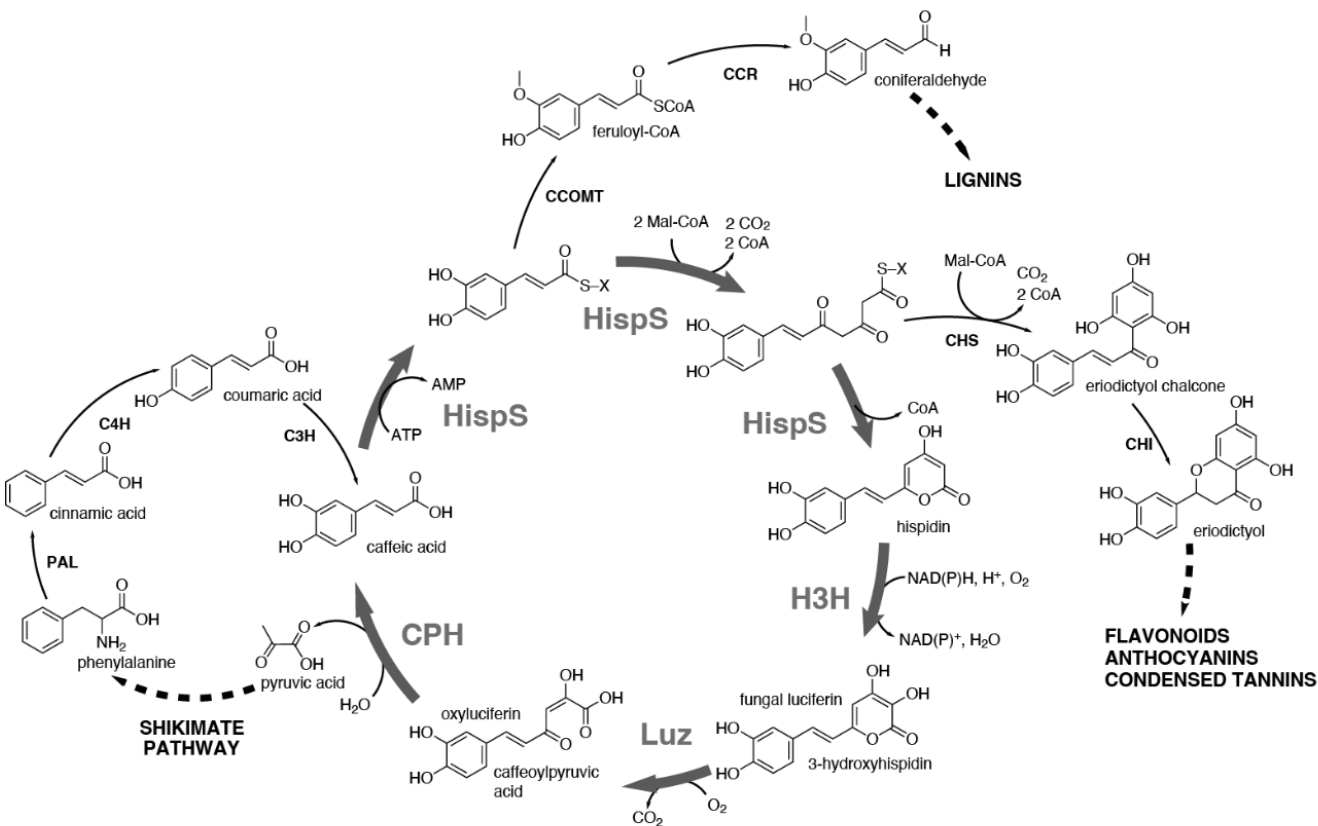


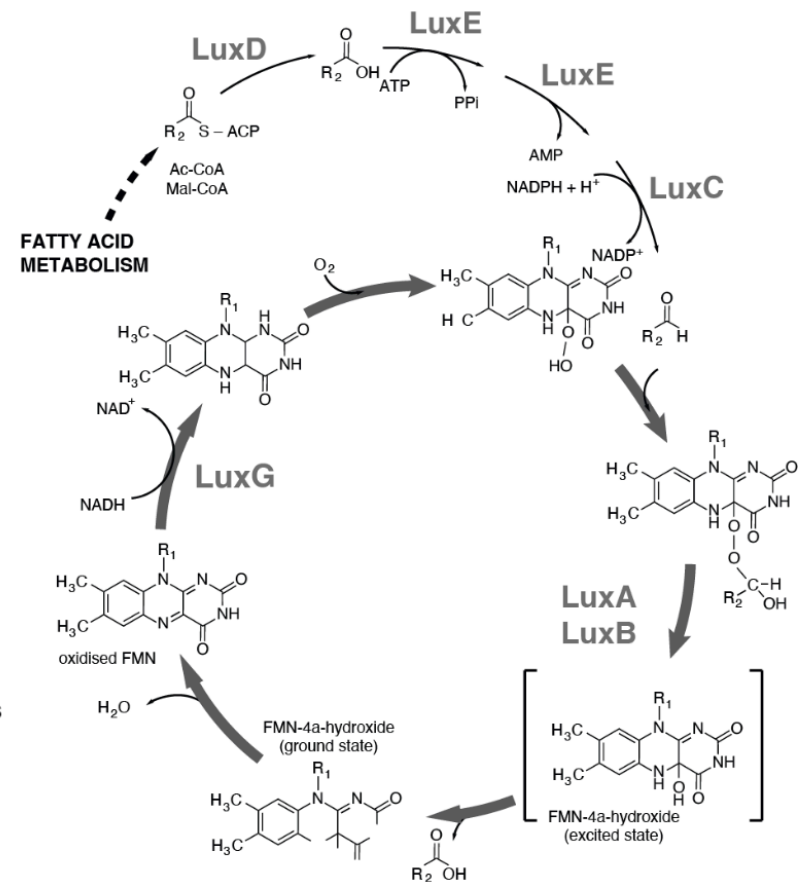
Metabolically robust ratiometric autoluminescent reporters

In the format provided by the
authors and unedited

A FUNGAL BIOLUMINESCENCE

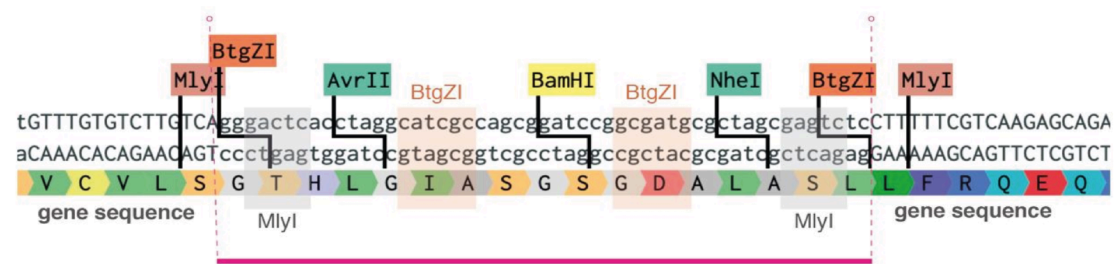


B BACTERIAL BIOLUMINESCENCE

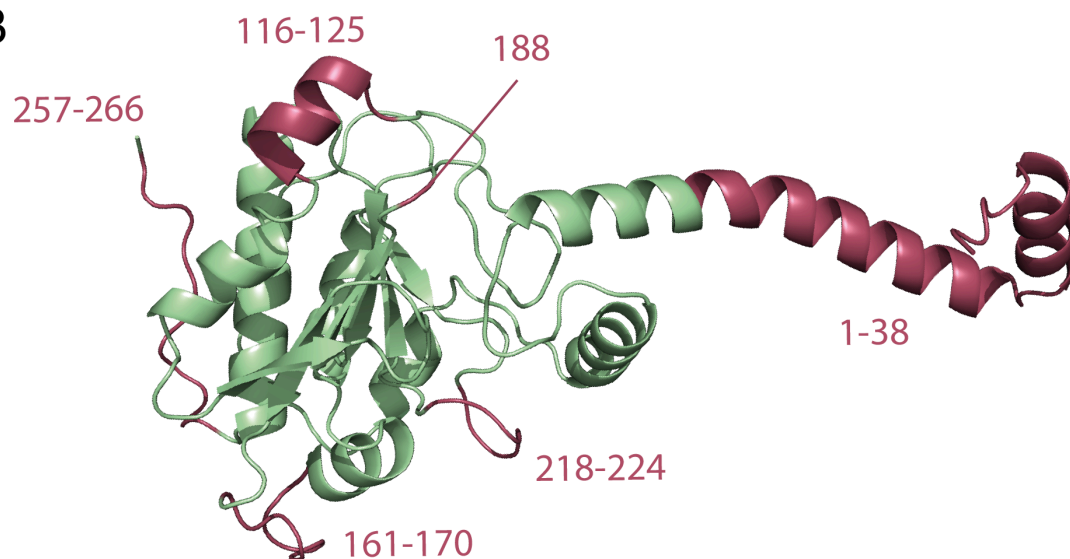


Supplementary Figure 1. Bioluminescent pathways in fungi and bacteria. A. Schematic of the fungal bioluminescence pathway in the context of plant metabolism. Shikimate and phenylpropanoid pathways lead to biosynthesis of caffeic acid, which is subsequently used to generate hispidin and fungal luciferin. In bioluminescent plants, key bioluminescence-specific enzymes such as HispS (hispidin synthase), H3H (hispidin-3-hydroxylase), Luz (fungal luciferase), and CPH (caffeoylpyruvate hydrolase) are expressed. Competing branches lead to lignin and flavonoid biosynthesis. **B.** Overview of the bacterial bioluminescence pathway. The fatty acid metabolism-derived aldehyde is processed through the LuxCDABE pathway. LuxCDE enzymes generate the long-chain fatty aldehyde substrate; LuxAB encodes luciferase; LuxG supplies reduced flavin mononucleotide (FMNH₂) required for light production.

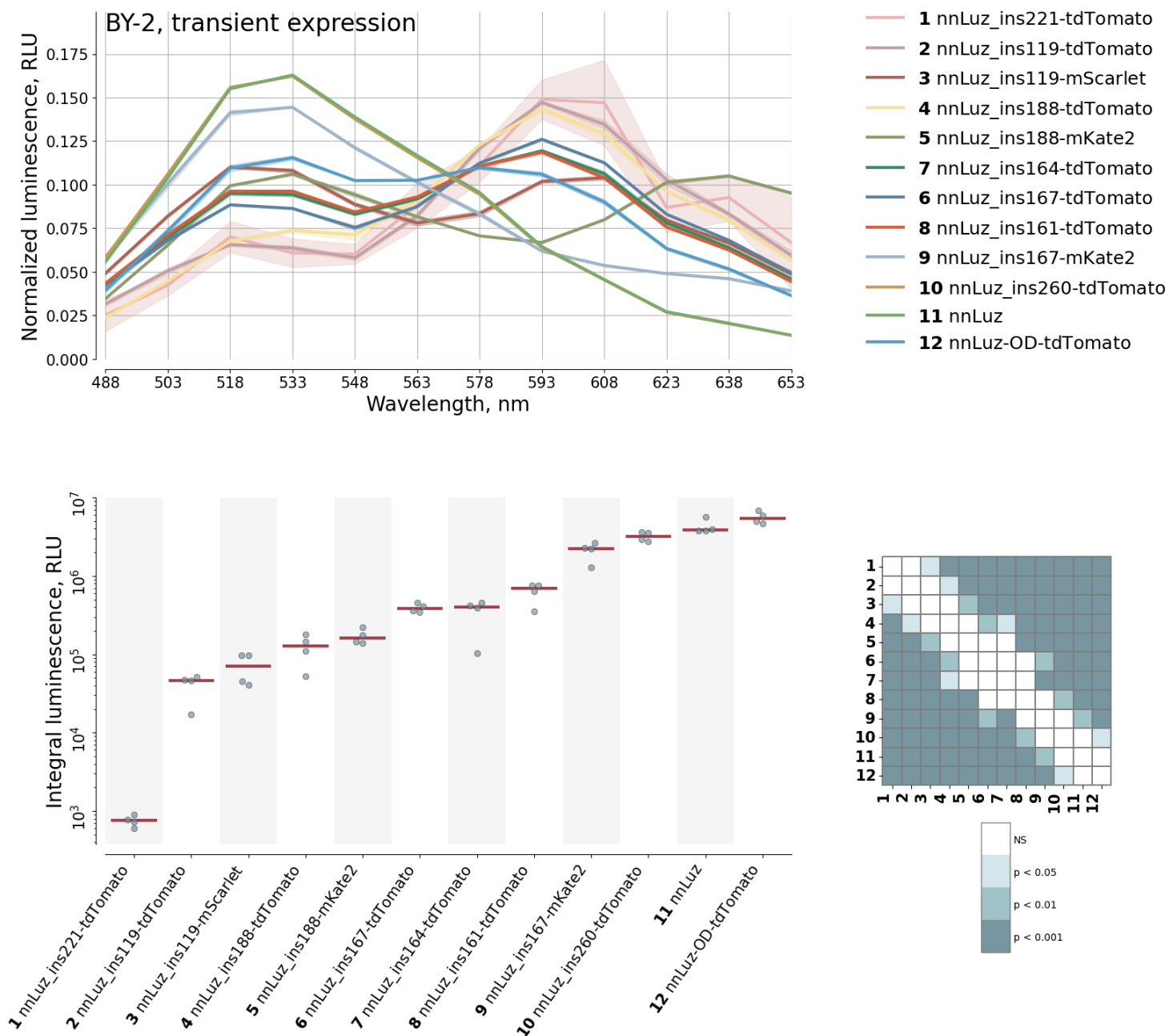
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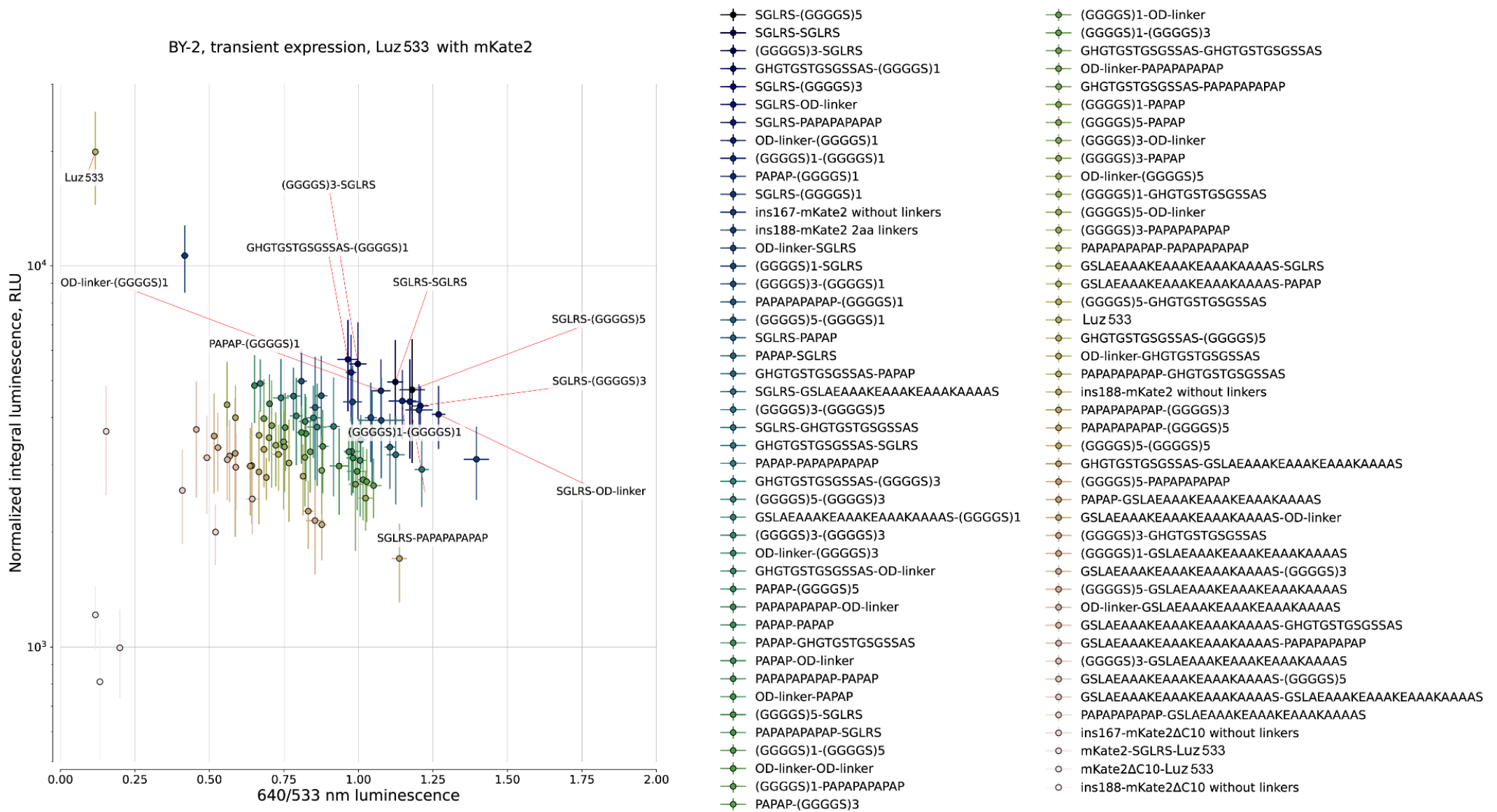
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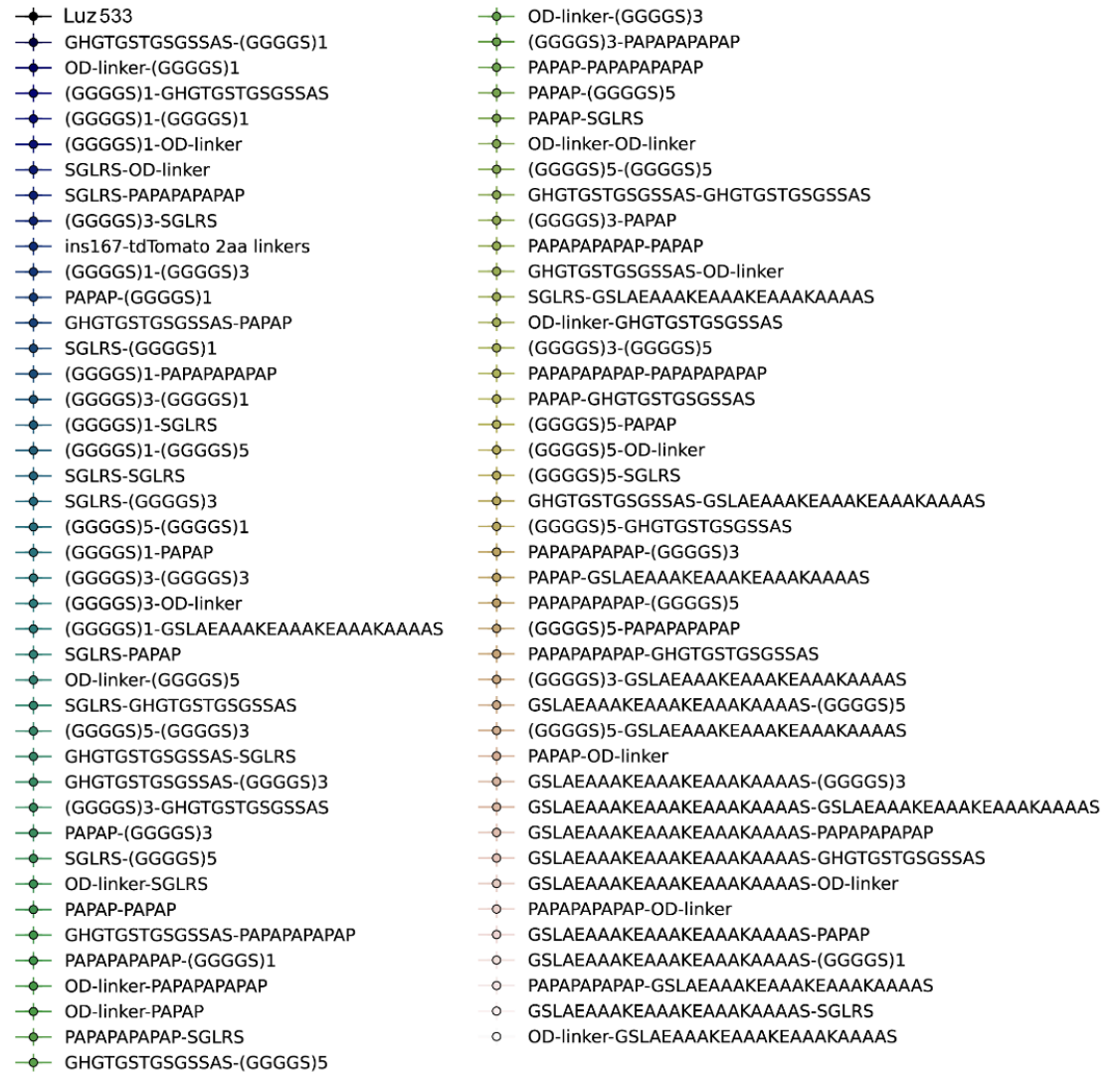
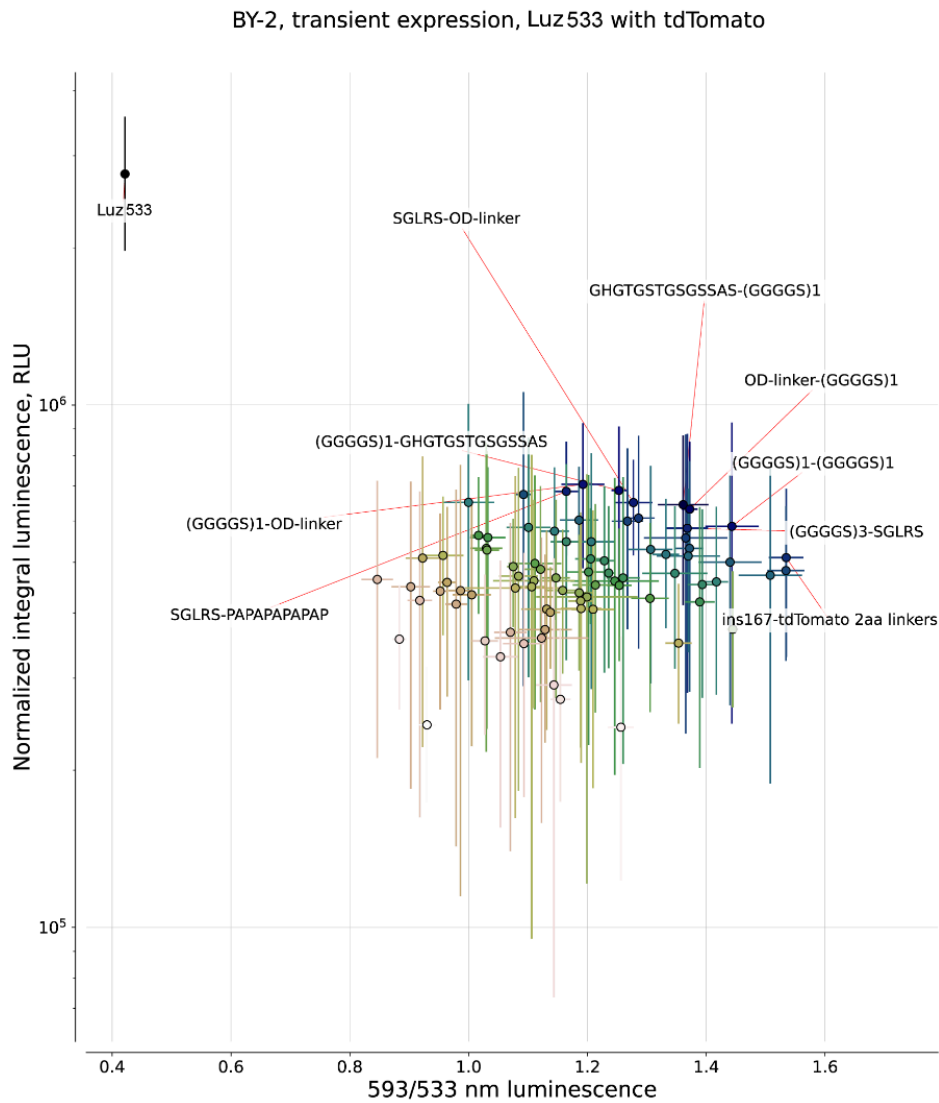
Supplementary Figure 2. Identification of sites within luciferase that are insensitive to insertions or deletions. A. Structure of the multipurpose insert in the synthetic fungal luciferase library designed for the generation of insertion and deletion libraries. B. Locations of identified positions suitable for the insertion of functional domains into fungal luciferase, indicated in pink.



