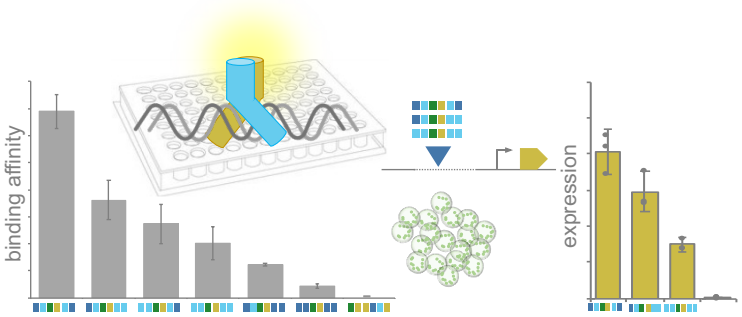
Tuning plant promoters using a simple split luciferase method for quantifying transcription factor binding affinity

Cai, Y-M[1], Witham, S[1], and Patron NJ[1*]
1. Engineering Biology, Earlham Institute, Norwich Research Park, Norwich, NR4 7UZ, UK
**Author for correspondence:* nicola.patron@earlham.ac.uk



Abstract: Sequence features, including the binding affinity of binding motifs for their cognate transcription factors, are important contributors to promoter behavior. The ability to predictably recode binding affinity enables the development of synthetic promoters with varying levels of response to known cellular signals. Here we describe a luminescence-based microplate assay for quantifying the relative affinity of transcription factors for short DNA probes. We then demonstrate how relative binding affinity data can be used to design synthetic plant promoters of varying strengths that respond to the same transcription factor.

Keywords: *protein binding, transcription factors, synthetic promoters, plant biotechnology, Arabidopsis*

Introduction: To initiate the transcription of synthetic genetic circuits, regulatory elements with predictable characteristics that respond to cellular signals are highly desirable. Similarly, the ability to rationally edit the sequence of endogenous sequences to alter the amplitude or timing of gene expression is useful for tuning quantitative phenotypes. Promoters play a role in initiating transcription through the recruitment of proteins, including transcription factors (TFs). While the availability of some proteins may influence expression patterns and levels[1], the sequence features of promoters that encode the binding locations and affinity for TFs are critical for defining function[2]. In previous work, we developed computationally designed minimal synthetic promoters (MinSyns) for plants with binding sites for known classes of endogenous TFs[3]. We reasoned that manipulating binding sites to alter binding affinity would enable us to produce variants of individual MinSyns that respond to the same endogenous signal. To do this rationally, we required a method to quantify TF-DNA binding.

Several methods exist for assessing the binding of TFs to DNA. Electrophoretic Mobility Shift Assays (EMSA), in which chemically labeled DNA probes are combined with purified protein and protein-DNA complexes are identified by electrophoresis, are widely used but can be challenging to quantify and are labor-intensive. Surface plasmon resonance (SPR) has the advantage of generating real-time data but requires expensive specialized equipment. Systematic evolution of ligands by exponential enrichment (SELEX) and protein binding microarrays (PBM) offer high throughput approaches but, as a result, though cost per probe is low, have high total experiment costs. Fluorescent or colorimetric microplate-based protein-DNA affinity assays require no specialized equipment and are reasonably scalable and quantifiable[4]. However, labeling of DNA and the conjugation of chemical groups can add substantial time and cost. Here, we describe a luminescence-based microplate assay for TF-DNA relative affinity measurement (TRAM). This method uses short, unlabeled DNA probes, which are cheaply obtained, and minimal quantities of recombinant protein with a genetically encoded minimal (11 amino acid) HiBiT Tag. We demonstrate the use of this assay to quantify the affinity of TFs to target sequences. We then apply knowledge of binding affinity to modulate the strength of minimal synthetic plant promoters (MinSyns).

