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Single molecule study of binding force between transcription factor TINY and its DNA responsive element

Junping Yu [a,1], Shan Sun [b,1], Yaxin Jiang [a], Xinyong Ma [a], Feng Chen [b], Guiyou Zhang [b], Xiaohong Fang [a,*]

[a] *Institute of Chemistry, Chinese Academy of Sciences, Center for Molecular Sciences, Beijing 100080, China*
[b] *Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China*

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Abstract

The interaction force between transcription factor TINY and its corresponding *cis*-element has been studied with single molecule force microscopy. TINY belongs to the AP2/EREBP family, which is the second largest transcription factor family in plant and plays an important role in the genetic regulation of plant development and growth. The results showed that TINY bound to dehydration-responsive element (DRE) with the core sequence A/GCCGAC efficiently. The single molecule forces between TINY and DRE A/GCCGAC measured by atomic force microscopy were 83.5 ± 3.4 and 81.4 ± 4.9 pN, respectively. Either the single base substitution of the DRE core sequence or point mutation of the key amino acid in TINY DNA-binding domain greatly reduced the binding strength. We have previously reported the method of using AFM to measure the interaction force between transcription factor ZmDREB1A, another AP2/EREBP family protein, and DRE responsive element. The results in this work further confirm the applicability and high sensitivity of AFM measurements in transcription factor–DNA interaction study. More interesting, it was found that 19th amino acid mutation of TINY resulted in the decrease of the binding force but the binding probability was the same as the wild type TINY. The reduced binding force was correlated with the reduced activity of TINY regulated reporter gene in the yeast one-hybrid experiment. It is thus expected that the single molecule force measurement offers valuable information on the analysis of transcription factor regulated gene expression.

Keywords: Atomic force microscopy; Single molecule; Transcription factor

1. Introduction

The regulation of gene expression controls many biological processes, such as maintaining physiological balance and responding to environmental signals [1,2]. At the level of transcription, gene regulation is mainly achieved by the interaction of the DNA-binding proteins, transcription factors, with their specific elements (promoters). Therefore, the understanding of the binding mechanism and binding property of transcription factors–DNA responsive element is crucial [3].

Several methods have been developed to study specific protein–DNA binding, and the most commonly used ones in biology are DNA footprint analysis, gel shift analysis (also called gel retardation analysis) and methylation interference [4,5]. Recently, we have developed a new method of using atomic force microscopy (AFM) to quantitatively study the interaction force between ZmDREB1A, a transcription factor from maize, and its DNA responsive element at single molecular level [6]. It is known that AFM can be used not only to image biological macromolecules with high-resolution, but also to measure inter/intra biomolecular interaction forces with pico Newton (pN) sensitivity [7–10]. Compared with the conventional methods, AFM is advantageous in direct force measurement, high sensitivity, minimum sample consuming, low experimental variations, easy quantitation and avoiding of radio-labeling. It is expected to be a simple, quick, sensitive and reliable method for the binding study of transcription factors–DNA element. In this paper, we have applied the method to measuring the single molecule force between another transcription factor from *Arabidopsis*, TINY [11], and DRE element. More importantly, we further investigated the relationship between the binding force measured by AFM and the downstream gene activity regulated by the transcription factor.

* Corresponding author. Tel./fax: +86 10 62650024.
E-mail address: xfang@iccas.ac.cn (X. Fang).
[1] The first two authors should be regarded as joint First Authors.

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Like ZmDREB1A, TINY also belongs to the second largest plant-specific transcription factors family, AP2/EREBP [6,11]. According to the number of AP2 domain they contained, the proteins in this family are classified into two subfamilies, AP2 and EREBP. While AP2 subfamily members with two AP2 DNA-binding domains regulate plant developmental process of blossoming and seeding, EREBP subfamily proteins contain only one AP2 domain and regulate the gene expression to tolerate environmental stress. EREBP subfamily is further classified into several subgroups with different affinity to different DNA elements. For example, previously reported ZmDREB1A belongs to the DREB subgroup of EREBP subfamily, which recognizes dehydration-responsive element (DRE) [6]. This subgroup transcription factors regulate expression of genes related to resist drought, high-salt and cold stress, by specifically interacting with DRE element, or other DNA elements containing DRE core sequences such as C-repeats. They play an important role in the genetic improvement of agriculturally important crops. The *TINY* gene was first isolated through a transposon-mutagenesis experiment designed to recover dominant gain-of-function alleles in *Arabidopsis* [11]. The phylogenetic analysis of its AP2 domain showed that TINY belongs to the DREB subgroup [12,13]. The yeast one-hybrid assay has demonstrated that TINY could bind with dehydration-responsive element (DRE) [14]. Although it is reported the overexpression of *TINY* suppressed cell proliferation and exhibited pleiotropic effects in *Arabidopsis*, the biological function of TINY is not clear yet, e.g. whether it may act a more important role in plant development and growth under unfavorable environment. Efforts are being made to understand more on the interaction of TINY with different DNA elements.

