

Metabolically robust ratiometric autoluminescent reporters

Corresponding Author: Dr Karen Sarkisyan

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

(Comments for the Author)

The manuscript entitled “Metabolically robust autoluminescent reporters” is an important advance on a captivating realm of innovation – autoluminescent plants and their uses. Since the first robustly autoluminescent plants were developed (including the work of authors here), the idea to use luminescence as a reporter output was a clear possibility that had not been achieved. The present study nicely demonstrates the amount and frequency of luminescence changes that occur in autoluminescent plants – even independently of apparent transcriptional control of the enzymatic component luciferase. As such large and frequent fluctuations are not observed when luciferin substrate is provided in excess, the interpretation is that the biochemistry of substrate synthesis is highly variable. To avoid this variation, which confounds reporter/sensing functions, the authors set out to create relevant orange/red shifted luciferase constructs by fusion to appropriate fluorescent proteins. The screening approach is clever and efficient and appears to have revealed highly successful new BRET proteins compatible with autoluminescent plants and opening the chance for ratiometric reporters that can be used even when raw luminescence fluctuates. The authors apply the principle to transcriptional reporters because transcription is a general mechanism with myriad important applications. The present manuscript is somewhat light on characterisations and descriptions (see comments below). Nonetheless, I think other groups would find this contribution exciting and prompt wider use of autoluminescence in plants.

Major comments:

“In contrast, when treated with cytokinin, the ratio showed expected dose-dependent response (Figure 3d, Supplementary Figure 10), providing quantitative readout amid metabolic fluctuations.”

This is a crucial point of validation – is the dose-dependency as expected? A comparison with TCSn-fluorescence protein readout is warranted here. And an honest discussion of quantitative must be included here as it is not dose-dependent quantitation in the strictest sense (e.g. 10x more signal for 10x more hormone). This is NOT a fundamental critique of the new approach – promoters are not truly quantitative under the best circumstances and no technique is perfect – just a comment that it is more useful to be specific about what has been achieved. If the quantitative nature is similar to YFP – that’s useful to know. It could even be superior. The only way to know is to complete a dose-response curve in TCSn-LUZ vs TCSn-GFP or YFP (even better to use a ratiometric TCSn fluorescent system). It could be a minimized experiment with a few more concentrations in the dynamic range of TCSn in the given cell/plant system with the compound used. In Supp Fig 12 the side-by-side comparison with Q-PCR is great. Here it should be discussed that transcript detects a low dose a bit better.

The nature of transgenes and promoters in plants precludes confidence in use of a second promoter-reporter as a truly stable ‘control’. All promoters have idiosyncrasies in practice and can also be differentially susceptible to stimuli and gene silencing. These issues are worsened if the two constructs are delivered on separate transgenes (insertion site effects). All of these issues will need to be discussed as limitations of the approach.

Additional comments:

1. Out of curiosity, what do the images of the mixed expression (ratiometric reporter) leaves look like before/after stimulus? Is the color ratio change detectable at all – I’m thinking of Figure 2B.
2. Additionally, it would be nice (not required) to see the Alphafold predictions of the new fusion proteins.

3. "Co-expression of red Luz608 and green Luz533 in the plant cell culture (Supplementary Figures 10) or *Nicotiana benthamiana* leaves (Figure 3a, Supplementary Figures 11-12), led to a major stabilisation of the signal, compared to intensity-based measurements."
-As this is a key finding, it deserves a more substantive explanation in the text (not just in figure 3A legend). For example, why is the non-ratiometric signal so variable after thidiazuron?
4. Figure 3C lacks SA treatment and 3D lacks mock treated negative control.
5. Figure 3D comes before C in the text.
6. Figure 3D should include the multiple doses as stated in the text.
7. All y-axes should have clear labelling of which metric is being quantified. Some only have info in title and others (Fig 3D) lack the information.
8. proWRKY70 is not an SA reporter – it is responsive to many stimuli (including defense signalling upstream of SA) and cannot be discussed as an SA reporter with any specificity or quantitative power. I suggest rephrasing as reporter gene and immune regulator whose expression that integrates defense signals.
9. proORCA3 is more characterised and specific for early JA signalling, but all native promoters are indirect and, at least quantitatively, integrate multiple signals. Even synthetic and simplified (i.e. TCSn) reporters aren't perfectly specific and often fail to respond in a variety of tissues/contexts. This doesn't mean proWRKY70 and proORCA3 aren't valid tools for proof of principle, but better to be more specific about what is being detected.
10. Figure 3E – what is the arrow representing? How much of the leaf was infiltrated?
11. This work is analogous to a body of literature on BRET constructs (e.g. NanoLuc based red-shifts). These will need to be discussed and cited. Is there no previous work with LUZ? Also, why aren't any of the existing BRET constructs suitable at all?
12. Matryoshka type ratiometric fluorescent proteins are also useful parallel in the fluorescence realm.