Supplementary Figure 3. Initial screening of fluorescent proteins at the identified positions. Spectral characteristics of the fusion proteins are shown at the top (all plots show mean values and standard deviation); brightness is shown at the bottom (median values are indicated by red lines in the swarm plots. All the values are depicted in the swarm as grey dots). Pairwise differences between groups were assessed using two-sided Mann–Whitney U tests as a post hoc analysis. The nnLuz label refers to the nnLuz_v4 gene variant. N = 4.

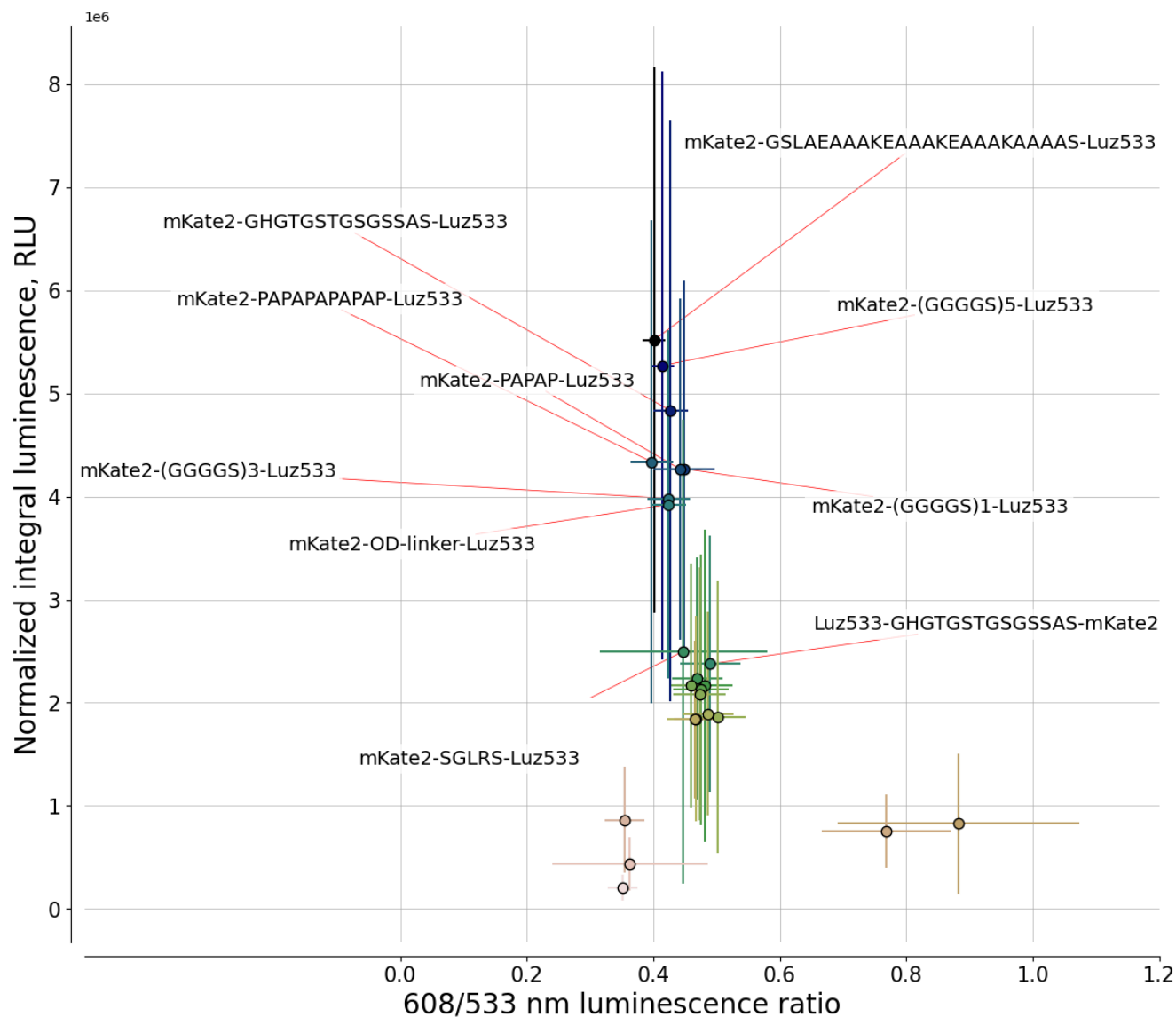


Supplementary Figure 4. Optimization of bioluminescence resonance energy transfer in the Luz533_ins188 and mKate2 pair by varying the linkers. In the legend, linker pairs are indicated as 'N-terminal to mKate2 linker – C-terminal to mKate2 linker'. The top 10 fusions, ranked by brightness and the 640-to-533 ratio, are labeled on the plot. Each point represents the mean, with horizontal and vertical error bars indicating ± 1 SD for the x- and y-axis variables, respectively. N = 5. The raw data is indicated in Supplementary Table 2.



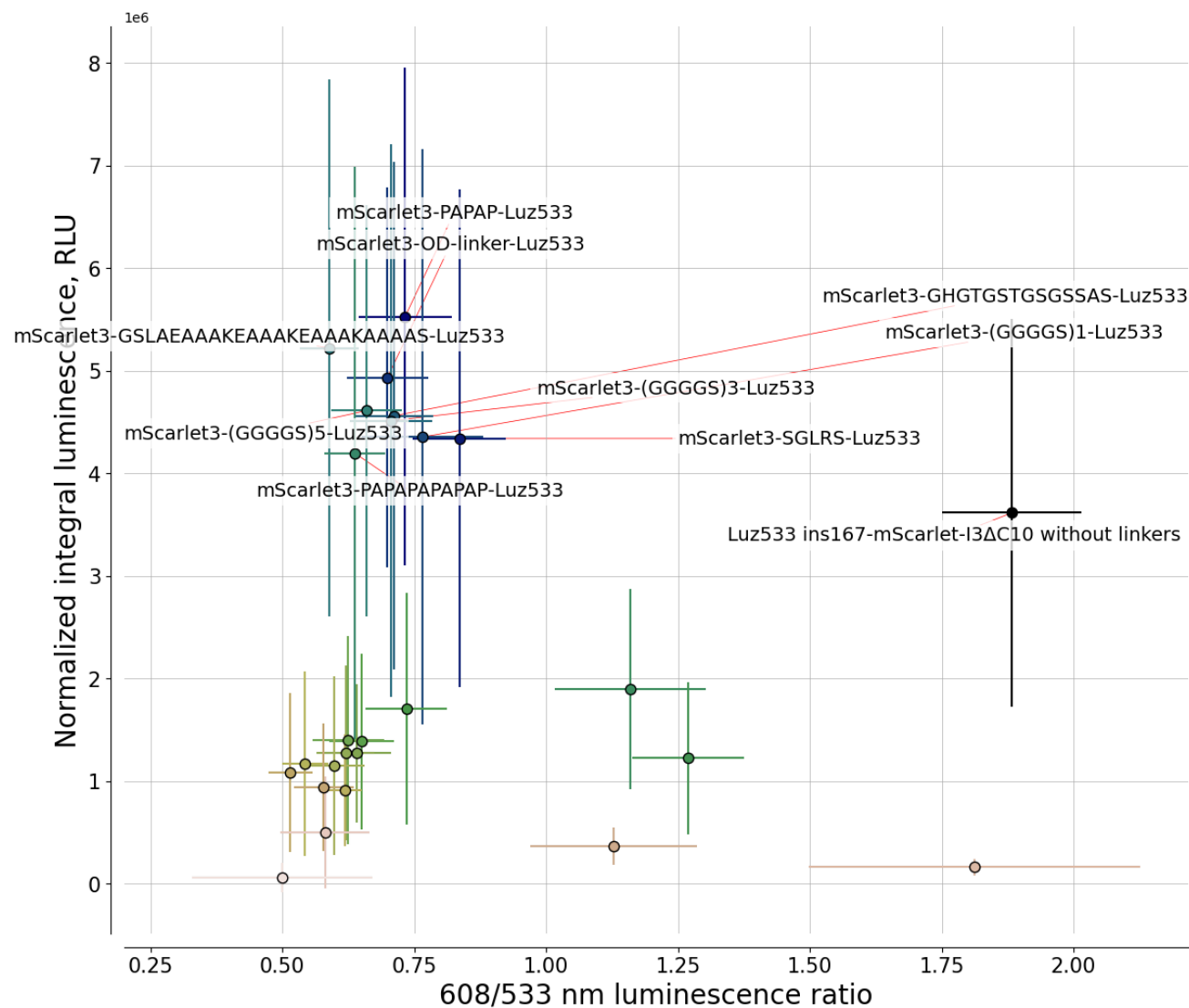
Supplementary Figure 6. Optimization of bioluminescence resonance energy transfer in the Luz533_ins167 and tdTomato pair by varying the linkers. In the legend, linker pairs are indicated as 'N-terminal to mScarlet linker – C-terminal to mScarlet linker'. The initial fusion of Luz533_ins167 with tdTomato, flanked by 2-amino-acid linkers, is indicated as 'ins167-tdTomato 2aa linkers'. The top 10 fusions, based on brightness and the 593-to-533 ratio, are labeled. Each point represents the mean, with horizontal and vertical error bars indicating ± 1 SD for the x- and y-axis variables, respectively. N = 5. The raw data is indicated in Supplementary Table 4.

BY-2, transient expression, Luz533 with mKate2



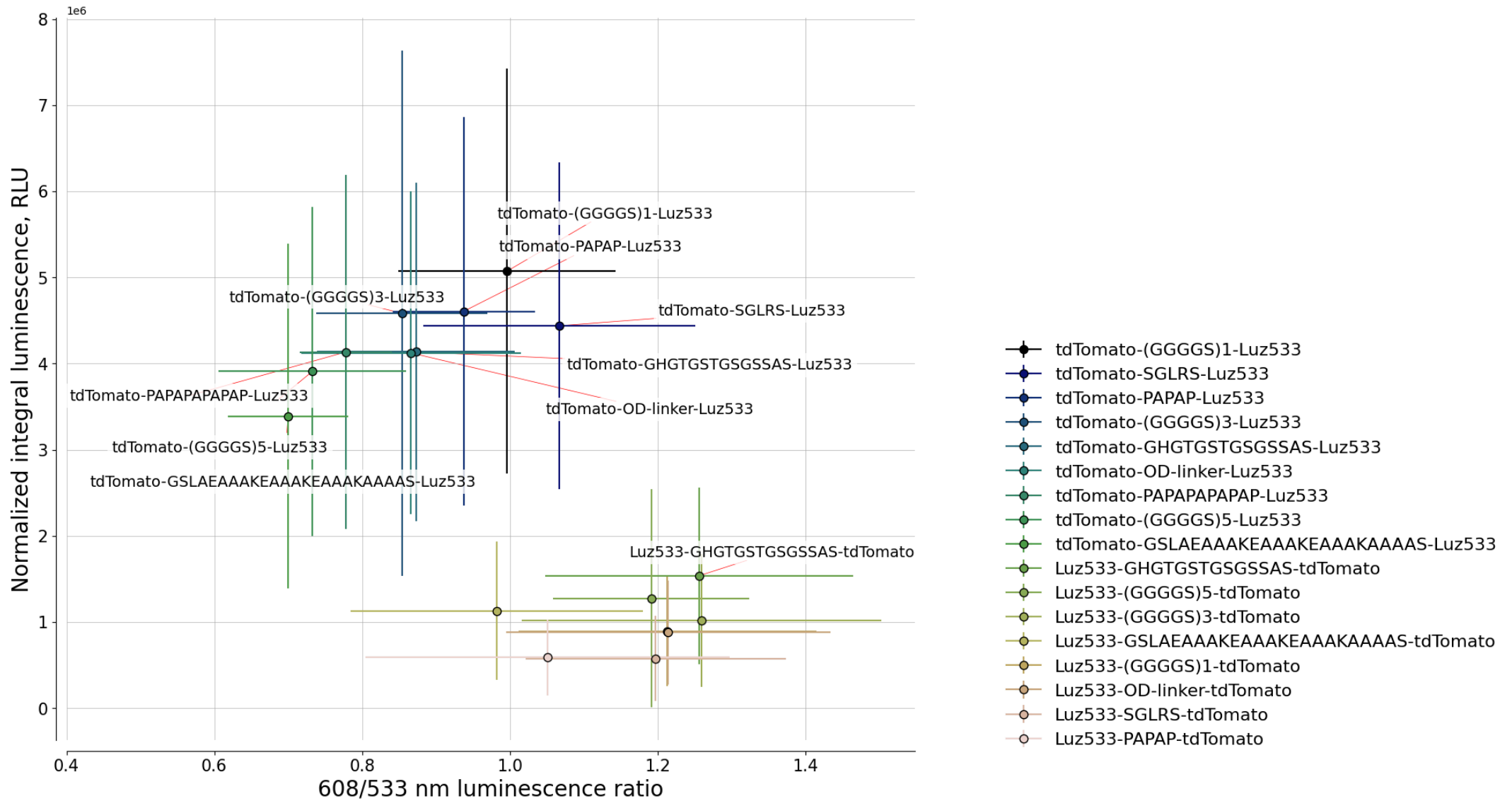
- mKate2-GSLAEAAAKEAAAKEAAKAAAAS-Luz533
- mKate2-(GGGGS)5-Luz533
- mKate2-GHGTGSTGSGSSAS-Luz533
- mKate2-(GGGGS)1-Luz533
- mKate2-PAPAP-Luz533
- mKate2-PAPAPAPAPAP-Luz533
- mKate2-(GGGGS)3-Luz533
- mKate2-OD-linker-Luz533
- Luz533-GHGTGSTGSGSSAS-mKate2
- mKate2-SGLRS-Luz533
- Luz533-GSLAEAAAKEAAAKEAAKAAAAS-mKate2
- Luz533-(GGGGS)3-mKate2
- Luz533-OD-linker-mKate2
- Luz533-(GGGGS)5-mKate2
- Luz533-(GGGGS)1-mKate2
- Luz533-PAPAP-mKate2
- Luz533-PAPAPAPAPAP-mKate2
- Luz533-SGLRS-mKate2
- Luz533 ins167-mKate2 without linkers
- Luz533 ins188 mKate2 (2 aa linkers)
- Luz533 ins188-mKate2 without linkers
- Luz533 ins167-mKate2ΔC10 without linkers
- mKate2ΔC10-Luz533
- Luz533 ins188-mKate2ΔC10 without linkers