In this work, we measured the interaction forces between single TINY–DNA element pairs. The binding forces of TINY and DRE with core sequence A/GCCGAC were determined by AFM. Single base-pair mutation of DRE core sequence or single point mutation of 14th amino acid in the DNA binding domain of TINY greatly reduced the binding strength, confirming the high sensitivity of AFM measurements. Moreover, it is found that the point mutation of TINY in 19th amino acid resulted in decreased binding force but the same binding probability as the wild type TINY. We further compared the single molecule force value of TINY–DRE with the expression quantity of the gene regulated by TINY in yeast one-hybrid experiments. The results demonstrated that the weaker the force was, the less the reporter gene expressed. This indicated that AFM can not only be used as a direct, valid and simple method to study the binding between transcription factors and DNA elements, but also offer the quantitative information on their regulation capabilities.

2. Experiments and methods

2.1. Experiments

2.1.1. Materials

The DNA sequences used in all force measurements were synthesized from SBS Genetech Co. Ltd (Beijing, China). These included the DRE element sequence (ACCGAC), 5′-NH_2-GATATACT*ACCGAC*ATGAGTTC-3′, and its complementary ssDNA, 3′-CTATATGA*TGGCTG*TACTCAAG-5′; the DRE element sequence (GCCGAC), 5′-NH_2-GATATACT*GCCGAC*ATGAGTTC-3′, and its complementary ssDNA, 3′-CTATATGA*CGGCTG*TACTCAAG-5′; the mutant DRE element sequence (ACCGAG), 5′-NH_2-GATATACT*ACCGA*[*G*]ATGAGTTC-3′, and its complementary ssDNA, 3′-CTATATGA*TGGCT*[*C*]TACTCAAG-5′. 3-Aminopropyltriethoxysilane (APTES), (3-mercaptopropyl) trimethoxysilane (MPTMS) and toluene (99.99%, HPLC grade) were purchased from ACRO (USA). *N*-hydroxysuccinimide–polyethylene glycol–maleimide (MW 3400) (NHS–PEG–MAL) was obtained from Nektar Therapeutics (Huntsville, AL, USA). Other reagents used in the experiments were all analytical grade. Milli-Q purified water (18.2 MΩ) was used for all experiments.

2.1.2. Preparation of TINY fragments

The *TINY* gene [11] was kindly provided by Dr George Coupland (Department of Molecular Genetics, John Innes Centre, UK). The point mutation of 14th or 19th amino acid residue in TINY was obtained by singly replaced valine by alanine or glutamic acid by aspartic acid, and named V14A and E19D, respectively. PCR strategy was used to introduce point mutations into the wild-type sequence by mutant primer pairs, in which the mutation sites were italized: 14-For 5′-GAAAATGGG*C*GTCCGAGATAC-3′; 14-Rev 5′-GTATCTCGGAC*G*CCCATTTTC-3′; 19-For 5′-CGTGA*C*CCTAGGAAAAAATC-3′; 19-Rev 5′-GATTTTTTCCTAGGG*T*CACG-3′. Each of the above reverse mutant primers were paired with the *TINY* full-length forward primer 5′-AAAAGAATTCATGATAGCTTCAGAGAGTAC-3′ to generate a 5′-terminal smaller fragment of *TINY*. Each of the forward mutant primers was paired with *TINY* full-length reverse primer 5′-AAAAGTCGACGACTTAATAATTATACAGTCCT-3′ to produce a 3′-terminal larger fragment of *TINY*. These pairs of fragments were used as the second PCR templates to generate the mutant *TINY* fragments. The wild-type and mutated DNA sequences were cloned into pBluescriptII SK vector with *Eco*RI and *Sal*I restriction enzyme sites for sequencing.

