nature portfolio

Peer Review File

Predictive genetic circuit design for phenotype reprogramming in plants

Corresponding Author: Dr Shuyi Zhang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Kong et al. describes the development of improved predictive genetic circuit design for plants. The methodology described here builds on previously developed methods for the rapid characterization of plant genetic parts (repressors, activators, promoters) using Arabidopsis protoplasts. Despite the inherent variability of protoplast transient assays, this work demonstrates that calculating relative promoter units (RPU) to quantify the output of each promoter significantly reduces the variability observed among different batches of protoplasts, allowing these data to be used to model the behavior of these parts. The authors then use this methodology to characterize repressor-promoter pairs by deriving their input-output functions, and build 1- and 2-input logic gates to integrate input signals in plants. The predictability of genetic circuit behavior is demonstrated in stable transgenic Arabidopsis roots, as well as by transient gene expression using Agroinfiltration of *N. benthamiana* leaves.

This manuscript provides a significant contribution to the predictability of genetic circuit design in plants, is well written and provides sufficient methodological details for the majority of the work. I believe this approach can significantly improve the application of genetic circuits to reprogram plant function.

Although the parts characterization and modeling portions of the manuscript are robust, my main concern lies with the *in planta* demonstration of the genetic circuits. For example, in Fig. 5, both the A IMPLY B and NAND logic gates appear to function as predicted in transgenic Arabidopsis roots. However, additional transgenic lines (shown in Extended Data Fig. 6) do not display the same behavior, and in fact, the NAND logic gate does not show NAND logic in the Extended Data. Therefore, I wonder if the results shown in Fig. 5 are actually the exception and not the norm. I suggest testing multiple additional independent transgenic lines to verify if this behavior is indeed observed.

Another main concern is with the methodology for testing the OR logic operation in N. benthamiana leaves. It is not clear from the described methods how a single leaf was treated with no hormones, NAA only, tZ only and both hormones. Were these hormones infiltrated into the leaf? In which case, the hormones probably diffuse to other parts of the leaf and may confound the results. More details are needed for these experiments to properly assess the behavior of the circuit.

Finally, the discussion section should include some comments regarding possible reasons for why some of the more complex gates did not match the predictions as well as the simpler ones, both in terms of clear distinction between on and off states, as well as the magnitude of the signals. Clear distinction between states becomes very important when logic gates are layered.

Minor concerns:
Lines 112-114: what is meant by "replacing" the DNA sequence in the promoter? Did you remove the corresponding number of nucleotides from the 35S promoter and inserted the operator sequence? Or was the operator added to the promoter at a specific location? This is an important detail for future research on building repressible and inducible plant promoters.

Line 263: autoactive

Line 450: what is meant by biological replicates? Does this mean different batches? What about the technical replicates within an experiment? Please clarify.

Lines 457-458: Position 2 is mislabeled either in panel a or in c.

Lines 460-461, c.: It seems surprising that placing multiple copies of Lmr RE in the promoter does not affect much of the 35S promoter activity (w/out LmR-NLS). This may be related to a point made earlier regarding replacement or addition of operator sequences on the 35S promoter.

Lines 465-466, e: why are some promoters labeled with a .1? Please explain these (in methods, perhaps).

Lines 490-491, h: tZ arrow has to point to the PTCSn promoter.

Line 501: The NIMPLY, NAND and AND experimental results do not show robust gate function. The on and off states seem barely distinguishable (although significant). A discussion of these results should be included in the Discussion section of the manuscript.

Line 516: spell out PI – propidium iodide.

Lines 535-536, Fig. 6d: Conductivity also seems to be increasing for the empty treatments, however, because it is initially lower, the increase doesn't seem as pronounced. Also, why is the conductivity so much higher in the TRUE gate compared to empty at 0h post induction? What is the empty treatment here? It should be Agrobacterium carrying no plasmids (or an empty plasmid, that doesn't have a gate). Please clarify these issues in the text.

Line 585: incorrect figure reference. Perhaps Fig. 3d?

Line 651: Agrobacterium

Line 665: provide references for Golden Gate and Gibson Assembly.

Line 786: Leica.

Line 797: Fig. 6a.

Line 844: Extended Data Table 4 doesn't seem to be mentioned in the text.

Lines 848-851: There are no red or blue colored letters in the table.

Reviewer #2

(Remarks to the Author)
In this work, Kong and colleagues present a predictive framework for designing synthetic genetic circuits in plants. The authors created various versions of synthetic, repressible promoters and tested their performance in the presence of repressors from the TetR family. They then created a library of NOT gates with inputs driven by auxin- and cytokinin-responsive promoters. Using this data, they constructed a predictive model based on the Hill equation and subsequently assembled a suite of two-input Boolean logic gates in protoplasts. Finally, the authors demonstrated the functionality of A IMPLY B and NAND gates in Arabidopsis roots and an OR gate for programmable control of cell death in Nicotiana benthamiana.

