

Novel cell surface luciferase reporter for high-throughput yeast one-hybrid screens

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ABSTRACT

Gene-centered yeast one-hybrid (Y1H) screens provide a powerful and effective strategy to identify transcription factor (TF)-promoter interactions. While genome-wide TF ORFeome clone collections are increasingly available, screening protocols have limitations inherent to the properties of the enzymatic reaction used to identify interactions and to the procedure required to perform the assay in a high-throughput format. Here, we present the development and validation of a streamlined strategy for quantitative and fully automated gene-centered Y1H screens using a novel cell surface *Gussia* luciferase reporter.

INTRODUCTION

The yeast one-hybrid system (Y1H) provides one of the few straightforward strategies that is commonly used to identify transcription factor (TF)-promoter interactions focusing on a single promoter region. The approach requires two main components: a reporter construct (bait) that carries a promoter region driving the expression of a reporter gene (e.g. *lacZ*), typically integrated into the genome of a yeast strain (e.g. YM4271) and an effector construct (prey) that carries a yeast constitutive promoter driving the expression of a TF fused to a transcriptional activation domain (AD) (e.g. Gal4-AD). Following transformation of effector constructs into the reporter strain, TF-promoter interactions are revealed by an increase in the reporter gene activity that depends on the DNA binding affinity and specificity of the TF-AD effector for the promoter bait. As initially designed, the effector constructs for Y1H screens were part of cDNA libraries. However, because TFs are typically expressed at low levels and often in a specific tissue, developmental stage or physiological condition (1–3), these cDNA libraries only provided a partial and biased pool of potential DNA binding proteins. With the advent of the genomic era, several ef-

forts were made to develop global TF-effector libraries for different species (4–10). Adapting the Y1H system to these TF ORFeome clone collections provided a remarkable improvement to the approach that enhanced the discovery of functional TF-promoter interactions in an unbiased and comprehensive manner (8,11–15).

Recently, we established a gene-centered high-throughput Y1H (HT-Y1H) screening approach using a clone collection encompassing 80% (1956 clones) of all predicted TFs in the plant model organism *Arabidopsis thaliana* (Arabidopsis) (8). This strategy, performed in 384-well plates and entirely in liquid format, evaluated all 1956 potential TF-promoter interactions individually (one interaction per well) using the *lacZ* gene as reporter (8,16). Importantly, this work indicated that ranking TF-promoter interactions based on the Y1H reporter activity could provide an effective mean for identifying and prioritizing TFs more likely to be involved in biologically meaningful interactions (8). Although gene-centered Y1H screens already identified a number of novel TF-promoter interactions in Arabidopsis (8,14,15,17–22), we found that quantification of the β -galactosidase activity is rapidly saturated when performed in a high-throughput format (i.e. 384-well plates). This issue limits our ability to detect positive interactions when background reporter levels are high (8), and to accurately sort positive interactions to select and prioritize potentially relevant TF candidates for further characterization *in vivo*.

As reported previously the ONPG (2-nitrophenyl- β -D-galactopyranoside)-based method performed in a liquid format provides an accurate quantification of the β -galactosidase activity in yeast cells but often requires of either multiple dilutions of the initial samples or an optimized reaction time for each sample to adjust the colorimetric signal within the linear range (23,24). While these adjustments are possible when individual samples are handled, they are impracticable in a high-throughput format where several thousand reactions are processed simultaneously (8,16). For this reason, all enzymatic reactions in HT-

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Y1H screens performed in a liquid format are stopped at a fixed time regardless of differences among individual reaction kinetics (16), resulting in a short linear range and rapid saturation of the β -galactosidase assay (Supplementary Figure S1). Furthermore, because each promoter bait usually drives a different background reporter activity, the progression of the colorimetric reaction has to be continually monitored and the reaction time subjectively determined by an operator in each experiment. This step could be theoretically automated, however it would require of a sophisticated system to continuously process multiple microplates and determine the optimal incubation time for the enzymatic reaction in each well. Importantly, similar limitations were reported for an alternative HT-Y1H screening method that relies on β -galactosidase-mediated color development in yeast cells spotted on agar plates (25). While it might be possible to improve either β -galactosidase assay using fluorescent or luminescent reaction products, such reagents would significantly increase the cost of HT-Y1H screens and likely limit its widespread usage (24).

lacZ-based HT-Y1H screens are additionally impacted by technical factors that affect either result analysis or automation efforts. Regarding the former, we found that incubation of liquid β -galactosidase reactions at 30–37°C in 384-well plates results in faster enzymatic reaction kinetics for the wells located at the periphery of the microplate, likely due to uneven temperature distribution. This problem introduces a bias in the calculation of the baseline β -galactosidase activity used to discriminate between positive and negative TF–promoter interactions. Likewise, the β -galactosidase assay in agar spotted yeast cells is subject to false positive calls due to color signal diffusion from strong positives into neighboring areas (25). Finally, quantification of the β -galactosidase activity in a liquid format requires yeast cell permeabilization to allow an efficient enzyme-substrate encounter (24). Currently used freeze and thaw cycles provide a simple, reproducible and cost-effective method for yeast cell lysis (26); however, this is a labor-intensive and rate-limiting step for HT-Y1H screens that is incompatible with automation (16).

Considering all these factors, we reasoned that HT-Y1H screen optimization would require of a reporter activity that could be reliably quantified at a fixed time over a wide range of concentrations, and an assay compatible with a fully automated procedure and performed at room temperature. Here, we analyzed the activity of several luciferase reporters in yeast cells and found that cell-surface expressed gLUC (gLUC59) activity can be analyzed directly in a yeast cell culture aliquot at room temperature. We then exhaustively characterized the quantitative capabilities of the gLUC59 assay in yeast cells and determined that has a significantly expanded linear range compared to a fixed-time β -galactosidase assay currently used for HT-Y1H screens. Finally, we used the gLUC59 reporter to establish an optimized Y1H system that is suitable for multiplexed and fully automated gene-centered screens.

MATERIALS AND METHODS

Plasmid constructs and yeast strains

All primers and the corresponding polymerase chain reaction (PCR) products are listed in Supplementary Table S1.

To build pY1 Δ Rep (pLacZi vector [Clontech] carrying the *Sna*BI and *Nhe*I restriction sites instead of the *lacZ* gene), the region upstream of the *lacZ* gene in pLacZi was PCR amplified and digested with *Xho*I/*Sna*BI, and the region downstream of the *lacZ* gene in pLacZi was PCR amplified and digested with *Sna*BI/*Ahd*I. These fragments were ligated into the *Xho*I/*Ahd*I sites of pLacZi. To generate pY1-RLUC, pY1-LUC+ and pY1-gLUC, the coding sequence for each luciferase flanked by *Sna*BI and *Nhe*I sites was PCR amplified and cloned into pY1 Δ Rep.