Results and Discussion

A low-cost assay for quantifying protein-DNA binding affinity: To avoid the use of expensive, surface-modified microplates and labeled DNA probes, we employed a commercially available DNA coating solution previously used for chemiluminescence immunoassay of DNA adducts[5]. This is used to bind short (60-80 bp) double-stranded probes to the plate. The quantity of immobilized probe (F_{DNA}) is quantified using PicoGreen. To enable the detection of bound protein, we expressed recombinant TF proteins with a C-terminal HiBiT tag[6]. Luciferase activity proportional to the level of HiBiT-tagged protein (L_{TF}) is enabled by the addition of the LgBiT polypeptide. Protein binding is calculated as L_{TF}/F_{DNA} and relative binding affinity for the target probe (rB) is calculated by normalizing this to the value obtained for the same TF to a random sequence (**Figure 1A**).

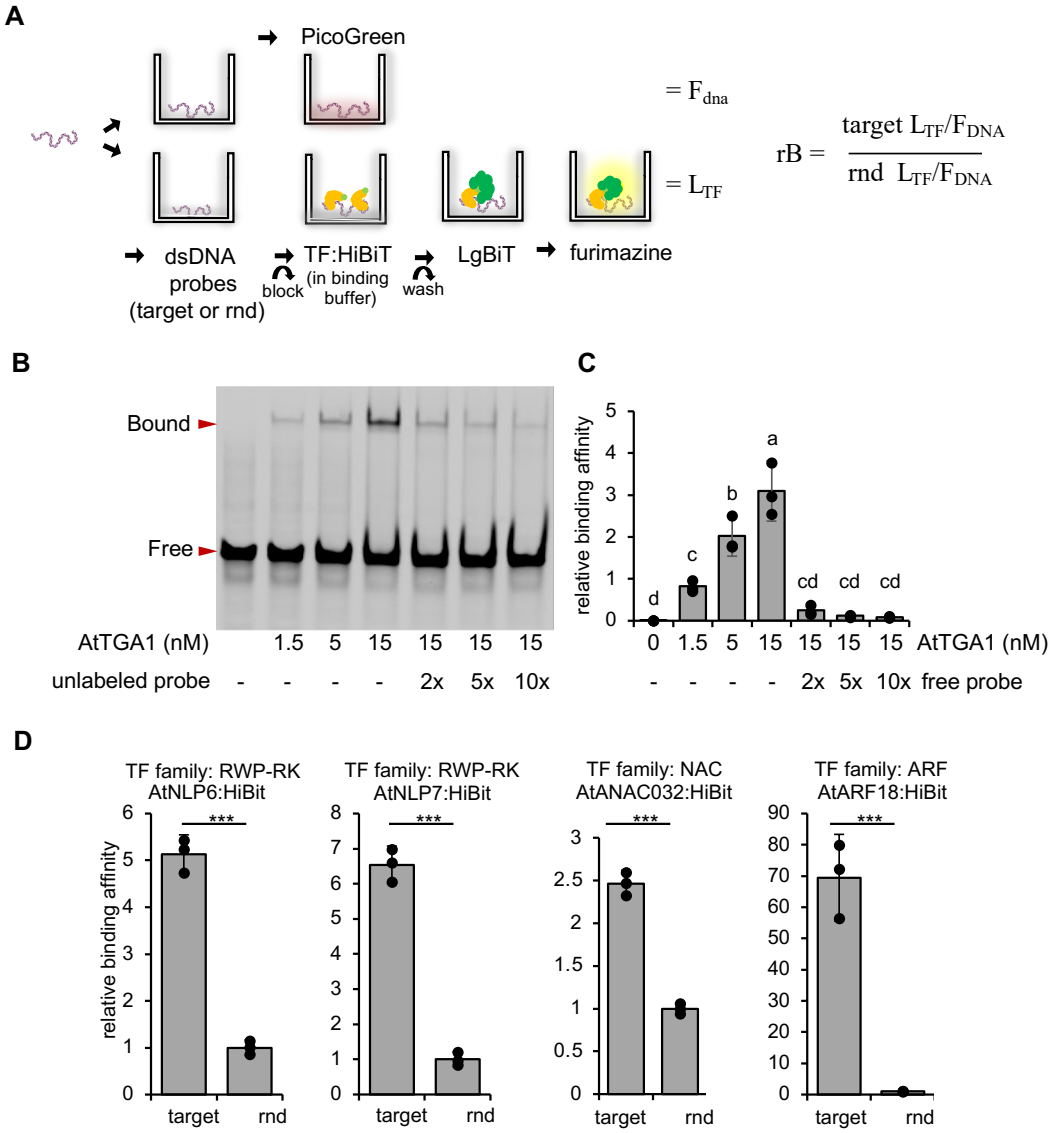


Figure 1: A low-cost assay for quantifying protein-DNA binding affinity A. Schematic of the transcription-factor relative affinity measurement assay (TRAM). DNA probes are added to six wells (three replicates for protein binding and three for quantifying immobilized DNA). Purified TF:HiBiT proteins are added to the former and, following removal of unbound protein, luminescence (L_{TF}) is quantified by addition of the LgBit polypeptide and fumarizine substrate. Immobilized DNA is quantified with PicoGreen (F_{DNA}). Protein binding is calculated as L_{TF}/F_{DNA} and relative binding affinity (rB) is calculated by normalizing to values obtained for random (rnd) DNA **B.** An electromobility shift assay (EMSA) showing binding of AtTGA1 to an 80 bp labeled probe. The presence of an unlabeled competitor probe reduces binding to the labeled probe. **C.** Relative binding affinity of AtTGA1:HiBiT to the same probe relative to random DNA obtained using TRAM**.** The inclusion of unbound competitor probe in the binding buffer reduces binding to the immobilized probe. Values are the mean and 2 x standard error of 3 replicates. Different letters indicate significant differences (TukeyHSD). **D**. Relative binding affinity of plant transcription factors to probes containing previously reported binding sites. Values are the mean and 2 x standard error of 3 replicates. *P*-values were calculated using a t-test: ***, $P \leq 0.001$.