Auxin and cytokinin responsive promoters are used as sensors for the circuits, and the hypersensitive response as the output of an OR gate in N. benthamiana. It was nice to see the use of the hypersensitive response to show that the circuits have so little leak that a toxic product can be adequately turned off by it. A common complaint about circuits is the leak and if it is too much for toxic product repression - this goes some ways to address this. Separation of the work into hard-coded circuits, circuits with sensors, and circuits with an actuator is useful.

Overall the model used is very simple, most of the TetR family proteins with similar designs have been in the study by Brophy et al (PMID: 35951698), and the protoplast system isn't very reproducible, such that it required further normalization to come up with Relative Promoter Units (RPU). As such the novelty of the study in terms of new innovations in plant circuits is somewhat limited. The modeling that is performed is very simple, however no one else has done anything like this so it is a valuable addition. The authors admit that the model lacks certain parameters, so the limitations are acknowledged.

Comments:

1. Lines 25-26: The authors have claimed the construction of "a library of orthogonal sensors…" while in reality, the authors have used previously reported two inducible promoters: responsive to auxin and cytokinins. As such two sensor components cannot be called a library.

2. Line 44: It is wrong to refer to some organisms as "higher" than others. You could describe them as more complex or as multicellular, but higher is meaningless in this context.

3. The authors claim to have established a rapid, quantitative, and predictive framework in plants that takes only ~5 days for the design and testing of synthetic circuits in plants. However, in reality, it will take more time for Golden Gate cloning, including sequencing, to get to the test constructs (Level 2). Can the authors please outline a realistic timeline for the full experiment, or give clearer constraints on what is being achieved within the 5 days?

4. On lines 73-74, the authors mention that precise quantification of genetic parts in transient systems (protoplasts) has not been established previously. However, there are some well-established protoplast based assays available that are more robust, high-throughput, and reproducible without the need of extra normalization (such as RPU in this study). Furthermore, the quantity of plasmid used in these transfections is unusually high (30 µg).

5. Line 78: the 100 and 200 bp of 35S promoters are the truncated versions of the wild-type 35S promoters, and as such cannot be called synthetic promoters. A suitable name would be “truncated” or “truncated versions” of the 35S promoter.

6. As the transfection efficiency in protoplasts varies from 30-80%, what were the reasons for not using a normaliser in the first place?

7. Extended Fig1a: The promoters shown are native promoters and not synthetic, so the label in the Figure should be changed accordingly.

8. The number of stably transformed Arabidopsis lines used for screening at T2 stage to determine the functionality of A IMPLY B and NAND gates is missing from the manuscript. This is important to describe.

9. Some of the circuits I would not call circuits (direct sensor-output fusions).

10. Fig 2D: what do the gray bars mean? Please define it with a legend.

11. The authors should release the full plasmid sequences in Genbank or similar format.

12. Line 91-92: Cite paper on batch variation of luciferase assays in protoplasts (Schaumberg et al., 2016 Nature Methods: https://www.nature.com/articles/nmeth.3659)

13. Lines 175-177: Can the authors please explain the meaning/consequences of the change in the Hill coefficient when >2 in the context of circuits.

14. Line 219: Why would lack of cell division be bad for circuit propagation? This is also an issue in stably transformed plant cells that are non-dividing (mature leaf or root tissue)

15. Lines 287-289: who has held this long-held belief? Can you offer a citation?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you for addressing my comments and concerns.

Reviewer #2

(Remarks to the Author)
In their revised manuscript the authors have addressed most of my prior comments suitably.

Regarding point 9, I would argue that a direct fusion of a promoter (e.g. a hormone induced promoter) to the output (Rluc) doesn't really count as a Buffer (or YES) gate. To me this is still too simple to call a gate. An enormous number of studies have made direct fusions of cell type- or treatment-specific promoters to GFP and other genes for many years, and this is not usually called a Buffer gate. Regarding the authors' OR gate, it is also questionable as to whether I would really call it an OR gate, as it is just two output genes (Rluc) each driven by a different sensor promoter. In this respect, these designs are somewhat simple compared to various other studies in recent years, and these could be reworded in the manuscript.

We thank all the reviewers for their time and effort in reading and offering insightful comments on our manuscript. We believe we have addressed all the comments and strengthened the quality of the manuscript. Point-by-point responses are given below **in blue text**, and the manuscript has been updated accordingly. Any modifications or additions are highlighted **in red text** in the revised manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Kong et al. describes the development of improved predictive genetic circuit design for plants. The methodology described here builds on previously developed methods for the rapid characterization of plant genetic parts (repressors, activators, promoters) using Arabidopsis protoplasts. Despite the inherent variability of protoplast transient assays, this work demonstrates that calculating relative promoter units (RPU) to quantify the output of each promoter significantly reduces the variability observed among different batches of protoplasts, allowing these data to be used to model the behavior of these parts. The authors then use this methodology to characterize repressor-promoter pairs by deriving their input-output functions, and build 1- and 2-input logic gates to integrate input signals in plants. The predictability of genetic circuit behavior is demonstrated in stable transgenic Arabidopsis roots, as well as by transient gene expression using Agroinfiltration of *N. benthamiana* leaves.

