the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2009) **59**, 851–858 doi: 10.1111/j.1365-313X.2009.03903.x

TECHNICAL ADVANCE

The analysis of entire gene promoters by surface plasmon resonance

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Received 23 February 2009; revised 6 April 2009; accepted 23 April 2009; published online 27 May 2009.

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SUMMARY

We demonstrate that the biophysical technique of surface plasmon resonance (SPR) analysis, which has previously been used to measure transcription factor binding to short DNA molecules, can also be used to characterize interactions involving entire gene promoters. This discovery has two main implications that relate, respectively, to novel qualitative and quantitative uses of the SPR technique. Firstly, SPR analysis can be used qualitatively to test the capacity of any transcription factor to interact physically with its putative target genes. This application should prove particularly useful for the confirmation of predicted transcriptional interactions in model species, and for comparative studies of non-model species in which transcriptional interactions are not amenable to study by other methods. Secondly, SPR may be used quantitatively to characterize interactions between transcription factors and gene promoters containing multiple *cis*-acting sites. This application should prove useful for the detailed dissection of promoter function in known target genes. The qualitative and quantitative applications of the SPR analysis of whole promoters combine to make this a uniquely powerful technique, which should prove particularly useful in systems biology, evolutionary developmental biology and various branches of applied biology.

Keywords: surface plasmon resonance, transcription factor, transcriptional regulation, DNA–protein interaction, promoter.

INTRODUCTION

Transcriptional regulation lies at the heart of most biological processes in plants and other organisms. This form of regulation depends on physical interactions that take place between transcription factors and DNA binding sites present in the *cis*-regulatory regions (promoters etc.) of their target genes. However, the binding site preferences of transcription factors are often poorly defined or unknown, rendering difficult or impossible the direct characterization of their DNA binding interactions using existing *in vitro* methods. Furthermore, transcriptional regulation often depends on the positions and binding affinities of multiple sites present in gene promoters. Even in cases where binding site preferences are known, the existing *in vitro* techniques are largely incapable of characterizing interactions involving multiple binding sites.

Surface plasmon resonance (SPR) has been used to quantify many types of molecular interaction, including transcription factor binding to individual target sites. In this method, short DNA molecules are typically attached to the surface of a gold-coated chip, in contact with a solution containing a transcription factor of interest. Molecular interactions between these two components are then measured, using a dedicated SPR analyser, from the angular deflection of a band of extinction that occurs within a beam of plane-polarized light reflected from the surface of the chip. Under typical experimental conditions, the coupling of this optical deflection to molecular interactions extends for approximately 200 nm into the solution (Lukosz, 1997; Kunz and Cottier, 2006). We reasoned that this range should be sufficient to measure interactions involving immobilized

DNA molecules of considerable length, given that the non-linear average conformation of long DNA molecules would tend to bring interaction sites closer to the surface of the chip.

We present here a proof of concept for the use of SPR analysis to measure transcription factor binding to long DNA molecules, such as entire gene promoters. We show this technique to be capable of both qualitative use, to discriminate between target and non-target genes, and quantitative use, to integrate the effects of binding to multiple sites within long DNA molecules. SPR analysis can be performed using recombinant proteins and promoters from any species, and can thus be used to study transcriptional interactions in any organism, including non-models. We discuss the potential qualitative and quantitative uses of this novel application of SPR in various fields of plant biology in which transcriptional regulation is of key importance.

RESULTS

SPR analysis distinguishes qualitatively between target and non-target genes

In many cases, it would be useful to determine whether a gene of interest may be directly regulated by a given transcription factor. It is frequently not possible to predict such transcriptional interactions from the presence of consensus binding sites in promoter regions, firstly because binding preferences for the factor of interest may be unknown, and secondly because not all consensus sites occur in a DNA context that will permit binding. Conversely, cryptic sites may also occur that bind transcription factors with high affinity, but do not conform to the known consensus.

