other 'affinity' measurements (21–25).

Previous study has indicated that the core of the DRE sequence could be both ACCGAC and GCCGAC (33,34,37). Under the same experimental conditions, the ZmDREB1A–GST-modified tip was also found to interact with the GCCGAC element-modified substrate, and their specific single molecular interaction force was 106 ± 4 pN deriving from the force distribution histogram (Figure 2d). We then did a series of repeated experiments with different ZmDREB1A–GST-modified tips and DRE A/GCCGAC-modified substrates. The results revealed a good reproducibility of our AFM force measurements. ZmDREB1A protein bound to both ACCGAC and GCCGAC efficiently with the standard deviation of the mean force value < 5 pN for each pair in 8 measurements. The averaged interaction forces were 101 ± 5 pN and 108 ± 3 pN for ZmDREB1A/ACCGAC and ZmDREB1A/GCCGAC respectively, and their binding probabilities are comparable (about 0.8). It is known that in *Arabidopsis*, DREBs bind to DRE A/GCCGAC equally, but recently, the study of one rice DREB protein, OsDREB1, suggested that this protein bound to GCCGAC more efficiently than ACCGAC (13). Here we measured the single molecular interaction forces of the new transcription factor ZmDREB1A with the A/GCCGAC element by AFM. Our results showed that the maize DREB, like the DREBs from *Arabidopsis*, could bind these two sequences with about the same affinity.

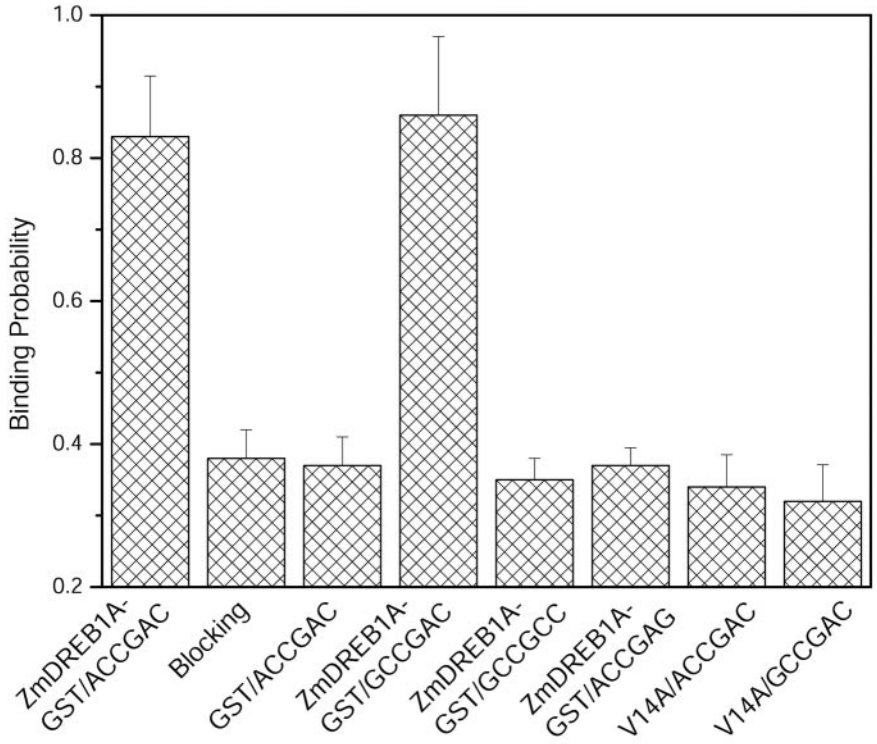

Figure 3. The binding probabilities obtained with different protein-modified tips and different DNA-modified substrates.

Interaction of ZmDREB1A with the ERE sequence

To test the specificity of the AFM measurements, another 20 bp sequence containing the ERE core sequence (GCCGCC) from the promoter region of *Arabidopsis HOOKLESS1* (35) was immobilized on the silicon wafers to interact with the ZmDREB1A–GST-modified tip. The ERE sequence lies in the upstream of many ethylene-inducible pathogenesis-related genes in response to the ethylene signal and resists pathogen attacks (9,10). Its binding factors (EREBPs) also belong to the same AP2/EREBP transcription factor family as DERBs. The ERE core sequence, GCCGCC, and the DRE core sequence, GCCGAC, are quite similar. However, according to the low binding probability (shown in Figure 3) and the random force distribution similar to that with the GST-modified tip and ERE-modified substrate (data not shown), we concluded that there was no specific interaction between ZmDREB1A–GST and the ERE sequence.