Reviewer #2

(Comments for the Author)

This manuscript describes a convenient approach to make autoluminescent reporters less sensitive to metabolic perturbations that impact luciferin biosynthesis or cofactor availability. The use of fluorescent proteins as FRET acceptors is a well-established method for altering luciferase emission wavelength and sometimes improving brightness, and ratiometric indicators are widely accepted as the most quantitative, so the combination of these approaches represents a tidy addition to the optical toolkit. The nnLuz fusions in this manuscript are the first described FRET constructs using this enzyme, to my knowledge.

The rationale and engineering approaches used are clearly articulated in the manuscript, and the insertion/deletion mapping done on nnLuz will be useful for future engineering efforts beyond the reporters generated in this work. The FRET efficiency for the new Luz constructs could certainly be improved, but is more than sufficient for the application described. The additional demonstrations using the bacterial Lux system and NLX FRET constructs corroborates the nnLuz results nicely and provides a strong motivation for future improvements to the bacterial autoluminescent system. Cytokinin and jasmonic acid sensor results are highly convincing and are good demonstrations of the advantages of this ratiometric approach.

This work is well suited to publication in *Nature Plants*. I recommend addressing the following as minor revisions:

1. If possible, the authors should comment on why they chose not to make any insertions of FP FRET acceptors in the N-terminal region of the protein, since this region appears especially insensitive to both in their initial screen.
2. Salicylic acid reporter results are not represented well in Supp. Fig. 13. The dynamics are easily apparent in Supp. Video 1, but very hard to discern in the figure.

I suggest adding more time points to Supp. Fig. 13, zooming to one or both regions of interest rather than showing the entire image frame, and possibly adding a line diagram of the plant anatomy being imaged to help orient the reader. As it is currently, it's difficult to tell where the leaf boundaries are, so a diagram or even an overlaid outline on the first image would be helpful.

The frame rate for Supp. Video 2 could be slower to make it easier to discern the rapidly changing speckles. For this sensor especially, higher-resolution imaging (higher magnification, single leaf, higher time resolution) would be very nice to see included, if feasible.

Finally, for the videos of the salicylic acid reporter, an accompanying Supp. Figure should be included with time plots of

red:green ratio for several representative regions of interest, at least over a subset of the time points, as feasible given the amount of motion between frames.

3. If possible, the supplementary data sets would be more useful if provided as .xlsx or .csv formatted tables rather than as PDFs.

4. Figure 3d: The colors are too similar for the two plotted lines. A more varied color palette would be beneficial for this and other panels in this figure.

Reviewer #3

(Comments for the Author)

In this paper the authors present some exciting technological improvements to their prior work using the fungal bioluminescence pathway to enable the study of gene expression dynamics in plants. Their key innovation is the development of a red shifted version of the luciferase to enable ratiometric normalization as a means to disambiguate the impact of fluctuations in metabolism that affect luciferin synthesis from true changes to reporter expression. While the authors present some exciting data, the description and replication provided of the work they report are not sufficient to substantiate the claims they make. Additionally, the rationale for the various protein engineering tasks they undertake to design this novel Luz are not explained or justified in any depth, making it challenging for future groups to build on this work. Finally, the authors do very little to characterize these reporters beyond their spectral separability. This limits the utility this tool may have to the broader community.