To test the use of SPR to discriminate qualitatively between target and non-target DNA, we studied DNA–protein interactions involving the well-characterized transcription factor LEAFY (LFY) from *Arabidopsis thaliana*. LFY controls floral patterning by inducing the expression of genes encoding several members of the MADS-box transcription factor family (Parcy *et al.*, 1998; Busch *et al.*, 1999; Lamb *et al.*, 2002). We compared the interactions of the C-terminal DNA binding domain of LFY (LFY-C) (Hamès *et al.*, 2008) with the promoters of two of its MADS-box target genes, *APETALA1* (*AP1*) and *APETALA3* (*AP3*), and with three negative control DNAs derived from the Epstein bar virus gene *BHLF1*, and from the *A. thaliana* genes *GAPA-2*, encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and *LONG HYPOCOTYL IN FAR-RED* (*HFR1*) (Fairchild *et al.*, 2000). The *AP1* and *AP3* DNA molecules used in these assays contained two and one consensus binding motifs for LFY, CCANTG(G/T), respectively. One of these sites in *AP1*, and the unique site in *AP3*, has been experimentally verified to bind LFY (Busch *et al.*, 1999). Among the negative control DNAs used, *GAPA-2* and *HFR1* contained four and one LFY-consensus motifs (as above), respectively, although these genes are not known or suspected to be regulated by LFY. Equal quantities, in arbitrary SPR response units (RU), of biotinylated DNA samples were immobilized in separate channels on streptavidivin-coated SPR chips. Interactions between LFY-C and immobilized DNA molecules were then monitored by SPR over a range of protein concentrations (Figure 1a–e). These analyses were performed in the constant presence of non-specific competitor DNA in solution, so as to avoid signal saturation by non-specific binding.

Differences between the target and non-target genes of LFY were visually apparent in the SPR interaction curves obtained: low concentrations of LFY-C (lower traces in Figure 1a–e) gave higher SPR responses to target (Figure 1a,b) than to non-target (Figure 1c–e) DNA. Moreover, at the end of protein injection, when LFY-C was removed from DNA by washing (shown by the decreasing traces), this dissociation occurred more rapidly from target than non-target DNA. The apparent equilibrium constant for dissociation (K_D^{App}) values were calculated from real-time interaction data using dedicated SPR analysis software (Table 1), assuming a 1:1 (Langmuir) binding model. Chi-squared test values of less than two for specific interactions (Table 1) indicated a good fit of this model to the observed data. As an independent verification, K_D^{App} values were also calculated from the linear gradients of graphs based on the equilibrium condition (Figure 1f). The K_D^{App} values obtained using both methods of calculation were in good agreement, and fell within the 10-nM range for both the *AP1* and *AP3* target genes (Figure 1f and Table 1). The measurement of interactions with non-target DNA yielded K_D^{App} values of between 1.3 and 40 μM, some two to three orders of magnitude higher than those for target genes (Table 1). In the case of LFY, SPR analysis thus proved capable of easily distinguishing between target and non-target DNA strands of up to 3.1 kb in length (Table 1). We conclude that the consensus motifs for LFY present in the *GAPA-2* and *HFR1* non-target DNAs used in this study do not occur in a DNA context that allows high-affinity binding to LFY. SPR analysis was thus able to discriminate between target genes containing consensus sites of biological significance from non-target genes containing very similar sites, also corresponding to the LFY consensus motif.

To test the ability of SPR to discriminate between target and non-target genes using a second type of transcription factor, we analysed the interactions of the human heat shock factor, HSF1, with the promoter of its predicted target gene *hsp70* (Morgan *et al.*, 1987), and with a negative control DNA derived from the *BHLF1* gene. The *hsp70* DNA fragment used in these assays measured