We benchmarked the TRAM assay by comparing it to an electromobility shift assay (EMSA). *Arabidopsis thaliana* TGA1 (AtTGA1) was previously shown to bind to the *CaMV35S* promoter[7]. In EMSA, the intensity of the protein-bound band increased with protein concentration from 1.5 to 15 nM (**Figure 1B**). The relative binding affinity detected by TRAM mirrored this data (**Figure 1C**). The inclusion of unlabeled (EMSA) or unbound (TRAM) competitor probes reduced binding (**Figure 1B and C**). We then demonstrated that TRAM works across TF families by quantifying affinity for previously reported targets of four TFs from the RWP-RK (or nodule inception (NIN)-like) family[8], NAC (NAM, ATAF and CUC) family[9] and ARF (auxin response factor) families (**Figure 1D**)[10].

Tuning plant promoters using transcription factor binding affinity: Previously, we demonstrated the function of plant minimal synthetic promoters (MinSyns) containing three tandem pairs of a TGA1 TF binding site[3]. To tune expression of this promoter, we used publicly available data to identify six *Arabidopsis thaliana* genes for which a change in expression has been detected in direct response to nuclear localization of AtTGA1[11,12]. We used the FIMO software[13] to identify candidate AtTGA1 binding sites within these target genes (**Figure 2A**) and found a range of affinities (**Figure 2B**). TGA proteins are known to bind to DNA as dimers and, importantly, the binding of such dimers has been shown to be stabilized by the presence of other TGA homodimers at the site[14]. We therefore hypothesized that combinations of sites with weak and strong binding affinity could be used to tune the activity of promoters activated by AtTGA1. We selected two sites with strong and weak relative affinities (TFBS 02 and 12) and, maintaining the architecture of the TGA1-binding sites in our existing MinSyn[3], we constructed promoters with different combinations (**Figure 2C).** These promoters were used to a luciferase reporter gene (LucF) and normalized expression was determined following transfection in Arabidopsis mesophyll protoplasts. Three copies of the high-affinity element gave strong activity while expression from MinSyns with three copies of the weak element was non-significant. Interestingly, MinSyns with one high-affinity but two weak elements also had significant activity, with expression correlating with the relative position of the high-affinity site to the TSS (**Figure 2D**). This is consistent with the observation that TGA-DNA complexes can stabilize nearby dimers. In summary, we developed a luciferase-based microplate quantitative protein DNA binding assay. We demonstrate the utility of this method for tuning expression of minimal synthetic plant promoters that respond to the same TF. The ability to experimentally identify low and high affinity sequence variants for different TFs, will also be useful for informing the rational editing of endogenous genes to tune expression patterns and levels.