2.1.3. GST fusion protein preparation

The wild type and mutants of 363-bp (1–363) fragments of *TINY* containing the DNA-binding domain were prepared by the primer pairs: 5′-AAAAGAATTCATGATAGCTTCAGAGAGTAC-3′ (forward); 5′-AAAAGTCGACTTAGGTCTCCATGTGTGCGGCTTTG-3′ (reverse), respectively. Each of these fragments was cloned into the *Eco*RI–*Sal*I sites of the pGEX-4T-1 vector (Pharmacia Biotech) and transformed into *Escherichia coli* to produce the GST-fusion proteins. The GST fusion proteins were separated using a Glutathione Sepharose 4B column (Pharmacia Biotech) according to the manufacturer's instructions.

2.2. Methods

2.2.1. AFM substrates and tips preparation

Chemical modification of substrates was carried out according to previously reported procedures [15]. Single-crystal silicon wafers were cut into 1.5 cm × 1.5 cm square and cleaned firstly. The cleaned wafers were transferred to a solution containing 1.0% (v/v) APTES in toluene, incubated for 2 h at room temperature, and then rinsed thoroughly with toluene. The silanized wafers were activated by incubation in a 0.1% (v/v) 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. The activated wafers were immersed in a DNA solution (1.0×10^{-7} M) for 10 h at 4 °C. After rinsing with a buffer, the functionalized wafers were stored in PBS at 4 °C until use.

AFM silicon nitride (Si_3N_4) tips (type: NP with a radius of 20–60 nm, from Veeco, Santa Barbara, CA, USA) were used in the experiments. The spring constants of the tips, calibrated by the thermal fluctuation method [16], were in the range of 0.040–0.065 N/m. The tips were first cleaned according to previously reported procedures [15]. The cleaned tips were transferred to a solution of 1.0% (v/v) MPTMS in toluene, incubated for 2 h at room temperature, and then rinsed thoroughly with toluene. The silanized tips were activated by incubation in 1 mg/ml NHS–PEG–MAL in dimethyl sulfoxide for 3 h at room temperature [17], and then rinsed thoroughly with dimethyl sulfoxide to remove any unbound NHS–PEG–MAL. The activated tips were immersed into a protein (TINY, GST or mutants of TINY) solution (2 μg ml^{-1} in PBS) and incubated at room temperature for 0.5 h. After rinsing with PBS, the protein-modified tips were stored in PBS at 4 °C until use.

2.2.2. AFM force measurement

All AFM force measurements were performed with Nano Scope IV AFM (Veeco, Santa Barbara, CA). Force–distance curves were obtained in a liquid cell filled with the freshly prepared PBS buffer. The loading rates of the force–distance measurements were in the range of 3.19×10^{4}–5.18×10^{4} pN/s. The force curves were recorded and analyzed by the Nanoscope 5.30b4 software (Veeco, Santa Barbara, CA).

2.2.3. Yeast one-hybrid assay

Construction of DRE reporter plasmids and selection of the yeast reporter system were performed as described previously [18]. The full-length coding region of wild type and mutants of *TINY* were excised with EcoRI and SalI from pBluescriptII SK plasmids and ligated into the corresponding sites of pGAD424 vector (MATCHMAKER one-hybrid system, Clontech). The yeast YM4271/$His^{+}Ura^{+}$ reporter containing dual *HIS* and *lacZ* reporter genes was transformed by LiAc-polyethylene-glycol method according to the protocol of the One-Hybrid System (Clontech) by using 1 μg of plasmid with pGAD424, wild type *TINY*, V14A or E19D, respectively. Yeast cells were grown on a selective medium of SD/$His^{-}Ura^{-}Leu^{-}$ for 18 h. Quantitative β-galactosidase activity, expressed in Miller units, was measured as described in the Yeast Protocols Handbook (Clontech) using *o*-nitrophenyl-*b*-D-galactopyranoside as a substrate. Each value of β-galactosidase activity were averaged from six experiments.