I suggest that the authors revise this manuscript in the following ways:

1. The authors should do more to explain the rationale for the engineering approaches they used. For example, why did they focus on internal insertions rather than terminal ones, what was the rationale for the linker lengths and compositions they screened and what is the explanation for the optimal one chosen. What hypothesis do they have for why one linker was better or worse than another and are there any general insights there? In what way is the V5 luz better? Why was an additional FP fused to this protein? And finally, what was the impact of each of these changes and was one particularly impactful? As it stands now, the results are presented without any detail.
2. In figures 2 b and c the degree of variation in the spectrum plots is not characterized. These experiments must be replicated.
3. For figure 2C it is not clear what the authors actually expressed in planta, and how these constructs were built (promoters and terminators used etc). This makes it challenging to know if this is an apples to apples comparison with the fungal bioluminescence pathway. This should be more clearly articulated to substantiate the claim the authors make, that the fungal bioluminescence pathway was brighter than the bacterial one. They should also provide some rationale for why, i.e. relative expression, relative turn over of luciferase, luciferin biosynthesis etc.
4. In figure 3 the authors describe experiments to demonstrate the capacity for their dual luz system for normalization, however, it is important to see secondary evidence to justify that the stable light output correlates with stable levels of their reporter luz (qPCR of the gene or western blots).
5. This work would be more impactful if the authors characterized the off-rates of their reporters. They do a good job of demonstrating how rapidly the reporters can record increases in gene expression, but the off rates will depend on protein degradation. This is especially relevant as their engineered red luz might have significantly different stabilities than the green luz, which might impact normalization.
6. The experiments the authors present on whole plants in figure 3 C and E need some more work. They do not present sufficient details on replication, especially on 3E. Answering the following questions would help address these issues. Are these plants from a single insertion event or more than 1? What generation are they and are they homozygous for the transgenes? What is the variability of performance of normalization across plants, i.e. do they see consistent responses across lines? How tied is the brightness of the reporters tied to expression of the transgenes? Did the authors need to adjust the relative expression of red shifted to green luz? Does the expression of the rest of the pathway matter?
7. How dose responsive is this system? The data to determine this seems to be in Supplementary Figure 12, but the authors have not performed the necessary statistical correlation analysis to be able to make a strong claim.
8. In supplementary Figure 8 the authors should use an Anova with the appropriate correction to make multiple comparisons rather than what appears to be multiple separate t-tests.
9. The authors should provide more detailed information about the sequences of their constructs and their experimental protocols so the broader community can build on this work.

Version 1:

Reviewer comments:

Reviewer #1

(Comments for the Author)

The authors have addressed all my concerns. This work is a much appreciated advance for the field.

Reviewer #2

(Comments for the Author)

I do not have any additional comments, this manuscript is suitable for publication in its revised form. The authors have sufficiently addressed all my original requests.

Reviewer #3

(Comments for the Author)

I believe the authors have adequately addressed all my concerns.

I commend them on the rigour and clarity with which they put together the supplementary note regarding their protein engineering strategy. The addition of multiply validated dose dependency also significantly improves the utility of this tool.

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The manuscript entitled “Metabolically robust autoluminescent reporters” is an important advance on a captivating realm of innovation – autoluminescent plants and their uses. Since the first robustly autoluminescent plants were developed (including the work of authors here), the idea to use luminescence as a reporter output was a clear possibility that had not been achieved. The present study nicely demonstrates the amount and frequency of luminescence changes that occur in autoluminescent plants – even independently of apparent transcriptional control of the enzymatic component luciferase. As such large and frequent fluctuations are not observed when luciferin substrate is provided in excess, the interpretation is that the biochemistry of substrate synthesis is highly variable. To avoid this variation, which confounds reporter/sensing functions, the authors set out to create relevant orange/red shifted luciferase constructs by fusion to appropriate fluorescent proteins. The screening approach is clever and efficient and appears to have revealed highly successful new BRET proteins compatible with autoluminescent plants and opening the chance for ratiometric reporters that can be used even when raw luminescence fluctuates. The authors apply the principle to transcriptional reporters because transcription is a general mechanism with myriad important applications. The present manuscript is somewhat light on characterisations and descriptions (see comments below). Nonetheless, I think other groups would find this contribution exciting and prompt wider use of autoluminescence in plants.