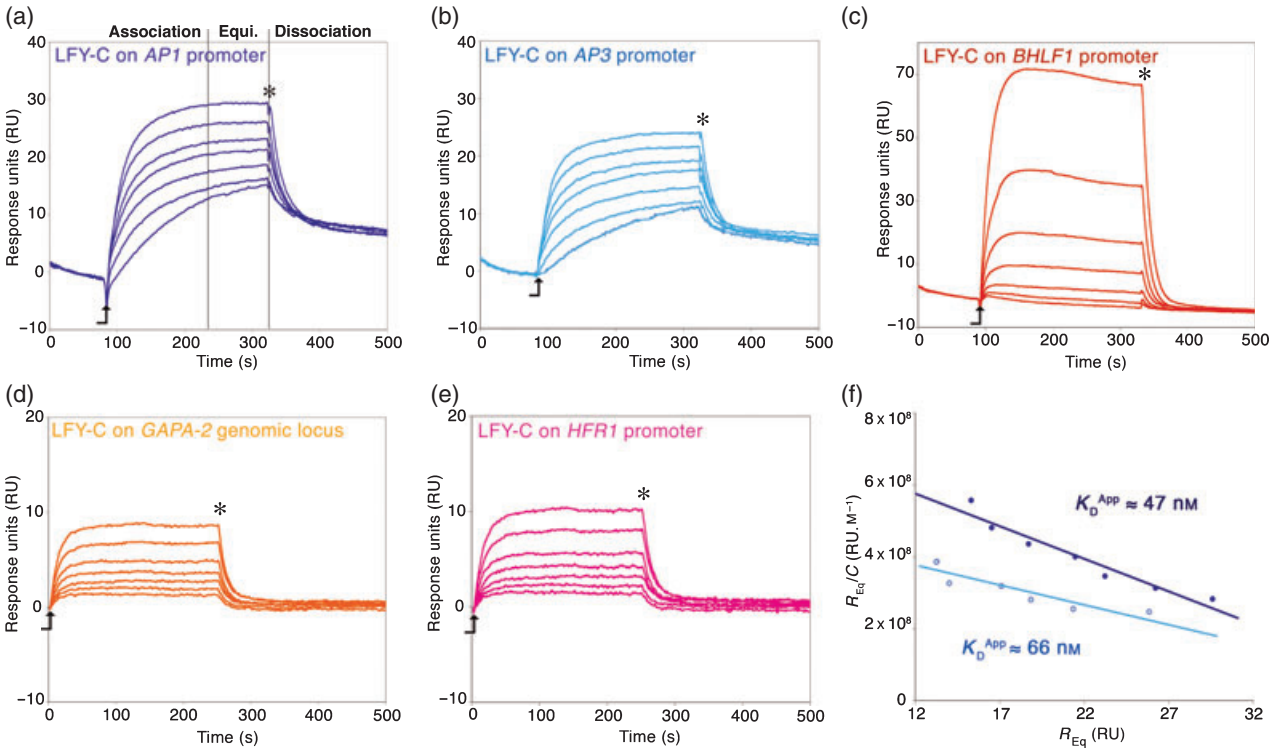

Figure 1. Simple discrimination between target and non-target genes of LFY.
(a–e) Surface plasmon resonance (SPR) curves for interactions of LFY-C with the regulatory regions of two target (a and b) and three non-target genes (c–e), showing specific interactions with target genes. The protein concentrations used, in descending order of response units (RU), were: 105, 84, 67, 54, 43, 34 and 28 nM (or 104, 69, 46, 31, 21, 14 and 9.1 nM, in the case of BHLF). The start and end of protein injections are marked with an arrow and an asterisk, respectively.
(f) Linear relationship of R_{Eq}/C against R_{Eq} for interactions of LFY-C with target promoter (a and b), where R_{Eq} corresponds to the SPR response (RU) at equilibrium for a given protein concentration (C). The linearity of the plots provides an independent validation of the 1:1 binding model. The apparent equilibrium constant for dissociation (K_D^{App}) values (shown), derived from the inverse negative of the linear gradients (Majka and Speck, 2007), are in agreement with the calculated values (Table 1).

Table 1 Kinetic constants for interactions of LFY-C with target and non-target genes

DNA tested	Length (bp)	Number of LFY consensus sites [CCANTG(G/T)]	LFY target	Apparent rate constant for dissociation k_{off}^{App} (sec^{-1})	Apparent rate constant for association k_{on}^{App} (M^{-1} sec^{-1})	Apparent equilibrium constant for dissociation K_D^{App} (nM)	χ^2
AP1	2386	2	Yes	$2.52 \times 10^{-3} \pm 0.5 \times 10^{-4}$	$1.59 \times 10^{5} \pm 0.5 \times 10^{4}$	15.8 ± 0.9	0.249
AP3	3050	1	Yes	$2.38 \times 10^{-3} \pm 0.2 \times 10^{-5}$	$1.11 \times 10^{5} \pm 0.7 \times 10^{4}$	21.4 ± 2.6	0.200
BLHF1	917	0	No	$2.96 \times 10^{-1} \pm 0.1 \times 10^{-5}$	$6.76 \times 10^{3} \pm 2 \times 10^{3}$	>40 000	6.2
GAPA-2	2444	4	No	$5.99 \times 10^{-2} \pm 0.5 \times 10^{-3}$	$2.55 \times 10^{3} \pm 0.2 \times 10^{3}$	~24 000	0.763
HFR1	2150	1	No	$5.12 \times 10^{-2} \pm 0.4 \times 10^{-3}$	$3.56 \times 10^{3} \pm 0.2 \times 10^{3}$	~15 000	1.05