This manuscript provides a significant contribution to the predictability of genetic circuit design in plants, is well written and provides sufficient methodological details for the majority of the work. I believe this approach can significantly improve the application of genetic circuits to reprogram plant function.

Although the parts characterization and modeling portions of the manuscript are robust, my main concern lies with the *in planta* demonstration of the genetic circuits. For example, in Fig. 5, both the A IMPLY B and NAND logic gates appear to function as predicted in transgenic Arabidopsis roots. However, additional transgenic lines (shown in Extended Data Fig. 6) do not display the same behavior, and in fact, the NAND logic gate does not show NAND logic in the Extended Data. Therefore, I wonder if the results shown in Fig. 5 are actually the exception and not the norm. I suggest testing multiple additional independent transgenic lines to verify if this behavior is indeed observed.

We sincerely appreciate your recognition of our work's contribution to enhancing the predictability of genetic circuits in plants and your insightful comments on the *in planta* demonstrations. We agree that verifying consistent behavior across multiple independent transgenic lines is crucial for confirming circuit robustness.

Regarding the performance of the NAND circuit, we would like to clarify that the output values of the NAND circuit are significantly lower than those of the IMPLY circuit. This difference likely accounts for the overall lower fluorescence intensity in NAND lines, making state-specific

differences less visually distinct. The IMPLY circuit exhibited higher output than the NAND circuit as predicted by our models (2-fold) and validated in protoplast experiments (2.8-fold, Fig. 4a). In the main figure, we adjusted the brightness to facilitate visualization, whereas in the extended data figure, we included the unadjusted root image.

To improve clarity and facilitate visual inspection, we have now also applied consistent brightness adjustments across the four states for each NAND line in Extended Fig. 6b. This adjustment demonstrates and confirms the circuit functionality (Extended Fig. 6e) in different lines. Thus, we believe the results observed in Fig. 5 represent the norm rather than an exception.

We apologize for not clearly stating this in the original manuscript. These adjustments have now been explicitly detailed in the figure legend for transparency:
(Line 653): "Brightness adjustments were applied only for visualization purposes."

Another main concern is with the methodology for testing the OR logic operation in N. benthamiana leaves. It is not clear from the described methods how a single leaf was treated with no hormones, NAA only, tZ only and both hormones. Were these hormones infiltrated into the leaf? In which case, the hormones probably diffuse to other parts of the leaf and may confound the results. More details are needed for these experiments to properly assess the behavior of the circuit.
We appreciate your comment and agree that clarifying the hormone treatment methodology is essential for accurately assessing circuit behavior. In our study, we infiltrated each hormonal treatment into separate, spatially distinct regions on the same leaf. This infiltration method of small molecule inducers is a commonly used approach for gene expression induction (e.g., Ngou et al., J Exp Bot, 2020; Wang et al., Mol Plant, 2023). Furthermore, previous studies have demonstrated that such localized infiltration treatments limit the inducer's effect to the treated area, preventing diffusion to other parts of the leaf and thus minimizing confounding effects (Roberts et al., PLOS Genet, 2013).

We have revised and added these details to the Methods section to provide greater transparency and reproducibility for this aspect of the experiment:
(Lines 842-844): "For inducers treatments, we applied the infiltration method for gene expression induction, which has been used in several studies[64-66]."
(Lines 848-852): "After 24 hours post-infiltration, the infiltrated leaves were treated with different hormonal inputs to test the OR logic operation. Specifically, we infiltrated water, NAA, tZ, and both hormones into separate, spatially distinct regions on the same leaf, with sufficient spacing between each treatment to minimize the risk of hormone diffusion between regions."

Finally, the discussion section should include some comments regarding possible reasons for why some of the more complex gates did not match the predictions as well as the simpler ones, both in terms of clear distinction between on and off states, as well as the magnitude of the signals. Clear distinction between states becomes very important when logic gates are layered.

Thank you for your insightful suggestion. The discrepancies between the predicted and observed performance of more complex gates, compared to simpler ones, may be attributed to three main factors: cellular resource competition, protein turnover dynamics, and limitations in our modeling (Brophy and Voigt, Nat Methods, 2014; Shakiba et al., Cell Syst, 2021; Şimşek et al., Trends Biotechnol, 2023).

