

A modified sensor chip for surface plasmon resonance enables a rapid determination of sequence specificity of DNA-binding proteins

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Abstract A novel method is described which rapidly determines specificity of DNA-binding proteins using a surface plasmon resonance (SPR) sensor chip. An oligohistidine-tagged DNA-binding domain of a transcription factor, NtERF2, was immobilised via nitrilotriacetic acid ligands to a sensor chip with an attenuated degree of carboxymethylation. DNA molecules were selected from a pool of randomised oligomers through binding to the immobilised protein and amplified by PCR. After several cycles of selection, during which binding was monitored by SPR, DNA sequences containing a consensus sequence were determined. The time necessary for one cycle is ~ 50 min, which is shorter than existing methods.

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Key words: Random oligonucleotide selection; Surface plasmon resonance; Sensor chip; GCC-box

1. Introduction

After the completion of whole genome sequences, a great number of genes encoding transcription factor homologues have been identified [1]. To reveal the functions of those transcription factors, and to identify the networks for transcriptional control, it is essential to determine their DNA-binding specificity. For that purpose, nucleic acids containing recognition sequences have been selected from a randomised oligomer pool through binding to their target proteins, in which the nucleic acids forming the complexes with proteins are separated and amplified by polymerase chain reaction (PCR) [2–7]. The separation has been carried out by various methods such as immunoprecipitation [2,5], affinity separation by columns or beads [4], polyacrylamide gel electrophoresis (PAGE) [3,6], and nitrocellulose membrane filtration [6,7], which are, however, generally time-consuming. To deal with a large number of DNA-binding proteins, development of rapid and/or automated systems is preferable. Recently, a pipetting robot was utilised in an automated protocol for selecting RNA ap-

tamers [8]. It is also important to develop a method with the least requirement for protein, in order to apply the method to proteins with limited expression. For that purpose, chip-based methods, which are currently utilised for microscopic array experiments [9–11], would be suitable.

The surface plasmon resonance (SPR) method is a chip-based method widely used for the detection of interactions between biomolecules [12]. Notable advantages of this method are: quantitative and simultaneous detection of binding; high sensitivity which enables detection of molecules in the picogram range; semi-automated protocols for solution injection and washing; capability of determining rate constants of binding and dissociation, as well as equilibrium binding constants; reproducibility of the experiments after removing the molecules from the chip surface; etc. Therefore, SPR is likely to be a suitable approach for theoretically designed, rapid and semi-automated analyses, which are applicable to proteins with limited expression.

Here we report a method for rapidly determining sequence specificity of DNA-binding proteins by using a SPR sensor chip. By using the method, the sequence specificity of the tobacco NtERF2 protein, formerly called EREBP2 [13], was determined.

NtERF2 is a member of the ethylene-responsive element-binding factors (ERFs) which are plant-specific transcription factors involved in a variety of signal transduction systems including that for ethylene-induced responses [13]. A highly conserved DNA-binding domain (DBD) of tobacco ERFs has been shown to recognise a sequence containing the AGCCG-CC motif termed the GCC-box [13,14]. The tertiary structure of the DBD of *Arabidopsis* AtERF1 in complex with the GCC-box DNA fragment has been determined, in which the three-stranded β -sheet of AtERF1 binds to the DNA major groove by forming specific hydrogen bonds [15]. Single-base substitution and in vitro binding experiments showed that the first A base of the GCC-box does not seem to be recognised by ERFs [14], although it is highly conserved among the GCC-box sequences identified in the plant genomes [16]. Therefore, it is necessary to determine the recognition sequence of the ERFs by selection from randomised oligomers and to compare the results with previous ones.

2. Materials and methods

2.1. Preparation of the oligohistidine-tagged DBD of NtERF2 and double-stranded DNA containing randomised sequences

The coding region for the DBD fragment (Thr88–Pro164) of NtERF2, which includes its N- and C-terminal flanking regions of 10 and eight amino acids, respectively, was synthesised by PCR and

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Abbreviations: DBD, DNA-binding domain; dsDNA, double-stranded DNA; EMSA, electrophoresis mobility shift assay; ERF, ethylene-responsive element-binding factor; FITC, fluorescein isothiocyanate; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SPR, surface plasmon resonance; ssDNA, single-stranded DNA

cloned into the pET16b plasmid (Novagen). Expression was induced in *Escherichia coli* strain BL21 (DE3) pLysS (Novagen) at 37°C by addition of IPTG to a final concentration of 1 mM. The expressed DBD fragment (MW 11 kDa) was then purified using the His Trap[®] protein purification kit (Pharmacia Biotech), which gave a purity of more than 98% as confirmed by sodium dodecyl sulphate–PAGE. The protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce), and further confirmed by the method of Gill and von Hippel [17].