2.4 kb and contained one HSF1 binding site [GAA(C/T)NTTC; Kroeger and Morimoto, 1994]. The K_D^{App} for the interaction between HSF1 and *hsp70* (Figure 2a), either calculated using dedicated SPR software (Table 2) or calculated from plots of equilibrium data (Figure 2b), fell within the nanomolar range. No interaction was observed between HSF1 and non-target *BHLF1* DNA (Figure 2a). Hence, in the case of HSF1, SPR was able to completely discriminate between target and non-target DNA, even without quantitative analysis. Experiments using both LFY-C and HSF1 have thus shown SPR analysis to provide a simple test of whether a given promoter may be bound by a transcription factor of interest.

SPR quantifies binding to multiple sites in *cis*-regulatory regions

In many cases, the level of transcription of a given gene will depend quantitatively on the binding affinity of

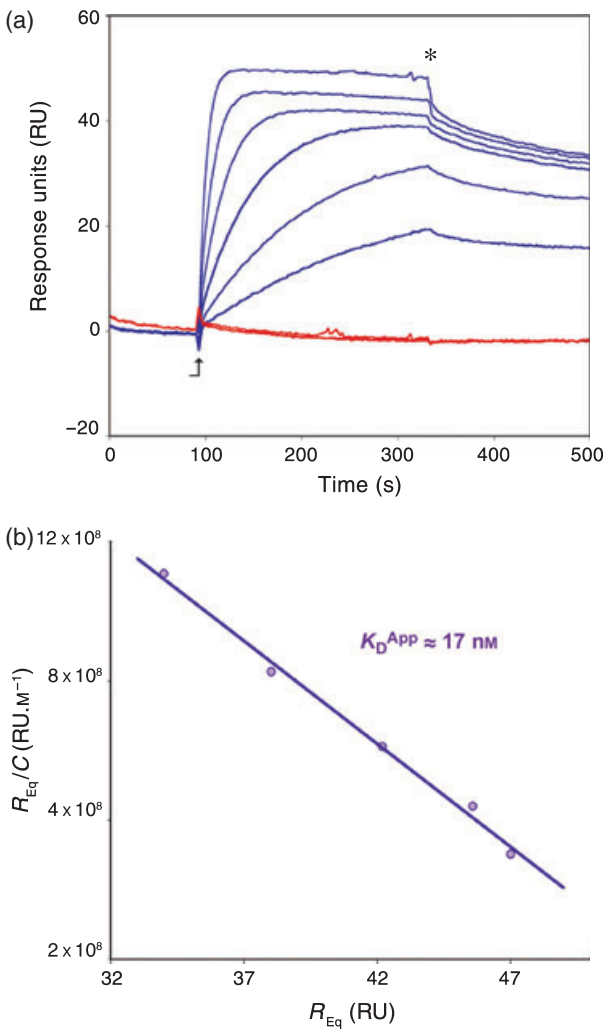

Figure 2. Simple discrimination between target and non-target genes of HSF1.
(a) Surface plasmon resonance (SPR) curves for the interaction of HSF1 with the *hsp70* target gene (blue traces) and *BHLF1* (red traces) non-target gene, showing the specific interaction with *hsp70*. The HSF1 concentrations used, in descending order of SPR response, were: 155, 104, 69, 46, 31 and 21 nM. The start and end of protein injections are marked with an arrow and an asterisk, respectively.
(b) Linear relationship of R_{Eq}/C against R_{Eq}, where R_{Eq} corresponds to the SPR response (RU) at equilibrium for a given protein concentration (C), for the interaction between HSF1 and *hsp70* provides an independent validation of the 1:1 binding model and an estimate of apparent equilibrium constant for dissociation (K_D^{App}, shown), in good agreement with the calculated value (Table 2).

transcription factors to multiple sites in its promoter region. SPR analysis is known to represent one of the most quantitative techniques available for the measurement of physical interactions involving individual binding sites (Majka and Speck, 2007). To test whether these quantitative characteristics are conserved when long DNA molecules containing multiple sites are analysed, we used SPR to investigate interactions involving the second intron of *AGAMOUS* (*AG*), which is necessary for the transcriptional regulation of this gene by LFY. The *AG* second intron measures 3.0 kb and contains four consensus LFY binding sites (Hong *et al.*, 2003). We used the classical technique of electrophoretic mobility shift assay (EMSA) to compare the individual binding of LFY-C to these four sites, BS1–4 (Figure 3a). This study identified BS1 as the site with the highest affinity for LFY, followed by BS2, BS4 and finally BS3 (Figure 3b).