Major comments:

1. *“In contrast, when treated with cytokinin, the ratio showed expected dose-dependent response (Figure 3d, Supplementary Figure 10), providing quantitative readout amid metabolic fluctuations.”*

This is a crucial point of validation – is the dose-dependency as expected? A comparison with TCSn-fluorescence protein readout is warranted here. And an honest discussion of quantitative must be included here as it is not dose-dependent quantitation in the strictest sense (e.g. 10x more signal for 10x more hormone). This is NOT a fundamental critique of the new approach – promoters are not truly quantitative under the best circumstances and no technique is perfect – just a comment that it is more useful to be specific about what has been achieved. If the quantitative nature is similar to YFP – that’s useful to know. It could even be superior. The only way to know is to complete a dose-response curve in TCSn-LUZ vs TCSn-GFP or YFP (even better to use a ratiometric TCSn fluorescent system). It could be a minimized experiment with a few more concentrations in the dynamic range of TCSn in the given cell/plant system with the compound used.

Thank you, we agree. For the revised manuscript, we have created plasmids encoding TCSn-driven fluorescent proteins (pTCSv2-EGFP + p35S-mScarlet-I3ΔC10), and performed back-to-back experiments with the luminescence systems. The new results are shown on **Suppl. Figure 16**, with raw data on **Suppl. Figure 17-19**.

Briefly, we observed a roughly similar dose-dependence of the signal in fluorescent and luminescent reporters. Both reporter types started responding to thidiazuron at 0.1 nM, and both showed the major signal change between 1 and 10 nM. This is in agreement with previously reported data ¹. All reporters displayed a sigmoidal concentration-response relationship, but the dynamic range of the luminescent reporters was somewhat narrower. Nevertheless, the overall responsiveness of the systems was comparable.

2. In Supp Fig 12 the side-by-side comparison with Q-PCR is great. Here it should be discussed that transcript detects a low dose a bit better.

We agree and now discuss these results and better qPCR performance in the revised manuscript.

3. The nature of transgenes and promoters in plants precludes confidence in use of a second promoter-reporter as a truly stable 'control'. All promoters have idiosyncrasies in practice and can also be differentially susceptible to stimuli and gene silencing. These issues are worsened if the two constructs are delivered on separate transgenes (insertion site effects). All of these issues will need to be discussed as limitations of the approach.

We agree with this comment as well, we now mention these limitations in the Discussion. To clarify, in our experiments we delivered two luciferase-encoding transcription units as a single transgene, and we agree with the reviewer that should they have been delivered independently, that'd likely cause extra signal variability.

Additional comments:

1. Out of curiosity, what do the images of the mixed expression (ratiometric reporter) leaves look like before/after stimulus? Is the color ratio change detectable at all – I'm thinking of Figure 2B.

We provide these photos on the Supplementary Figure 14a – transient expression, cytokinin-responsive ratiometric Luz608-based sensor, bottom infiltration spot of the leaf. The left image was taken before infiltration with 1 mM thidiazuron, and the right image – after. Note the boundary thidiazuron infiltration area: the yellow region on the left was not efficiently infiltrated and remained uninduced, whereas the rest of the spot was infiltrated and appears green. This strong green signal indicates activation of the TCSv2 reporter.

2. Additionally, it would be nice (not required) to see the AlphaFold predictions of the new fusion proteins.

We predicted the structures with AlphaFold3, they are now shown on Supplementary Figure 9 and provided as .pse files in Supplementary Data 2.

3. "Co-expression of red Luz608 and green Luz533 in the plant cell culture (Supplementary Figures 10) or *Nicotiana benthamiana* leaves (Figure 3a, Supplementary Figures 11-12), led to a major stabilisation of the signal, compared to intensity-based measurements."