A 60-mer single-stranded DNA (ssDNA) containing 10 randomised bases in the centre (5'-CTGTCAGTGATGCATATGAACGAATNNNNNNNNNAATCAACGACATTAGGATCCTTAGC-3') and the 5' and 3' primers (5'-CTGTCAGTGATGCATATGAA-3' and 5'-GCTAAGGATCCTAATGTCGT-3', respectively) were synthesised chemically by Espec Oligo (Tsukuba, Japan). The 60-mer double-stranded DNA (dsDNA) containing randomised base pairs (average MW 40 kDa) was produced using the ssDNA and the 3' primer driven by the large fragment of DNA polymerase I (Boehringer Mannheim). The produced dsDNA was purified using the QIAquick Nucleotide Removal Kit (Qiagen), and dissolved in reaction buffer (25 mM HEPES pH 7.0, 40 mM KCl, 0.2 mM EDTA, and 0.005% Tween 20). The concentration of the dsDNA was estimated by A_{260} to be 1.7 μ M.

2.2. Outline of selection procedure

The selection scheme is summarised in Fig. 1, and consists of immobilisation of an oligohistidine-tagged protein to the SPR sensor chip, binding of DNA oligomers containing randomised sequences to the chip, washing, release and collection of the protein–DNA complex, PCR amplification, and sequence analysis of the DNA. The processes were repeated several times, during which the degree of binding was monitored by SPR measurement.

2.3. SPR experiments

A BIACORE X (Biacore) device was used for the real-time detection of SPR as the 'sensorgram' (Fig. 2). All the reactions in the SPR flow cells were carried out at 25°C. A nitrilotriacetic acid (NTA)-coupled sensor chip was prepared by the amine coupling protocol [18], essentially according to Gershon and Khilko [19]. *N*-(5-Amino-1-carboxypentyl)iminodiacetic acid (50 mM, Dojindo, Kumamoto, Japan) dissolved in 50 mM borate buffer (pH 8.5) was chemically bound to a B1 sensor chip (Biacore) by using an amine coupling kit (Biacore). Since BIACORE X contains two flow cells for a sensor chip, NTA was coupled to one of the flow cells so that the other was treated as the control.

Solutes described below were dissolved in the reaction buffer described above unless otherwise stated. After 10 μ l of 500 μ M NiSO₄, 20 μ l of 50 nM oligohistidine-tagged protein (2 pmol or 22 ng, including an excess volume of 20 μ l to fill the internal route space) was injected into the flow cell connected with the NTA-coupled sensor chip at a flow rate of 10 μ l/min (Fig. 2). To remove proteins weakly associated to the chip, probably through electrostatic attraction, 20 μ l of 1 M KCl was injected. The quantity of the protein finally immobilised on the chip was approximately 1000 response units, corresponding to ~ 1.0 ng/mm² [18].

dsDNA oligomers were injected over the protein-immobilised chip surface for 2 min at a flow rate of 10 μ l/min, followed by constant flow of the buffer for 4 min including a wash process. The protein–DNA complex was eluted by injecting 10 μ l of 350 mM EDTA, and was collected from the outlet port of the flow cell.

2.4. PCR amplification of the selected DNA and determination of the binding sequence

The selected DNA (1 μ l solution) was amplified using pyroBest DNA polymerase (Takara) in a reaction volume of 100 μ l, to which an extra amount of MgCl₂ was added to a final concentration of 7 mM in order to compensate the EDTA brought over from the elution of the protein–DNA complex. The 5' and 3' primers described above were used for amplification, while the same primers labelled with fluorescein isothiocyanate (FITC) at their 5' ends were used for electrophoretic mobility shift assay (EMSA). A 15-cycle PCR protocol was used which consisted of 95°C for 1 min, 55°C for 0.5 min, and 72°C for 0.5 min at each cycle. The PCR products were purified using the QIAquick Nucleotide Removal Kit (Qiagen), and eluted into 40 μ l of the reaction buffer ready for the next cycle of selection. The population of selected DNA was monitored either by SPR or by

EMSA on 8% polyacrylamide gels. In EMSA, 0.1 mg/ml bovine serum albumin and 5 mM dithiothreitol were added to the reaction buffer. After the majority of population of the amplified DNA was purified by gel electrophoresis through cutting out of the corresponding band, and cloned into the pUC119 plasmid, 40–50 insert-containing colonies were selected for sequence determination. Approximately 30 successful sequences were aligned using the program Clustal X [20]. The frequency of each nucleotide appearing in the aligned position of the selected sequences was calculated leading to the establishment of the recognition sequence motif.