Following the analysis of individual LFY binding sites, we made mutant versions of the *AG* second intron in which either the two highest affinity sites, BS1 and BS2, or all four sites, BS1–4, were mutated to eliminate their capacity to bind LFY (Figure 3a). We then performed SPR analyses to derive K_D^{App} values for interactions of LFY-C with the wild-type and two mutated versions of the *AG* second intron produced (Table 3). A K_D^{App} value of 0.6 nM was obtained for the wild-type intron, compared with values of 42 and 270 nM for the doubly and quadruply mutated introns, respectively. K_D^{App} values for the overall interaction with the *AG* second intron thus increased progressively with the elimination of LFY binding sites, showing a 70-fold proportional increase on mutation of the two highest affinity binding sites, and a further sixfold increase on mutation of the two remaining, lower affinity sites. These results clearly demonstrate SPR analysis to be capable of the quantitative comparison of transcription factor binding to long DNA molecules possessing multiple binding sites, indicating the usefulness of the SPR technique for the *in vitro* dissection of promoter function. Interestingly, the K_D^{App} value of the quadruply mutated intron (Table 3) remained somewhat lower than the values measured for interactions of LFY-C with non-target DNAs (Table 1), which may indicate the presence of a cryptic binding site in the *AG* second intron, which does not conform to the known consensus.

Table 2 Kinetic constants for interactions of HSF1 with target and non-target genes

DNA tested	Length (bp)	Number of HSF1 consensus sites [GAA(C/T)NTTC]	HSF1 target	Apparent rate constant for dissociation k_{off}^{App} (sec^{-1})	Apparent rate constant for association k_{on}^{App} (M^{-1} sec^{-1})	Apparent equilibrium constant for dissociation K_D^{App} (nM)	χ^2
hsp70	2532	1	Yes	$6.48 \times 10^{-4} \pm 0.4 \times 10^{-5}$	$3.03 \times 10^{5} \pm 0.3 \times 10^{4}$	3.5 ± 0.033	0.255
BHLF1	917	0	No	No interaction	No interaction	No interaction	No interaction

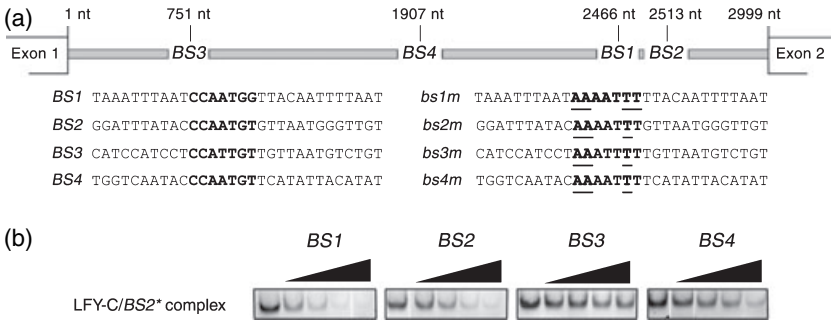

Figure 3. Quantitative measurement of interactions with multiple binding sites in *cis*-acting regions.
(a) Positions and sequences of wild-type (BS1–4) and mutant (bs1–4m) LFY binding sites in the *AG* second intron, as used in electrophoretic mobility shift assay (EMSA) and surface plasmon resonance (SPR) analyses. LFY consensus sites are indicated in bold, and mutated bases are underlined.
(b) EMSA assays showing the relative affinities of LFY consensus motifs BS1–4, by the competition of different quantities of unlabelled target oligonucleotides (0-, 5-, 25-, 100- and 500-fold excess, from left to right) with a fluorescently labelled BS2 complex with LFY-C.