-As this is a key finding, it deserves a more substantive explanation in the text (not just in figure 3A legend). For example, why is the non-ratiometric signal so variable after thidiazuron?

We agree, and now added a reference to ² that discusses how thidiazuron perturbs metabolism.

4. Figure 3C lacks SA treatment and 3D lacks mock treated negative control.

We have added mock controls in Figure 3c (previously Figure 3d) and treatment with ASA on Figure 3d. The full figure is available at Supplementary Figure 23.

5. Figure 3D comes before C in the text.

Thank you, corrected.

6. Figure 3D should include the multiple doses as stated in the text.

Our intent was to use Figure 3c to illustrate signal stabilisation, without overcrowding it with multiple experimental conditions. We provide all concentrations on Supplementary Figure 12. We now corrected the references to the figures.

7. All y-axes should have clear labelling of which metric is being quantified. Some only have info in title and others (Fig 3D) lack the information.

Thank you, we corrected the labels.

8. proWRKY70 is not an SA reporter – it is responsive to many stimuli (including defense signalling upstream of SA) and cannot be discussed as an SA reporter with any specificity or quantitative power. I suggest rephrasing as reporter gene and immune regulator whose expression that integrates defense signals.

We agree. We now refer to it as pWRKY70-nnLuz reporter.

9. proORCA3 is more characterised and specific for early JA signalling, but all native promoters are indirect and, at least quantitatively, integrate multiple signals. Even synthetic and simplified (i.e. TCSn) reporters aren't perfectly specific and often fail to respond in a variety of tissues/contexts. This doesn't mean proWRKY70 and proORCA3 aren't valid tools for proof of principle, but better to be more specific about what is being detected.

Thank you, we corrected the description of pORCA3 promoter in the text.

10. Figure 3E – what is the arrow representing? How much of the leaf was infiltrated?

The arrow points to the area of infiltration (which coincides with the area of reaction of the plant to the pathogen). We now commented on it in the Figure 3e legend.

11. This work is analogous to a body of literature on BRET constructs (e.g. NanoLuc based red-shifts). These will need to be discussed and cited. Is there no previous work with LUZ? Also, why aren't any of the existing BRET constructs suitable at all?

Yes, Nanoluc is a more mature technology, and not much has been done with nnLuz. We added extra references to some of the previous BRET studies, and to our early nnLuz BRET designs with suboptimal brightness and BRET efficiency. In parallel to our work, Diego Orzaez' and Marta Vazquez-Vilar' groups developed BRET-based designs that allow tracking of viral infections with the fungal pathway (<https://www.biorxiv.org/content/10.1101/2025.08.05.668616v1>).

12. Matryoshka type ratiometric fluorescent proteins are also useful parallel in the fluorescence realm.

We agree and now cite these in the revised text.

1. Antoniadis, I. *et al.* Cell-surface receptors enable perception of extracellular cytokinins. *Nat Commun* **11**, 4284 (2020).
2. Erland, L. A. E., Giebelhaus, R. T., Victor, J. M. R., Murch, S. J. & Saxena, P. K. The Morphoregulatory Role of Thidiazuron: Metabolomics-Guided Hypothesis Generation for Mechanisms of Activity. *Biomolecules* **10**, (2020).

Reviewer #2

This manuscript describes a convenient approach to make autoluminescent reporters less sensitive to metabolic perturbations that impact luciferin biosynthesis or cofactor availability. The use of fluorescent proteins as FRET acceptors is a well-established method for altering luciferase emission wavelength and sometimes improving brightness, and ratiometric indicators are widely accepted as the most quantitative, so the combination of these approaches represents a tidy addition to the optical toolkit. The nnLuz fusions in this manuscript are the first described FRET constructs using this enzyme, to my knowledge.

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This work is well suited to publication in Nature Plants. I recommend addressing the following as minor revisions:

1. If possible, the authors should comment on why they chose not to make any insertions of FP FRET acceptors in the N-terminal region of the protein, since this region appears especially insensitive to both in their initial screen.