3. Results

3.1. Optimisation of the NTA-coupled sensor chip

Before selection of the randomised oligomers, preliminary experiments were conducted for the binding of DNA to the protein-immobilised sensor chip. Double-stranded 13-mer GCC-box DNA (5'-GCTAGCCGCCAGC-3') did not appear to bind NtERF2 DBD immobilised to a standard NTA sensor chip (Biacore) (Fig. 3). This is probably due to the electrostatic repulsion between the negatively charged carboxymethyl groups of the chip surface and DNA molecules. To overcome this problem, sensor chip B1 (Biacore), which has a surface dextran matrix with an attenuated degree of carboxymethylation, was modified so that it possesses NTA groups. The 13-mer DNA significantly bound NtERF2 DBD immobilised through the NTA group on the B1 chip (Fig. 3). An apparent binding constant of 5.2×10^6 M⁻¹ was determined by SPR equilibrium analysis (data not shown). Although the response value decreased after the injection of DNA, it is negligible compared with the total immobilisation level (~ 1000 RU). The immobilised NtERF2 DBD remains stably bound on the chip surface with a half-life of 10 h, which is long enough for the present purpose.

3.2. Selection of DNA

Statistically a sequence containing 10 randomised nucleotides will give $4^{10} \approx 10^6$ different molecules. Since the initial DNA solution resulting from the Klenow reaction was estimated to be 1.7 μ M as described in Section 2, the quantity of the DNA initially injected onto the SPR sensor chip (20 μ l) is ~ 35 pmol, corresponding to $\sim 2 \times 10^{13}$ molecules, which is much higher than the number of possible different molecules.

In the selection scheme (Fig. 1), binding, washing, and elution procedures in a selection cycle take less than 10 min (Fig. 2b). It should be emphasised that real-time monitoring by SPR indicates that the appropriate amount of protein was immobilised on the chip (Fig. 2a) and that the DNA was bound significantly and dissociating gradually (Fig. 2b). The information greatly facilitated the correct setting of the period for each procedure. It is also important to note that the procedures were conducted on a single chip in a semi-automated process. Including PCR (~ 30 min) and purification (~ 5 min), the total duration for one cycle is approximately 45 min. Although immobilisation of the protein is necessary for every cycle, it does not make the duration longer since it can be done in parallel with the PCR and purification processes. Therefore, a selection procedure consisting of five to seven cycles can be carried out in a single day, which will be followed by cloning and sequencing for another 2 days. The used protein quantity is 22 ng for a cycle, which will enable application to proteins with limited expression.

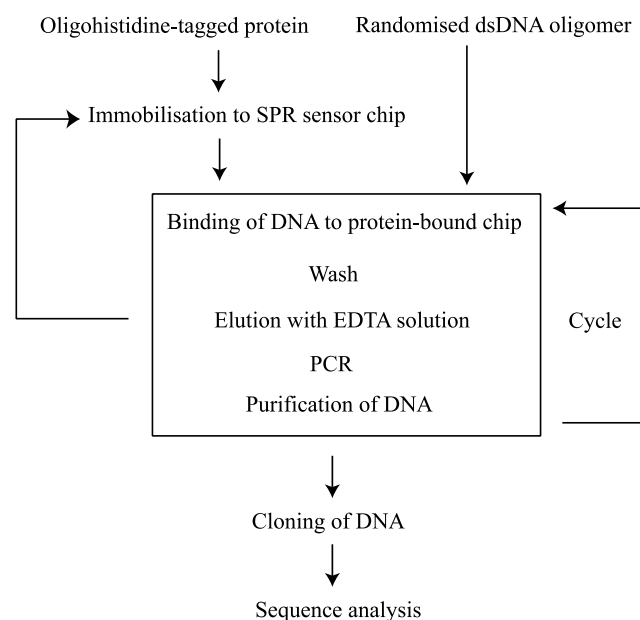


Fig. 1. Summary of the selection scheme.