Table 3 Kinetic constants for interactions of LFY-C with wild-type and mutated versions of the *AG* second intron

DNA tested	Length (bp)	Number of LFY consensus sites [CCANTG(G/T)]	Apparent rate constant fordissociation k_{off}^{App} (sec^{-1})	Apparentrate constant for association k_{on}^{App} (M^{-1} sec^{-1})	Apparent equilibrium constant for dissociation K_D^{App} (nM)	χ^2
AG WT	3176	4	$7.04 \times 10^{-5} \pm 0.2 \times 10^{-5}$	$1.18 \times 10^{5} \pm 2 \times 10^{3}$	0.6 ± 0.02	1.20
AG bs12m	3176	2	$2.38 \times 10^{-3} \pm 0.4 \times 10^{-5}$	$5.68 \times 10^{4} \pm 0.9 \times 10^{3}$	41.9 ± 0.7	0.735
AG bs1234m	3176	0	$1.49 \times 10^{-2} \pm 0.5 \times 10^{-3}$	$5.57 \times 10^{4} \pm 0.5 \times 10^{4}$	267.5 ± 30.5	0.654

DISCUSSION

Why length is so important

Despite the availability of commercial SPR analysers for around 20 years, SPR analysis has not been widely taken up by researchers working on transcription factors. This lack of widespread interest has probably been because of the perception that SPR, as applied to transcriptional interactions, was limited to the analysis of short DNA fragments. Accordingly, this technique has mostly been used for detailed, quantitative studies of transcription factor binding to individual *cis*-acting sites, rather than to relate such biophysical events to their higher level biological effects. In the present work, we demonstrate two things: firstly that SPR can be used to detect transcription factor binding to much longer DNA fragments than was previously believed possible, and secondly that this technique retains its quantitative value when applied to such long DNA molecules. The first of these findings means that SPR can be used as a simple and rapid, qualitative test of whether a gene of interest may be the direct target of a given transcription factor. As a general rule of thumb, any interaction yielding a K_D^{App} of below 100 nM is probably worthy of further investigation. The second finding opens the possibility of more subtle uses of SPR in the analysis of complex transcriptional interactions involving multiple binding sites, and/or multiple transcription factors. Both the qualitative and quantitative uses of SPR should find many applications to biological problems, as described below.

To bind or not to bind: novel qualitative applications of SPR analysis

A major objective of systems biology is to describe how networks of transcriptional regulators control complex biological processes. A first requirement of such studies is the identification of the direct target genes of transcription factors participating in the networks of interest, which can be achieved using such *in vivo* techniques as microarray analyses (Gomez-Mena *et al.*, 2005) and chromatin immunoprecipitation (ChIP) (Weinmann, 2004). These procedures yield lists of putative target genes, which must then be verified by independent techniques including the *in vitro* analysis of DNA binding. Previously, such *in vitro* analyses have only been possible for transcription factors for which binding sites could be found within *cis*-regulatory regions. The SPR analysis of entire gene promoters, as demonstrated in the present work, will provide a rapid means of verifying such predicted transcriptional interactions, even in cases in which binding sites cannot be predicted from the gene sequences under analysis. This feature is important not only for transcription factors for which binding site preferences are currently unknown, but

also for the many cases in which transcription factors bind to cryptic sites that show little similarity to their known consensus sequences. SPR analysers of the latest generation are capable of measuring binding to several hundred DNA samples simultaneously, and should therefore prove ideal for the rapid verification of lists of direct target genes identified using large-scale approaches such as microarray and ChIP-seq (Robertson *et al*., 2007) analyses. Indeed, with the possibility of analysing large numbers of bound DNA molecules simultaneously, SPR could also be used for the *in vitro* characterization of entire transcriptional networks by sequentially passing all transcription factors in a network over the complete set of the promoters of that network. In this way, the transcriptional relationships linking all the components of a system could be identified.

Differences in transcriptional control relationships between organisms account for much of the biodiversity of the natural world, as demonstrated on a micro-evolutionary scale by many of the molecular causes underlying the domestication of crop plants (Doebley *et al*., 2006). However, our current knowledge of transcriptional interactions derives exclusively from the study of a few model organisms that are amenable to genetic analysis. To identify the differences in transcriptional interactions that are responsible for biodiversity in plants and other organisms, a means is needed to test whether interactions are conserved between the well-studied model species and others chosen for their key phylogenetic positions or strategic importance. The SPR analysis of entire gene promoters should prove ideal for this purpose as it can be performed using recombinant transcription factors and putative target genes from any species. The use of SPR to characterize transcriptional relationship in non-model species will thus be of great importance to both evolutionary developmental (evo-devo) biology (Frohlich and Chase, 2007) and to agricultural science and other branches of applied biology.