Surface reporter vectors for yeast genomic integration were built using the pY1-PGA59emp backbone, which consists of the pLacZi vector (Clontech) carrying the coding and terminator sequences of the *PGA59* gene (from the *Cip10::ACT1p-gLUC59* vector (27)) instead of the *lacZ* reporter gene. To build pY1-PGA59emp (pLacZi vector [Clontech] carrying the coding and terminator sequences of the *PGA59* gene from *Cip10::ACT1p-gLUC59* (27) instead of the *lacZ* gene), the region upstream of the *lacZ* gene in pLacZi followed by the *PGA59* signal peptide (+first 2 aa of *PGA59*) were amplified by PCR. Next, the *PGA59* coding sequence followed by the *PGA59* terminator were cloned upstream of the region containing the origin of replication of pLacZi by using two PCR reactions. The final PCR products were digested using *Sma*I/*Bbu*I and *Bbu*I/*Ahd*I, respectively and ligated into the *Sma*I/*Ahd*I of pLacZi. To build pY1-gLUC59, the gLUC59 coding sequence was excised from *Cip10::ACT1p-gLUC59* (27) by digestion with *Bam*HI/*Pf*MI and ligated into the corresponding sites of pY1-PGA59emp. To generate pY1-RLUC59 and pY1-LUC+ 59, each luciferase coding gene (without ATG and stop codons) flanked by *Bbu*I and *Pf*MI (RLUC) or *Bam*HI and *Pf*MI (LUC+) sites were PCR amplified, digested with *Bbu*I/*Pf*MI or *Bam*HI/*Pf*MI and ligated into the corresponding sites of pY1-PGA59emp.

To generate the Gateway™ cloning (Life Technologies) compatible versions of cytosolic and surface luciferase reporter plasmids, an attR1/attR2 recombination cassette was excised from pBluescript (Stratagene) (28) using *Eco*RV restriction digestion and blunt end ligated into the *Sma*I site of pY1-RLUC, pY1-LUC+, pY1-gLUC, pY1-RLUC59, pY1-LUC+ 59 and pY1-gLUC59.

pGLacZi is a Gateway-compatible version of pLacZi (Clontech) (28).

Promoter fragments were PCR amplified from *Saccharomyces cerevisiae* genomic DNA (*ADH1* and *ADH1* Δ promoters), pBridge plasmid (Clontech) (*MET25* promoter), pCRBII-CCA1pr Δ and pCRBII-CCA1pr Δ (TBSmut) plasmids (14) (*CCA1* -363/-192 wild-type and TBS-I mutant promoters), and cloned into pENTR/D-TOPO according to the manufacturer's protocol (Life Technologies).

To generate reporter constructs, promoter fragments in pENTR/D were transferred to pGLacZi, pY1-gLUC_GW, pY1-LUC+_GW, pY1-RLUC_GW, pY1-gLUC59_GW, pY1-LUC+ 59_GW and/or pY1-RLUC59_GW using LR

clonase II according to the manufacturer's protocol (Life Technologies).

YM4271 reporter strains were generated by recombination of reporter plasmids into the *URA3* locus of the yeast genome (Clontech). While single or multiple reporter construct copies may be integrated, stable genomic insertions equalize reporter background levels for each bait strain (11). Briefly, reporter plasmids (unable to replicate in yeast) were transformed into YM4271 cells (*ura3-52*). Integration of the reporter plasmid carrying the wild-type *URA3* gene restores the ability of these cells to grow SD medium without uracil. The genomic integration was confirmed by PCR after several passages onto YPD medium as previously described (16).

pDEST22-TCP vectors were obtained from the pDEST22-TF clone library available at the Arabidopsis Biological Resource Center (ABRC) (<http://abrc.osu.edu>) under stock #CD4-89 (8).

Quantification of reporter gene activities in yeast

To quantify the activity of different luciferases in yeast, reporter strains were grown in YPD to saturation overnight at 30°C, then diluted five times with fresh YPD medium and grown in the same conditions for six additional hours. For phosphate-buffered saline (PBS) washed cells, a 500- μ l aliquot of this cell culture was centrifuged 5 min at 1000 \times *g*, resuspended in 500 μ l of 1 \times PBS pH 7.4, centrifuged again and resuspended in 500 μ l of 1 \times PBS pH 7.4. Reporter gene activity was quantified in 100 μ l (96-well format) or 25 μ l (384-well format) of the cell culture or PBS-washed cells. For all luciferases, flash luminescence emission was determined immediately after addition of 100 μ l (96-well format) or 25 μ l (384-well format) of the enzyme substrate (detailed below). Glow emission was measured every 2 min thereafter up to 60 min after substrate addition. Integration time for the luminescence detection was 1 s/well. For LUC+ activity we used: (i) two commercial assays, the Dual-Luciferase[®] Reporter Assay System (using only the Luciferase Assay Reagent II, LAR II) (Promega) and the Bright-Glo[™] Luciferase Assay System (Promega), and (ii) two lab-made substrate solutions, D-luciferin (A) (25 mM Glycylglycine, 15 mM MgSO₄, 1 mM dithiothreitol, 1 mM D-luciferin potassium salt [Gold Biotechnology]) and D-luciferin (B) (0.01% triton X-100, 1 mM D-luciferin potassium salt [Gold Biotechnology]). For RLUC activity we used: (i) three commercial assays, the *Renilla* Luciferase Assay System (Promega), the *Renilla*-Glo[®] Luciferase Assay System (Promega) and the BioLux[®] *Gaussia* Luciferase Assay Kit (New England Biolabs), and (ii) two lab-made substrate solutions, coelenterazine native (A) (1 \times PBS pH 7.4, 5 mM NaCl, 20 μ M coelenterazine [Promega]) and coelenterazine native (B) (1 \times PBS pH 7.4, 5 mM NaCl, 20 μ M native coelenterazine [Biosynth]). Coelenterazine was dissolved in acidified methanol (10 μ l of 1N HCl per ml of solution) at a concentration of 1 mg/ml (100 \times). For gLUC activity we used: (i) two commercial assays, the *Renilla* Luciferase Assay System (Promega) and the BioLux[®] *Gaussia* Luciferase Assay Kit (New England Biolabs), and (ii) the same lab-made substrate solutions used to quantify RLUC activity.

To quantify the β -galactosidase activity, yeast reporter strains were grown in YPD to saturation (overnight at 30°C), then diluted five times with fresh YPD medium and grown in the same conditions for six additional hours. A 500 μ l aliquot of this cell culture was transferred to an eppendorf tube and centrifuged for 3 min at 1000 \times *g*. The supernatant was discarded and the cell pellet was resuspended in 500 μ l of Z buffer (60 mM Na₂HPO₄, 30 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄) (pH 7.0). Tubes were centrifuged, the supernatant discarded, and the cell pellet resuspended in 500 μ l of Z buffer. Cells were lysed by performing four freeze/thaw cycles (liquid nitrogen/30°C water bath) and 100 μ l of the lysate were transferred to a 96-deep well plate. The β -galactosidase reaction was started by adding 170 μ l of ONPG substrate solution (170 μ l Z buffer, 68.8 nl of 2-mercaptoethanol and 28 μ g of 2-Nitrophenyl- β -D-galactopyranoside [Sigma]) to each well. Plates were incubated at 30°C between 0.5–24 h until color development. The enzymatic reaction was stopped by adding 80 μ l 1 M Na₂CO₃ per well and cleared by centrifugation for 8 min at 1000 \times *g*. OD₄₂₀ was measured in 100 μ l of the supernatant using 96-well plates.