3. Results and discussion

3.1. Measuring single molecule force of TINY and DRE element

The force measurement was carried out with the protein immobilized AFM tips and the DNA element modified substrates. As previously reported, the density of the protein on the tips was controlled to be low by using the solution with low protein concentration during the immobilization [6]. This ensured only one pair of protein and DNA molecules was measured. In addition, a PEG chain (20–40 nm long) was used to link the protein to the tip, which was intended to differentiate the specific binding from nonspecific interaction [19–21]. The typical force–distance curves are shown in Fig. 1A. While the random appearances of the first peaks were caused by the nonspecific interaction between the AFM tip and substrate [19], the second peaks, which appeared about 20–40 nm away from separation of the tip and substrate, represented the specific force between TINY and its DNA responsive elements. When the solution of free TINY (2 μg/ml) or the DNA element (1×10^{-7} mol/l) was injected for the blocking experiment, the second peaks were hardly detected, but the random appearance of the first peaks did not change much (shown in Fig. 1B).

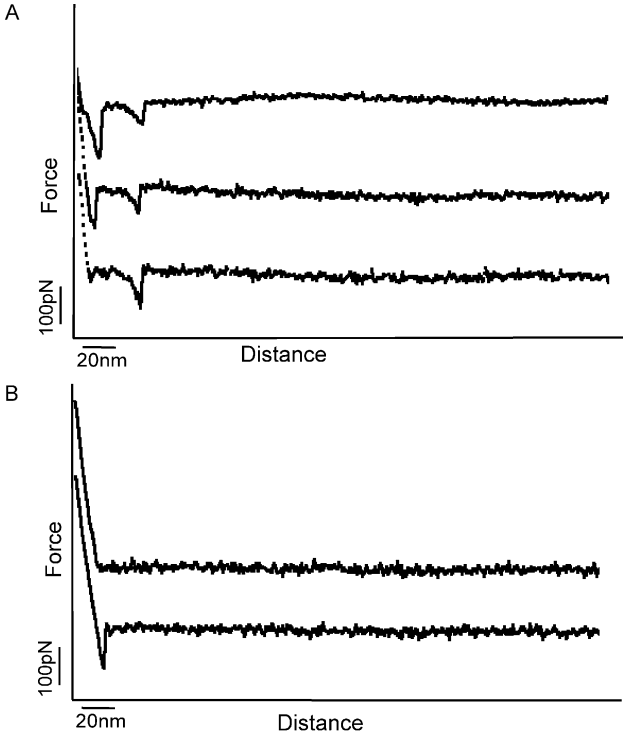

Fig. 1. (A) Representative force–distance curves between TINY modified AFM tip and DRE element (ACCGAC) modified substrate in the PBS buffer. (B) Representative force–distance curves after blocking the specific binding of TINY to DRE with the solution of free TINY.

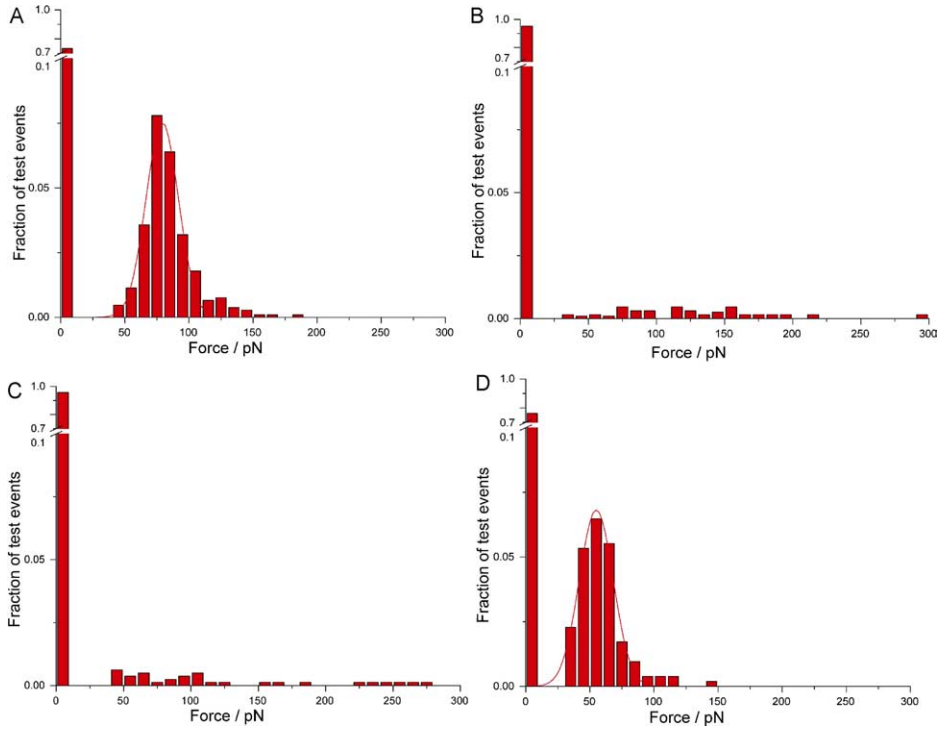

Fig. 2. Histograms of the binding forces of TINY-GST/ACCGAC (A), TINY-GST/ACCGAC after blocking with TINY (B), V14A-GST/ACCGAC (C), and E19D-GST/ACCGAC (D). (▭, experimental data; —, theoretical Gaussian distribution curve).