3.2.1. Monitoring the DNA binding during the selection cycles. In the SPR measurement, the response after injection of the DNA solution is apparently larger than before the injection in flow cell 2 where the protein is immobilised, but not in flow cell 1 (Fig. 2b). The difference sensorgram indi-

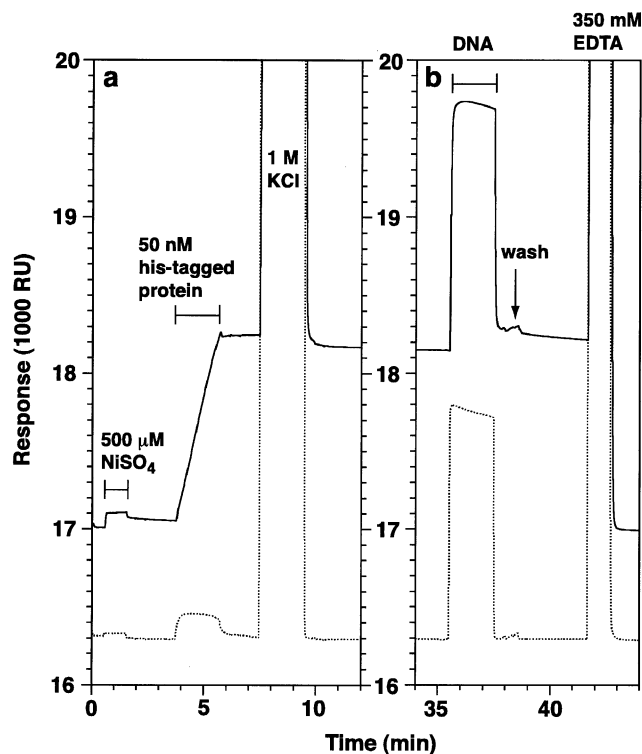


Fig. 2. Sensorgrams of the two flow cells during the seventh cycle of selection, i.e. protein immobilisation (a), DNA binding, and release of the protein–DNA complex (b). NTA is coupled to flow cell 2 (solid lines), but not to flow cell 1 (dotted lines). 1000 response units corresponds to approximately 1.0 ng/mm² [18].

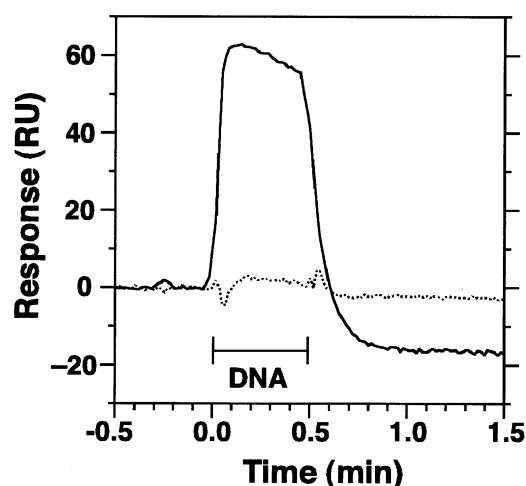


Fig. 3. Binding of DNA to two types of protein-immobilised chips, the NTA-coupled B1 sensor chip (solid line) and the standard NTA chip (broken line), shown by difference sensorgrams (response in flow cell 1 was subtracted from that in flow cell 2). During the period from 0 min to 0.5 min, 1 μM solution of 13-mer dsDNA (5'-GCTAGCCGCCAGC-3') was injected.

cates that DNA binds to the chip with the apparent half-life of 4.4 min (Fig. 4a, solid line). This shows that the complex of the protein and the 60-mer dsDNA is much more stable than that with the 13-mer GCC-box DNA (Fig. 3), probably because of the enhanced electrostatic attraction between the protein and DNA. The apparent DNA binding just before elution, shown by the arrow in Fig. 4a, increases during the selection procedure (Fig. 4b), which suggests that the DNA oligomers that bind to the NtERF2 DBD were successfully amplified following the selection cycles. Although the apparent increase over a single cycle is ~1.3-fold until the response reaches ~160 RU (Fig. 4b), the efficiency of the amplification of the selected sequence should be much higher since the process also involves the decrease in the contribution of non-specific binding, as well as the generation of PCR artefacts, as described below.

The binding of the selected DNAs was also examined by PAGE (Fig. 5a). During cycles 1–6, the density of the bands shifted by the binding of the protein gradually increased while those of the free DNA bands decreased (Fig. 5a). This clearly indicates that the DNA molecules containing the recognition sequence are successfully selected. After cycle 7, the density of the shifted bands appears to reach a saturated level. It should be noted that bands with a larger molecular weight appeared after cycle 8 (Fig. 5a). In the control without protein (Fig. 5b), however, similar bands for DNA fragments with a larger molecular weight also appeared, probably due to an artefact during the PCR reaction. Therefore, the dependence of the apparent binding in SPR on cycle numbers (Fig. 4b) can be explained by the amplification of selected DNA, a decrease in non-specific binding, and generation of PCR artefacts. It is still useful to observe the increment in the SPR response during the selection cycles since the information can be obtained instantly. The PCR product from the seventh cycle showed the saturated level of the shifted band density, and is free of PCR artefacts, which was subjected to sequence determination.