All enzymatic activities were normalized to the cultures' OD₆₀₀ and the β -galactosidase activities further normalized by the reaction time. Luminescence and absorbance measurements were performed at room temperature using a Synergy 2 (96-well) or a SynergyH1 (384-well) multi-mode microplate readers (BioTek) equipped with an injector device.

To determine the dynamic range for the quantification of β -galactosidase and gLUC59 activities we used YM4271 cells carrying the *ADH1::lacZ* and *ADH1::gLUC59* reporter constructs. These strains were grown in YPD overnight at 30°C, then diluted five times with fresh YPD medium and grown in the same conditions for six additional hours. After this incubation, OD₆₀₀ was determined, cells were harvested by centrifugation and resuspended to a calculated OD₆₀₀ = 5 in YPD. Serial dilutions of this cell suspension were generated using wild-type YM4271 cells grown and treated equally. Aliquots of each dilution (100 μ l) were used to determine the β -galactosidase or gLUC (using the lab-made coelenterazine native "B" substrate) activities in a 96-well format as described above. A representative image of gLUC emitted light for each dilution was obtained using a VIM photon counting camera (Hamamatsu Photonics). The linear range of the calibration curve was addressed by means of the Lack-of-Fit test and R², using GraphPad Prism version 6.00 (GraphPad Software, www.graphpad.com). Only datapoints statistically different than background levels were considered (multiple comparisons using one way ANOVA).

Yeast one-hybrid assays

pDEST22-TCP plasmids and the pEXP-AD empty vector control (Life Technologies) were transformed directly into the reporter strains (haploid cell experiments) or into the YU yeast strain (8) (diploid cell experiments) in a 96-well format as described previously (29). Transformants were selected in SD medium without tryptophan (SD-W).

For experiments using haploid cells, transformed YM4271 strains were resuspended in 70 μ l of sterile water (keeping the 96-well format) and 3 μ l of this cell suspension were used to inoculate 96-well deep plates containing liquid SD-W medium (100 μ l/well). Plates were incubated at 30°C for 24–36 h with agitation. Then, 400 μ l of YPD were added to each well and incubation continued for six additional hours. A 100 μ l aliquot of this short-term culture was used to determine the OD₆₀₀. A second 300 μ l aliquot of the short-term culture was transferred to a new deep well plate and centrifuged for 3 min at 1000 \times g. The supernatant was discarded and the cell pellet was resuspended in 150 μ l of Z buffer. Plates were centrifuged for 3 min at 1000 \times g and the cell pellet was resuspended in 25 μ l of Z buffer. Cells were lysed by performing four freeze/thaw cycles (liquid nitrogen/30°C water bath), and the β -galactosidase reaction was performed as indicated above. The β -galactosidase activity was calculated as $[\text{OD}_{420} \times 1000]/[\text{OD}_{600} \times \text{time (min)} \times \text{culture volume (ml)}]$.

For the experiments using diploid cells, YU-TF effector strains (MAT α) were mated with YM4271-reporter strains (MAT α) as reported previously (8,16). Briefly, effector strains were resuspended in 70 μ l of sterile water (keeping the 96-well format) and 5 μ l of this cell suspension were used to inoculate medium lacking tryptophan (600 μ l/well). Plates were grown for 24–36 h at 30°C with agitation using microplate shakers (700 rpm). Simultaneously, promoter strains were grown in 250 ml flasks containing 50 ml of YPD. After incubation, 10 μ l of each culture (promoter and TF strains) were transferred into a new 96-well plate containing 90 μ l of YPD per well. Plates were incubated at 30°C for 24–36 h without agitation. After mating, cells were washed using SD medium without tryptophan and uracil (SD-WU) (selection medium for diploid yeast cells) and resuspended in 180 μ l of SD-WU. A 3 μ l aliquot of this cell suspension was transferred to a new 96-deep well plate containing 100 μ l of SD-WU and incubated at 30°C for 24–36 h with agitation. Then, 400 μ l of YPD were added to each well and incubation continued for six additional hours. Finally, growth (OD₆₀₀) and β -galactosidase activity (OD₄₂₀) were determined as described above for haploid cells.

For gLUC assays in haploid and diploid cells, we followed the same steps described above to determine the OD₆₀₀ of each well. A second 100 μ l aliquot of the short-term culture was transferred to a 96-well white plate. Flash and glow luminescence were determined after the addition of 100 μ l of the lab-made coelenterazine native ‘B’ substrate (1 \times PBS pH 7.4, 5 mM NaCl, 20 μ M native coelenterazine [Biosynth]). The gLUC activity was calculated as Lum/OD₆₀₀.

β -galactosidase and gLUC activities were then normalized to the average value obtained for control wells (pEXP-AD). Binding cut-off was set at 2 fold over the mean of the control value.

Sequence analyses

Protein sequence alignment and phylogeny trees were generated using the Geneious software version 6.0.6 (<http://www.geneious.com>) (30).

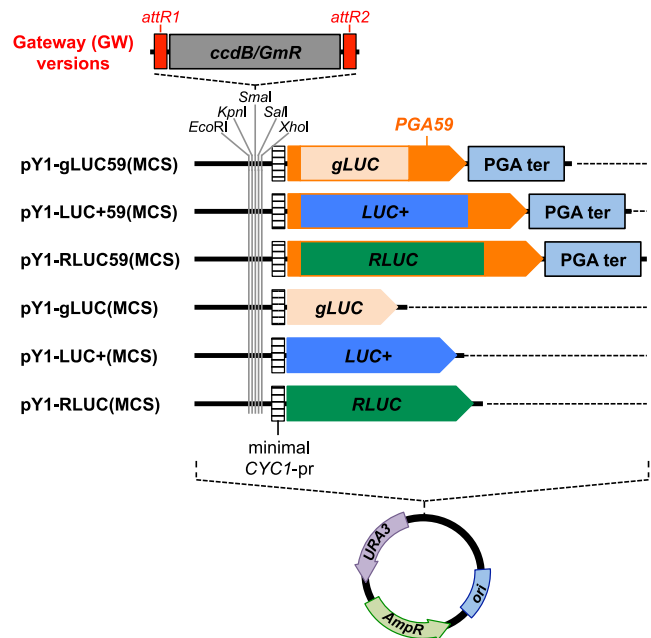


Figure 1. Schematic representation of yeast integration vectors for cytosolic and surface-displayed luciferase reporters. All reporter constructs were built using the pLacZi vector backbone and replacing the *lacZ* gene by each luciferase reporter gene. Surface display vectors also include the *PGA59* gene terminator of *Candida albicans* (see ‘Materials and Methods’ section). For each construct restriction endonuclease-based cloning (MCS) and gateway-based cloning (GW) versions were generated.