Moreover, the histogram of the detected forces did not fit to Gaussian distribution either (shown in Fig. 2B). Therefore, the single molecular interaction force between TINY and the DRE element was directly extracted from the force curves by measuring the force difference between the break point (the lowest point) of the second peak and baseline.

For the measurement of each set of protein–DNA, such as TINY–ACCGAC element, about 300 force curves were recorded to form the histogram of the force distribution, as shown in Fig. 2A. The single maximum in the histogram by Gaussian fitting further demonstrated that the single molecule force was measured [22–25]. The mean values of most probable single molecular interaction force (from three experiments) of TINY–DRE ACCGAC and TINY–DRE GCCGAC were determined as 83.5 ± 3.4 and 81.4 ± 4.9 pN, respectively. The binding probabilities of TINY and the DRE element were ranged from 20 to 25% (Fig. 3). The measured single molecule forces of TINY–DRE element and their binding probabilities all fell within the ranges obtained in the previously reported single molecule force study of noncovalent binding between two biomolecules [24,26–27].

Besides the blocking experiment, we also measured the force between GST-modified AFM tip and DRE element modified substrate as a control. In those cases, the peak represented the specific interaction of protein and DNA rarely appeared and the similar force–distance curves like the blocking experiment shown in Fig. 1B were obtained most of the time. The binding probability decreased to about 6% (Fig. 3), and the histogram of the detected forces did not fit to Gaussian distribution either. This further confirmed that although the GST fusion TINY was used, only the specific binding force between TINY and the DNA elements was measured. Like ZmDREB1A, which belongs to the same EREBP subfamily as TINY, TINY binds with both ACCGAC and GCCGAC with similar affinity.

3.2. Effect of single base mutation in DRE core sequence

It is known that mutation experiments are often needed in studying the interaction of a transcription factor and its DNA responsive element [6]. Under the same experimental conditions mentioned above, the force measurement with one point mutation of DRE core sequence was carried out. When

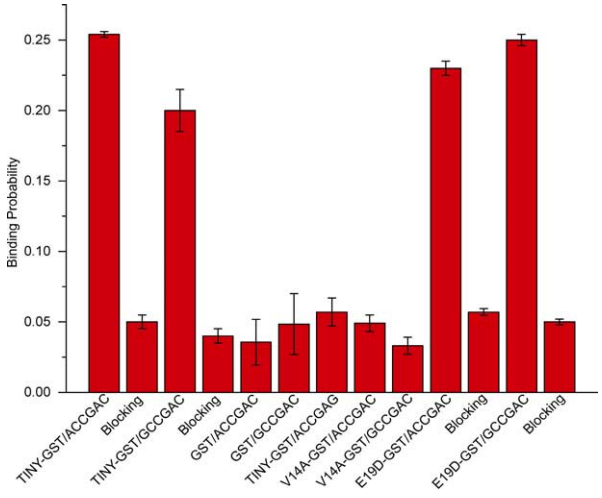

Fig. 3. The binding probabilities obtained with different proteins modified tips and different DNA modified substrates.

Table 1
Different *LacZ* expression in yeast reporter cells transformed with wild type and mutants of *TINY*

Reporter strains	(pGAD424 + TINY)/ DRE(ACCGAC)	(pGAD424 + E19D)/ DRE(ACCGAC)	(pGAD424 + V14A)/ DRE(ACCGAC)	pGAD424/DRE(ACCGAC)
β-Galactosidase activity (u[a])	0.240 ± 0.015	0.091 ± 0.009	0.035 ± 0.001	0.028 ± 0.002

[a] Miller units.

the DRE core sequence was mutated from ACCGA*C* to ACCGA*G*, or from GCCGA*C* to GCCGA*G*, no specific interaction was detected, according to the greatly decreased binding probability (Fig. 3) and randomly distributed force histogram. This further demonstrated the high sensitivity of AFM in measuring the interaction force between a transcription factor and its DNA element as we reported earlier [6].