1. Cellular resource competition:

Complex circuits require more cellular resources for transcription and translation, which intensifies resource competition. This competition can attenuate the signal strength of each single gate and reduce the dynamic range, impairing the distinction between on and off states in the final circuit (Mather et al., Biophys J, 2013).

2. Protein turnover dynamics:

The absence of cell division in protoplasts leads to challenges in signal propagation through genetic circuits. In non-dividing protoplast systems, repressor proteins accumulate over time, disrupting signal propagation in layered NOT gates. This buildup keeps the first layer suppressed, reducing activation of subsequent layers and blurring on/off states (Jadhav et al., Open Biol, 2022). In dividing cells, the repressor is diluted over time by cell division to realize signal propagation, whereas in non-dividing cells, the repressor protein persists longer, thus affecting the circuit performance. Incorporating protein degradation tags in non-dividing cells could improve protein dynamics, improving circuit performance (Chassin et al., Nat Commun, 2019).

3. Modeling limitations:

Our models did not fully consider essential factors such as resource competition and protein degradation dynamics. These simplifications led to optimistic predictions, particularly for multi-layered circuits. Future models integrating part characterization and dynamic approaches could improve predictive accuracy and circuit optimization (Schreiber et al., Mol Syst Biol, 2016; Yazdani et al., PLOS Comput Biol, 2020; Gómez-Schiavon et al., ACS Synth Biol, 2020).

We have added this section in the discussion to address this point:

(Lines 222-229): “These discrepancies could be attributed to three main factors[31,32]: (1) cellular resource competition, as complex circuits demand substantial transcriptional and translational resources, potentially leading to signal attenuation[33]; (2) protein turnover dynamics, where non-dividing protoplast system permits the accumulation of repressors, hindering effective signal propagation in layered circuits[34]; and (3) simplifications in our predictive modeling, such as ignoring resource constraints and degradation dynamics, which may have led to inaccuracies, particularly for more complex, multi-layer circuits[35].”

(Lines 312-319): “While our current modeling framework is robust, it oversimplifies certain factors, such as cellular resource competition and protein turnover dynamics, that may influence circuit performance in plants. These aspects are particularly important in more complex circuits, where multi-layered gates require precise regulation and efficient signal propagation[31,32,35]. Future efforts should include incorporating protein degradation tags to mitigate accumulation issues[48], alongside multidimensional characterization of parts[49] and the

integration of both mechanistic and machine learning models[50,51], to enhance model accuracy and optimize complex synthetic genetic circuits[52-56].”

Minor concerns:

Lines 112-114: what is meant by "replacing" the DNA sequence in the promoter? Did you remove the corresponding number of nucleotides from the 35S promoter and inserted the operator sequence? Or was the operator added to the promoter at a specific location? This is an important detail for future research on building repressible and inducible plant promoters.

Yes, we removed the corresponding number of nucleotides from the 35S promoter and inserted the operator sequence to maintain the appropriate spacing between the CAAT and TATA boxes. We have updated the manuscript to reflect this detail clearly:

(Lines 116-119): “We then replaced the DNA sequence with either one or two copies of operators from the TetR family repressor at five different sites of the 200-bp 35S promoter, maintaining the overall length of the promoter sequence.”

Line 263: autoactive

Thank you for pointing this out. We have changed the typo from " atuoactive " to "autoactive" on Line 273.

Line 450: what is meant by biological replicates? Does this mean different batches? What about the technical replicates within an experiment? Please clarify.

Thank you for your comment. By "biological replicates," we are referring to different experimental batches, where each replicate is an independent biological sample. In our study, each biological replicate was subjected to two distinct LUC gain settings for data collection. Within each setting, we used two technical replicates. We have clarified this in the manuscript to avoid any confusion:

(Lines 492-494): “Three biological replicates (different protoplasts batches, B1, B2, B3) of protoplasts were tested under two LUC gain settings (S1, S2), with two technical replicates per setting for each biological replicate.”

Lines 457-458: Position 2 is mislabeled either in panel a or in c.

Thank you for pointing this out. Position 2 refers to the region between B1 and the first CAAT box. In the LmrA operator-based promoter design (panel c), position 2 is located immediately upstream of the first CAAT box. We have corrected this mislabeling in the revised Fig. 2c to ensure consistency.

Lines 460-461, c.: It seems surprising that placing multiple copies of Lmr RE in the promoter does not affect much of the 35S promoter activity (w/out LmR-NLS). This may be related to a point made earlier regarding replacement or addition of operator sequences on the 35S promoter.

Thank you for your comment. As we mentioned previously, we removed the corresponding number of nucleotides from the 35S promoter and inserted the operator sequence to maintain the appropriate spacing between the CAAT and TATA boxes, thus preserving the

overall promoter length. Additionally, in our design, we carefully avoided modifying the TATA and CAAT boxes throughout the entire promoter region, including the core promoter, by preserving their sequences and maintaining the proper spacing. This approach ensured that the promoter activity remained unaffected.