RESULTS

gLUC59 outperforms other luciferase reporters in *S. cerevisiae*

The activity of most luciferase enzymes can be quantitatively measured over a wide range of concentrations at room temperature. Furthermore, yeast cells are permeable to some luciferase substrates (e.g. coelenterazine) and luciferase enzymes can be expressed in the extracellular compartment (e.g. linked to the cell surface (27)), suggesting that luciferase quantification assays in yeast could be performed without a cell lysis step. Considering that HT-Y1H screen optimization would require a reporter assay with a long linear range, performed at room temperature and amenable for robotic automation, we envisioned that a luciferase reporter could be used to improve the assay. To determine the type of luciferase enzyme and expression format that would provide the best Y1H reporter for high-throughput screens, we generated reporter vectors for either cytosolic or surface expression of the *Photinus pyralis* (firefly) (LUC+), *Renilla reniformis* (RLUC) or *Gaussia princeps* (gLUC) luciferase reporter genes (Figure 1). Yeast surface expression was achieved by using a translational fusion between each luciferase reporter and the GPI-linked cell wall protein (PGA59) of *Candida albicans* (27). Two versions of each reporter vector were generated to allow the insertion of bait promoter sequences via restriction endonuclease digestion or recombination-based cloning (Figure 1). To evaluate the performance of these luciferase reporters, the constitutive *Alcohol Dehydrogenase I (ADH1)* gene pro-

moter of *S. cerevisiae* was cloned into the cytosolic or surface LUC+, RLUC and gLUC reporter vectors (Figure 1). These constructs were integrated into the chromosome of YM4271 yeast cells and the luciferase activity of each resulting strain was quantified in 96-well plates using a suite of commercially available and lab-made luciferase assays (Figure 2). Both flash and glow luminescence, respectively emitted immediately or between 2 and 60 min after addition of the substrate, were quantified directly from a yeast cell culture aliquot. Results of this experiment indicated that the LUC+ reporter activity was higher in glow than flash bioluminescence assays but exhibited a similar performance when expressed either in the cytosol or the cell surface (Figure 2A and B). RLUC activity was also greater in glow than flash bioluminescence assays, however glow light emission was significantly higher when the enzyme was expressed in the cytosol (Figure 2A and B). Conversely, the gLUC activity was significantly higher in flash than glow bioluminescence assays, especially when the reporter was expressed in the cell surface (Figure 2A and B). Comparing the performance of all reporters, our results indicated that while their activity was similar in glow bioluminescence assays, the activity of cell surface expressed gLUC (gLUC59) was significantly better than the other reporters when flash luminescence was measured. It is important to note that parallel experiments, performed with wild-type YM4271 cells, indicated that these cells did not display an intrinsic bioluminescence activity with any of the enzymatic assays used (Figure 2). Since quantification of the luciferase activity was evaluated directly in a cell culture aliquot, we reasoned that growth medium components could affect the activity of some luciferase reporters, especially those expressed at the cell surface. To evaluate this hypothesis we repeated all luminescence measurements using PBS-washed cells. Interestingly, the luciferase activity for all the reporters tested was significantly lower after the washing step (Figure 2C and D), indicating that the luciferase signal displays higher intensity and better signal to background ratio when measured directly in a yeast cell culture aliquot. Taken together, our initial results indicated that gLUC59 would be a reporter of choice for gene-centered Y1H screens. To determine its performance in a higher throughput format, we ran the gLUC59 assay in 384-well plates measuring both flash and glow luminescence. Importantly, we observed similar luminescence values as those obtained using 96-well plates (Supplementary Figure S2), indicating that the gLUC59 reporter was indeed a suitable alternative for automated gene-centered HT-Y1H screens.

gLUC59 reporter activity correlates with promoter function in intact *S. cerevisiae* cells

To further explore the feasibility of using cell surface expressed gLUC for HT-Y1H screens, we evaluated the linear range of gLUC59 bioluminescence measurements using increasing concentrations of an *ADHI::gLUC59* yeast cell culture. Results of this experiment indicated that the gLUC59 activity linear range extended to four and three orders of magnitude, for flash and glow luminescence measurements, respectively (Figure 3A and B; Supplementary Figure S3A). This represented a significant improvement

compared to the quantification range observed for the fixed-time β -galactosidase assay (1–1.5 orders of magnitude) (Supplementary Figure S1), suggesting that the *gLUC59* reporter activity could be uniformly and accurately quantified in samples with different reaction kinetics. Having established that luminescence measurements were well correlated with gLUC59 concentrations (provided by different concentrations of *ADHI::gLUC59* cells), we next analyzed the ability of the cell surface reporter to detect different expression levels at constant cell concentrations, the latter being the most likely scenario encountered in Y1H screens. To do this, an additional yeast reporter strain carrying a weaker truncated *ADHI* promoter (*ADHI Δ*) was generated (31). Reporter cells carrying either the full-length or truncated *ADHI* promoters were grown to the same density and the gLUC59 activity was quantified. Luminescence was about three–four times higher in reporter cells carrying the full-length *ADHI* promoter for both flash and glow measurements (Figure 3C and Supplementary Figure S3B). To test a conditional promoter, we generated a yeast strain carrying the methionine repressed *MET25* promoter (32) driving the expression of the *gLUC59* reporter. In agreement with the results using the constitutive *ADHI* and *ADHI Δ* promoters, *MET25::gLUC59* cells grown in methionine-depleted medium exhibited higher gLUC59 activity compared to cells grown in methionine-containing medium using both flash and glow luminescence measurements (Figure 3D and Supplementary Figure S3C). These experiments indicated that the differential expression of *gLUC59* translated into quantitative differences of the emitted bioluminescence. As noted previously, while the fixed-time β -galactosidase assay used in HT-Y1H screens has a limited quantitative capability, a time-optimized assay for each individual sample provides an accurate quantification of the β -galactosidase activity (24). Since our goal was to implement a reporter system with quantitative capabilities similar to the best performing time-optimized β -galactosidase assay, we decided to use this method as a reference. Therefore, we generated YM4271 strains carrying the *ADHI::lacZ*, *ADHI Δ ::lacZ* and *MET25::lacZ* reporter constructs and used them to perform the same experiments described above. Quantification of the β -galactosidase activity using the time-optimized assay (Figure 3E and F) showed similar results to those obtained using gLUC59 (Figure 3C and D). Importantly, these experiments revealed that the gLUC59 assay measuring luminescence emission for only 1-s displayed similar results as the ONPG-based β -galactosidase assay using an optimal incubation time for each sample.