We have also carried out gel shift mobility assays for the above binding study of the transcription factors and DNA elements. As shown in Figure 4, only the mixture of ZmDREB1A–GST and the DRE (ACCGAC) probe displayed two bands in the right lane of the native PAGE gel, with the lower band for the free DRE probe and the upper band for their binding complex. No complex band was detected for either the mixture of GST/DRE probe or ZmDREB1A–GST/ERE (GCCGCC). These results are in a good agreement with those from the above AFM force measurements, demonstrating the reliability of the AFM study. Comparing with the gel shift mobility assays, AFM requires much less protein sample and no radio-isotopic labeling, and offers advantages in easy quantitation, binding preference study and future binding screen for DNA element identification as discussed in the conclusion part.

Figure 4. Gel mobility shift assay for GST/DRE, ZmDREB1A–GST/ERE and ZmDREB1A–GST/DRE.

Sensitivity of the force measurements in the mutation experiments

In the binding mechanism study of the transcription factors to DNA promoters, mutation experiments are often performed to determine which base or amino acid is critical in their binding. We have further tested whether AFM is sensitive enough to detect the change of binding affinity when there is a mutation either in the protein or the DNA element sequence.

First, we have studied the effect of a single base mutation in the DNA element on its binding capability with the transcription factor. When we mutated the DRE core sequence ACCGAC to ACCGAG (the position of the mutation is underlined), the binding probability of the protein and the DNA element decreased greatly even when we used the same ZmDREB1A–GST-modified tips (Figure 3). Moreover, the force distribution of ZmDREB1A–GST/ACCGAG did not change when a free ZmDREB1A–GST or ACCGAG solution was injected in the blocking experiments (data not shown). It is noteworthy that compared to the ZmDREB1A–GST/ACCGAC system, there is only one base pair change in the total 22 bp promoter sequence chosen for our AFM force measurement. Since this single base substitution was taken in the core region of the element that is critical for its interaction with ZmDREB1A, its affinity for the transcription factor greatly reduced. This result agrees with that reported for the binding DRE A/GCCGAC with the *Arabidopsis* DREB proteins (33).

Second, the effect of a single amino acid mutation of the transcription factor on the binding was studied. Some recent researches suggested that the 14th amino acid in the DNA-binding domain of DREBs in *Arabidopsis* determines their specific interaction with the DNA sequence using yeast one-hybrid method (39) and gel shift mobility assay (33). Whether this conclusion is also applicable to this kind of transcription factors from maize is not known yet. We point mutated the 14th valine in ZmDREB1A to alanine in its DNA-binding domain and modified this mutated protein (V14A) on the AFM tips to study its interaction with different A/GCCGAC-modified wafers. The results repeatedly showed that not only did the binding probability between V14A and the A/GCCGAC element decrease significantly to 0.3–0.4 (Figure 3), but also the force distribution of the rare unbinding events due to the non-specific interaction could not be fitted to Gaussian distribution. This demonstrated that the key point mutation in the DNA-binding domain of ZmDREB1A resulted in the loss of its binding capability to the DRE sequence.

CONCLUSION

In this paper, we have applied successfully atomic force microscopy to the study of specific interactions between the transcription factor and DNA promoter. The single molecular interaction forces of the transcription factor, ZmDREB1A, with its DRE A/GCCGAC sequence were determined to be 101 and 108 pN, respectively. It is the first time that the individual interaction force between a transcription factor and its target DNA element has been quantitatively measured. Moreover, we have shown the reliability and sensitivity of this method using the point mutated protein and single base substituted DNA sequence. The key point mutation in the DNA-binding domain of ZmDREB1A or single base substitution of the core sequence of DRE results in the loss of their binding affinity. These results provide valuable information on the study of the recognition and interaction of maize DRE transcription factors with their DNA promoters. The method developed in this work should also be widely applicable to other transcription factor/DNA promoter interaction study.

In the study of the functional genome, it is of great significance to demonstrate the biological functions of every transcription factor. To this end, the identification of the responsive DNA element for each transcription factor is ultimately important. Recent achievements in genomics have suggested that there are about 1533 transcription factors in *Arabidopsis*, and maybe less in yeast, *Drosophila* and *Caenorhabditis elegans* (2). With the commonly used techniques in molecular biology, it is possible to express every transcription factor *in vitro*, but it is quite difficult to identify their responsive elements in the genome. With the advantages of simplicity and high sensitivity, AFM might be a powerful tool in the high-throughput DNA elements identification in future. This can be achieved, e.g. by using transcription factor-modified AFM tips to interact with arrayed DNA sequences from a synthetic DNA library. Moreover, the slight difference in the binding preference of a transcription factor to different DNA core sequences can be quantitatively measured by AFM to give clues for the detailed interaction study. Compared to the traditional gel electrophoresis method and the currently developed biochip technique using optical (fluorescence) detection strategy, single molecule AFM force measurement is advantageous in less protein sample consumption, not requiring sample labeling and simultaneous quantitation for binding property study. Therefore, AFM is expected to be a quick, sensitive and reliable method for potential DNA elements identification and transcription factor characterization in the functional genomics research.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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