Putting a figure on it: novel quantitative applications of SPR analysis

In the present work, we have shown that the quantitative value of SPR analysis is conserved for interactions involving long DNA molecules. Accordingly, the SPR analysis of transcription factor binding to a given target promoter may yield a single K_D^{App} value that quantifies the overall binding interaction between those two components. By repeating SPR analyses using mutated versions of a promoter of interest, it should be possible to determine the importance to the overall binding interaction of the positions and affinities of all the individual binding sites present. Such an approach would rapidly indicate the presence of such phenomena as cooperative binding, where several sites of individually low affinity may, for example, combine to produce a strong overall binding interaction.

Transcription factors frequently bind to DNA as complexes, and these may also interact simultaneously with several *cis*-acting sites positioned at considerable distances along the target gene. For example, the MADS-box transcription factors encoded by three of the target genes analysed in the present work, *AP1*, *AP3* and *AG*, are hypothesized, on the basis of genetic evidence, to form various combinations of tetramers, known as floral quartets, (Theissen and Saedler, 2001) with a further class of MADS-box protein, SEPALLATA (Pelaz *et al*., 2000; Honma and Goto, 2001). According to this hypothesis, four different tetrameric complexes of MADS-box proteins would interact with multiple *cis*-acting sites in four different sets of target genes to specify the four types of floral organ: sepals, stamens, petals and carpels. Several studies have identified putative MADS-box targets in specific floral organs (Sablowski and Meyerowitz, 1998; Ito *et al*., 2004; Gomez-Mena *et al*., 2005; Sundstrom *et al*., 2006). Furthermore, a recent study employing the classical EMSA method has shown SEP proteins to be capable of interacting as tetramers with pairs of precisely spaced consensus MADS-box transcription factor binding sites in a short, artificial DNA molecule (Melzer *et al*., 2009). However, classical techniques such as EMSA cannot be used to characterize the binding of MADS-box complexes to sets of native target promoters measuring up to several kilobases in length. SPR analysis may thus prove the ideal technique to finally demonstrate or refute the floral quartet hypothesis, and to investigate the many other cases of transcription factor binding to multiple target sites in plants and other organisms.

EXPERIMENTAL PROCEDURES

Expression and purification of recombinant protein

The *A. thaliana* LFY DNA binding domain (LFY-C), corresponding to amino acids 223–424 of the full-length LFY protein, was inserted into the *pETM11* expression vector (Dummler *et al*., 2005) to yield the *pCH28* plasmid (Hamès *et al*., 2008), thereby permitting the production of LFY-C fused to an N-terminal 6x histidine tag. Cell cultures were generated in the *Escherichia coli* Rosetta Blue DE3-pLysS strain (Novagen, Merck, http://www.merckbiosciences.co.uk/html/NVG/home.html), and the production of recombinant protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Following incubation overnight at 22°C, cell cultures were lysed by sonication. Recombinant protein was then purified by affinity chromatography on Ni-NTA resin (Qiagen, http://www.qiagen.com). Protein-containing fractions were pooled and subjected to size-exclusion chromatography using a Hi-load Superdex-200 16/60 preparation grade column (GE Healthcare, http://www.gehealthcare.com) to eliminate protein aggregates.

Preparation of biotinylated DNA molecules for SPR analysis

DNA fragments containing *cis*-acting regulatory regions were obtained by PCR amplification using the primers shown in Table S1,

on templates of genomic DNA from *A. thaliana* plants of the Columbia ecotype or from human, except for *AP1*, which was amplified from a plasmid supplied by Dr R. Benlloch (Umeå Plant Science Centre, Sweden). The DNA fragments obtained were ligated into the PCR cloning site of *pGEM-T-Easy* (Promega, http://www.promega.com). A *pBluescriptI*-derived plasmid containing part of *BHLF1*, corresponding to bases 52401–53092 of the Epstein–Barr virus genome (Genbank accession V01555), was obtained from Dr Henri Gruffat (École normale supérieure de Lyon, http://www.ens-lyon.eu). Mutant versions of the second intron of *AG*, containing mutations to disrupt the function of LFY binding sites, as shown in Figure 3(a), were constructed by sequential site-directed mutagenesis of this sequence in *pGEM-T-Easy* (Kirsch and Joly, 1998). Double-stranded DNA molecules for SPR analysis were synthesized by PCR amplification from recombinant *pGEM-T-Easy* plasmids using T7 and SP6 primers, or from recombinant *pBluescriptI* plasmids using M13 forward and reverse sequencing primers, one primer of each pair being biotinylated in each case, and subsequently purified using a NucleoSpin® Extract II kit (Macherey-Nagel, http://www.macherey-nagel.com).