Lines 465-466, e: why are some promoters labeled with a .1? Please explain these (in methods, perhaps).

Thank you for your suggestion. Specifically, these labels indicate two distinct replacements of the operator sequence within Position 5, a 25-nt region located between the TATA box and the transcription start site (TSS). For operators with shorter sequences (e.g., the 20-nt BM3R1 operator), we hypothesized that positioning the operator either closer to the TATA box (".1") or the TSS (".2") might affect transcriptional regulation differently. Thus, both upstream and downstream placements were tested to evaluate potential differential effects on promoter activity (Fig. 2d, Extended Data Fig. 2 and 3). We have added more explanation in the methods to clarify this point:

(Lines 720-723): "Promoter variants labeled with '.1' and '.2' correspond to different replacements of the operator sequence within a 25-nt region between the TATA box and the transcription start site, with '.1' placed closer to the TATA box and '.2' closer to the TSS."

Lines 490-491, h: tZ arrow has to point to the PTCSn promoter.

Thank you for catching this. We have corrected the arrow in the figure to ensure it accurately points to the PTCSn promoter as intended.

Line 501: The NIMPLY, NAND and AND experimental results do not show robust gate function. The on and off states seem barely distinguishable (although significant). A discussion of these results should be included in the Discussion section of the manuscript.

Thank you for your suggestion. As discussed in our response to your Major Concern #3, the observed issues with the NIMPLY, NAND, and AND experimental results likely arise from cellular resource competition, which limits transcriptional and translational resources, and protein turnover dynamics, particularly in non-dividing protoplasts where repressor accumulation affects signal propagation for multiple-layer circuit. We have added the discussion on Lines 222-223.

Line 516: spell out PI – propidium iodide.

Thank you for the suggestion. We have spelled it out for clarity.

Lines 535-536, Fig. 6d: Conductivity also seems to be increasing for the empty treatments, however, because it is initially lower, the increase doesn't seem as pronounced. Also, why is the conductivity so much higher in the TRUE gate compared to empty at 0h post induction? What is the empty treatment here? It should be Agrobacterium carrying no plasmids (or an empty plasmid, that doesn't have a gate). Please clarify these issues in the text.

Thank you for raising this point. For the electrolyte leakage assays, the "empty" treatment refers to *Agrobacterium* infiltration with an empty vector, which does not contain a functional

circuit construct. The "0 hours post-induction" measurement reflects conductivity 24 hours after the *Agrobacterium* infiltration, before the external inducers were added.

Regarding the specific issues raised:
1. Increase in conductivity for empty treatments: The gradual increase in conductivity likely results from mild stress caused by the infiltration and *Agrobacterium*, not the specific constructs, reflecting general physiological responses in the plant.
2. Higher conductivity in the TRUE gate at 0h post-induction: This is likely due to basal expression of the gate constructs during the 24-hour pre-induction period, causing early ion leakage and higher conductivity compared to the empty treatment.

These points have been addressed and integrated into the revised manuscript, ensuring that the experimental design and observations are clearly communicated:
(Lines 569-571): "The same genetic circuit was introduced through *Agrobacterium tumefaciens* infiltration, followed by inducers infiltration 24 hours later. Conductivity was measured 24 hours after inducer treatment."
(Lines 576-577): "Empty refers to *Agrobacterium* infiltration with an empty vector."

Line 585: incorrect figure reference. Perhaps Fig. 3d?
Thank you for pointing this out. Upon review, the correct reference is indeed Fig. 3d. We have updated the manuscript accordingly to reflect this correction.

Line 651: Agrobacterium
Thank you for catching this typo. The incorrect spelling has been corrected in the revised manuscript. We appreciate your attention to detail.

Line 665: provide references for Golden Gate and Gibson Assembly.
Thank you for pointing this out. References for the methods mentioned have been added in the revised manuscript. Specifically, Golden Gate Assembly (Engler et al., PLOS ONE, 2008) and Gibson Assembly (Gibson et al., Nat Methods, 2009) are now cited in the Methods section.

Line 786: Leica.
Thank you for catching this typo. We have corrected it in the revised manuscript.

Line 797: Fig. 6a.
Thank you for pointing this out. We have updated the manuscript accordingly.

Line 844: Extended Data Table 4 doesn't seem to be mentioned in the text.
Thank you for pointing this out. Extended Data Table 4 is included to provide a clearer description of the induction conditions used for the protoplast-based logic gates, supplementing the experimental details in the Methods section. We inadvertently omitted referencing this table in the text. We have now added the appropriate citation to the table in the revised manuscript:

(Lines 772-773): “The concentrations of inducers, unless otherwise indicated, are provided in Extended Data Table 4.”