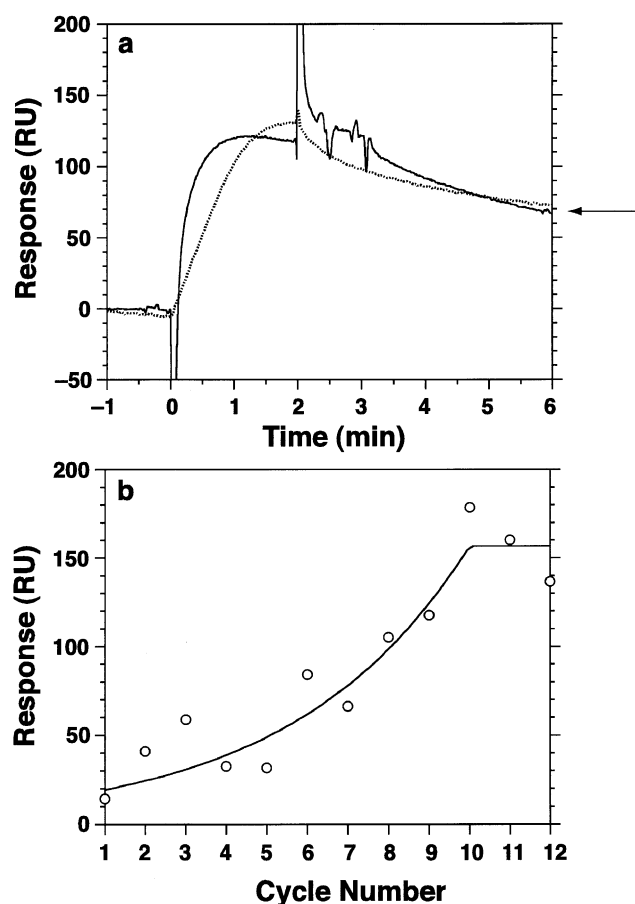


Fig. 4. Apparent binding of DNA to the SPR sensor chip. The difference sensorgram (flow cell 2 minus flow cell 1) (a). During the period from 0 min to 2 min, the DNA mixture at the seventh cycle of the selection (solid line) which is equivalent to Fig. 2b or 5 nM solution of synthesised 60-mer dsDNA containing a finally selected sequence (GGCGCCGCCG) (broken line) was injected. The response values were calibrated with reference to the values before the injection. The value just before the elution (arrow in a) is shown as a function of cycle number (b). The data were non-linearly fitted to an exponential curve with a limit.

3.3. Selected sequences

The determined sequences of 33 DNA oligomers are shown in Table 1. The selected sequences are highly G/C-rich with a GC content of $\sim 90\%$. A computational alignment using the Clustal X program [20] deduced a consensus sequence of CGCCGCC (Table 1). This is very similar to the putative GCC-box sequence of AGCCGCC, although there is C at the beginning instead of A, which will be further discussed below.

One of the selected dsDNAs (5'-CTGTCA GTGATGCA-0TATGAACGAATGGCGCCGCCGAATCAACGACATT-AGGATCCTTAGC-3'; selected sequence underlined, which is equivalent to sequences 21 and 22 in Table 1) was chemically synthesised and subjected to SPR analysis of binding to the NtERF2 DBD (Fig. 4a, broken line). The binding profile is similar to that of the DNA mixture of the seventh cycle of the selection. The kinetic association and dissociation rate constants are estimated to be $2.4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $2.1 \times 10^{-3} \text{ s}^{-1}$, respectively. Then, the equilibrium binding constant is $1.2 \times 10^9 \text{ M}^{-1}$, which is of the same order as, but somewhat less than, a reported value between NtERF2 DBD and a

GCC-box DNA, $9.1 \times 10^9 \text{ M}^{-1}$ [21]. This is probably due to the electrostatic repulsion between the negative charges in the DNA molecule and chip surface matrix, despite an attenuated degree of carboxymethylation in the B1 chip.