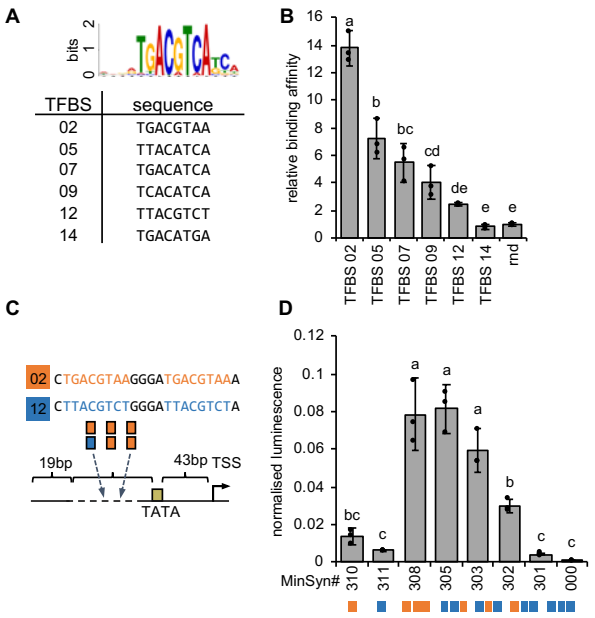


Figure 2: Tuning synthetic promoters by modulating TF binding affinity A. The position weight matrix consensus logo for AtTGA1 (above) and candidate binding sites from six Arabidopsis promoters that change expression in response to AtTGA1. **B**. Relative binding affinity of AtTGA1:HiBiT to probes containing AtTGA1 binding sites. Error bars indicate the mean and 2 x standard error of 3 replicates. Different lowercase letters indicate significant difference (ANOVA, P = 2e-16, TukeyHSD, α = 0.05). **C.** Three pairs of AtTGA1 binding sites are inserted into the variable region of the minimal synthetic promoter chassis (MinSyn000). **D.** Expression from MinSyns with combinations of AtTGA1 binding sites. Error bars indicate the mean and 2 x standard error of 3 replicates. Different lowercase letters indicate significant difference (ANOVA, P = 2.79e-11, TukeyHSD, α = 0.05).

Methods

Plasmid construction: Constructs were designed in Benchling (San Francisco, CA). TF coding sequences were synthesized (Twist Biosciences, San Francisco, CA) and HiBiT tag, stop codon (VSGWRLFKKIS*) and attB sites for Gateway cloning introduced by PCR. Amplicons were cloned into pDONOR207 using BP Clonase (ThermoFisher, Waltham, MA) and subcloned into pH9GW by LR-clonase (ThermoFisher). All other plasmids were assembled in BsaI-mediated Golden Gate reactions as previously described[6]. Details of all standard parts and assembled expression constructs are provided as Supporting Information. Plasmids and sequences are available at Addgene.

Recombinant protein production: Bacteria harboring expression vectors were grown at 37°C, 220 rpm in 20 mL LB with (100 mg/L kanamycin) to OD_{600} 0.6-0.8. Expression was induced with 0.2 mM IPTG and cultures incubated at 18°C, 200 rpm for 18-20 h. Cells were collected in lysis buffer (50 mM Tris-HCL pH8.0, 500 mM NaCl, 20 mM Imidazole, 10% Glycerol, 0.05% Tween-20) and lysed by 20 cycles of sonication at 2s on, 5s off, 11 µS amplitude (Soniprep 150, MSE). Proteins were purified on 50 µL Ni-NTA resin and eluted in 100 µL elution buffer (50 mM Tris-HCL pH8.0, 500 mM NaCl, 300 mM Imidazole, 10% Glycerol, 0.05% Tween-20). Protein was quantified using the Nano-Glo® HiBiT Extracellular Detection System (N2420, Promega, Madison, WI) using the HiBiT Control Protein (20 µM, N3010, Promega) as a standard.

EMSA: EMSA was performed as previously described [6] except that probes were produced by PCR using cy5 labeled oligonucleotide primers.