Lines 848-851: There are no red or blue colored letters in the table.
Thank you for highlighting this detail. We have corrected this issue and restored the intended color coding in the revised version for clarity.

Reviewer #2 (Remarks to the Author):

In this work, Kong and colleagues present a predictive framework for designing synthetic genetic circuits in plants. The authors created various versions of synthetic, repressible promoters and tested their performance in the presence of repressors from the TetR family. They then created a library of NOT gates with inputs driven by auxin- and cytokinin-responsive promoters. Using this data, they constructed a predictive model based on the Hill equation and subsequently assembled a suite of two-input Boolean logic gates in protoplasts. Finally, the authors demonstrated the functionality of A IMPLY B and NAND gates in Arabidopsis roots and an OR gate for programmable control of cell death in Nicotiana benthamiana.

Auxin and cytokinin responsive promoters are used as sensors for the circuits, and the hypersensitive response as the output of an OR gate in N. benthamiana. It was nice to see the use of the hypersensitive response to show that the circuits have so little leak that a toxic product can be adequately turned off by it. A common complaint about circuits is the leak and if it is too much for toxic product repression - this goes some ways to address this. Separation of the work into hard-coded circuits, circuits with sensors, and circuits with an actuator is useful.

Overall the model used is very simple, most of the TetR family proteins with similar designs have been in the study by Brophy et al (PMID: 35951698), and the protoplast system isn’t very reproducible, such that it required further normalization to come up with Relative Promoter Units (RPU). As such the novelty of the study in terms of new innovations in plant circuits is somewhat limited. The modeling that is performed is very simple, however no one else has done anything like this so it is a valuable addition. The authors admit that the model lacks certain parameters, so the limitations are acknowledged.

Comments:

1. Lines 25-26: The authors have claimed the construction of “a library of orthogonal sensors…” while in reality, the authors have used previously reported two inducible promoters: responsive to auxin and cytokinins. As such two sensor components cannot be called a library.
We appreciate your comment. We used the term "library" to describe a group of orthogonal sensors, modular synthetic promoters, and NOT gates, which were constructed and quantitatively characterized in our study. However, we recognize that referring to it as a

"library" might imply a more extensive collection than what was presented. To clarify, we have revised the manuscript to describe these components as a "group," which more accurately reflects their scope in this study.

2. Line 44: It is wrong to refer to some organisms as “higher” than others. You could describe them as more complex or as multicellular, but higher is meaningless in this context.
We appreciate your comment and agree with your suggestion. We have revised the text to avoid using the term "higher organisms" and have instead described them as "more complex organisms" to more accurately convey the intended meaning.

3. The authors claim to have established a rapid, quantitative, and predictive framework in plants that takes only ~5 days for the design and testing of synthetic circuits in plants. However, in reality, it will take more time for Golden Gate cloning, including sequencing, to get to the test constructs (Level 2). Can the authors please outline a realistic timeline for the full experiment, or give clearer constraints on what is being achieved within the 5 days?
We appreciate your insightful comment. The 5-day timeline is based on the assumption that Level 2 plasmids have already been constructed and verified, which is usually how we perform circuit construction. With this starting point, the subsequent steps—including Gibson assembly for circuit constructs, sequencing verification, plasmid preparation, protoplast transient transformation, and functionality testing—can be completed within this period. This timeline is intended to highlight the rapid pace at which circuit testing can be achieved, especially in comparison to the significantly longer durations required for stable transformation. As you rightly pointed out, if the construction of both Level 1 and Level 2 plasmids is factored in, it would add approximately five additional days, bringing the total time to around 10 days. We have updated the text and figure accordingly.

4. On lines 73-74, the authors mention that precise quantification of genetic parts in transient systems (protoplasts) has not been established previously. However, there are some well-established protoplast based assays available that are more robust, high-throughput, and reproducible without the need of extra normalization (such as RPU in this study). Furthermore, the quantity of plasmid used in these transfections is unusually high (30 µg).
We thank you for your comment. Regarding existing protoplast-based assays, we agree that they are robust and high-throughput, often utilizing fluorescence or luminescence signals normalized by plasmid quantity or protein levels. These methods provide metrics like relative light units (RLU) for gene expression analysis (e.g., Feike et al., Plant Biotechnol J, 2019; Belcher et al., Nat Chem Biol, 2020). Our approach enhances this framework by introducing normalization through relative promoter units (RPUs), which accounts for both plasmid input and variability across samples (Fig. 1d). This additional step ensures more accurate and quantitative comparisons of promoter activities in transient systems, addressing variability challenges more effectively than traditional methods. Additionally, using RPUs facilitates the connection and exchange of parts to enable circuit design. We have modified the text to clarify our point:
(Lines 75-77): “…and precise quantification of genetic parts in such transient systems has not been established to facilate gate normalization and connection for circuit design.”