3.3. Effect of single amino acid mutation of TINY

In the family of AP2/EREBP transcription factors, the amino acid residues at the 14th Val and 19th Glu are known to be absolutely conserved in the DNA binding domain [28]. To assess the functional significance of these two residues in TINY, we mutated the 14V to Ala and 19E to Asp, respectively. The effects of the mutations on TINY binding to the DRE element were examined by AFM. The results showed that the 14th site mutation (from Val to Ala) seriously inhibited the binding ability of TINY (Figs. 2C and 3). The binding probability between V14A and DRE A/GCCGAC dramatically decreases to about 5% and 3.3% from about 25% and 20%, respectively. This result was also in agreement with that from the gel shift assays (data not shown).

However, under the same condition, the specific binding of E19D to DRE element was detected. This was in contrast to V14A mentioned above and the mutant of ZmDREB1A [6] we previously reported, where the single amino acid mutation resulted in the total loss in binding affinity between the mutants and the DRE element. The single molecule forces between E19D and DRE A/GCCGAC were 54.1 ± 5.6 and 56.8 ± 4.7 pN, respectively. Their binding probabilities were about 20%, which was much higher than those with V14A and in the control experiments, and the histograms of force distribution fitted well to Gaussian distribution (Figs. 2D and 3). Comparing to the wild type TINY, the binding force was smaller, but the binding probability was about the same.

3.4. Relationship between the binding force and the gene activation activity

To investigate whether there is a relationship between the measured binding force and the gene activation regulated by the transcription factor, β-galactosidase activity assay was performed with wild type or mutants of TINY using yeast one hybrid experiment. If the transcription factor expressed in yeast binds to the target sequence (i.e. DRE element) and then activates the transcription of downstream reporter gene of *lacZ*, the activity of β-galactosidase (the product of *lacZ* gene) can be detected. It is known that the activation capability of a transcription factor is affected by the binding ability of its DNA-binding domain with the target sequence, as well as the intrinsic characteristic of its activation domain. Since the wild type and the 19th or 14th mutant TINY should have the same activation domain, the weakened binding to the target DRE sequence would result in the lower activation of *lacZ* transcription, thus the lower β-galactosidase production and activity in yeast. As expected, our results (Table 1) showed the decreased the enzyme activity in the yeast transformed with E19D (about 38% of that with the wild type). However, it did show the higher β-galactosidase activity than the blank control where only pGAD424 was used. This correlated well with the single molecule force measurement that E19D had significant binding capability with DRE but the binding was weaker than that of the wild type TINY. Moreover, for the mutant V14A which showed no specific binding force with the DNA element, no significant β-galactosidase activity was detected either comparing to the blank control.

It is general believed the biological function of transcription factor is mediated by its specific interaction with the DNA responsive elements, including the binding strength. However, no direct force information of the transcription factor and DNA element has been obtained until the recent application of AFM measurement [6]. The results in this work provided, for the first time, the experiment evidence of the relationship between the strength of the binding force and the activation capability of the transcription factor. Therefore, the single molecular force measurement is not only expected to be a sensitive method in the quantitation of binding force of a transcription factor to the DNA element and the investigation of its DNA binding preference, but also a simple method in the predication of gene expression regulated by the transcription factor.

4. Conclusion

In this work, we have applied AFM to measure the single molecule specific interaction forces of the transcription factor TINY and its mutants with DRE element. This is based on our earlier work of single molecule force study of transcription factor ZmDREB1A and DRE [6]. Although a different immobilization strategy was used here, similar results were obtained on measuring force between the protein and DRE element, and their mutants having a point mutation in the key binding sites. This result confirmed that single molecule force microscopy is a simple, quick, sensitive and reliable method for the characterization of binding between transcription factors and the DNA responsive elements as claimed in our previous work [6]. More

importantly, through the single amino acid mutation of the DNA binding domain in TINY, it is found that the stronger the binding force, the stronger the activation capability of TINY. Our results revealed the relationship between the single molecule force measured in vitro and the biological function of a transcription factor in vivo. As single molecule measurement by AFM could offer the quantitative interaction force information to analyze the specific interaction of transcription factor–DNA sequence, and to discriminate the difference in binding capability and gene activation capability, it is expected to have wide application in functional genomics research.

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