4. Discussion

4.1. Comparison with other existing methods

Major advantages of the present selection method using the SPR sensor chip are likely to be its rapidity, the small quantity of protein required, real-time monitoring, and semi-automated protocols. A single cycle takes approximately 10 min for the binding, washing, and elution procedures on the SPR chip, approximately 30 min for PCR, and 5 min for the DNA purification, which is less than 50 min in total. Since all the following methods involve PCR, it is better to compare the duration without PCR, for simplicity, which is approximately 15 min for the present method. The necessary time was compared with those in other methods (Table 2).

The selection methods with immunoprecipitation [2,5,22,23] typically involve a binding reaction for the target protein and DNA, that for protein and its antibody, that for antibody and beads, centrifugation to recover the complexes, washing, and elution of DNA. Each binding reaction takes 15 min to hours, or even overnight, which makes the total duration for the binding reactions 80 min to overnight [2,5,22,23] (Table 2). Washing and elution involving several centrifugation and resuspension steps probably takes at least 10 min, although there is no apparent description in the literature. Therefore

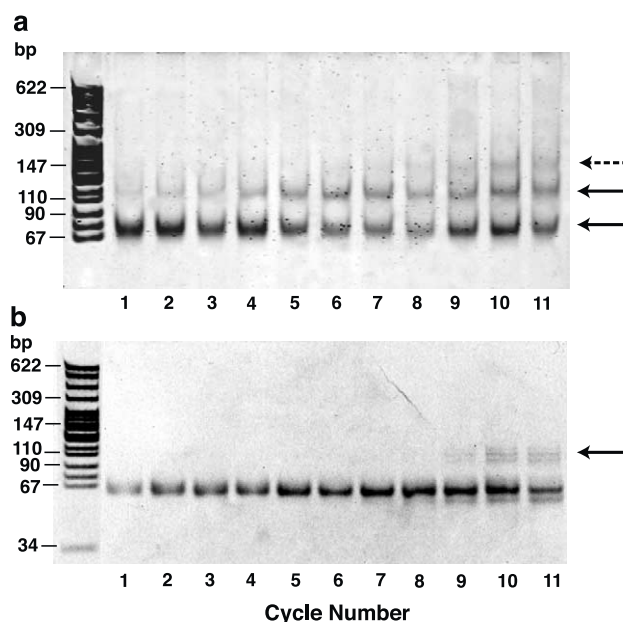


Fig. 5. PAGE analyses of the PCR products in the presence of 100 nM protein (a) or in the absence of the protein (b). In panel a bands for $\sim 20 \text{ nM}$ DNA solutions were detected by fluorescence emission of FITC. In panel b bands for $\sim 4 \mu\text{M}$ DNA solutions were detected by ethidium bromide staining. The molecular markers in panel a were also detected by ethidium bromide staining. The upper solid arrow in panel a shows the DNA bands shifted by the protein binding while the lower solid arrow shows those of unbound DNAs. The dashed arrow in panel a and the solid arrow in panel b show bands of concatenated DNAs with larger molecular weight caused by PCR.

Table 1
Selected oligonucleotides

Randomised Sequence		---NNNNNNNNNN---
Selected Sequences	1	--1-3-5-7-9-11-
	2	--CNGCGCGCC--
	3	--CAGCGCGCC--
	4	---GTCCGCGCC-
	5	---GGCGCGCCN-
	6	---CGCGCGCC-
	7	--TCCGCGCGCC--
	8	---NCGCGCGC--
	9	--CGACTGCGCC--
	10	---TGCGCGGACN-
	11	---GGCGCGCCAN-
	12	---GGCGCGCCNN-
	13	---AGATGACAGG
	14	---TCCGCGCATC
	15	--CGCGCGCGCA--
	16	--CGCGCGCGCC--
	17	--CGCGCGCGCG--
	18	--AGCGCGCGCC--
	19	--TGCGCGCGCC--
	20	--GGCGCGCGCA--
	21	--GGCGCGCGCG--
	22	--GGCGCGCGCG--
	23	--CGCGCGCGCG--
	24	---ACGCGCGCGT-
	25	--CGCGCGCGCC--
	26	--CACCGCGCGC--
	27	--CACCGCGCGC--
	28	--CGCGCGCGCC--
	29	--TCCGCGCGCC--
	30	---CGCGCGCGCG--
	31	--GGCGCGCGCG--
	32	---GTGGCGCGCG--
	33	---ACATGCGCGG--
Frequency (%)		Position
		4 5 6 7 8 9 10
Nucleotide		
A		6.1 0 3.0 0 3.0 9.1 0
G		3.0 93.9 18.2 6.1 97.0 6.1 3.0
T		12.1 3.0 3.0 3.0 0 0 0
C		78.8 3.0 78.8 90.9 0 84.8 97.0
Consensus		C G C C G C C

the total duration for a single cycle, excluding PCR, should be approximately 100 min to overnight.