BY-2, transient expression, Luz533 with mScarlet

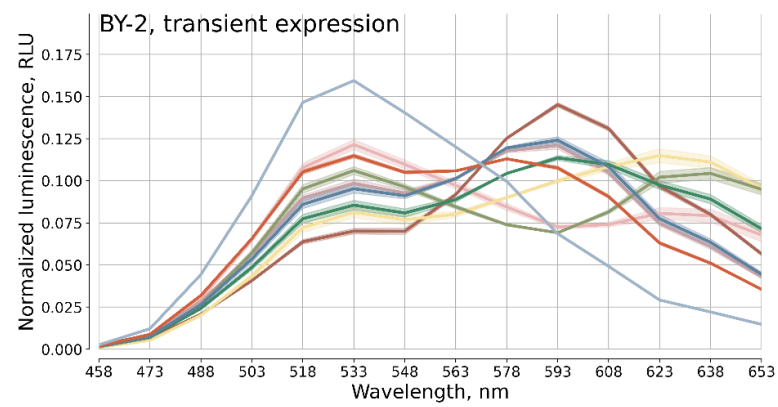


- Luz533 ins167-mScarlet-I3ΔC10 without linkers
- mScarlet3-PAPAP-Luz533
- mScarlet3-SGLRS-Luz533
- mScarlet3-OD-linker-Luz533
- mScarlet3-(GGGGS)1-Luz533
- mScarlet3-GHGTGSTGSGSSAS-Luz533
- mScarlet3-(GGGGS)3-Luz533
- mScarlet3-GSLAEAAAKEAAAKEAAKAAAAS-Luz533
- mScarlet3-(GGGGS)5-Luz533
- mScarlet3-PAPAPAPAPAP-Luz533
- Luz533 ins167-mScarlet-I3 without linkers
- Luz533 ins188-mScarlet-I3 without linkers
- Luz533-GHGTGSTGSGSSAS-mScarlet3
- Luz533-(GGGGS)3-mScarlet3
- Luz533-(GGGGS)5-mScarlet3
- Luz533-(GGGGS)1-mScarlet3
- Luz533-SGLRS-mScarlet3
- Luz533-OD-linker-mScarlet3
- Luz533-PAPAP-mScarlet3
- mScarlet-I3-SGLRS-Luz533
- Luz533-PAPAPAPAPAP-mScarlet3
- Luz533-GSLAEAAAKEAAAKEAAKAAAAS-mScarlet3
- Luz533 ins119 mScarlet3 (2 aa linkers)
- Luz533 ins119-mScarlet-I3ΔC10 without linkers
- mScarlet-I3ΔC10-Luz533
- Luz533 ins188-mScarlet-I3ΔC10 without linkers

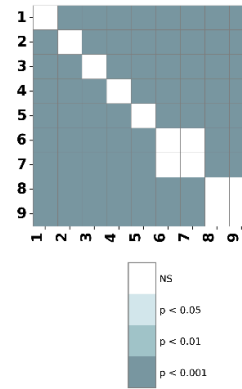
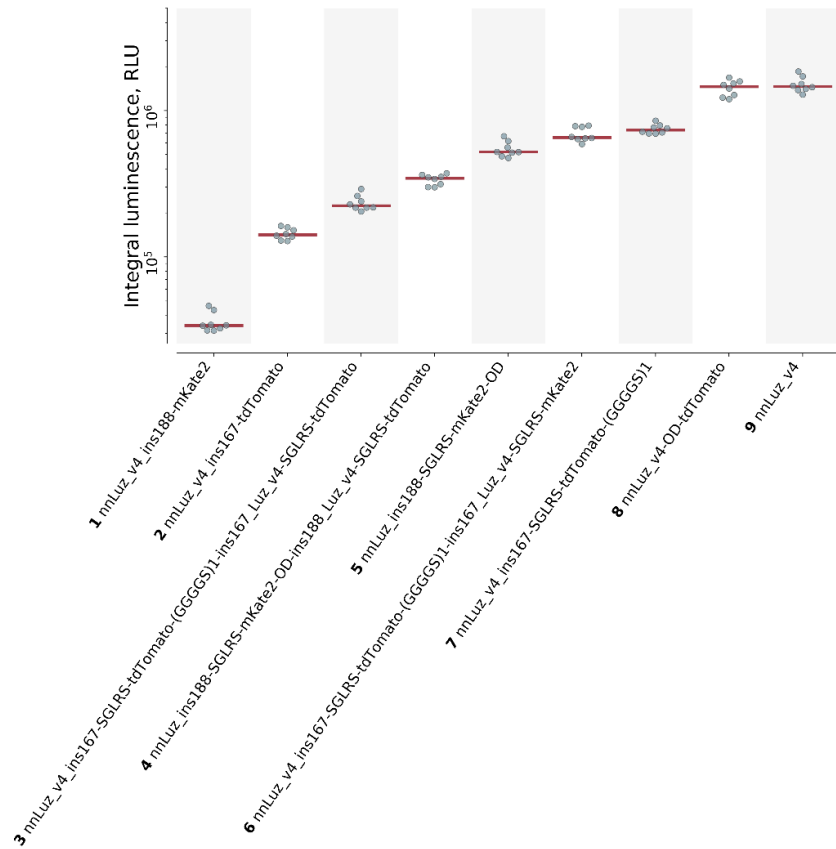
BY-2, transient expression, Luz533 with tdTomato



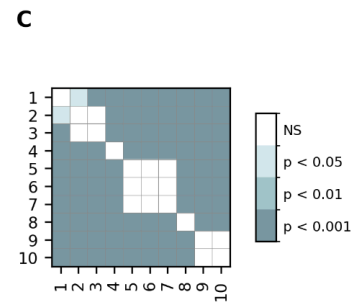
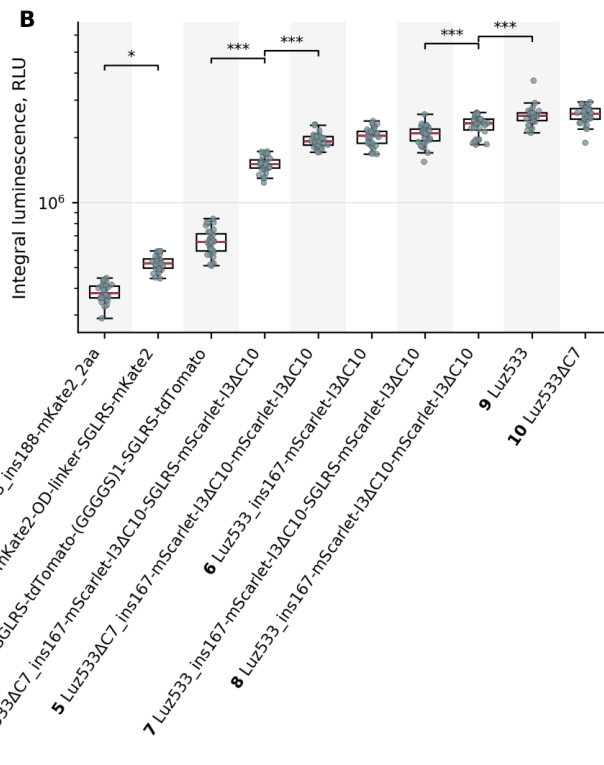
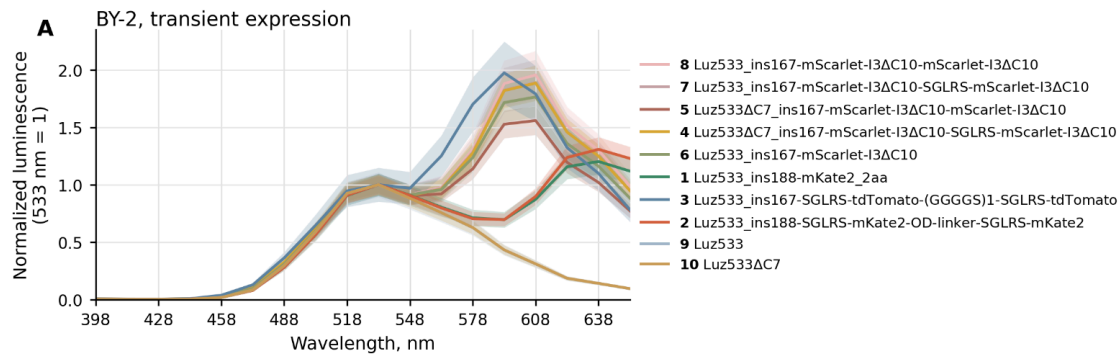
Supplementary Figure 7. Optimization of bioluminescence resonance energy transfer in Luz533 through fusion with fluorescent proteins at either the C- or N-terminus of the luciferase. The top 10 best-performing pairs for each fluorescent protein are labeled. Each point represents the mean, with horizontal and vertical error bars indicating ± 1 SD for the x- and y-axis variables, respectively. N = 5. The raw data is indicated in Supplementary Table 5.



- 1 nnLuz_v4_ins188-mKate2
- 2 nnLuz_v4_ins167-tdTomato
- 3 nnLuz_v4_ins167-SGLRS-tdTomato-(GGGGG)1-ins167_Luz_v4-SGLRS-tdTomato
- 4 nnLuz_ins188-SGLRS-mKate2-OD-ins188_Luz_v4-SGLRS-tdTomato
- 5 nnLuz_ins188-SGLRS-mKate2-OD
- 6 nnLuz_v4_ins167-SGLRS-tdTomato-(GGGGG)1-ins167_Luz_v4-SGLRS-mKate2
- 7 nnLuz_v4_ins167-SGLRS-tdTomato-(GGGGG)1
- 8 nnLuz_v4-OD-tdTomato
- 9 nnLuz_v4

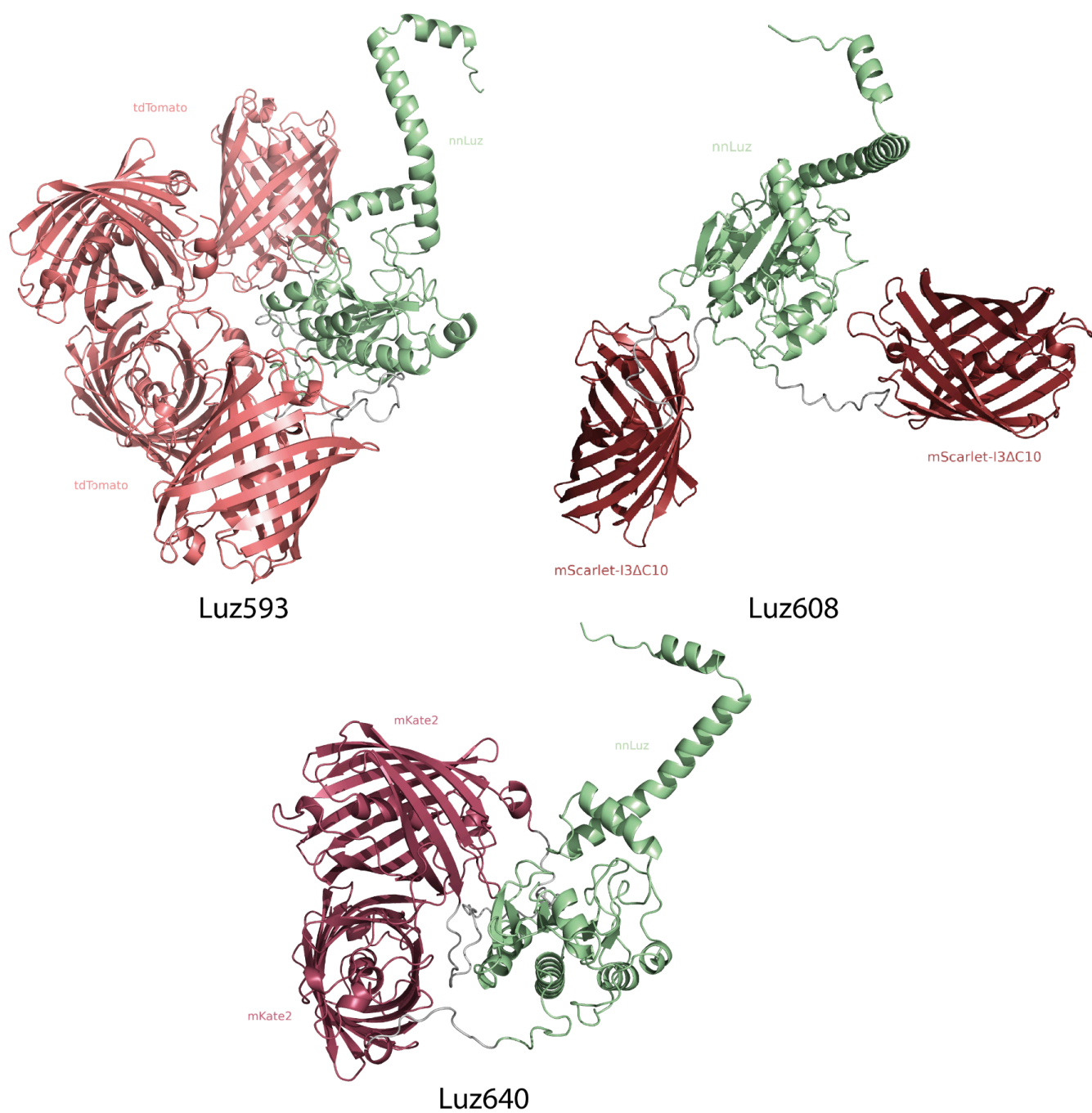


Supplementary Figure 8. Optimization of bioluminescence resonance energy transfer in already existing BRET pairs through fusion with additional copy of fluorescent proteins at C-terminus of the luciferase. Spectral characteristics of the fusion proteins are shown at the top (the plot shows mean values and standard deviation); brightness is shown at the bottom (median values are indicated by red lines in the swarm plots. All the values are depicted in the swarm as grey dots). N = 8. Pairwise differences between groups were assessed using two-sided Mann–Whitney U tests as a post hoc analysis.

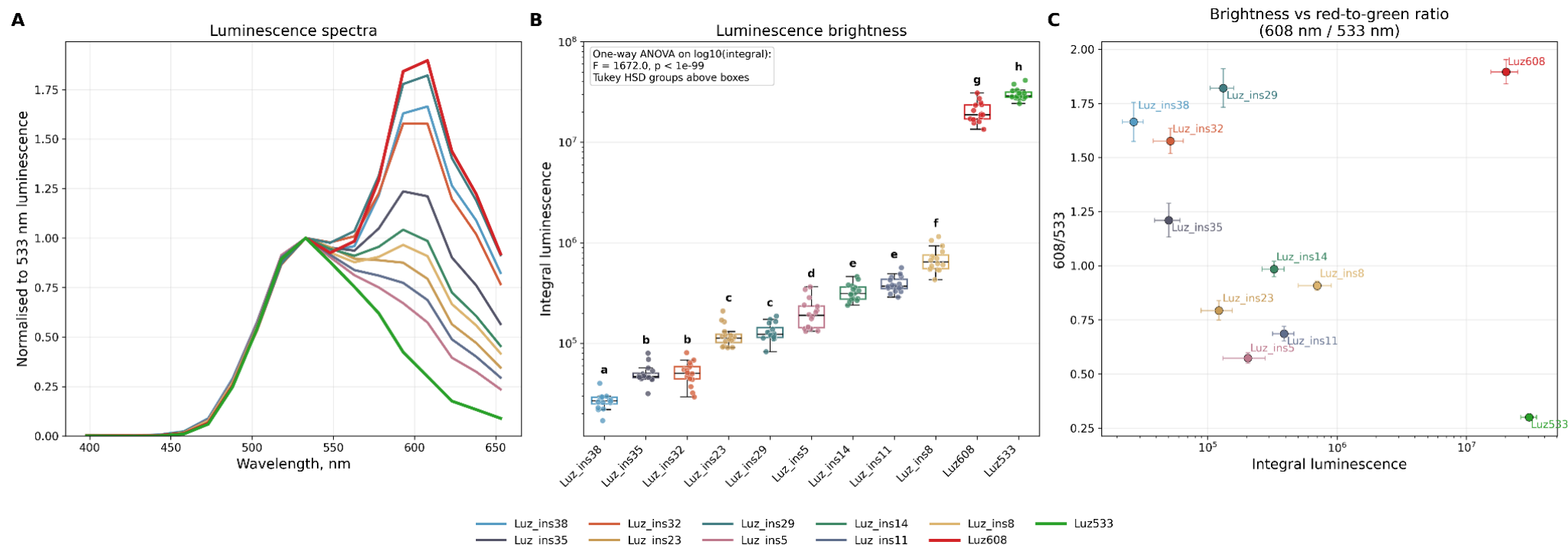


Luz593 = 3 Luz533_ins167-SGLRS-tdTomato-(GGGGGS)1-SGLRS-tdTomato
Luz608 = 8 Luz533_ins167-mScarlet-I3ΔC10-mScarlet-I3ΔC10
Luz640 = 2 Luz533_ins188-SGLRS-mKate2-OD-linker-SGLRS-mKate2

Supplementary Figure 9. Optimization of bioluminescence resonance energy transfer in BRET pairs through fusion with an additional copy of mScarlet3ΔC10 at C-terminus of the luciferase. **A**. Emission spectra of luciferase variants (mean $\pm$ s.d.), normalized to the signal intensity at 533 nm for each individual curve. The plot shows mean values and standard deviation. **B**. Integral luminescence of each variant displayed as box plots with individual data points (log scale). Median values are indicated by red lines in the swarm plots. All the values are depicted in the swarm as grey dots. Box plot shows median, interquartile range (IQR, box), $1.5 \times$ IQR (whiskers). Variants are ordered by median luminescence. Statistical significance was assessed using one-way ANOVA followed by Tukey's honestly significant difference (HSD) test; significant pairwise differences between adjacent groups are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). **C**. Heatmap representation of pairwise comparisons based on Tukey's HSD test, with color coding corresponding to p-values, $N = 16$.