TRAM assay: Double-stranded probes (DSPs) of random sequence or containing candidate binding sites were made by combining 2 µL of 100 µM forward and reverse oligos and 10 µL 2X annealing buffer (20 mM Tris-HCl pH8.0, 100 mM NaCl, 2 mM EDTA) in a 20 µL reaction. After heating to 95°C, the temperature was reduced by 0.1°C/s to 25°C. 5 µL 40 µg/µL of each DSP was combined with 45 µL DNA coating solution (17250, ThermoFisher Scientific) and added to six wells of a medium-binding microplate (655076, Greiner Bio-One, Kremsmünster, Germany) and incubated for 20 hrs at room temperature in the dark. Unbound probe was removed by three washes with 1X PBS. 270 µL of 3% bovine serum albumin (BSA) in PBS was added to three wells per probe and incubated at room temperature for 30 mins. After one wash with 1X PBS, 1.5 - 50 nM purified protein in binding buffer (25mM Tris-HCL pH8.0, 100 mM KCl, 2 mM DTT, 1 mM EDTA, 0.1 % BSA, 500 ng Poly(dI-dC), 5% Glycerol, 0.05% IGEPAL CA630) was added and plates incubated for 1-3 h at room temperature. Unbound protein was removed by three washes of wash buffer (20 mM Tris-HCL pH8.0, 100 mM KCl, 10% Glycerol, 0.01% IGEPAL CA630). Bound protein was quantified using the Nano-Glo® HiBiT Extracellular Detection System (N2420, Promega). To quantify immobilized DNA in wells without protein, 0.25 µL PicoGreen (P7581, ThermoFisher Scientific) in 50 µL 1x TE buffer (10 mM Tris-HCL pH8.0, 1 mM EDTA) was incubated for 2 min at room temperature. Luminescence and fluorescence were quantified in a CLARIOstar Plus plate reader (BMG LABTECH, Ortenberg). Sequences of probes are provided as Supporting Information.

Protoplast preparation and transfection: Mesophyll protoplasts were prepared from *A. thaliana* leaves and transfected as previously described[3]. To quantify expression from MinSyns, 10 µg plasmid DNA comprising equal molar ratios of plasmids encoding MinSyn:LucF:*AtuOCSt* and a calibrator plasmid (*AtuNOSp*:LucN:*AtuNOSt*) was used to transfect 200 µL protoplasts (10^4 -10^5 /mL) and expression was normalized to an experiment calibrator (*AtuMASp*:LucF:*AtuOCSt* + *AtuNOSp*:LucN:*AtuNOSt*) as previously described[3].

Supporting Information: Details and sequences of plasmids and DNA probes are provided as supporting information files.

Acknowledgements: pH9GW was a gift from Paul O'Maille. We thank Will Nash, Wilfried Haerty, Eftychios Frangedakis, Susana Sauret-Gueto, Tufan Oz, Siobhan Brady and Gozde Demirer for helpful discussions during the development of TRAM. We thank the John Innes Centre Horticultural services team for help with plant husbandry.

Funding: We gratefully acknowledge the support of the United Kingdom Research and Innovation's Biotechnology and Biological Sciences Research Council (UKRI BBSRC); this research was funded by a Strategic Programme Grant to the Earlham Institute (BB/CSP1720/1) - work package 'Regulatory Interactions and Complex Phenotypes' (BBS/E/T/000PR9819). Additional funding was provided by the OpenPlant Synthetic Biology Research Centre (BB/L014130). SW was supported by the BBSRC Norwich Research Park Doctoral Training Partnership (BB/M011216/1 Project No. 2116916).

Author contributions: YC and NP conceptualized the study. YC and SW analyzed sequences and produced constructs and protein. YC performed TF-binding and expression assays. YC and NP drafted the manuscript and all authors edited and approved.

Conflicts of Interest: The authors have no conflicts of interests to declare.

References

(1) Martin, E.; Sung, M.-H. Challenges of Decoding Transcription Factor Dynamics in Terms of Gene Regulation. *Cells*. 2018, p 132. https://doi.org/10.3390/cells7090132.