Thank you for your comments. We previously observed that the N-terminal region of the luciferase is membrane-associated ([Kotlobay et al., PNAS 2018](#)). Both types of terminal fusions displayed substantially lower BRET efficiency than internal fusion variants carrying insertions at positions 167 or 188 (**Supplementary Figures 7-8**).

We now added our early data on N-terminal insertions / fusions to the revised manuscript (**Supplementary Figure 10**). Briefly, insertions of mScarlet-I3ΔC10 into positions within the transmembrane region generated several variants with a good red-to-green ratio of approximately 1.8, comparable to that of Luz608. However, these variants were 2-3 orders of magnitude dimmer than Luz608 and Luz533, making them suboptimal candidates for further development.

2. Salicylic acid reporter results are not represented well in Supp. Fig. 13. The dynamics are easily apparent in Supp. Video 1, but very hard to discern in the figure.

I suggest adding more time points to Supp. Fig. 13, zooming to one or both regions of interest rather than showing the entire image frame, and possibly adding a line diagram of the plant anatomy being imaged to help orient the reader. As it is currently, it's difficult to tell where the leaf boundaries are, so a diagram or even an overlaid outline on the first image would be helpful.

We agree, and now updated **Supplementary Figure 21**.

The frame rate for Supp. Video 2 could be slower to make it easier to discern the rapidly changing speckles. For this sensor especially, higher-resolution imaging (higher magnification, single leaf, higher time resolution) would be very nice to see included, if feasible.

We agree and now provide the updated **Supplementary Video 2**.

Finally, for the videos of the salicylic acid reporter, an accompanying Supp. Figure should be included with time plots of red:green ratio for several representative regions of interest, at least over a subset of the time points, as feasible given the amount of motion between frames.

We prepared a separate **Supplementary Figure 22** depicting dynamics of different leaves in green, red and ratio channels.

3. If possible, the supplementary data sets would be more useful if provided as .xlsx or .csv formatted tables rather than as PDFs.

Thank you, we now provide Supplementary tables as .xlsx.

4. Figure 3d: The colors are too similar for the two plotted lines. A more varied color palette would be beneficial for this and other panels in this figure.

We agree. We updated the colors.

Reviewer #3:

In this paper the authors present some exciting technological improvements to their prior work using the fungal bioluminescence pathway to enable the study of gene expression dynamics in plants. Their key innovation is the development of a red shifted version of the luciferase to enable ratiometric normalization as a means to disambiguate the impact of fluctuations in metabolism that affect luciferin synthesis from true changes to reporter expression. While the authors present some exciting data, the description and replication provided of the work they report are not sufficient to substantiate the claims they make. Additionally, the rationale for the various protein engineering tasks they undertake to design this novel Luz are not explained or justified in any depth, making it challenging for future groups to build on this work. Finally, the authors do very little to characterize these reporters beyond their spectral separability. This limits the utility this tool may have to the broader community.

I suggest that the authors revise this manuscript in the following ways:

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Thank you, we agree that we should have provided more details on all these points. We now describe intermediate results and our reasoning in detail in the **Supplementary Note 1**.

2. In figures 2 b and c the degree of variation in the spectrum plots is not characterized. These experiments must be replicated.

We agree that it does look like this information is missing. In fact, it is there, but the variation was just very small (N=24-30) and thus hard to spot. It's best visible for Luz640.

3. For figure 2C it is not clear what the authors actually expressed in planta, and how these constructs were built (promoters and terminators used etc). This makes it challenging to know if this is an apples to apples comparison with the fungal bioluminescence pathway. This should be more clearly articulated to substantiate the claim the authors make, that the fungal bioluminescence pathway was brighter than the bacterial one. They should also provide some rationale for why, i.e. relative expression, relative turn over of luciferase, luciferin biosynthesis etc.