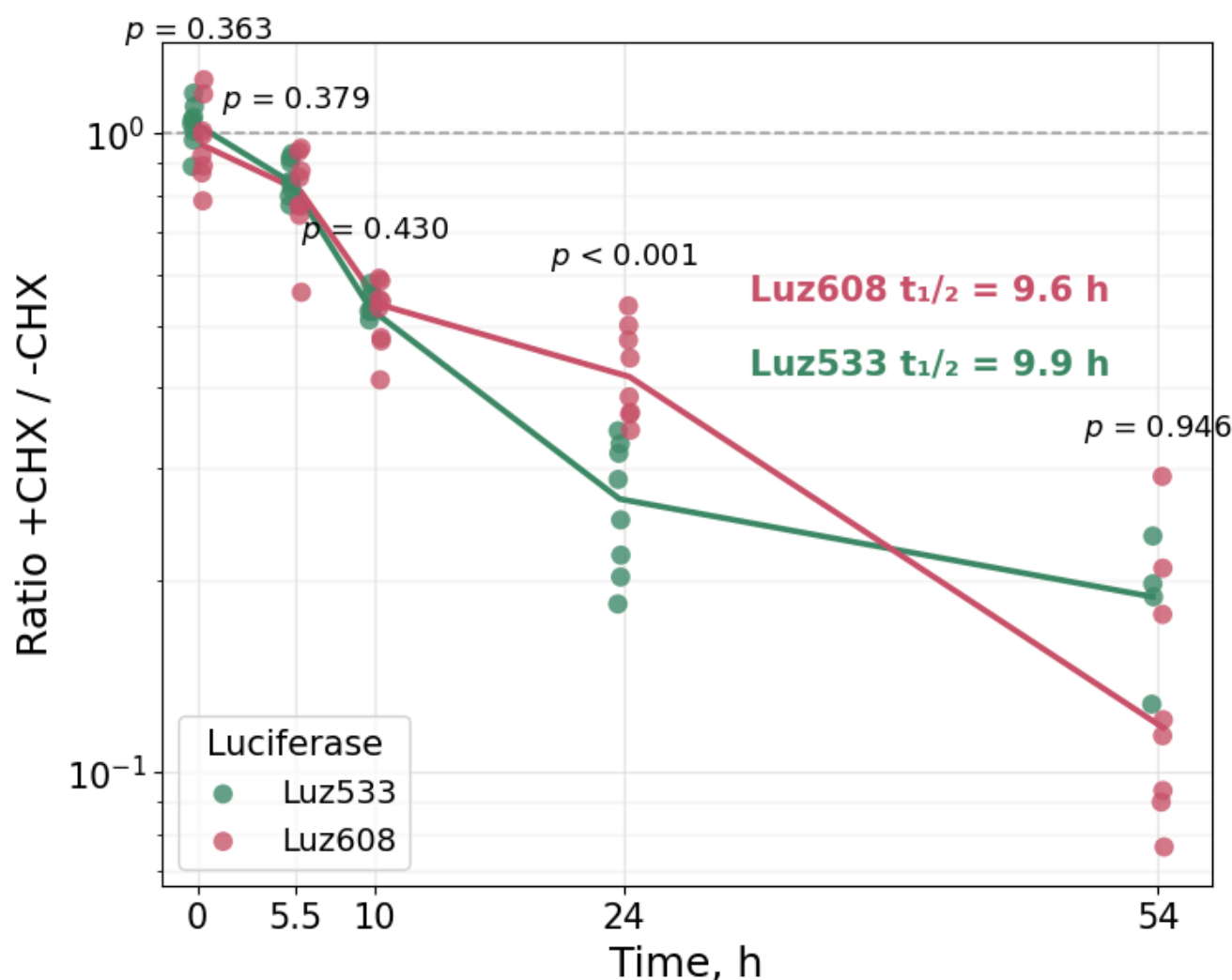
Supplementary Figure 10. Structures of luciferase–fluorescent proteins fusions Luz593, Luz608, Luz640. Predictions were performed in Alphafold3.



Supplementary Figure 11. Insertion of mScarlet-I3ΔC10 at the transmembrane region of Luz533, validation in BY-2 cell packs.

A. Emission spectra of Luz533 insertion variants normalised to luminescence at 533 nm. Spectra are shown as mean values across replicate measurements. The solid lines indicate the mean. **B.** Integral luminescence, calculated as the sum of luminescence across all measured wavelengths, for each variant ranked from dimmest to brightest. Luz608 was used as a best-performing BRET pair – positive control. Points represent individual replicate measurements (N = 16); boxes show the median and interquartile range, with whiskers indicating the 1.5x data range excluding outliers. Statistical differences were assessed by one-way ANOVA on log10-transformed integral luminescence followed by Tukey's HSD test; letters above boxes denote Tukey groups. **C.** Relationship between mean integral luminescence and the red-to-green emission ratio, calculated as luminescence at 608 nm divided by luminescence at 533 nm. Points indicate variant means and error bars show the s.d. across replicate measurements. Luminescence was measured 72 hours post transformation. N = 16.

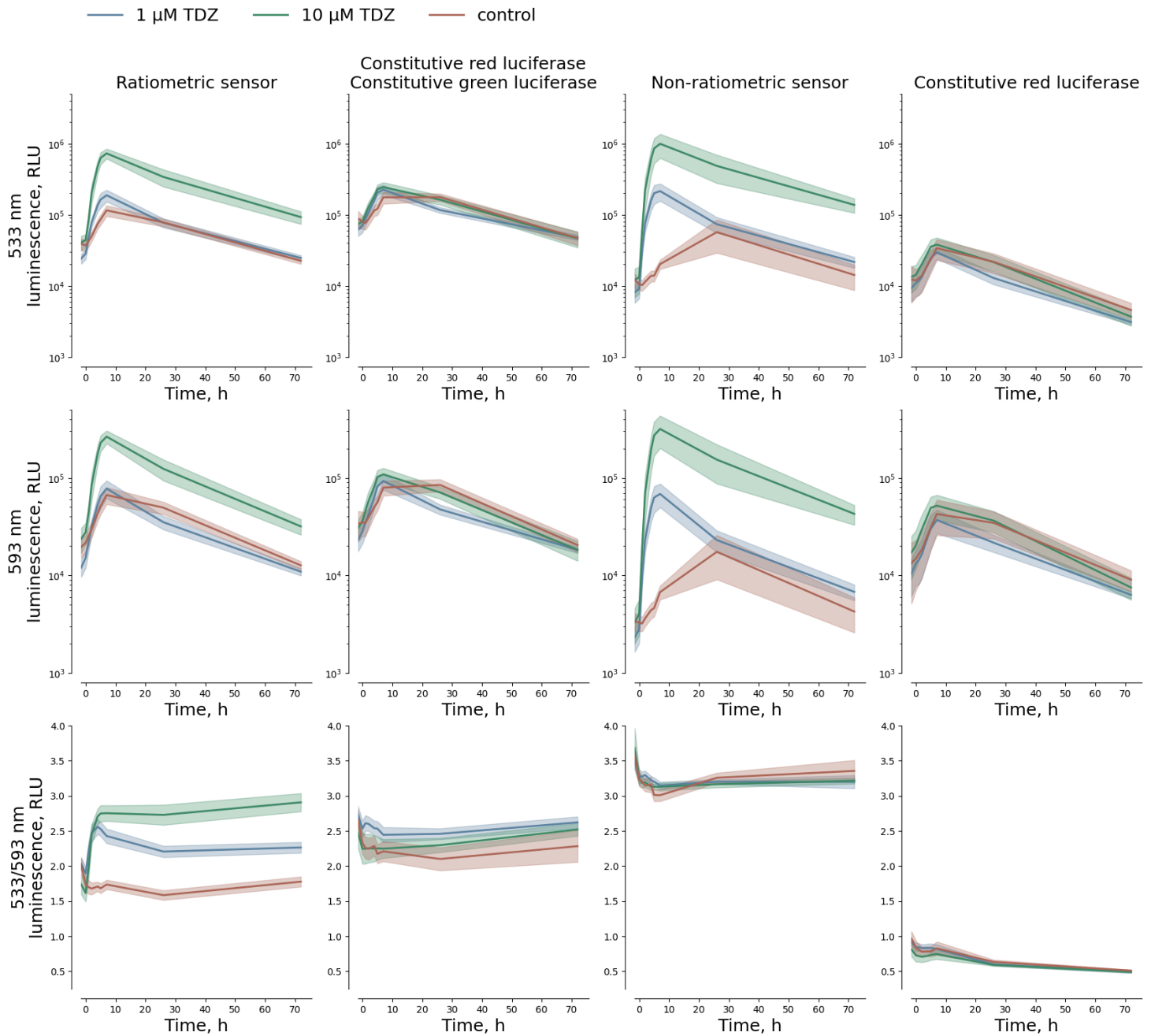
Lifetime of fungal luciferases – Luz533 and Luz608



Supplementary Figure 12. Comparative decay kinetics of the fungal luciferases Luz533 and Luz608.

Luminescence signals were measured over time in samples supplied with luciferin in the presence or absence of cycloheximide (+CHX or -CHX). For each luciferase, signal values were calculated as the ratio of +CHX/-CHX at each time point and plotted on a logarithmic scale. Dots represent individual biological replicates; lines indicate median values at each time point. Estimated half-lives, calculated from the decay of the +CHX samples, are shown on the plot. Differences between Luz533 and Luz608 at each time point were assessed by one-way ANOVA followed by Tukey's HSD test; p values are indicated. Only the 24 h time point showed a significant difference between the two luciferases ($p < 0.001$). $N = 8$.

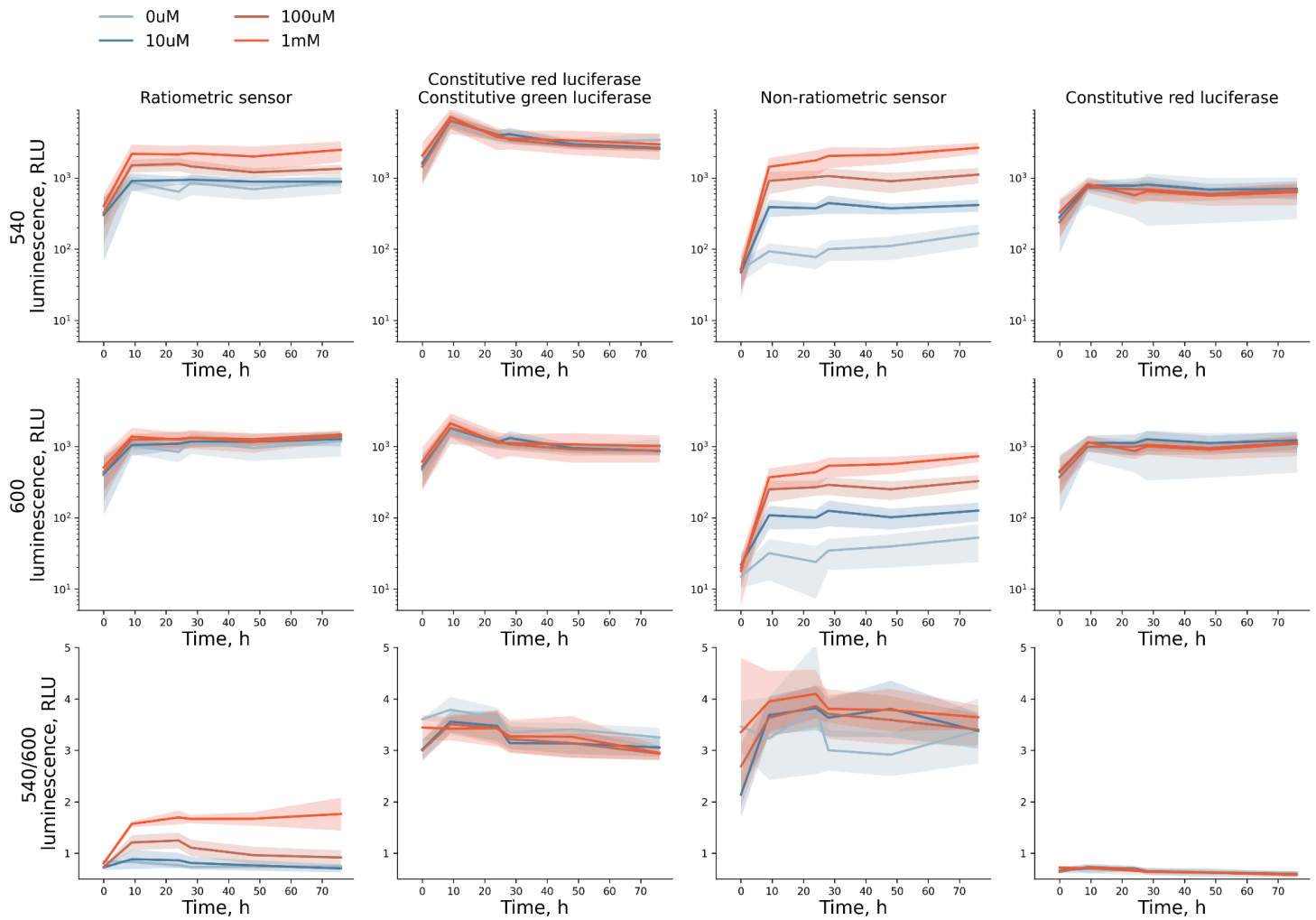
BY-2, transient expression



Supplementary Figure 13. Effect of thidiazuron (TDZ) treatment on the transient expression of fungal ratiometric and non-ratiometric luminescence reporters in *Nicotiana tabacum* BY-2 cell packs.

Time-course luminescence data showing expression of Luz533 and Luz593 variants driven by different promoter combinations: pTCSv2-Luz533 + MinSyn108-Luz593 (ratiometric sensor), AtTCTP-Luz533 + MinSyn108-Luz593 (constitutive red luciferase + constitutive green luciferase), pTCSv2-Luz533 (non-ratiometric sensor), and MinSyn108-Luz593 (constitutive red luciferase) (columns, left to right). Rows represent measurements of luminescence at 533 nm (top), 593 nm (middle), and the 533/593 nm emission ratio (bottom). Cell packs were infiltrated (time point 0h) with either 1 μ M TDZ (blue), 10 μ M TDZ (green), or control buffer (brown), and luminescence was monitored over a 72-hour period. Absolute luminescence (RLU) shows TDZ concentration-dependent induction in constructs containing the pTCS2 promoter. Ratiometric (533 nm/593 nm) measurement (lower left panel) shows clear dose-dependent response without decline between 10h and 76h, in contrast with single-channel detection (upper left panel). The plots show mean values and standard deviation. Measurements were performed using Tecan Spark. N = 8.