The methods involving affinity columns [4,24] or beads [25,26] are basically similar to the above methods, which involve a reaction for the binding of the nucleotide to the protein-immobilised column or beads, washing, and elution of the nucleotide. The necessary periods that have appeared in the literature are described in Table 2. Likewise, the necessary protocols and periods for the methods utilising gel electrophoresis [3,6,27,28] and nitrocellulose membranes [6,7,29–31] seen in the literature are summarised in Table 2.

Therefore, the present method with the SPR sensor chip, which requires a duration of approximately 15 min except for PCR, is much faster than the above existing methods (Table 2). The necessary protein quantity is similar to the lowest one in the literature, and smaller than the typical quantities required for the existing methods. Real-time monitoring is possible only in the present method. Together with the programmable protocols on a single chip and the reproducibility of the experiments after removing the molecules from the chip surface, it is likely to be suitable for an automated system. Another version of the BIACORE devices, BIACORE 2000, is equipped with an auto-sampler which can deal with a number of samples sequentially. Therefore, if it is further combined with a PCR unit, a fully automated system similar to the robotics system [8] but also enabling the real-time monitoring convenient for the appropriate design of the experiments, would be possible in the future.

We should also note that there is a disadvantage in the present method, caused by the electrostatic property of the sensor chip. The electrostatic repulsion between the negative charges of the chip matrix and DNA attenuated the binding. The electrostatic repulsion or attraction will be reduced when the salt concentration of the buffer is increased. This is not applicable, however, to protein–DNA interaction, since increasing the ionic strength will also attenuate the binding of

Table 2
Comparison of the selection methods

Method	Protocol	Time ^a	References
SPR	Binding	2 min	Present study
	Washing	4 min	
	Elution	1 min	
	DNA purification	5 min	
		(total: ~15 min) ^b	
Immunoprecipitation	Binding	80 min–overnight	[3,6,22,23]
	Washing	N.A.	
	Elution	N.A.	
Affinity column or beads		(total: ~100 min–1 day) ^b	[5,24–26]
	Binding	10–30 min	
	Washing	N.A.	
	Elution	10 min	
Gel electrophoresis		(total: ~30–60 min) ^b	[4,7,27,28]
	Binding	15 min	
	Electrophoresis	2.5–4 h	
	Drying	3 h	
	Film exposure	4–5 h	
	Elution	3 h	
Nitrocellulose membrane		(total: ~1 day) ^b	[7,8,29–31]
	Binding	4 min–overnight	
	Filtering	N.A.	
	Washing	15 min	
	Elution	10 min	
		(total: ~30 min–1 day) ^b	

^aValues for a single cycle, obtained from the literature.

^bAn approximate value of the total time for the listed protocols, which excludes PCR. N.A.: has not appeared in the literature.

the molecules [32]. In the present study, the sensor chip with an attenuated degree of carboxymethylation enabled the selection of DNA molecules to which the NtERF2 protein binds. It is still probably better to keep the surface charges slightly negative, so that the non-specific binding of the DNA to the chip is minimised.

4.2. Sequence specificity of NtERF2 protein

The present selection experiment indicated that NtERF2 possesses specificity for the 'CGCCGCC' sequence (Table 1), although the putative GCC-box possesses a conserved 'AGCCGCC' sequence. The present result is consistent with our single-base substitution experiment, which showed that the first A of the GCC-box does not contribute to the stability of the protein–DNA complex [14]. This is also consistent with the tertiary structure of the AtERF1/GCC-box complex [15], i.e. although the first A has hydrophobic contacts with the Trp154 side chain, these are likely to still exist even when A is replaced by other bases. Therefore, the sequence 'GCCGCC' should be considered the core sequence of the GCC-box.

It should be noted that in the base substitution experiment, C in the first position is slightly preferred to the original A, although within the standard deviation [14]. It is also notable that a 'CGCCGCC' sequence appears in a promoter of an *Arabidopsis* chitinase gene [16]. At the position preceding CGCCGCC, i.e. position 3 in Table 1, ~80% of the selected sequences possess G or C. Also at position 2 in Table 1, ~60% are G or C. This suggests the possibility that an additional three G/C bases contribute to an elongation of the six-base GCC-box core, which increases the binding constant and/or binding rate constant through the apparent increase in the initial binding region.