Regarding the quantity of plasmid in protoplast transformation systems, in a widely used protocol, approximately 10–20 µg of plasmid DNA is typically employed in a 100 µL reaction with 2×10^4 protoplasts (e.g., Yoo et al., Nat Protoc, 2007; Wu et al., Plant Methods, 2009). The reaction setup we described in Methods section was scaled to a 200 µL system with 30 µg of DNA, maintaining proportionality and staying within reasonable and established ranges for effective transformation.

5. Line 78: the 100 and 200 bp of 35S promoters are the truncated versions of the wild-type 35S promoters, and as such cannot be called synthetic promoters. A suitable name would be “truncated” or “truncated versions” of the 35S promoter.
Thank you for your suggestion. We have revised the terminology to refer to the 100 and 200 bp promoters as "truncated promoters".

6. As the transfection efficiency in protoplasts varies from 30-80%, what were the reasons for not using a normaliser in the first place?
Thank you for your comment. We believe you are referring to the normalization of transfection efficiency. In our assay, in addition to use the normalization promoter (RPU), we also used GUS as a normalizer, alongside the LUC reporter on the same plasmid, to account for variations in transfection efficiency. Therefore, transfection efficiency normalization was indeed considered in our study.

7. Extended Fig1a: The promoters shown are native promoters and not synthetic, so the label in the Figure should be changed accordingly.
We appreciate your suggestion. The promoters used in Extended Fig. 1a are indeed truncated promoters, not synthetic. We have updated the figure label to accurately describe them as "truncated" promoters.

8. The number of stably transformed Arabidopsis lines used for screening at T2 stage to determine the functionality of A IMPLY B and NAND gates is missing from the manuscript. This is important to describe.
We appreciate your suggestion. We have updated the manuscript to include this information: (Lines 247-249): “For both circuits, we observed the expected and input-responsive logical behavior within *Arabidopsis* roots of three T2 lines for A IMPLY B and four for NAND.”

9. Some of the circuits I would not call circuits (direct sensor-output fusions).
We appreciate your comment. While BUFFER gates may seem simplistic, they still qualify as two-input circuits. In these gates, the two inputs are two external inducers, which regulate the activity of a single sensor promoter. The output is determined by how the inputs influence the promoter, enabling the circuit to respond to two signals. This satisfies the definition of a circuit, where distinct inputs are processed to produce a specific output. This classification is consistent with previous work in synthetic biology, where even simple systems like BUFFER gates are recognized as functional circuits (Brophy et al., Science, 2022; Khan et al., Nat Biotechnol, 2024).

10. Fig 2D: what do the gray bars mean? Please define it with a legend.
We appreciate your suggestion. The gray bars in Fig. 2D represent the RPUs after repression, and we have defined this in the figure legend. To enhance clarity, we have now also included a label directly in the revised Fig. 2d.

11. The authors should release the full plasmid sequences in Genbank or similar format.
We appreciate your suggestion. We have released all plasmids in the Inventory of Composable Elements at https://registry.jgi.doe.gov/ . After logging in, the plasmids can be found under the ACS Synbio Registry tab. Additionally, we have provided all plasmid sequences as a supplemental zip file.

12. Line 91-92: Cite paper on batch variation of luciferase assays in protoplasts (Schaumberg et al., 2016 Nature Methods: https://www.nature.com/articles/nmeth.3659)
Following your suggestion, we have cited the paper in our revised text:
(Lines 95-96): "…, consistent with the batch variation reported previously[13]."

13. Lines 175-177: Can the authors please explain the meaning/consequences of the change in the Hill coefficient when >2 in the context of circuits.
Thank you for your comment. In the context of synthetic genetic circuits, a higher Hill coefficient enables the system to exhibit a steep, nonlinear response to input changes. This is crucial for circuits requiring sharp "on" and "off" transitions and robust signal propagation. It has been demonstrated in cascaded and multi-layered circuits that a Hill coefficient greater than 2 helps amplify small inputs across layers (Ferrell and Ha, Trends Biochem Sci, 2014). Specifically, it was shown that the fold-change in the output is greater than the fold-change in the input if the Hill coefficient (n) is greater than 2, and smaller if n is less than 2 (Ferrell and Ha, Trends Biochem Sci, 2014). This property is key to enabling effective signal propagation in layered circuits. We have updated the manuscript to incorporate these clarifications:
(Lines 181-184): "A higher Hill coefficients means the system exhibits a steep, nonlinear response to input signals. And a Hill coefficient greater than 2 ensures effective signal amplification of the input signals, which is critical for maintaining reliable signal propagation in multi-layered circuit designs[30]."