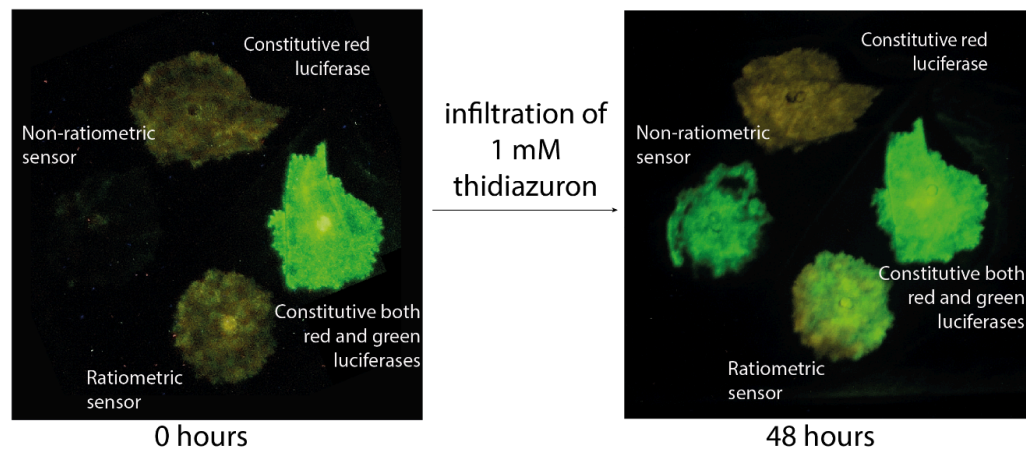
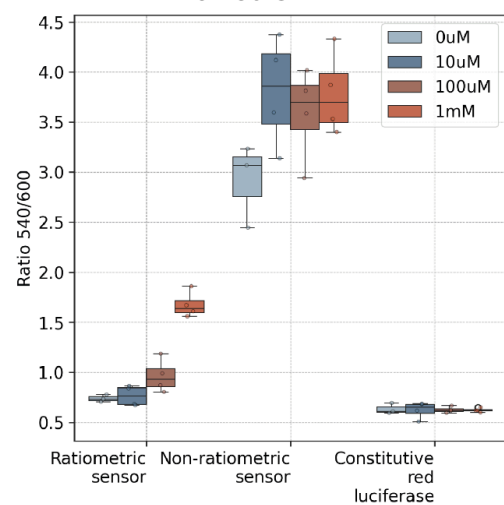
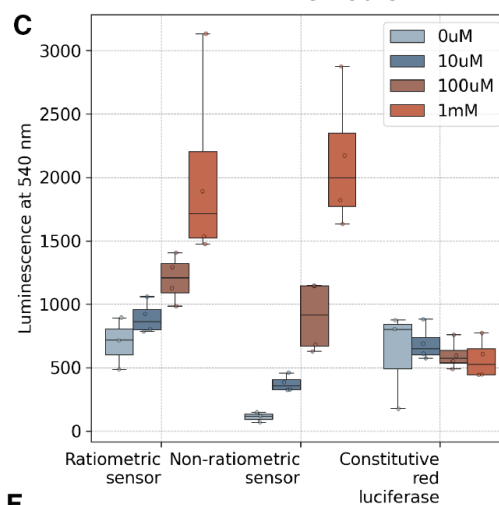
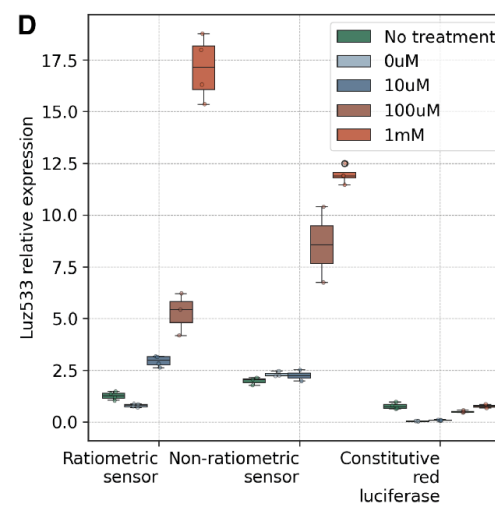
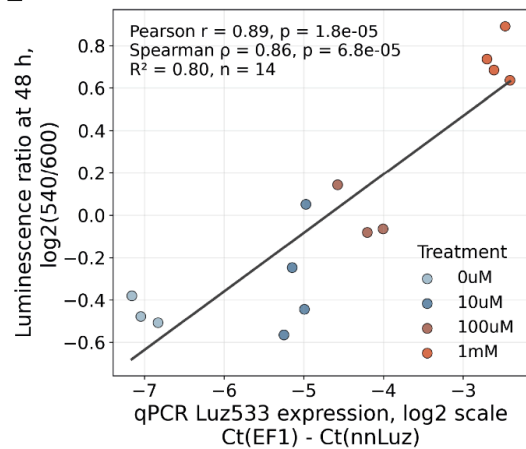
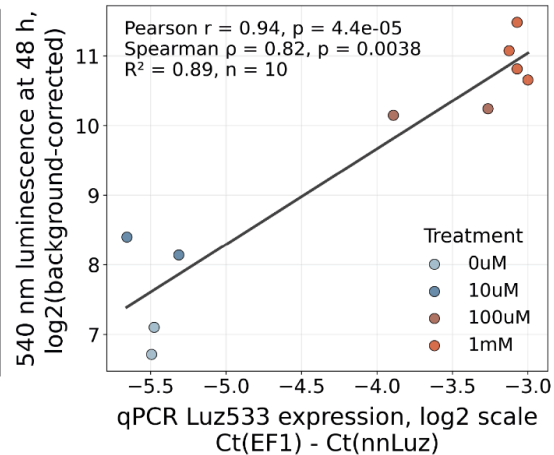
Nicotiana benthamiana, transient expression



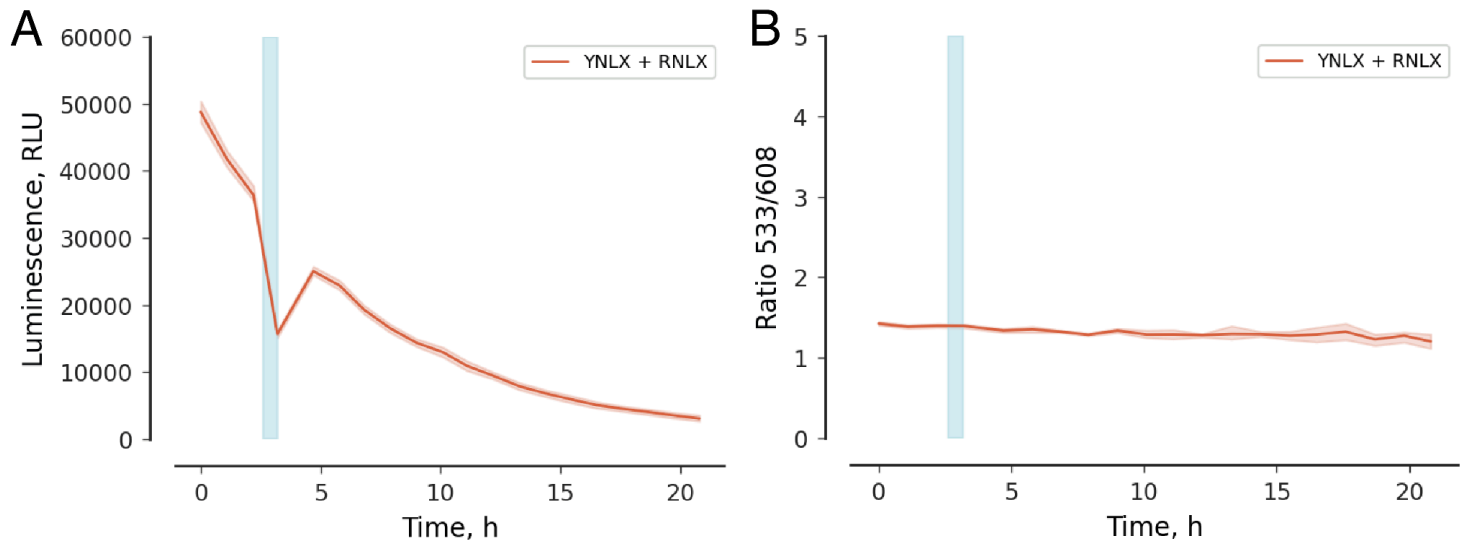
Supplementary Figure 14. Effect of thidiazuron (TDZ) treatment on the transient expression of fungal ratiometric and non-ratiometric luminescence reporters in *Nicotiana benthamiana* leaves.

Time-course luminescence data showing expression of Luz533 and Luz608 variants driven by different promoter combinations (columns left to right): pTCSv2-Luz533 + p35S-Luz608 (indicated as “Ratiometric sensor”), p35S-Luz533 + p35S-Luz608 (indicated as “Constitutive red luciferase, constitutive green luciferase”), pTCSv2-Luz533 (indicated as “Non-ratiometric sensor”), and p35S-Luz608 (indicated as “Constitutive red luciferase”). Rows represent measurements of luminescence at 540 nm (top), 600 nm (middle), and the 540/600 nm emission ratio (bottom).

The leaves were infiltrated with either 1 mM TDZ (light red), 100 μ M (brown), 10 μ M TDZ (blue), or control buffer (light blue), and luminescence was monitored over a 76-hour period. Absolute luminescence (RLU) shows TDZ concentration-dependent induction in constructs containing the pTCS2 promoter. Ratiometric (540 nm/600 nm) measurement (lower left panel) shows dose-dependent response, in contrast with single-channel detection (upper left panel). The plots show mean values and standard deviation. Measurements were performed using IVIS Spectrum. N = 4.

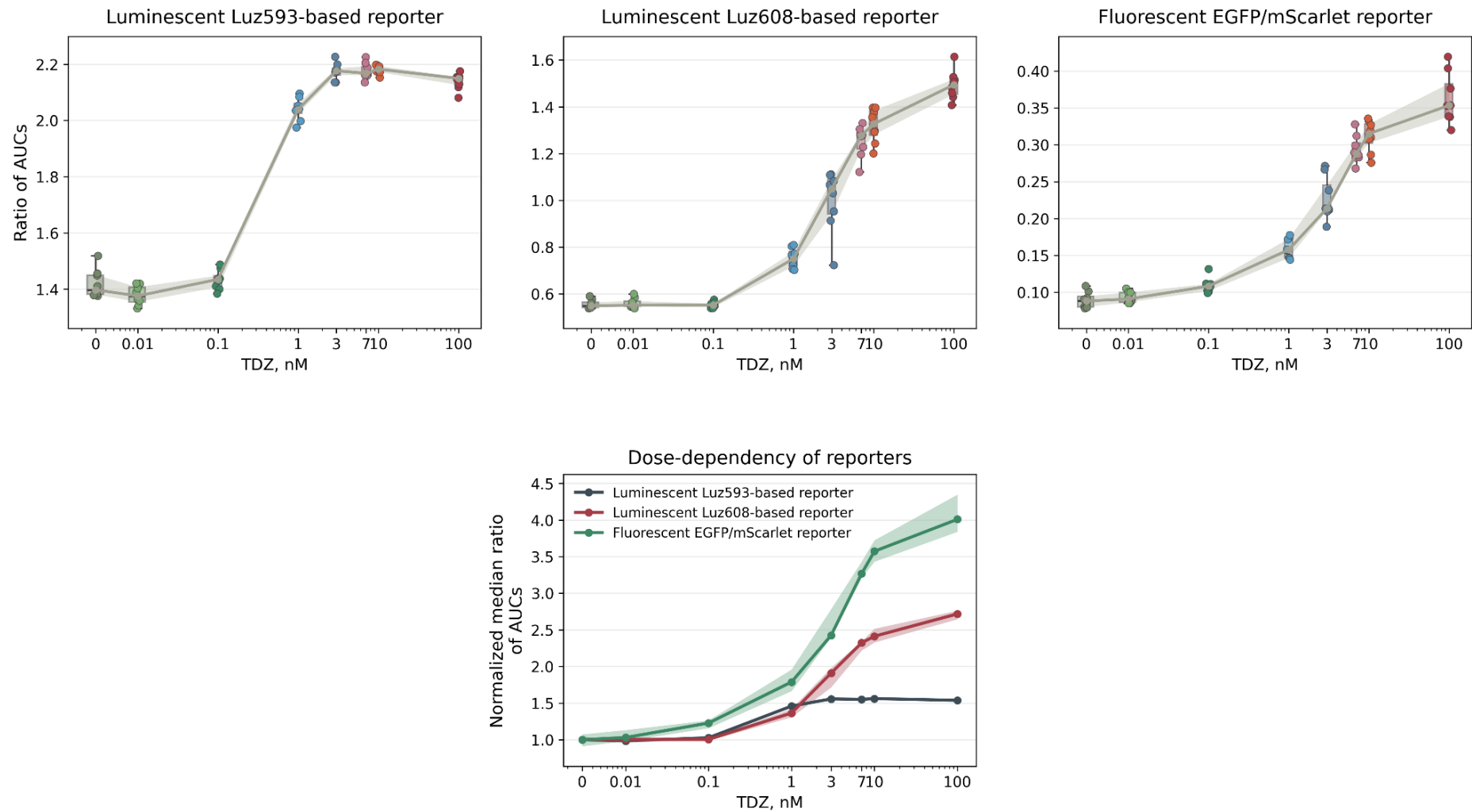
A**B****C****D****E****F**

Supplementary Figure 15. Induction of ratiometric (pTCS2-Luz533 + p35S-Luz608), non-ratiometric (pTCS2-Luz533) cytokinin sensors, and controls (constitutive red luciferase – p35S-Luz608; constitutive red and green luciferases – p35S-Luz608 + p35S-Luz533) in *Nicotiana benthamiana* leaves by infiltration with 1 mM thidiazuron solution. **A.** Representative image of leaves captured using a Sony ILCE-7m3 camera (luminescent signals, ISO 3200, 30-second exposure) before infiltration ('0 hours') and 48 hours later ('48 hours'). N = 4. **B.** Ratio of luminescence values at the reporting wavelength (540 nm) to values at the normalizing wavelength (600 nm). Measurements were performed using IVIS Spectrum. **C.** Absolute luminescence at the reporting wavelength (540 nm). Measurements were performed using IVIS Spectrum. **D.** Luciferase expression levels relative to the housekeeping EF1a gene for the ratiometric and non-ratiometric sensors and the constitutive control. For B-D box plots show median, interquartile range (IQR, box), $1.5 \times \text{IQR}$ (whiskers). All the values are depicted in the swarm as dots. **E.** Correlation between qPCR-derived Luz533 expression and the 540/600 luminescence ratio at 48 h in the ratiometric reporter line (pTCS2-Luz533, 35S-Luz608). Expression was calculated as $2^{-(\text{Ct}_{\text{EF1}} - \text{Ct}_{\text{nnLuz}})}$. Points represent matched biological replicates and are coloured by treatment concentration. The line indicates linear regression. **F.** Correlation between qPCR-derived Luz533 expression and 540 nm luminescence at 48 h in the non-ratiometric reporter line (pTCS2-Luz533). Expression was calculated as $2^{-(\text{Ct}_{\text{EF1}} - \text{Ct}_{\text{nnLuz}})}$, and luminescence values were background-corrected. Points represent matched biological replicates and are coloured by treatment concentration. The line indicates linear regression.



Supplementary Figure 16. Stability of ratiometric readout in the bacterial luminescence system despite fluctuations in absolute brightness. Effect of a 30-minute heat shock at 37°C (indicated as a light blue rectangle) on luminescence intensity (A) and ratio 533/608 (B) of luminescence emitted by BY-2 cell packs, expressing yellow (YNLX = Venus Δ C10-EL-iluxA), and red (RNLX = mScarlet-I Δ C10-EL-iluxA) variants of iluxA in the cytosol, along with the rest of enzymes of bacterial luminescence pathway. The plots show mean values and standard deviation. N = 4.

BY-2, dose-dependency of ratiometric cytokinin reporters



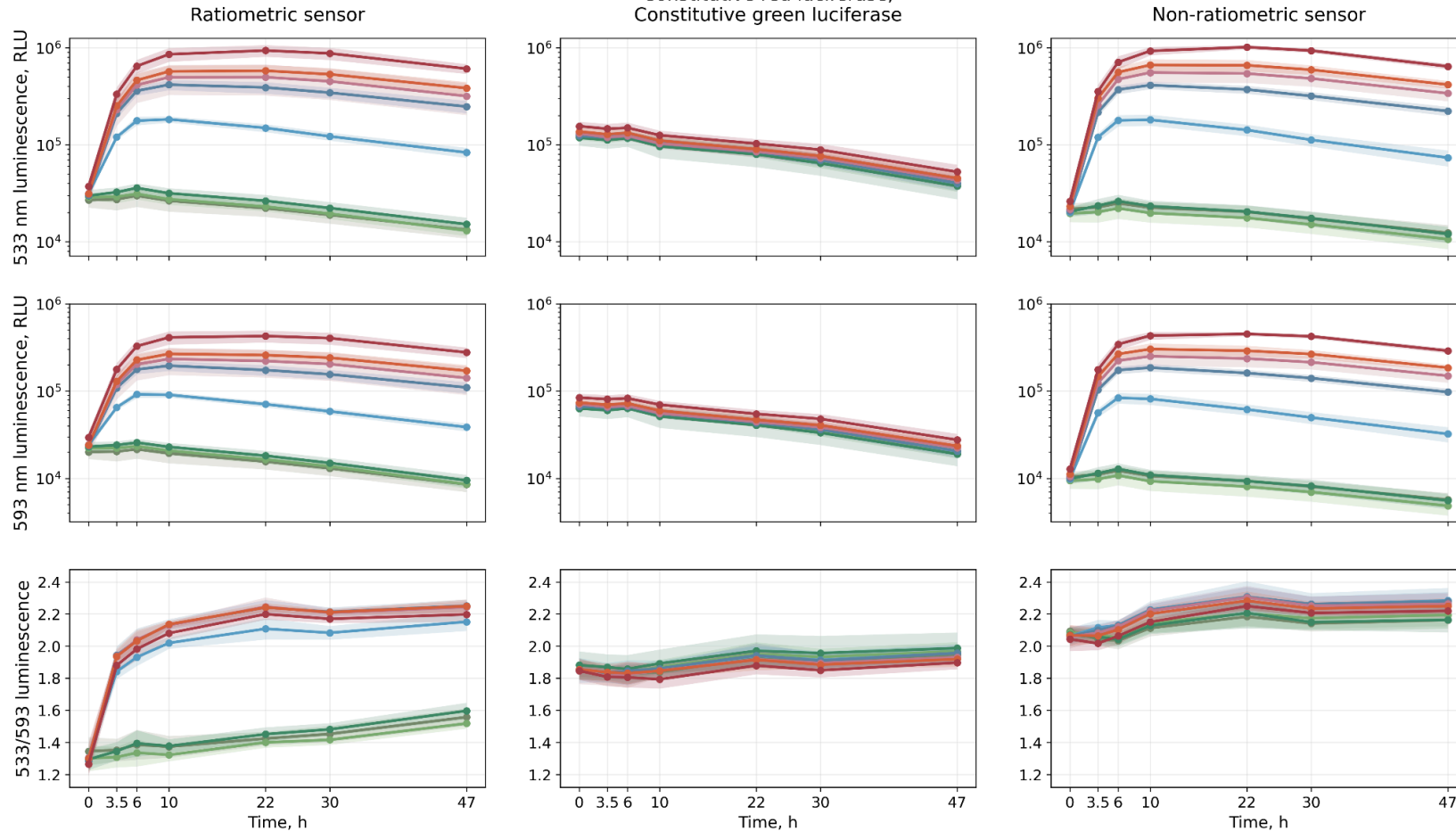
Supplementary Figure 17. Comparison of dose-dependencies and dynamic ranges of luminescent (Luz593- and Luz608-based) and fluorescent (EGFP/mScarlet-I3ΔC10) cytokinin reporters in *Nicotiana tabacum* BY-2 cell packs.

Time-course luminescence/fluorescence data were analyzed by calculating the areas under the curve (AUCs) and their ratios for the ratiometric luminescent (Luz593-based reporter, Supplementary Figure 17; Luz608-based reporter, Supplementary Figure 18) and fluorescence (Supplementary Figure 19) reporters (top), N = 12. Gray lines connect the median values. Shaded regions connect box plots. Box plots show median, interquartile range (IQR, box), $1.5 \times$ IQR (whiskers). To assess the dynamic range, median values were normalized to the 0 nM values for each reporter.