Given that the gLUC59 reporter had several advantages for HT-Y1H screens when compared to the commonly used *lacZ* reporter, we reasoned that an improved assay for quantitative HT-Y1H screens could be established using either gLUC59 flash or glow luminescence assays. However, quantifying glow luminescence in a large throughput format (i.e. 384-well plates) would require a stable light emission over time as it takes several minutes for a luminometer to process one microplate. Since in most luciferase assays the luminescence rapidly decays after addition of the enzyme substrate, we evaluated the kinetics of light emission at different gLUC59 concentrations using *ADHI::gLUC59* yeast cells. Our results confirmed a luminescence decrease over time

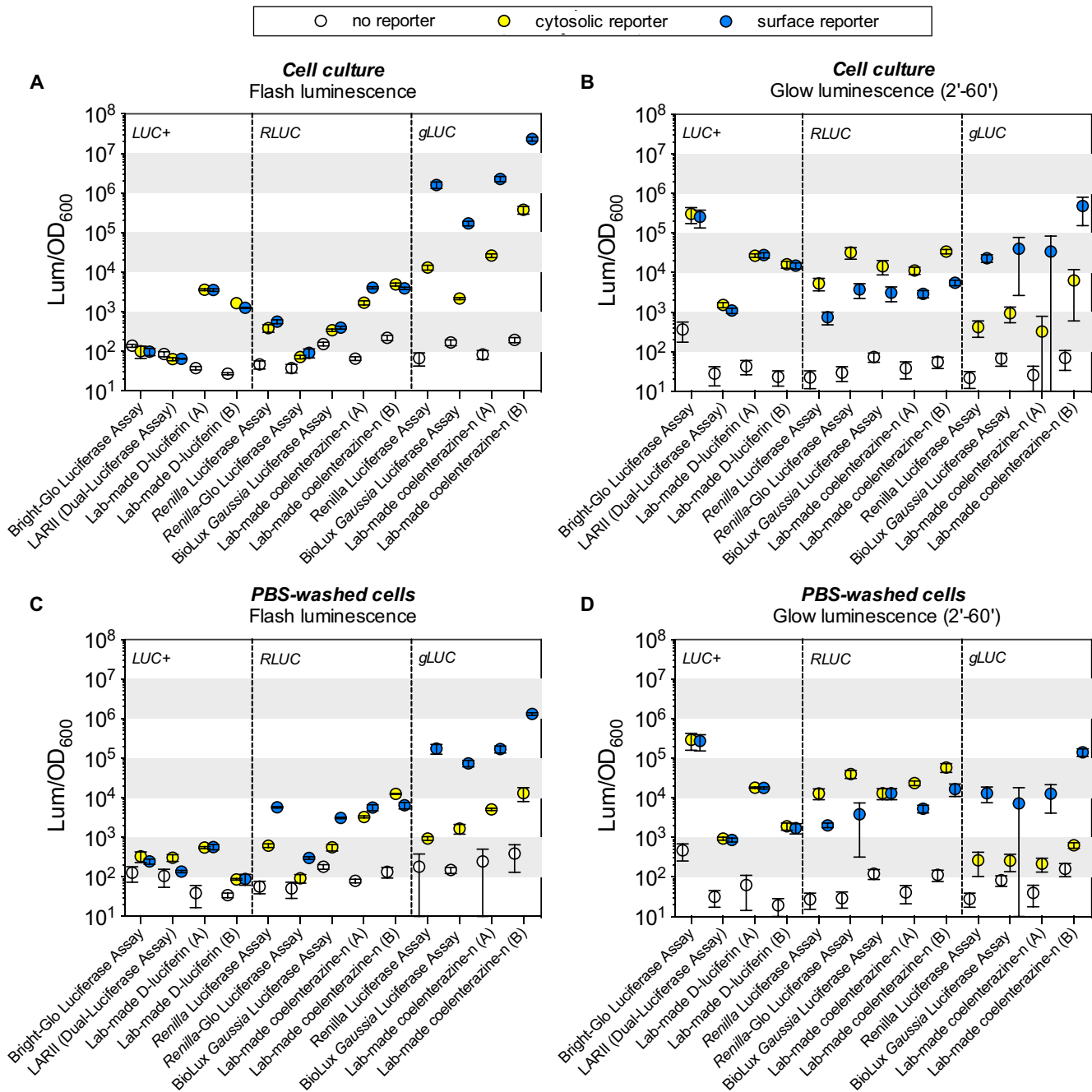


Figure 2. Luciferase activity for cytosolic and surface-displayed reporters expressed in *Saccharomyces cerevisiae* cells. Luciferase activity from YM4271 strains carrying a chromosomally integrated *ADH1::luciferase* (*LUC+*, *RLUC* and *gLUC* as indicated in each panel) reporter construct for either cytosolic (yellow symbols) or cell surface expression (blue symbols). Alternative substrate assay solutions indicated on the x-axis of each graph were tested (see 'Materials and Methods' section). Wild-type YM4271 strains were used as controls (white symbols). (A) Flash luminescence determined directly in a cell culture aliquot immediately after substrate addition ($n = 3$). (B) Glow luminescence determined directly in a cell culture aliquot between 2 and 60 min (2'-60') after substrate addition ($n = 30$). (C) Flash luminescence determined using phosphate-buffered saline (PBS) washed cells immediately after substrate addition ($n = 3$). (D) Glow luminescence determined using PBS washed cells between 2 and 60 min (2'-60') after substrate addition ($n = 30$). Results were normalized to their respective cell density (OD_{600}) and represent average values \pm SD ($n = 3$ independent experiments).