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References

- [1] Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C.-Z., Keddie, J., Adam, L., Pienda, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., Pilgrim, M., Broun, P., Zhang, J.Z., Ghandehari, D., Sherman, B.K. and Yu, G.-L. (2000) *Science* 290, 2105–2110.
- [2] Kinzler, K.W. and Vogelstein, B. (1989) *Nucleic Acids Res.* 17, 3645–3653.
- [3] Blackwell, T.K. and Weintraub, H. (1990) *Science* 250, 1104–1110.
- [4] Ellington, A.D. and Szostak, J.W. (1990) *Nature* 346, 818–822.
- [5] Pollock, R. and Treisman, R. (1990) *Nucleic Acids Res.* 18, 6197–6204.
- [6] Thiesen, H.-J. and Bach, C. (1990) *Nucleic Acids Res.* 18, 3203–3209.
- [7] Tuerk, C. and Gold, L. (1990) *Science* 249, 505–510.
- [8] Cox, J.C. and Ellington, A.D. (2001) *Bioorg. Med. Chem.* 9, 2525–2531.
- [9] Lueking, A., Horn, M., Eickhoff, H., Büssow, K., Lehrach, H. and Walter, G. (1999) *Anal. Biochem.* 270, 102–111.
- [10] MacBeath, G. and Schreiber, S.L. (2000) *Science* 289, 1760–1763.
- [11] deWildt, R.M.T., Mundy, C.R., Gorick, B.D. and Tomlinson, I.M. (2000) *Nat. Biotechnol.* 18, 989–994.
- [12] Szabo, A., Stols, L. and Granzow, R. (1995) *Curr. Biol.* 5, 699–705.
- [13] Ohme-Takagi, M. and Shinshi, H. (1995) *Plant Cell* 7, 173–182.
- [14] Hao, D., Ohme-Takagi, M. and Sarai, A. (1998) *J. Biol. Chem.* 273, 26857–26861.
- [15] Allen, M.D., Yamasaki, K., Ohme-Takagi, M., Tatenno, M. and Suzuki, M. (1998) *EMBO J.* 17, 5484–5496.
- [16] Hart, C.M., Nagy, F. and Meins, F.Jr. (1993) *Plant Mol. Biol.* 11, 121–131.
- [17] Gill, S.C. and von Hippel, P.H. (1989) *Anal. Biochem.* 182, 319–326.
- [18] Johnsson, B., Löfås, S. and Lindquist, G. (1991) *Anal. Biochem.* 198, 268–277.
- [19] Gershon, P.D. and Khilko, S. (1995) *J. Immunol. Methods* 183, 65–76.
- [20] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) *Nucleic Acids Res.* 18, 3203–3209.
- [21] Hao, D., Yamasaki, K., Sarai, A. and Ohme-Takagi, M. (2002) *Biochemistry* 41, 4202–4208.
- [22] Wright, W.E., Binder, M. and Funk, W. (1991) *Mol. Cell. Biol.* 11, 4104–4110.
- [23] Whyatt, D.J., deBoer, E. and Grosveld, F. (1993) *EMBO J.* 12, 4993–5005.
- [24] Liaw, P.C.Y. and Brandl, C.J. (1994) *Yeast* 10, 771–787.
- [25] Pierrou, S., Enerbäck, S. and Carlsson, P. (1995) *Anal. Biochem.* 299, 99–105.
- [26] Hyde-DeRuyscher, R.P., Jennings, E. and Shenk, T. (1995) *Nucleic Acids Res.* 23, 4457–4465.
- [27] de Pater, S., Greco, V., Pham, K., Memelin, J. and Kijne, J. (1996) *Nucleic Acids Res.* 24, 4624–4631.
- [28] Zhou, Z., Corden, J.L. and Brown, T.R. (1997) *J. Biol. Chem.* 272, 8227–8235.
- [29] Nørby, P.L., Pallisgaard, N., Pedersen, F.S. and Jørgensen, P. (1992) *Nucleic Acids Res.* 20, 6317–6321.
- [30] Nishikawa, J.-I., Kitaura, M., Matsumoto, M., Imagawa, M. and Nishihara, T. (1994) *Nucleic Acids Res.* 22, 2902–2907.
- [31] Sakai, Y., Nakagawa, R., Sato, R. and Maeda, M. (1998) *Biochem. Biophys. Res. Commun.* 250, 682–688.
- [32] Record Jr., M.T., Anderson, C.F. and Lohman, T.M. (1978) *Q. Rev. Biophys.* 11, 103–178.