BY-2, transient expression of Luz593-based cytokinin reporter

TDZ, nM

0 0.01 0.1 1 3 7 10 100

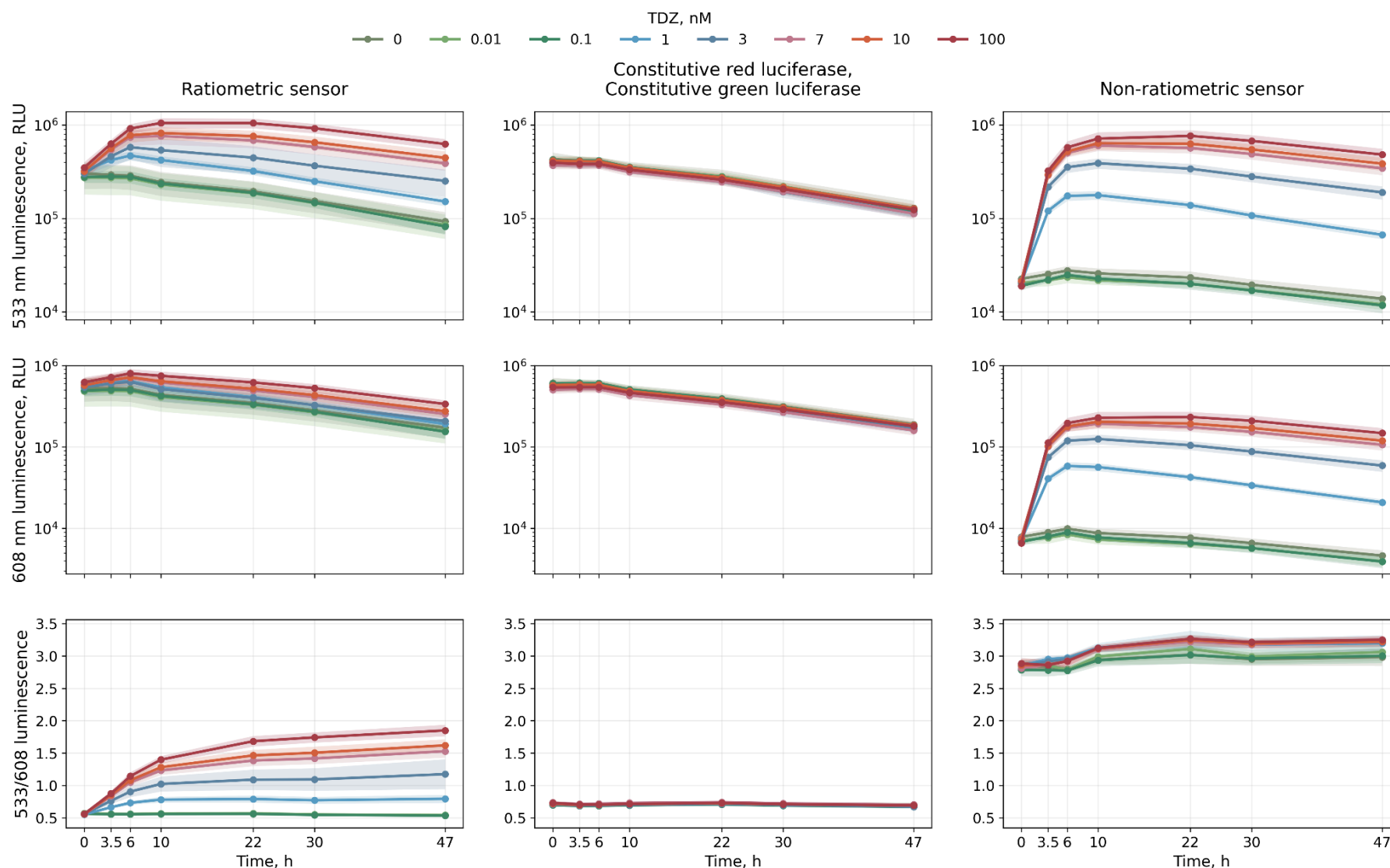


Supplementary Figure 18. Effect of thiazuron (TDZ) treatment on the transient expression of fungal ratiometric and non-ratiometric luminescence reporters in *Nicotiana tabacum* BY-2 cell packs.

Time-course luminescence data showing expression of Luz533 and Luz593 variants driven by different promoter combinations: pTCSv2-Luz533 + MinSyn108-Luz593 (ratiometric sensor), AtTCTP-Luz533 + MinSyn108-Luz593 (constitutive red luciferase + constitutive green luciferase), pTCSv2-Luz533 (non-ratiometric sensor)(columns, left to right). Rows represent measurements of luminescence at 533 nm (top), 593 nm (middle), and the 533/593 nm emission ratio (bottom). Cell packs were infiltrated with a range of concentrations, or control buffer (0 uM), and luminescence was monitored over a 47-hour period. Absolute luminescence (RLU) shows TDZ concentration-dependent induction in constructs containing the pTCS2 promoter. In contrast, the 533/593 nm emission ratio remains relatively

stable across treatments and time points, suggesting its potential as a ratiometric normalization method. The plots show mean values and standard deviation. N = 12. Measurements were performed using Tecan Spark.

BY-2, transient expression of Luz608-based cytokinin reporter



Supplementary Figure 19. Effect of thidiazuron (TDZ) treatment on the transient expression of fungal ratiometric and non-ratiometric luminescence reporters in *Nicotiana tabacum* BY-2 cell packs.

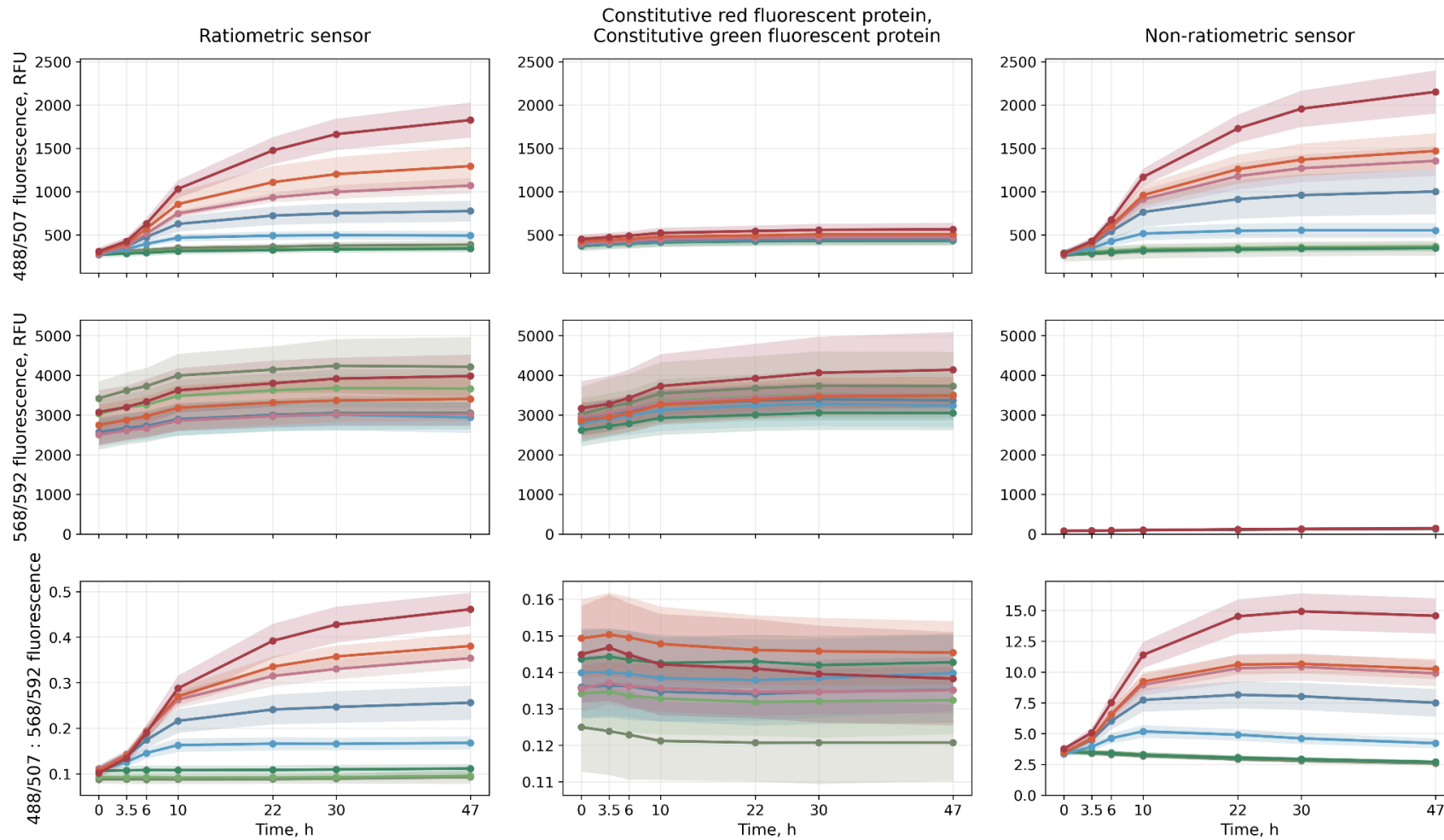
Time-course luminescence data showing expression of Luz533 and Luz608 variants driven by different promoter combinations: pTCSv2-Luz533 + p35S-Luz593 (ratiometric sensor), AtTCTP-Luz533 + p35S-Luz593 (constitutive red luciferase + constitutive green luciferase), pTCSv2-Luz533 (non-ratiometric sensor)(columns, left to right). Rows represent measurements of luminescence at 533 nm (top), 608 nm (middle), and the 533/608 nm emission ratio (bottom).

Cell packs were infiltrated with a range of concentrations, or control buffer (0 μ M), and luminescence was monitored over a 47-hour period. Absolute luminescence (RLU) shows TDZ concentration-dependent induction in constructs containing the pTCS2 promoter. In contrast, the 533/608 nm emission ratio remains relatively stable across treatments and time points, suggesting its potential as a ratiometric normalization method. N = 12. The plots show mean values and standard deviation. Measurements were performed using Tecan Spark.

BY-2, transient expression of fluorescent cytokinin reporter

TDZ, nM

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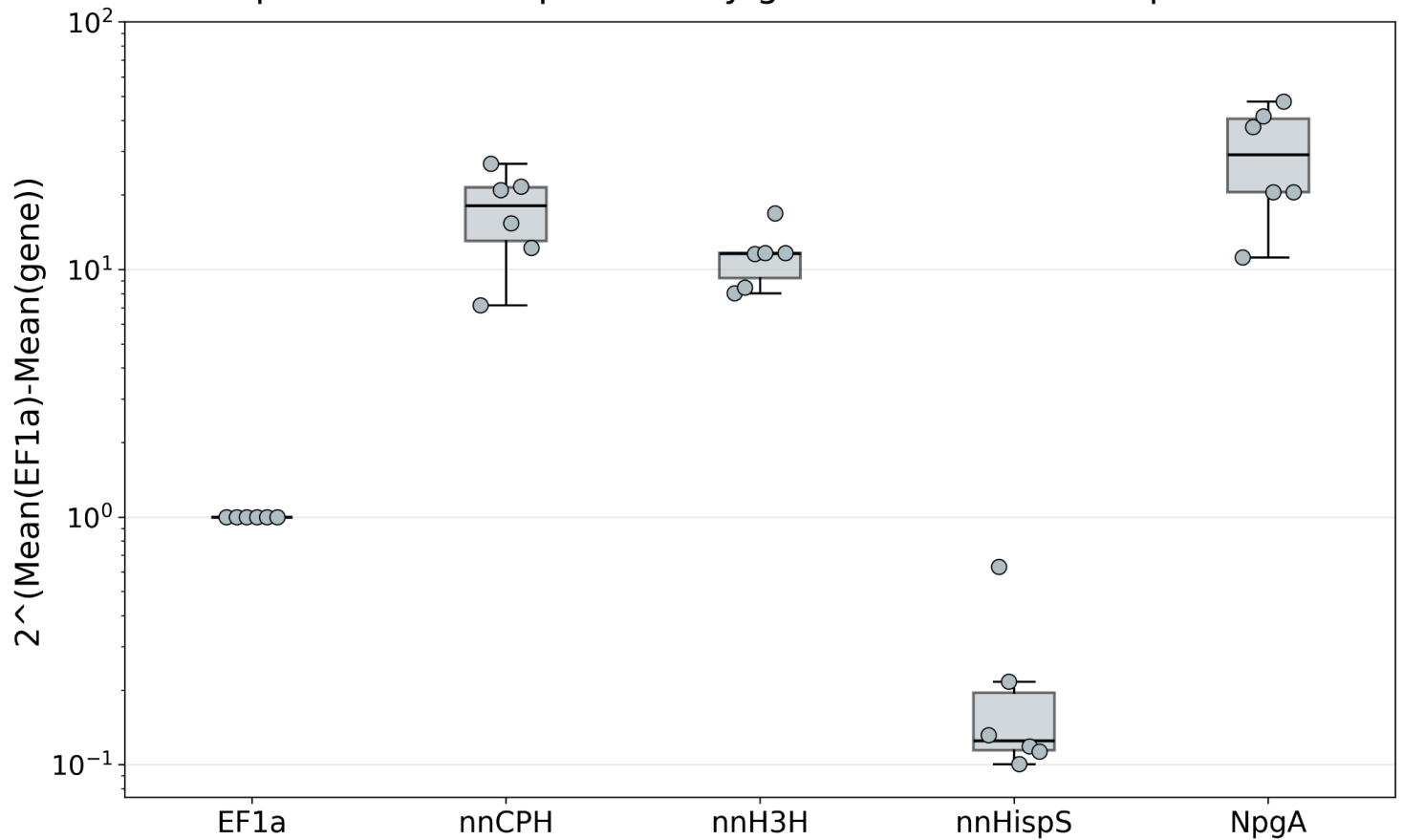


Supplementary Figure 20. Effect of thidiazuron (TDZ) treatment on the transient expression of ratiometric and non-ratiometric fluorescent reporters in *Nicotiana tabacum* BY-2 cell packs.

Time-course luminescence data showing expression of EGFP and mScarlet-I3ΔC10 variants driven by different promoter combinations: pTCSv2-EGFP + p35S-mScarlet-I3ΔC10 (ratiometric sensor), AtTCTP-EGFP + MinSyn108-mScarlet-I3ΔC10 (constitutive red luciferase + constitutive green luciferase), pTCSv2-EGFP (non-ratiometric sensor)(columns, left to right). Rows represent measurements of fluorescence at ex.488 nm em.507 nm (green fluorescence, top), ex.568 nm em. 592 nm (red fluorescence, middle), and the green-to-red fluorescence emission ratio (bottom). Cell packs were infiltrated with a range of concentrations, or control buffer (0

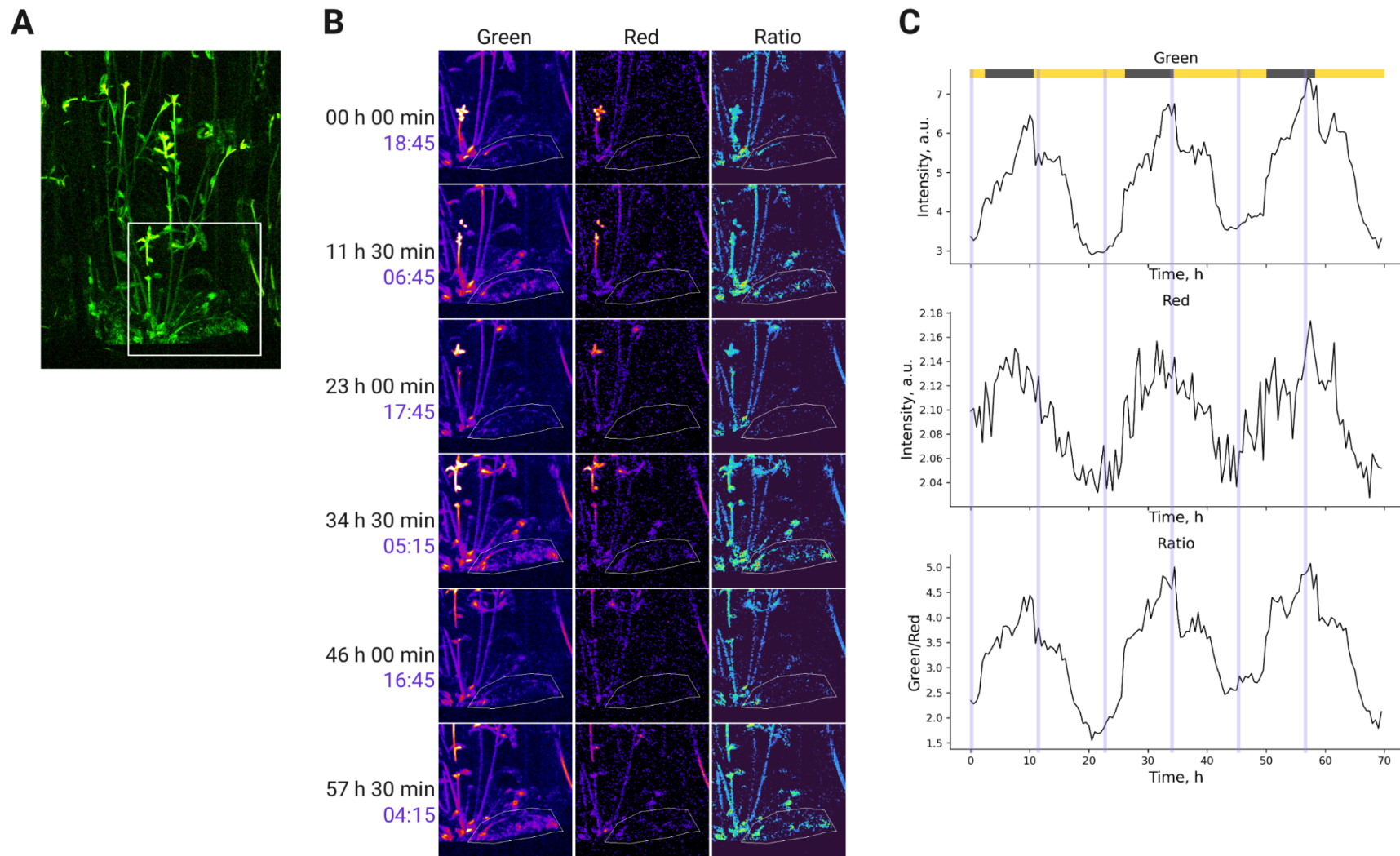
uM), and fluorescence was monitored over a 47-hour period. N = 12. The plots show mean values and standard deviation. Measurements were performed using Tecan Spark.