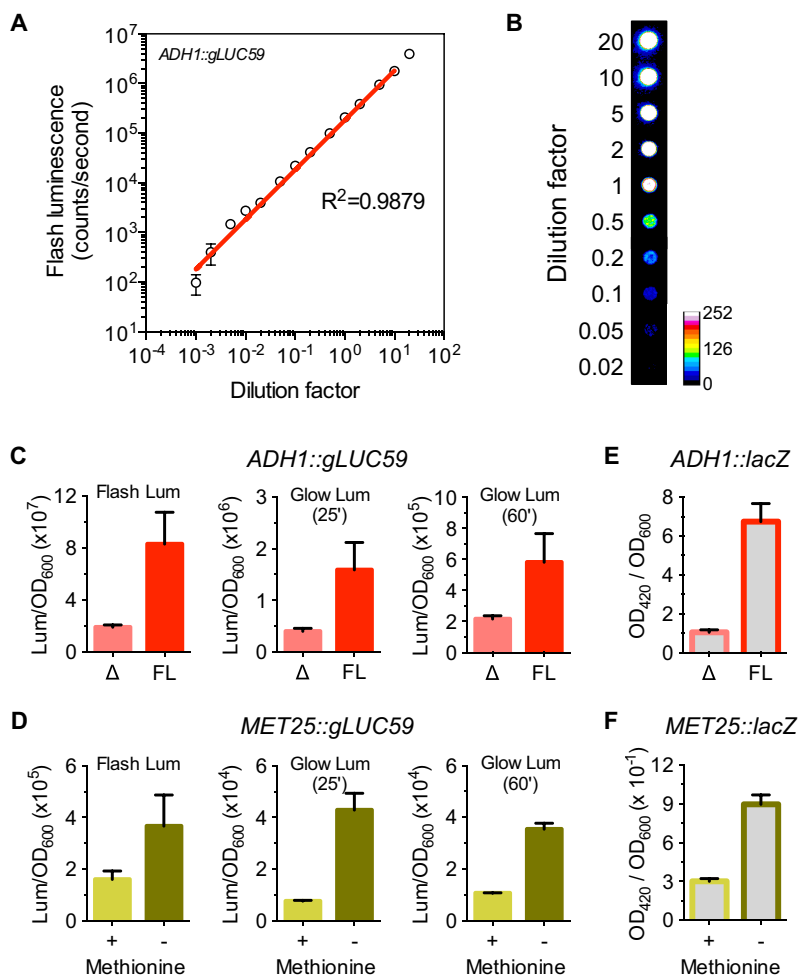


Figure 3. Quantitative performance of the gLUC59 reporter in *Saccharomyces cerevisiae* cells. (A) Analysis of the linearity for the quantification of gLUC59 activity using increasing *ADH1::gLUC59* reporter cell concentrations. The linear range is indicated by the red line (Lack-of-Fit test, $F = 0.4047$, $P = 0.9554$). Luminescence was measured immediately after addition of the enzyme substrate and the results are average values \pm SD ($n = 5$ independent experiments). (B) Representative pseudo-colored image of the dilution series used in C. (C–F) Evaluation of the quantitative capacity of the cell surface gLUC reporter system (C and D) in comparison to the *lacZ* reporter system (E and F) using two *ADH1* promoters of different strength (FL = full-length *ADH1* promoter and Δ = truncated *ADH1* promoter) (C and E), or the methionine repressed promoter *MET25* (+ and – indicates the presence or absence of methionine in the culture medium) (D and F). Results were normalized to their respective cell density (OD₆₀₀) and represent the average values \pm SD ($n = 3$ independent experiments).

following a two-step exponential decay kinetic for all the gLUC59 concentrations tested. After substrate addition the luminescence first decayed rapidly (half-life ~ 30 s) for about 2 min and then slowly (half-life ~ 2 min) for the next 10 min, and finally reached plateau levels that were maintained for the following 48 min (Figure 4A). These results indicated that glow luminescence in gLUC59-based HT-Y1H screens should be measured after the slow decay phase to get comparable quantitative results across multiple wells and plates.

gLUC59 provides an improved reporter system for Y1H screens

We previously established a HT-Y1H screening protocol using a genome-wide clone collection encompassing most Arabidopsis TFs (8). Following this protocol, we set a pilot Y1H experiment using the gLUC59 reporter to investigate the ability of Arabidopsis class-I TCP TFs (Supplementary

Figure S4) to interact with the promoter of the clock gene *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*). A pioneering gene-centered Y1H screen previously uncovered a class-I TCP (TCP21) named CHE (*CCA1* HIKING EXPEDITION), which binds to the *CCA1* promoter region and negatively regulates the *CCA1* promoter activity (14). Through this regulation, CHE modulates the proper period of clock-controlled rhythms (14). CHE shares high homology at the DNA binding domain with the other 12 Arabidopsis class-I TCPs suggesting that at least some members of the subfamily could also bind to the *CCA1* promoter (14) (Supplementary Figure S4). To evaluate the performance of the luciferase-based assay parallel Y1H experiments were performed using the *lacZ* reporter. Yeast reporter strains carrying a *CCA1* promoter region (-363/-192) that contains a canonical class-I TCP binding site (TBS-I) (GGNC-CCAC) were generated. These strains were independently