Immobilization of DNA samples for SPR analysis

CM5 SPR chips (Biacore, http://www.biacore.com) were activated in a Biacore T100 SPR Analyser to accept a streptavidin coating, using an amine coupling kit (Biacore) according to the manufacturer's instructions. Amine-coupling reagents were injected in a continuous flow of 5 μl min^{-1} of HBS-P+ buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.05% w/v P20 detergent). Aliquots of 50 μl of streptavidin (0.1 mg ml^{-1} in 10 mM sodium acetate, pH 4.2) were then injected, followed by 50-μl aliquots of ethanolamine (1 M, pH 8.5), to inactivate the residual carboxyl groups. The chips were then washed by two injections of 5 μl of HBS-P+ buffer. Double-stranded DNA molecules (5 ng μl^{-1} in HBS-P+ buffer, degassed before use) each carrying a single biotin moiety at its 3′ terminus, relative to the direction of transcription of the encoded gene, were immobilized in separate channels on SPR chips by injection at 10 μl min^{-1}, until 200 RU (arbitrary SPR units) of DNA had been added, corresponding to approximately 0.15 ng mm^{-2}. One channel was left empty on each chip as a reference. Unbound DNA was finally removed by injections of 10-μl aliquots of NaCl (1 M).

SPR analysis of DNA–protein interactions

SPR chips containing immobilized DNA samples were equilibrated in a Biacore T100 Analyser by injection of HBS-P+ buffer containing non-homologous DNA (40 ng μl^{-1}, molecular size >120 bp) from salmon testis (Roche, http://www.roche.com) until SPR responses were stable. Transcription factors, dissolved in the above buffer, were then injected into all channels of these chips for 250 sec at a flow rate of 50 μl min^{-1}, followed by an injection of further buffer for 300 sec to monitor protein dissociation. Transcription factor solutions were used in decreasing order of concentration, and SPR chips were regenerated between analyses by the sequential injection of guanidinium hydrochloride (3 M) and sodium dodecyl sulfate (0.03%, w/v), each for 60 sec at a flow rate of 50 μl min^{-1}.

SPR data analysis

Real-time SPR interaction curves were analysed using BIOEVAL T100 software (Biacore). For each analysis, the response of the reference channel was subtracted from the interaction curves obtained from the three experimental channels, and the resulting curves were then adjusted to zero at the start of transcription factor injection. These normalized curves were then fitted globally to a 1:1 (Langmuir) interaction model, permitting the determination of forward and reverse apparent rate constants (k_{on}^{App} and k_{off}^{App}). The validity of the interaction model was verified by data fitting, with a good fit indicated by $\chi^2 < 2$ (or <10 for low-affinity interactions). Equilibrium binding constants (K_D^{App}) were calculated from the ratio $k_{off}^{App}/k_{on}^{App}$, and also from SPR values (RU), at equilibrium over a range of protein concentrations.

EMSA assays

EMSA assays were performed as described in (Hamès *et al.*, 2008) using oligonucleotides (10 nM) labelled with 5-carboxytetramethylrhodamine, varying concentrations of unlabelled competitors and LFY-C protein at a concentration of 300 nM.

ACKNOWLEDGEMENTS

We thank Mike Frohlich for helpful discussions, Sylvie Ricard-Blum and Clément Faye for advice on SPR analysis, Reyes Benlloch and Henri Gruffat for supplying plasmid DNA and Hüseyin Besir (EMBL, Heidelberg) for providing the *pETM-11* vector. The SPR analysis was performed using equipment provided by the Institut Féderatif de Recherche-128 (Biosciences-Gerland, Lyon-Sud). We acknowledge grant funding from the French National Research Agency (Plant TF-Code/ANR-07-BLAN-0211-01) and doctoral studentships to EM (French Ministry of Research), CH and MR (Rhone-Alpes Region, Cluster 9).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Oligonucleotide sequences used in the PCR amplifications.

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