qPCR relative expression by gene in ratiometric reporters



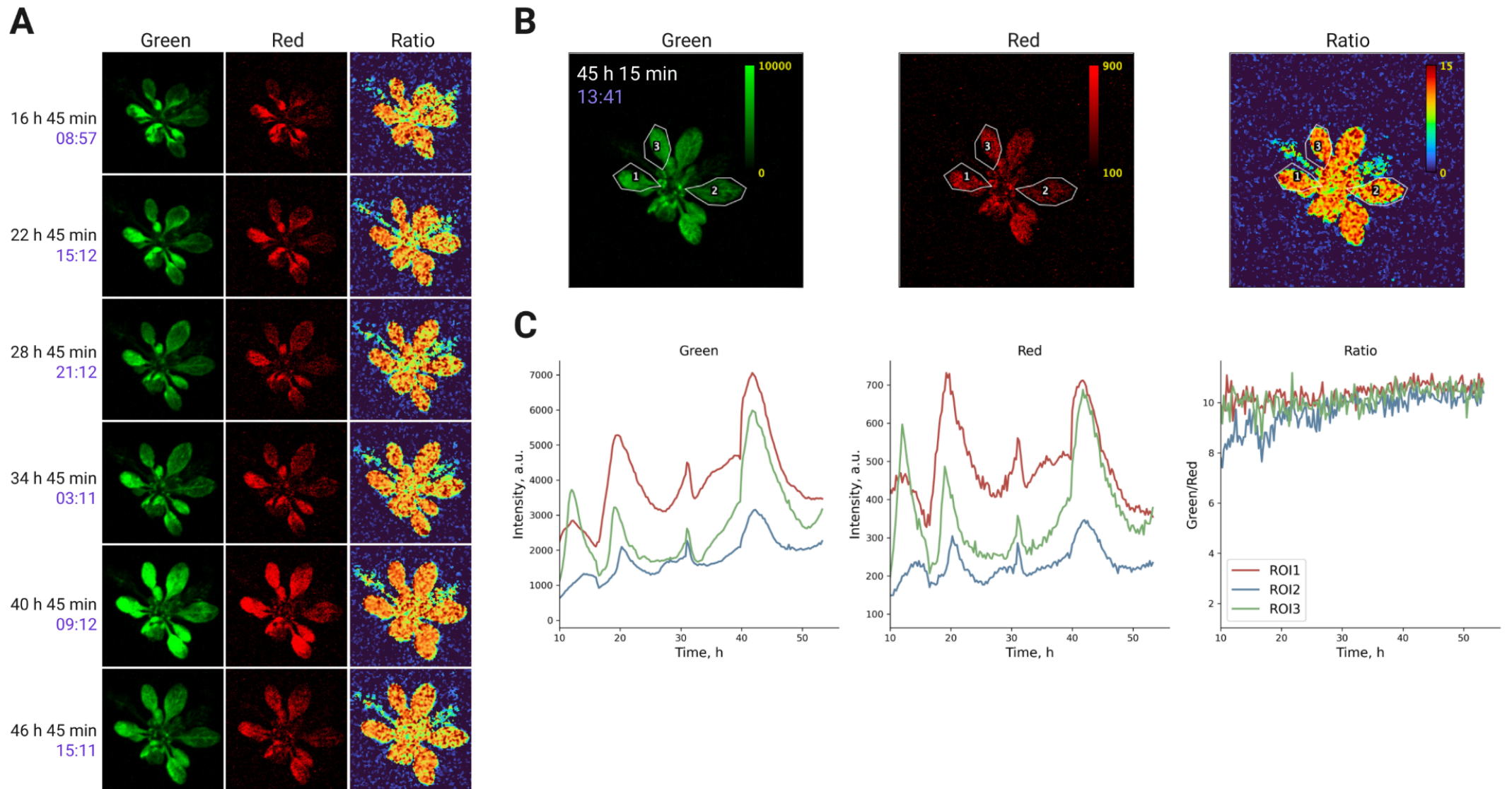
Supplementary Figure 21. Relative expression of fungal bioluminescence pathway enzymes in a luciferase-less master line AT9865 used for generation of transgenic reporter lines.

EF1a was used as a housekeeping gene for normalization. Data was obtained using 6 independent plants (N = 6, grey dots), 3 leaves each and 3 technical replicates. Box plot shows median, interquartile range (IQR, box), $1.5 \times \text{IQR}$ (whiskers).



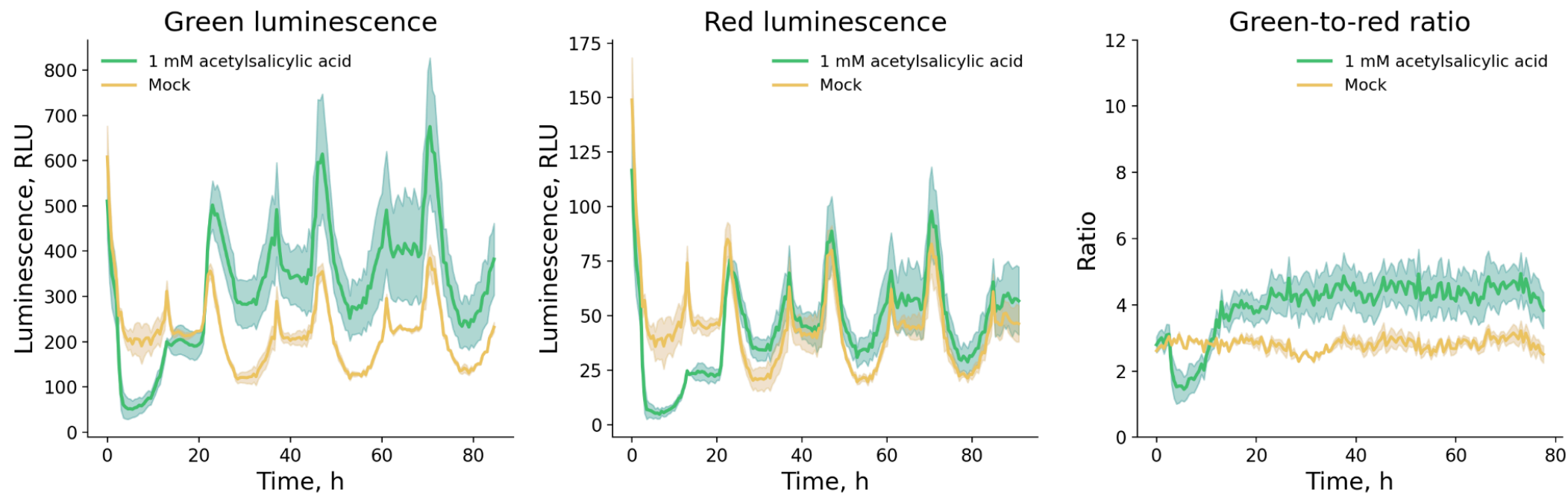
Supplementary Figure 22. Long-term time-lapse imaging of *Arabidopsis thaliana* plants stably expressing the pWRKY70-Luz533 + pMinSyn108-Luz593 reporter throughout the life cycle.

A. Region of interest selected for analysis from Supplementary Video 1. **B.** Dynamics of immunity-related oscillations in leaves. Time elapsed from the start of imaging is shown in black, and astronomical time is shown in purple. **C.** Oscillation dynamics in the green, red, and ratiometric channels. Daytime is indicated in yellow and nighttime in black. Shown for N = 3 plants.



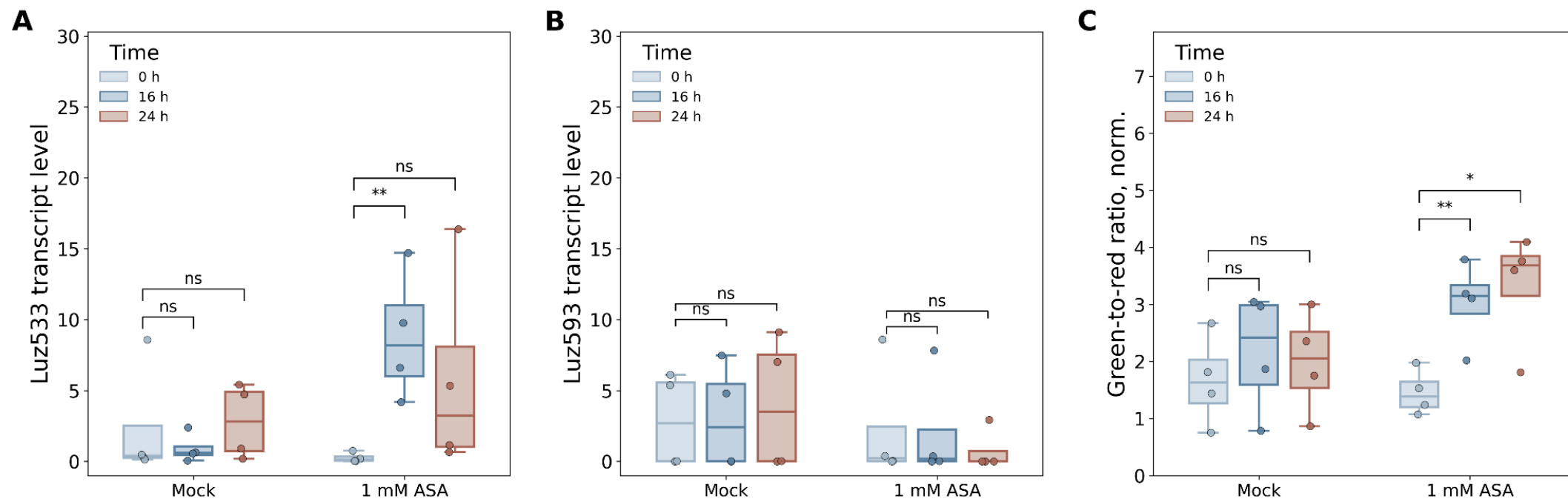
Supplementary Figure 23. Long-term timelapse snapshots of *Arabidopsis thaliana* plants stably expressing pWKRY70-Luz533 + pMinSyn108-Luz593 reporter.

A. Dynamics of luminescence intensity fluctuations in green and red channels and stability of the signal in ratio. Time elapsed from the start of imaging is shown in black, and astronomical time is shown in purple. **B.** Regions of interest (leaves) in green, red and ratio channels analyzed in C. **C.** Oscillation dynamics in the green, red, and ratiometric channels by leaves.



Supplementary Figure 24. Treatment of a ratiometric pWRKY70-Luz533 + pMinSyn108-Luz593 reporter with acetylsalicylic acid.

Green luminescence (reporter signal) and red luminescence (normalisation signal) over time following treatment with 1 mM acetylsalicylic acid or mock control. Green-to-red luminescence ratio is displayed on the right. Luminescence values were background-corrected by subtracting the mean signal of background wells at each time point. Data represent mean $\pm$ SD of independent technical replicates (N = 2 plants for treatment and control; N = 3 regions of interest for background). Measurements were acquired at 30-minute intervals.



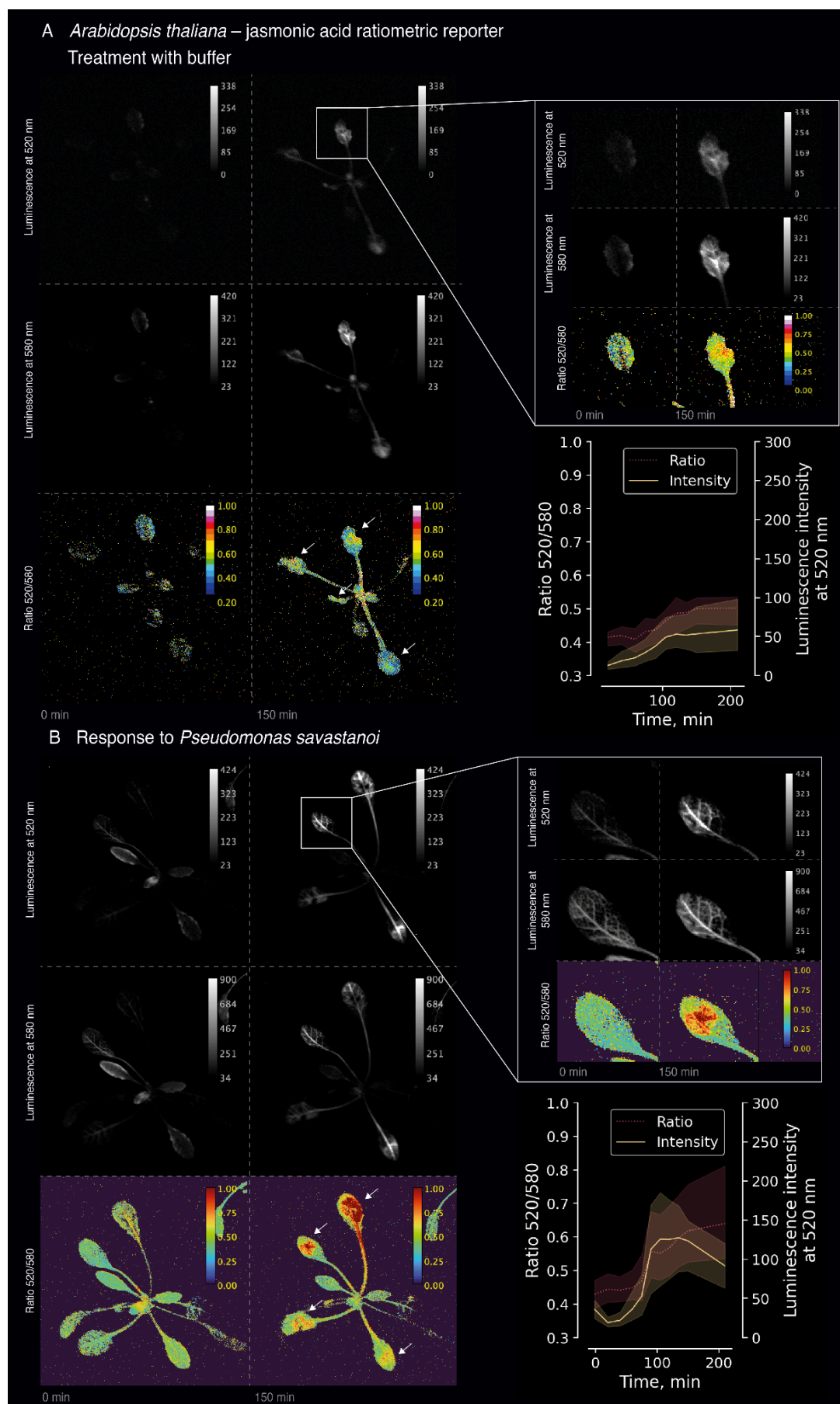
Supplementary Figure 25. Acetylsalicylic acid selectively induces *A.thaliana* stably expressing ratiometric pWRKY70-nnLuz reporter.

A. Plant-level RT-qPCR analysis of Luz533 transcript abundance in mock- and acetylsalicylic acid (ASA)-treated plants at 0, 16 and 24 h after treatment. Expression was calculated as $2^{(Ct_{EF1} - Ct_{Luz533})}$.

B. Plant-level RT-qPCR analysis of the constitutive Luz593 transcript in the same samples, calculated as $2^{(Ct_{EF1} - Ct_{Luz593})}$.

C. Green-to-red luminescence ratio in mock- and ASA-treated plants at 0, 16 and 24 h after treatment.

In all panels, boxes show the median and interquartile range, whiskers indicate the data range, and points represent individual plants or biological replicates. N = 4 individual plants, 2 leaves each. Statistical comparisons were performed within each treatment group between 0 and 16 h and between 0 and 24 h using one-way ANOVA. In acetylsalicylic acid-treated plants, Luz533 expression was significantly increased at 16 h, while Luz593 expression remained unchanged. The green-to-red luminescence ratio increased significantly at both 16 h and 24 h after acetylsalicylic acid treatment.



Supplementary Figure 26. Characterization of luminescence ratio and intensity in transgenic *Arabidopsis thaliana* lines expressing jasmonic acid ratiometric reporter expressing pORCA3-Luz533 + pMinSyn108-Luz593 treated with buffer (top) or *Pseudomonas savastanoi* (bottom). Red dotted lines indicate the ratio of signals at 520 nm to

580 nm, while yellow solid lines represent luminescence intensity at 520 nm. Shown for N = 3 plants. Measurements were performed using IVIS Spectrum.

Supplementary Video 1. Three-day-long timelapse of 42-44-day-old *Arabidopsis thaliana* plants. Luz593-expressing plant was used as a control, defence signals ratiometric reporter – pWRKY70 ratiometric reporter.

Supplementary Video 2. 53-hour-long timelapse of *Arabidopsis thaliana* plants expressing defence signals ratiometric reporter – pWRKY70 ratiometric reporter.

Supplementary Video 3. 76-hour-long timelapse of transgenic *Arabidopsis thaliana* plants expressing ratiometric jasmonic acid reporter pORCA3-Luz533, pMinSyn108-Luz593. The plants were infiltrated with buffer.

Supplementary Video 4. 76-hour-long timelapse of transgenic *Arabidopsis thaliana* plants expressing ratiometric jasmonic acid reporter pORCA3-Luz533, pMinSyn108-Luz593. The plants were infiltrated with *Pseudomonas savastanoi*.

Supplementary Table 1. Plasmids used in the study.