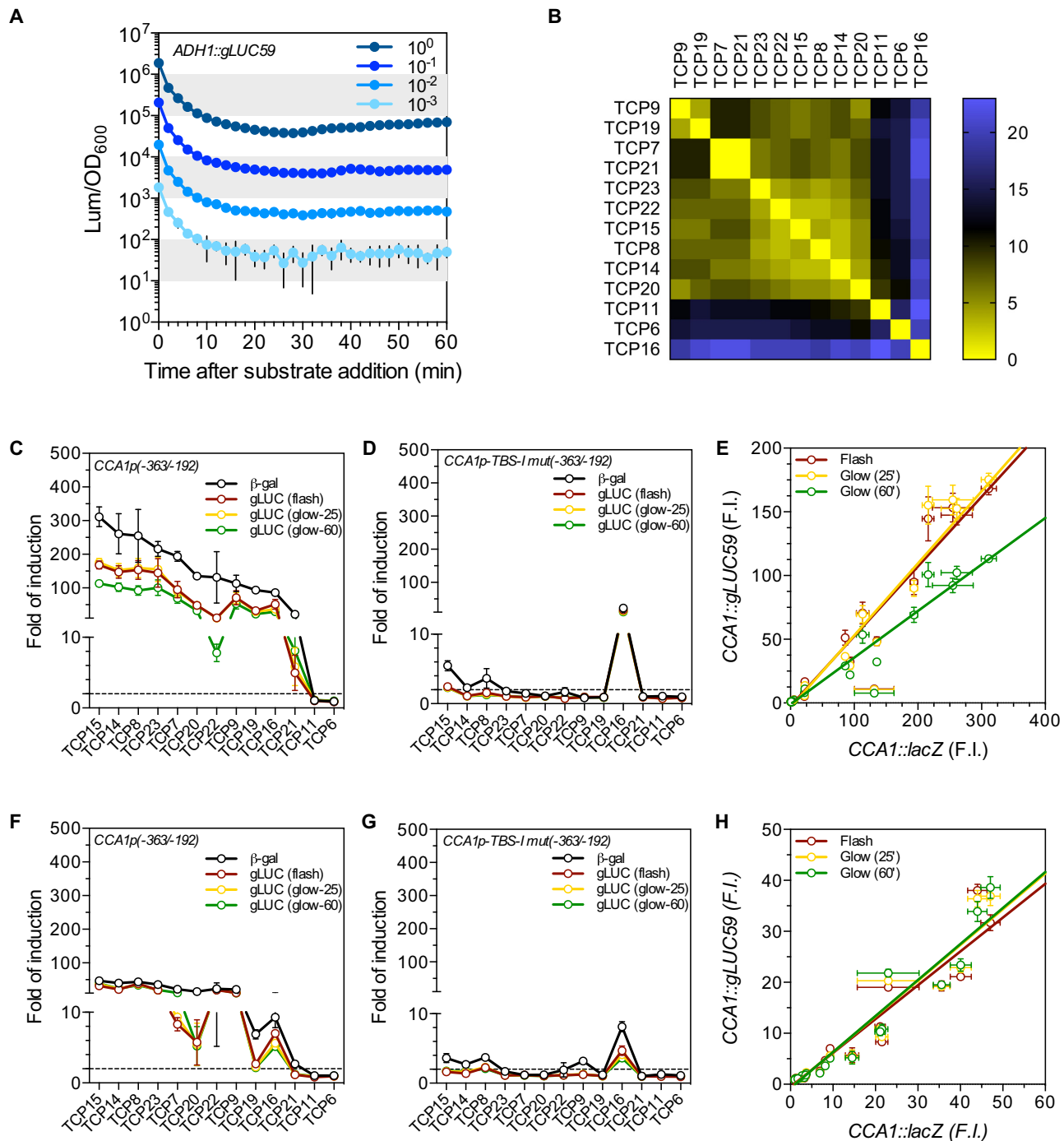


Figure 4. gLUC59-based yeast one-hybrid system. (A) Bioluminescence kinetics decay after substrate addition at increasing concentrations of YM4271 cells carrying the *ADH1::gLUC59* reporter construct. Results were normalized to their respective cell density (OD₆₀₀) and represent average values \pm SD ($n = 8$ independent experiments). (B) Heat map indicating the number of amino acid differences between the DNA binding domains of class-I TCPs. Rows and columns were sorted based on decreasing sequence identity scores. (C and F) gLUC59- and β -galactosidase-based Y1H screens to evaluate the binding of class-I TCP TFs to the -363/-192 *CCA1* promoter region. Experiments were performed in haploid reporter strains transformed with the effector constructs for each class-I TCP (C) or diploid cells after mating the reporter strain (MAT α) with YU cells (MAT α) carrying effector constructs for each class-I TCP (F). Results were normalized to the reporter activity obtained with an empty effector construct. Luminescence measurements were performed at 0 (flash) or 25 and 60 min (glow) after addition of the gLUC substrate. Each symbol represents the average fold of induction \pm SD ($n = 6$ independent experiments). (D and G) gLUC59- and β -galactosidase-based Y1H screens to evaluate the binding of class-I TCPs to the -363/-192 *CCA1* promoter region carrying a mutated class-I TCP binding site (TBS-I mut). Experiment and results were performed as indicated for (C) and (F). (E and H) Comparison of the gLUC59- and β -galactosidase-based Y1H screening results for the experiments shown in (C) and (D) (R^2 [flash] = 0.9276, R^2 [glow-25'] = 0.9177 and R^2 [glow-60'] = 0.9235), and in (F) and (G) (R^2 [flash] = 0.9232, R^2 [glow-25'] = 0.9327 and R^2 [glow-60'] = 0.9346).

transformed with prey plasmids that drive the constitutive expression of each class-I TCP fused to the Gal4 transcriptional activation domain (8). Quantification of the gLUC59 or β -galactosidase activity in the transformed yeast reporter cells revealed that in addition to CHE, 10 class-I TCPs (TCP7, 8, 9, 14, 15, 16, 19, 20, 22 and 23) were able to interact with the *CCA1* promoter (Figure 4C). TCP6 and TCP11, did not show interaction with the -363/-192 *CCA1* promoter fragment suggesting that either they were not properly expressed in yeast or that they did not bind to the TBS-I in the promoter bait. In support to the latter, a sequence analysis indicated that indeed the DNA binding domains for both TCP6 and TCP11 are less conserved compared to most other class-I TCPs (Figure 4B). To confirm that, as for CHE, the TBS-I mediated the interactions detected for TCP7, 8, 9, 14, 15, 16, 19, 20, 22 and 23, we performed Y1H assays using *gLUC59* and *lacZ* reporter strains carrying a mutated TBS-I (GGTCCCAC to TTGAAACA) within the -363/-192 *CCA1* promoter region (14). These assays revealed a significant reduction in the reporter activity for TCP7, 8, 9, 14, 15, 19, 20, 21, 22 and 23 transformed cells, indicating that these TFs interacted with the *CCA1* promoter through the TBS-I (Figure 4D). TCP16 was the exception showing a reduced but still significant induction of the reporter activity suggesting unique DNA binding abilities for TCP16 among class-I TCPs. Notably, we found a close correlation between the results obtained with the *gLUC59*-based Y1H system, using both flash or glow bioluminescence measurements, and the *lacZ*-based Y1H system, using the reference time-optimized ONPG-based β -galactosidase assay (Figure 4E). This further confirmed our initial observations regarding the quantitative capabilities of the *gLUC59* assay and indicated that the *gLUC59* reporter outperforms the *lacZ* reporter in gene-centered HT-Y1H screens.

While yeast transformation provides an effective mean to deliver TF prey constructs into reporter cells in a low throughput format, yeast mating provides a more convenient alternative when performing HT-Y1H screens using large TF collections (e.g. 1956 Arabidopsis TFs) and multiple yeast reporter strains. For mating-based Y1H screens, TF constructs are first transformed into a MAT α yeast strain, then these cells are mated with the Y1H reporter strains (MAT α), and finally diploid TF-reporter cells are selected and the reporter activity is quantified (8). To evaluate if the *gLUC59* reporter could be used in a mating-based approach, we transformed class-I TCP constructs into YU yeast cells (8) and mated these cells with the reporter strains carrying the wild-type or TBS-I mutated versions of the -363/-192 *CCA1* promoter region. Quantification of the *gLUC59* or β -galactosidase activities in the resulting diploid cells provided similar results, although with lower overall reporter activities (Figure 4F–H), as the ones obtained when TF constructs were directly transformed into the reporter cells (Figure 4C–E and Supplementary Table S2). These results further confirmed that the *gLUC59* reporter could be effectively used for mating-based HT-Y1H screens.

DISCUSSION

Gene-centered Y1H screens provide a straightforward, comprehensive and unbiased strategy to unveil the TF-interaction landscape of a single promoter region. By design, the Y1H system is not suited to accurately reveal the strength of TF–DNA interactions and essentially delivers positive or negative results based on the expression level of a reporter gene. Thus, establishing a reliable cut-off value for the reporter activity and confidently determining which are the TF–promoter interactions that result in reporter activities above or below this limit are critical for the interpretation of Y1H screen results. This is especially important for promoter baits that drive high expression of the reporter gene in the absence of effector constructs as high background reporter levels often confound the identification of positive interactions (8). The improved quantitative capability of the *gLUC59* reporter presented here provides a strengthened ability to establish cut-off values and to discern between positive and negative interactions with higher confidence. In addition, the larger linear range of *gLUC59* activity quantification allows proper ranking of positive interactions, which (as suggested by our previous work (8)) provides a useful criterion to prioritize TF candidates and guide follow-up studies.