14. Line 219: Why would lack of cell division be bad for circuit propagation? This is also an issue in stably transformed plant cells that are non-dividing (mature leaf or root tissue)
Thank you for your comment. Since proteins are usually stable, in non-dividing protoplast systems, once repressor proteins are produced, they will accumulate and persist over time, disrupting signal propagation in layered NOT gates. This buildup keeps the first layer suppressed, reducing activation of subsequent layers and blurring on/off states (Jadhav et al., Open Biol, 2022). In dividing cells, the repressor will be diluted by cell division over time to enable signal propagation, whereas in non-dividing cells, the repressor protein persists longer, thus affecting the performance of the circuit. Incorporating protein degradation tags in non-dividing cells could counteract this accumulation, improving circuit performance (Chassin et al., Nat Commun, 2019).

Specifically, regarding non-dividing stably transformed plant cells, both protoplast and stably transformed plants share the challenge of repressor accumulation due to the lack of cell division, which can impair signal propagation in multi-layered circuits. Our observations, as presented in Figures 5d and 5g, indicate that the circuit outputs in stably transformed plants are consistent with those observed in protoplasts, suggesting a similar effect of non-dividing cellular environments in both systems. To mitigate this, future design might introduce protein degradation tags to help increase the dynamic turn over of repressor, thereby improving circuit performance in both protoplasts and mature tissues of stably transformed plants.

15. Lines 287-289: who has held this long-held belief? Can you offer a citation?

Thank you for pointing this out. We acknowledge that our wording may have led to some misunderstanding. Our intention was to emphasize the challenges often discussed in the rational engineering of plants, such as the complexity of plant systems, long generation times, and variability in transgene expression, which have been highlighted in several reviews (e.g., Liu and Stewart, Trends Plant Sci, 2015; Medford and Prasad, Plant J, 2016; Patron, New Phytol, 2020; and Vazquez-Vilar et al., J Exp Bot, 2023).

To address your concern, we have revised the text and included citations to clarify:
(Lines 297-300): " The findings of our study suggest that, despite challenges associated with rational engineering in plants[44-47], predictive design and modeling of genetic circuits are feasible when leveraging repressor-synthetic promoter pairs and NOT gates. These results mark an incremental yet significant step toward developing synthetic biology tools tailored for plant systems."

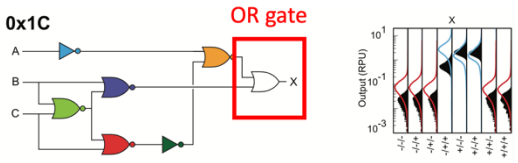
Reviewer #2 (Remarks to the Author):

In their revised manuscript the authors have addressed most of my prior comments suitably.

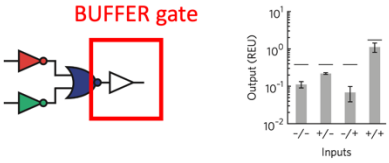
Regarding point 9, I would argue that a direct fusion of a promoter (e.g. a hormone induced promoter) to the output (Rluc) doesn't really count as a Buffer (or YES) gate. To me this is still too simple to call a gate. An enormous number of studies have made direct fusions of cell type- or treatment-specific promoters to GFP and other genes for many years, and this is not usually called a Buffer gate. Regarding the authors' OR gate, it is also questionable as to whether I would really call it an OR gate, as it is just two output genes (Rluc) each driven by a different sensor promoter. In this respect, these designs are somewhat simple compared to various other studies in recent years, and these could be reworded in the manuscript.

Response: Thank you for your comment. In our manuscript, we use the terms "BUFFER gate" and "OR gate" within the context of genetic circuits, drawing inspiration from their definitions in digital circuits. For the BUFFER gate, our design captures the logic of directly passing an input signal to an output, similar to an electronic buffer gate. Specifically, a non-zero input signal (e.g., activation of an induced promoter) produces a high output (expression of luc), while the absence of input results in no output. Similarly, the OR gate in our system follows its logical definition, producing an output when at least one input signal is present. This is implemented by two separate sensor promoters independently driving the same output gene, ensuring that either input signal triggers expression.

While these designs may appear simplistic, it is common in genetic circuit design to test and validate these gates, and they can also serve as essential building blocks for more complex circuits, as illustrated in circuit designs from two example papers.



Nielsen et al., 2016, Science
Genetic circuit design automation



Stanton et al., 2014, Nature Chemical Biology
Genomic mining of prokaryotic repressors for orthogonal logic gates

However, we acknowledge that these gates may seem basic compared to others. To enhance clarity and better reflect this point, we have added further discussion on the reasoning behind building and testing these simple gates:
(Lines 301-305): “Although some designs, such as the BUFFER and OR gates presented here, may appear relatively simple, they serve as foundational building blocks for more complex circuit construction. These basic logic gates provide essential proof-of-concept demonstrations, validating the application of logic gate principles in plant systems.”

We appreciate the opportunity to address this and hope the updated text resolves any concerns.