ID	Plasmid description	Plasmid map	Addgene ID
Y16990	p35S-Luz608-OCSt	https://benchling.com/s/seq-F6KSPNaZIWDatU6VUjvO?m=slm-zhdzzyjZUa3unN7LrtVm	254704
Y17788	p35S-Luz533-OCSt	https://benchling.com/s/seq-XUzVeTri5PJCwiYkDJzY?m=slm-SdUd3mkfc9oQfz04AHjs	254706
Y17790	p35S-Luz593-OCSt	https://benchling.com/s/seq-HINEE7tv00IqKjvVDP8L?m=slm-NKE7bCikLjNs0egij4E	254689
Y17791	p35S-Luz640-OCSt	https://benchling.com/s/seq-ayr1k0ZhaFReGwcbtntb2m=slm-FXLHbSUhsiVcSwDOFNUR	254701
Y13213	pTCS2-min35S-TMV-Luz533-act2t	https://benchling.com/s/seq-QZj4r48LSaY71kGNJcVQ?m=slm-ghLEspiuMXvRsP0HTS4H	
Y13080	pAtTCTP-Luz533-act2t	https://benchling.com/s/seq-Uh5EJBY0F4feUhKiWrw?m=slm-ZRZKmnzNklxzz04esDvi	
Y9646	MinSyn108-5'UTR_AtRBCS2B-Luz593-act2t	https://benchling.com/s/seq-daQAR79rc2hZV77IBUi?m=slm-v5S6UrUeaEliYCG89Mc	
Y17463	p35s-nnHisps-OCSt pCmYLCV-npgA-ATPt dummy p35s-nnCPH-OCSt pFMV-H3H-NOST	https://benchling.com/s/seq-JChGOcCVNiYRKbruZQ2z?m=slm-cN2aKkBzOeeBNZLMgSwe	
dna5168	p35s-iluxA-OCSt	https://benchling.com/s/seq-3J5UBiU9t6qMrphMhzLF?m=slm-KISCBeu8PcaiSB3acqN4	
dna5169	p35s-iluxB-OCSt	https://benchling.com/s/seq-I012dBJ0GTrutMoRZHcX?m=slm-Dtc7JE3w6H4BbRfRqymA	
dna5170	p35s-iluxC-OCSt	https://benchling.com/s/seq-XfMhkPSbxcCjkPPPWmDx?m=slm-bH4Zi0jLc7EsP6PcMovt	
dna5171	p35s-iluxD-OCSt	https://benchling.com/s/seq-T0SKpZl8wDVZdmEy4YbM?m=slm-SDlctnvae3Ceh7aER2zT	
dna5172	p35s-iluxE-OCSt	https://benchling.com/s/seq-E5d36yluhQLmwU33eC6y?m=slm-e7hGlht0iHXRNF0QuZry	
dna5173	p35s-ilux_frp-OCSt	https://benchling.com/s/seq-9k498FwHyNqd9iG4CRyw?m=slm-YAt9HDKsQAQUbZFvTBJM	
dna5174	p35s-VenusΔC10-EL-iluxA-OCSt	https://benchling.com/s/seq-JhbPVfNAnCJfWiHW6it6?m=slm-s97nsWAEjmBVf0U22sc6	
dna5175	p35s-mScarlet-ΔC10-EL-iluxA-OCSt	https://benchling.com/s/seq-yKbsmXHTIZHMz8J35uS2?m=slm-FqzGSjwq9s4J4TCzXpaP	
dna5177	p35s-sfGFPΔC10-EL-iluxA-OCSt	https://benchling.com/s/seq-KUwh8yW8sxDflw1py8Xn?m=slm-DKQjFkiYJah3Y8BpHodR	

Supplementary Table 6. Plant lines used in the study.

ID	Insert description	Seed generation
AT7805	[forward] pWRKY70 - Luz533 - WRKY70_T [reverse] MinSyn108-5'UTR AtRBCS2B - Luz593 - act2T [forward] HygR cassette	T1
AT7807	[reverse] MinSyn108-5'UTR - Luz593 - act2T [forward] HygR cassette	T1
AT7808	[forward] pORCA3 -Luz533 - ORCA3_T [reverse] MinSyn108-5'UTR AtRBCS2B - Luz593 - act2T [forward] HygR cassette	T1

Supplementary Table 7. Primers used for qPCR.

Gene	Forward primer	Reverse primer
Luz533	tagtctcttcggataattgggaatgctattgc	tgacggtctcCaatgAGGtctactATCTCTTTGTCT AGTCT
EF1a	CTGCAACAAGATGGATGCTAC	CAAAACCAGAGATGGGGACGAA
act2_T/tdTomato	GATCCCATTTCATAAAACCCCAGC	ACCTGTTCTGTACGGCATGGAC
Luz533/WRKY70_T	GCTGGTAAGATTGTCGAAAATGCC	TATGTTTTCCACTCTACATGGCC
nnCPH	AAATAATCGGAAGCGGCAGTGC	TTAGCTTCTGTGGCGTGGACC
nnHispS	TATCTCCAGTCCTCAAGTGCTG	ACATATCCGTCTGCATCAGTCG
NpgA	AACTCCAGATCCCCATAGAAGG	GAGTTGGGTGTAAATGCAGTGC
H3H	ATTTGTCTTGGAGGAGGACGTG	AGATTGTGGATTCTGGGGCGTA

Supplementary Note 1.

Engineering strategies used for development of red-shifted variants of fungal luciferase

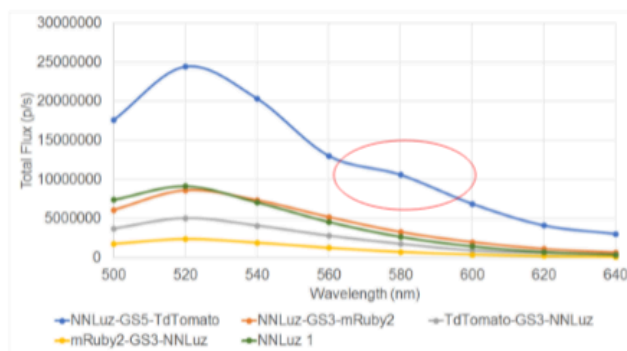
To guide engineering, we formulated the following working hypotheses:

- (1) efficient BRET requires favorable donor-acceptor orientation,
- (2) internal insertions may shorten distance between donor and acceptor, and provide better geometric constraints than terminal fusions,
- (3) linker composition and length modulate domain orientation and flexibility,
- (4) increasing the number of acceptors may enhance probability of energy transfer, and
- (5) truncation of fluorescent proteins may reduce the distance, improving coupling efficiency.

Subsequent experiments were designed to test these hypotheses.

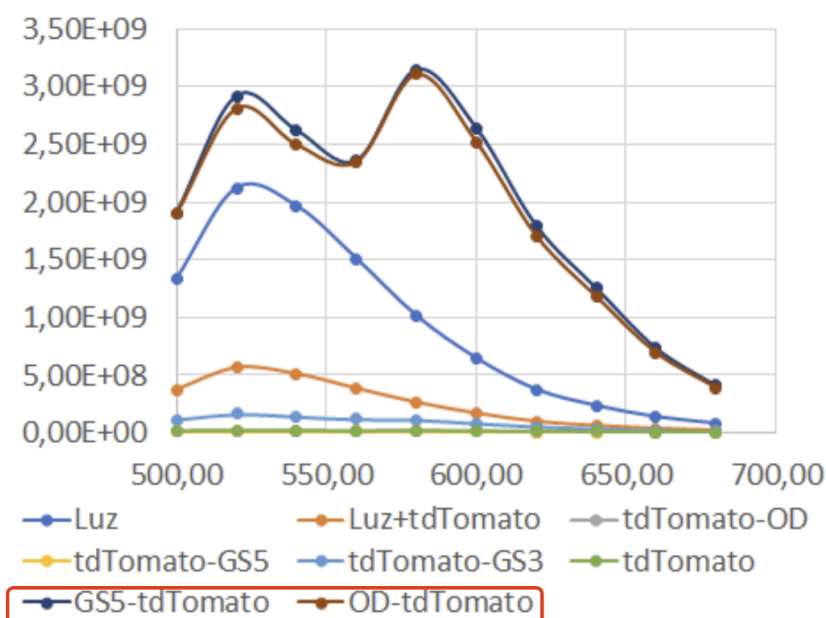
1. Early terminal fusions of fungal luciferase did not significantly shift the color of luminescence

We first generated N- and C-terminal fusions of the luciferase nnLuz_v4 with two fluorescent proteins selected based on spectral compatibility, such that the luciferase emission overlapped with the excitation spectra of the acceptors—mRuby2 and tdTomato. For initial screening, flexible linkers of varying lengths, (GGGGS)₃ and (GGGGS)₅, were used to connect the fluorescent proteins to either terminus of nnLuz. When expressed in HEK293 cells, mRuby2 did not produce detectable BRET, whereas C-terminal tdTomato fusions exhibited weak energy transfer (Supplementary Note 1, Figure 1). In contrast, N-terminal fusions showed no measurable energy transfer.



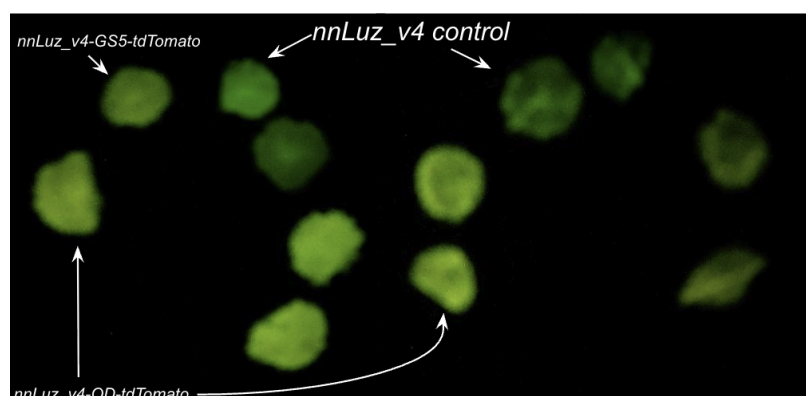
Supplementary Note 1, Figure 1. Initial screening of mRuby2 and tdTomato as C- or N-terminal fusions of nnLuz_v4 in HEK293 cells.

Based on these initial results, we concluded that C-terminal positioning of tdTomato, combined with a longer linker, provided the most promising configuration. We therefore generated an additional variant, nnLuz-OD-tdTomato, where OD represented a relatively flexible, moderately charged linker with the sequence SGSSLTGTRSDIGPSRAS. We evaluated its expression in plant BY-2 cells (Supplementary Note 1, Figure 2). The OD linker performed similarly to the (GGGGS)₅ linker, yielding comparable to the wild-type nnLuz brightness and a red-to-green emission ratio (593 nm / 533 nm) of approximately 1.



Supplementary Note 1, Figure 2. Initial screening of tdTomato as C- or N-terminal fusions with the luciferase nnLuz_v4 in BY-2 cells.

Despite this partial success, the observed performance was insufficient for the development of robust ratiometric sensors. In *Nicotiana benthamiana* leaves, the infiltrated cells still appeared predominantly green (Supplementary Note 1, Figure 3), indicating limited contribution of the red-shifted emission.



Supplementary Note 1, Figure 3. Color of early red-shifted variants in *N.benthamiana*.

The lack of energy transfer in N-terminal fusions and weak performance of C-terminal fusions indicated that terminal positioning did not ensure favorable donor-acceptor distance or orientation in the fungal luciferase. To improve BRET efficiency, we next focused on identifying suitable internal insertion sites within the luciferase.

2. Internal positions for insertions into fungal luciferase were identified

As described in the main text, we engineered a synthetic insertion and deletion library of luciferase variants (Supplementary Figure 1). Screening of both libraries in BY-2 cells identified several regions tolerant to insertions or deletions, notably around amino acid positions 119, 167, 188, and 221.

3. Initial screening of fluorescent proteins identifies novel BRET pairs in BY-2 cells

Building on the internal and C-terminal positions identified as permissive for insertions, we performed an initial screening of spectrally compatible red fluorescent proteins: tdTomato, which had already shown evidence of resonance energy transfer; mScarlet/mScarlet3/mScarlet-I3 – a set of the brightest and fastest-maturing red fluorescent proteins; and mKate2, a far-red fluorescent protein (**Supplementary Figure 2**).

In our DNA assembly design, insertion of fluorescent proteins resulted in introduction of an extra amino acid next to the insertion sites, due to overhangs required for Golden Gate assembly. Screening of the resulting fusion proteins revealed multiple functional BRET pairs with varying brightness and energy transfer efficiency. Among these, the best-performing constructs were nnLuz_ins167-tdTomato, nnLuz_ins188-mKate2, and nnLuz_ins119-mScarlet. Their brightness and red-to-green ratios summarized in **Supplementary Note 1, Table 1**.

4. Optimal orientation of of BRET donor and acceptor is achieved utilising the right linkers

Efficient resonance energy transfer requires a favorable relative orientation of donor and acceptor transition dipoles, which is determined by the spatial arrangement of the fused domains ¹⁻⁴. This arrangement is influenced by linkers connecting donor and acceptor proteins, including their length, composition, and charge.

Aiming to improve BRET efficiency, we designed and tested a panel of linkers varying in length, rigidity, and charge. Several of these linkers have previously been used in previously FRET/BRET-focused publications:

(GGGGS)₁ – short flexible linker ⁵
SGLRS – short flexible linker ⁶
(GGGGS)₃ – medium length flexible linker ⁵
SGSSLTGTRSDIGPSRAS – OD-linker, previously used in our early BRET pair with tdTomato
GHGTGSTGSGSSAS – from tdTomato ⁶
(GGGGS)₅ – long flexible linker ⁷
GSLAEAAAKEAAAKEAAAKAAAAS – rigid alpha-spiral ⁸
PAPAP – rigid short linker ⁹
PAPAPAPAPAP – rigid longer linker ⁹

As an additional strategy, we introduced truncation of ten C-terminal amino acids in mKate2 and mScarlet-I3 (mKate2 Δ C10 and mScarlet-I3 Δ C10) fluorescent proteins, following the approach previously shown to improve BRET performance ¹⁰⁻¹².

We then screened combinations of linkers and truncated fluorescent proteins in BY-2 cells using three representative BRET pairs: nnLuz_ins188-mKate2 (**Supplementary Figure 3**), nnLuz_ins119-mScarlet (**Supplementary Figure 4**), and nnLuz_ins167-tdTomato (**Supplementary Figure 5**).

For nnLuz_ins167-tdTomato, we were able to substantially improve brightness and slightly improve energy transfer efficiency. The optimal linker combination consisted of an N-terminal SGLRS linker and a C-terminal (GGGGS)₁ linker, yielding a red-to-green ratio of 1.25 and a ~5-fold increase in brightness (**Supplementary Note 1, Table 1**).

For mKate2-based fusions, the best-performing configuration included N-terminal SGLRS linker and a C-terminal OD-linker, resulting in a red-to-green ratio of ~1 and a several-fold increase in brightness (**Supplementary Note 1, Table 1**).

In contrast, for mScarlet-I3 Δ C10, the truncated variant without additional linkers showed a ~50% increase in BRET efficiency and a several-fold increase in brightness compared to non-truncated variants

(**Supplementary Figure 4**). We conclude that C-terminal truncation of mScarlet-I3ΔC10 was important towards achieving sufficiently bright red-shifted luciferase variants.

Interestingly, the short SGLRS linker repeatedly outperformed other designs, suggesting that moderate flexibility combined with limited length provides an optimal balance between structural constraint and mobility.

5. Linker optimization in N- and C-terminal fusions did not yield better BRET pairs

Given that linker optimization proved effective for internal fusions, we explored whether a similar strategy could enhance N- and C-terminal luciferase fusions with mKate2, mScarlet3, and tdTomato (**Supplementary Figure 6**), using same panel of linkers.

For mKate2-based constructs, linker optimization led to a several-fold increase in brightness; however, this was accompanied by a decrease in energy transfer efficiency. In the case of mScarlet3, brightness was also improved, but BRET efficiency remained substantially lower than that observed for the optimal internal fusion, nnLuz_ins167-mScarlet-I3ΔC10. For tdTomato, all tested variants performed similarly to the initial nnLuz-OD-tdTomato construct, with no notable improvement.

6. Extra repeat of fluorescent protein improved BRET

Previous studies showed that adding a second fluorescent acceptor can increase FRET efficiency. In particular, tandem-acceptor variants of the Epac sensor exhibited higher FRET efficiencies than the corresponding single-acceptor construct, and tandem fluorescent-protein designs have also been used to generate enhanced FRET acceptors⁶. Additionally, the Antares reporter incorporates NanoLuc fused to tandem copies of the fluorescent protein CyOFP1, illustrating how the use of multiple acceptors can enhance BRET efficiency and overall signal output¹³.

We applied the same design strategy to engineer red-shifted luciferases based on the best-performing variants identified at that stage (**Supplementary Figure 7**) with the replacement of nnLuz_v4 with improved nnLuz_v5 (Luz533). In a parallel study (unpublished), we applied directed evolution to develop Luz533, a variant of the fungal luciferase with increased brightness compared to previously published nnLuz_v4. The improved performance of Luz533 likely reflects enhanced catalytic activity or improved protein stability.

The best-performing BRET pairs for improvement included:

Construct A, Luz533_ins167-SGLRS-tdTomato-GGGGS, and
Construct B, Luz533_ins188-SGLRS-mKate2-OD-linker.

In the case of tdTomato, addition of a second copy increased the red-to-green ratio to ~2 while maintaining comparable brightness (relative to a ratio of 1.25 in Construct A), resulting in the variant Luz593. For construct B, incorporation of an additional tdTomato copy led to a broader, more red-shifted emission spectrum and a modest increase in brightness. Similarly, addition of a second mKate2 copy to construct B slightly improved energy transfer efficiency without affecting overall brightness, yielding the variant Luz640 (**Supplementary Figure 8**).

Motivated by previous observations that truncation of fluorescent proteins can enhance energy transfer efficiency, we generated various fusion proteins with and without truncation of seven C-terminal amino acids of Luz533, and with and without linkers. Truncation of the luciferase itself did not improve brightness or colour shift. However, addition of a second mScarlet-I3ΔC10 copy without a linker resulted in a red-to-green ratio of 1.9 (compared to 1.8 for nnLuz_ins167-mScarlet-I3ΔC10), while maintaining comparable brightness. We named this variant Luz608.

Summary

Among all tested modifications, the most impactful were:

- (1) identification of internal insertion site 167,
- (2) optimization of linkers in internal insertions,
- (3) truncation of fluorescent proteins,
- (4) tandem acceptor design.

Linker optimization in terminal fusions had minimal impact.

Overall, the best-performing red-shifted fungal luciferase variants, Luz593 and Luz608, exhibited red-to-green emission ratios of up to ~2. Luz608 retained brightness comparable to the wild-type Luz533, whereas Luz593 displayed a several-fold reduction in brightness.

Supplementary Note 1, Table 1. Characteristics of red-shifted versions of fungal luciferase.

Milestone version	Red-to-green ratio	Approximate brightness (absolute brightness varies across the experiments), %
Luz533	0.4	100
<i>mKate2-based fusion</i>		
nnLuz_v4_ins188-mKate2 (2aa linkers)	0.64	7
Luz533_ins188-SGLRS-mKate2-OD-linker	1.2	40
Luz640 (Luz533_ins188-SGLRS-mKate2-OD-linker-SGLRS-mKate2)	1.3	10
<i>tdTomato-based fusions</i>		
nnLuz_v4-OD-tdTomato	0.9	80
nnLuz_v4_ins167-tdTomato (2aa linkers)	1	12
Luz533_ins167-SGLRS-tdTomato-(GGGGS) ₁	1.38	60
Luz593 (Luz533_ins167-SGLRS-tdTomato-(GGGGS) ₁ -SGLRS-tdTomato)	2	20
<i>mScarlet-I3-based fusions</i>		
Luz533_ins167-mScarlet-I3ΔC10	1.7	67
Luz608 (Luz533_ins167-mScarlet-I3ΔC10-mScarlet-I3ΔC10)	1.9-2	77

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