We agree that we have not provided enough information. We have added extra information on constructs and expression conditions to the text. Both bacterial and fungal bioluminescence systems were expressed in BY-2 cells and *Petunia hybrida* under similar conditions. Coding sequences were co-optimized for expression in *Nicotiana sp*, *Petunia hybrida*, and *Arabidopsis thaliana*, and all the enzymes were delivered on the same vector backbone with the same regulatory elements (p35S, OCSt). Agrobacterium cultures carrying the respective constructs were adjusted to the same optical density prior to infiltration.

These measures were taken to ensure as close to an “apples-to-apples” comparison as possible. That said, direct comparison of brightness between the systems was not our objective; we also think that their relative brightness may be context-dependent, given that the pathways rely on distinct luciferins and distinct branches of metabolism, have different numbers of enzymes, and distinct co-factors.

4. In figure 3 the authors describe experiments to demonstrate the capacity for their dual luz system for normalization, however, it is important to see secondary evidence to justify that the stable light output correlates with stable levels of their reporter luz (qPCR of the gene or western blots).

Thank you, we agree. We have now compared luminescence output with transcript levels for two reporters under distinct conditions: cytokinin-responsive pTCSv2-based ratiometric reporter in transient expression assays (**Figure 3a, Supplementary Figure 14**) and defence-related pWKRY70-based ratiometric reporter in stable transgenic lines (**Supplementary Figure 24**).

In the transient assay, reporter luciferase transcript abundance showed a strong positive correlation with luminescence output (Pearson $r = 0.89$ for the ratiometric reporter; **Supplementary Fig. 14e,f**).

Results in transgenic lines also confirmed the link between expression and luminescence signal: qPCR-assayed expression of the reporter Luz533 was increased upon treatment with acetylsalicylic acid, while expression of the reference gene remained stable, following the observed luminescence changes (**Supplementary Figure 24**). To note, in these experiments, some of the 24h data points were not showing high expression – which we interpret as decay of the biological signal, in agreement with previously published observations that treatment with 20 μ M salicylic acid led to a quick WRKY70 mRNA increase after 1h, followed by a gradual decay over the 0-24h, even though the seedlings were kept on salicylic-acid-containing medium ¹.

Additionally, in this revision we performed a comparison of our luminescent system with a similar fluorescent-protein-based setup (pTCSv2-EGFP & p35S-mScarlet-I3 Δ C10; **Supplementary Figures 16-19**). Similar fluorescence-based systems were previously characterised, including their dose-dependency ^{2,3}. In our experiments, ratiometric *luminescent* cytokinin reporters were similar in their response to EGFP/mScarlet-I3 Δ C10 (**Supplementary Figures 19**).

5. This work would be more impactful if the authors characterized the off-rates of their reporters. They do a good job of demonstrating how rapidly the reporters can record increases in gene expression, but the off rates will depend on protein degradation. This is especially relevant as their engineered red luz might have significantly different stabilities than the green luz, which might impact normalization.

We agree with the reviewer. We have now measured the lifetime of the luciferase variants, using cycloheximide chase assay ⁴. The Luz608 variant is degraded at a rate similar to that of Luz533 (**Supplementary Figure 11**), with a half-life of about 9.6-9.9 hours.

6. The experiments the authors present on whole plants in figure 3 C and E need some more work. They do not present sufficient details on replication, especially on 3E.

We added information about the number of replicates to **Figure 3** legend.

Supplementary Figure 25 contains more detailed information that corresponds to **Figure 3e**.

Answering the following questions would help address these issues. Are these plants from a single insertion event or more than 1?

Bioluminescence reporter plants were generated by transforming a homozygous luciferin-producing master line (T3, line AT9865) encoding all enzymes of the pathway, except the luciferase. Because of that, in the *reporter lines* luciferin biosynthesis genes were present in the same genomic locations and at the same copy numbers, but we did not characterise copy numbers and insertion locations of AT9865 or the resulting bioluminescent lines. We believe that because genes encoding green and red luciferase were delivered on a single T-DNA, and because our results come from multiple lines, experimental models, and bioluminescence pathways, conclusions presented here will hold regardless of the genome integration copy number.

What generation are they and are they homozygous for the transgenes? What is the variability of performance of normalization across plants, i.e. do they see consistent responses across lines?