Assays to quantify the commonly used β -galactosidase reporter activity in a high-throughput format exhibit a short linear range and rapidly reach saturation. In addition, the procedures have several technical limitations, such as long and variable incubation times for color development, time-consuming freeze-thaw steps to lyse yeast cells, temperature distribution bias across microplates or well-to-well color signal diffusion. Here, we describe the characterization of a novel reporter system that improves the quantitative capabilities of gene-centered HT-Y1H screens and that additionally provides a simplified assay suitable for full automation. We evaluated six different luciferase reporters, including three novel cell surface expressed reporters and determined that *gLUC59* is the most versatile as it displays the best performance for both flash and glow bioluminescence measurements. The *gLUC59* assay additionally provides a cost effective option as the highest luminescence intensities were obtained using a lab-made substrate solution. We also found that, unlike the β -galactosidase activity, the *gLUC59* activity can be quantified directly in a yeast cell culture aliquot without additional washing or cell lysis steps. Furthermore, the assay is performed at room temperature and requires minimal incubation time after addition of the enzyme substrate. More importantly, in these conditions the *gLUC59* quantification assay displays a linear range that extends for up to four orders of magnitude and thus is significantly larger than the one obtained with a fixed-time β -galactosidase assay (16). This improved quantification capability allows, using a luminescence integration time of only 1 s per well, an accurate quantification of the reporter activity that is comparable to using a β -galactosidase assay where the reaction time is optimized to fit the specific reaction kinetics in each well. Thus, the *gLUC59* reporter enables a uniform Y1H screen procedure for the simultaneous quantification of multiple reactions with different enzymatic activities. Furthermore, the increased sim-

plicity of the gLUC59 assay reduces the processing time and enables fully automated gene-centered Y1H screens including experimental procedure and data analyses (Supplementary Figure S5 and Table S3). It should be noted that gLUC59 reporter strains are fully compatible with histidine auxotrophic selection, which could be used in combination with the cell-surface luciferase to call positive interactions (33).

Furthermore, we showed that the gLUC59 reporter could be used for mating-based Y1H assays although with a lower sensitivity compared to experiments where effector plasmids are directly transformed into the reporter strain. This observation is in line with previous reports that compared transformation- and mating-based Y1H screens (34) and suggests that the different sensitivity of the assay in haploid and diploid cells is not dependent on the reporter used but rather an inherent property of diploid cells that affects the overall expression of reporter genes. A transformation-based screen might be considered if a higher sensitivity is needed. In fact, our experiments indicate that the interaction of TCP21/CHE with the *CCA1* promoter is clearly revealed when haploid cells were used, which is consistent with TCP21/CHE being initially discovered in a small-scale gene-centered Y1H screen using haploid cells (14). However, transformation of a genome-wide clone collection (e.g. ~2000 Arabidopsis TF clones) into reporter strains is time-consuming and thus a mating-based approach would be of choice for gene-centered HT-Y1H screens. Given the increased quantitative power of the gLUC59 assay, we anticipate that by analyzing HT-Y1H screen results, where thousands of interactions are evaluated simultaneously, it will be possible to establish more accurate baseline and cut-off values, thereby improving our ability to define positive interactions and ultimately the sensitivity of mating-based Y1H screens. In support to this notion the Y1H experiment using diploid cells also revealed the TCP21/CHE-*CCA1* promoter interaction as indicated by the higher gLUC59 reporter activity compared to the background control (Supplementary Table S2).

Results of pilot experiments using the gLUC59-based Y1H assay confirmed its improved performance. In particular, these experiments indicate that TCP7, 8, 9, 14, 15, 16, 19, 20, 22 and 23 interact with the *CCA1* promoter suggesting that several class-I TCPs regulate the Arabidopsis clock function through *CCA1*. These results are in part validated by a recent report showing that TCP20 and TCP22 bind to the *CCA1* promoter and regulate *CCA1* expression *in planta*, and that *tcp20/22* loss-of function shortens the period of clock-controlled rhythms (35). We found that two class-I TCPs, TCP6 and TCP11, do not interact with the -363/-192 *CCA1* promoter region. In support to this finding, an amino acid sequence comparison of all class-I TCPs indicates that the DNA binding domain of TCP6 and TCP11 is significantly different compared to the other subfamily members (Figure 4B and Supplementary Figure S4). Furthermore, it was recently reported that TCP11 shows a different DNA-binding specificity, with preference for the GTGGGCCNNN sequence, due to a threonine residue at position 15 of the TCP domain (36) (Supplementary Figure S4C). The binding of TCP7, 8, 9, 14, 15, 19, 20, 22 and 23 was mediated by the TBS-I in the *CCA1* promoter (Fig-

ure 4D and G). However, this was not the case for TCP16 suggesting that this TF binds to the mutated TBS or to another element within the -363/-192 region of the *CCA1* promoter. In support to the latter, it is important to note that TCP16 is phylogenetically distant from all other class-I TCPs, has a DNA binding domain significantly different to most of them (~50% identity) (Figure 4B and Supplementary Figure S4), and was shown to preferentially interact with the consensus binding site for class-II TCP proteins (GTGGNCCCNN) (37). Taken together, our results indicate that most class-I TCPs bind to the *CCA1* promoter and thus likely regulate the clock function, and that TCP16 function is possibly associated to responses of both TCP classes. Given that class-I TCPs regulate plant responses to several signals such as light, and biotic and abiotic stress (38), our findings suggest that these TFs represent a regulatory hub that controls the Arabidopsis clock function by multiple environmental cues.

Here, we established a novel luciferase-based Y1H system that is faster, simpler and more powerful than the current methods used for gene-centered screens (Supplementary Figure S5 and Table S3). Importantly, the enhanced quantitative capabilities of the gLUC59 reporter assay improves the detection of positive interactions, and allows a uniform procedure and data analysis regardless of each promoter bait background activity. In addition, gLUC59-based Y1H screens have minimal pre-assay requirements, and demand less and shorter steps that are fully compatible with automation. Notably, these improvements did not create any concomitant disadvantage when compared to existing methods (Supplementary Table S3). Furthermore, the gLUC59 reporter could further contribute to develop future approaches that may require the isolation of yeast cells carrying positive interactions (i.e. Y1H coupled to next generation sequencing technologies) (33), as yeast expressing the cell-surface reporter could be immunolabeled and purified by cell sorting or other cell isolation methods. Given the success of gene-centered Y1H screens to unveil TF-promoter interactions and the continuous development of genome-wide TF clone collections, we anticipate that the upgraded approach presented here will be widely adopted and further contribute to the longstanding efforts toward disentangling the intricate mechanisms that regulate gene expression across species.

ACCESSION NUMBERS

Sequences for the pY1-gLUC59(MCS) and pY1-gLUC59(GW) plasmids are available on GenBank under accession numbers KY581738 and KY581739, respectively.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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the experiments. S.E.K. contributed to execute some experiments. J.L.P-P. and G.B. analyzed results for all experiments. J.L.P-P., K.B. and Z.L. wrote the manuscript. All authors contributed to discussions and edited the manuscript.

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