(2) Grossman, S. R.; Zhang, X.; Wang, L.; Engreitz, J.; Melnikov, A.; Rogov, P.; Tewhey, R.; Isakova,

A.; Deplancke, B.; Bernstein, B. E.; Mikkelsen, T. S.; Lander, E. S. Systematic Dissection of Genomic Features Determining Transcription Factor Binding and Enhancer Function. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114* (7), E1291–E1300.

(3) Cai, Y. M.; Kallam, K.; Tidd, H.; Gendarini, G.; Salzman, A.; Patron, N. J. Rational Design of Minimal Synthetic Promoters for Plants. *Nucleic Acids Res.* **2021**, *48* (21), 11845–11856.

(4) Zhang, Z.-R.; Palfrey, D.; Nagel, D. A.; Lambert, P. A.; Jessop, R. A.; Santos, A. F.; Hine, A. V. Fluorescent Microplate-Based Analysis of Protein-DNA Interactions. I: Immobilized Protein. *Biotechniques* **2003**, *35* (5), 980–982, 984, 986.

(5) Divi, R. L.; Beland, F. A.; Fu, P. P.; Von Tungeln, L. S.; Schoket, B.; Camara, J. E.; Ghei, M.; Rothman, N.; Sinha, R.; Poirier, M. C. Highly Sensitive Chemiluminescence Immunoassay for Benzo[a]pyrene-DNA Adducts: Validation by Comparison with Other Methods, and Use in Human Biomonitoring. *Carcinogenesis* **2002**, *23* (12), 2043–2049.

(6) Dudley, Q. M.; Cai, Y.-M.; Kallam, K.; Debreyne, H.; Carrasco Lopez, J. A.; Patron, N. J. Biofoundry-Assisted Expression and Characterization of Plant Proteins. *Synth. Biol.* **2021**, *6* (1), ysab029.

(7) Kanazawa, A.; O'Dell, M.; Hellens, R. P. The Binding of Nuclear Factors to the as-1 Element in the CaMV 35S Promoter Is Affected by Cytosine Methylation in Vitro. *Plant Biol.* **2007**, *9* (3), 435–441.

(8) Konishi, M.; Yanagisawa, S. Arabidopsis NIN-like Transcription Factors Have a Central Role in Nitrate Signalling. *Nat. Commun.* **2013**, *4*, 1617.

(9) Allu, A. D.; Brotman, Y.; Xue, G.-P.; Balazadeh, S. Transcription Factor ANAC032 Modulates JA/SA Signalling in Response to Pseudomonas Syringae Infection. *EMBO Rep.* **2016**, *17* (11), 1578–1589.

(10) Ulmasov, T.; Murfett, J.; Hagen, G.; Guilfoyle, T. J. Aux/IAA Proteins Repress Expression of Reporter Genes Containing Natural and Highly Active Synthetic Auxin Response Elements. *Plant Cell* **1997**, *9* (11), 1963–1971.

(11) Brooks, M. D.; Cirrone, J.; Pasquino, A. V.; Alvarez, J. M.; Swift, J.; Mittal, S.; Juang, C.-L.; Varala, K.; Gutiérrez, R. A.; Krouk, G.; Shasha, D.; Coruzzi, G. M. Network Walking Charts Transcriptional Dynamics of Nitrogen Signaling by Integrating Validated and Predicted Genome-Wide Interactions. *Nat. Commun.* **2019**, *10* (1), 1569.

(12) Swift, J.; Alvarez, J. M.; Araus, V.; Gutiérrez, R. A.; Coruzzi, G. M. Nutrient Dose-Responsive Transcriptome Changes Driven by Michaelis–Menten Kinetics Underlie Plant Growth Rates. *Proceedings of the National Academy of Sciences* **2020**, *117* (23), 12531–12540.

(13) Bailey, T. L.; Johnson, J.; Grant, C. E.; Noble, W. S. The MEME Suite. *Nucleic Acids Res.* **2015**, *43* (W1), W39–W49.

(14) Lam, E.; Lam, Y. K. P. Binding Site Requirements and Differential Representation of TGA Factors in Nuclear ASF-1 Activity. *Nucleic Acids Res.* **1995**, *23* (18), 3778–37785.