We used T1 reporter plants for all experiments; the plants with similar brightness were selected. For **Figure 3e**, the pathogen was infiltrated into 4-5 leaves per plant across three independent plants, all of which showed a similar response. **Figure 3e** shows a representative leaf; the full-plant images and a summary plot across all three plants are provided as **Supplementary Figure 25**. All these data are now present in the **Materials and Methods** section and in the legends of **Figure 3e** and **Supplementary Figure 25**. For **Figure 3d** the number of individual plants = 5, and there is little variation in luminescence. Overall, we observed consistency in brightness and green-to-red ratio across individual plants of each line.

How tied is the brightness of the reporters tied to expression of the transgenes?

Master line: the ratiometric reporter plants were generated by transforming a master line. We now provide data on similar expression of luciferin biosynthesis genes in individual plants of this line (**Supplementary Figure 20**).

Reporter systems: see the response to Q4 above. Briefly, we observed correlation between expression and bioluminescence signal in transient assays (**Supplementary Figure 14e,f**; Pearson $r = 0.89$), and a similar relation in stable lines (**Supplementary Figure 24**).

Did the authors need to adjust the relative expression of red shifted to green luz?

Yes. In transient expression assays, we adjusted copy numbers of red-shifted luciferases, as their high brightness obscured subtle changes in the Luz533 reporter signal (**Supplementary Figures 12, 13, 14, 17, 19**). This was mitigated by diluting *Agrobacterium* cultures 3–10-fold. No such issue was observed in stable lines.

Does the expression of the rest of the pathway matter?

We think it does not matter – as long as it is not too low so that luciferin availability does not become the bottleneck; and not too high so that it does not become toxic to the plant. For this revision, we double checked levels of expression of luciferase biosynthesis genes in individual plants of our master line (AT9865, T3 line), and found it to be consistent (**Supplementary Figure 20**; N = 6 plants, 3 leaves per plant, and 3 technical replicates for each leaf).

7. How dose responsive is this system? The data to determine this seems to be in **Supplementary Figure 12**, but the authors have not preformed the necessary statistical correlation analysis to be able to make a strong claim.

Thank you, we now report correlation data on qPCR-based reporter expression and luminescence (**Supplementary Figure 14**). Additionally, as mentioned above, we assayed dose-dependencies of luminescent reporters back-to-back with a similar fluorescence system (**Supplementary Figures 19**) and found the system to be most responsive to thidiazuron within 0.1-10 nM range, in agreement with published data (**Figure 3d** in ref. ³

8. In supplementary Figure 8 the authors should use an Anova with the appropriate correction to make multiple comparisons rather than what appears to be multiple separate t-tests.

We agree. We now corrected the figure using Anova. Statistical significance was assessed by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test for multiple comparisons.

9. The authors should provide more detailed information about the sequences of their constructs and their experimental protocols so the broader community can build on this work.

Thank you, we expanded the Materials and Methods section. All construct sequences are now provided as annotated GenBank (.gb) files in **Supplementary Data 1** and as accessible Benchling links in **Supplementary Table 1**. The Data Availability statement has also been updated: raw data have been deposited in Figshare, and the scripts used for data analysis are publicly available on GitHub.

1. Li, S. *et al.* The Arabidopsis Hypoxia Inducible Long Non-Coding RNA also Contributes to Plant Defense and Root Elongation Coordinating with WRKY Genes under Low Levels of Salicylic Acid. *Noncoding RNA* **6**, (2020).
2. Steiner, E. *et al.* Characterization of the cytokinin sensor TCSv2 in arabidopsis and tomato. *Plant Methods* **16**, 152 (2020).
3. Antoniadis, I. *et al.* Cell-surface receptors enable perception of extracellular cytokinins. *Nat Commun* **11**, 4284 (2020).
4. O'Quin, J. B. *et al.* Temperature-sensitive post-translational regulation of plant omega-3 fatty-acid desaturases is mediated by the endoplasmic reticulum-associated degradation pathway. *J Biol Chem* **285**, 21781–21796 (2010).