A protoplast-based bioassay to quantify strigolactone activity in Arabidopsis using StrigoQuant

Running head: Protoplast-based bioassay for strigolactone quantitative studies

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Abstract

Understanding the biological background of strigolactone (SL) structural diversity and the SL signaling pathway at molecular level requires quantitative and sensitive tools that precisely determine SL dynamics. Such biosensors maybe also very helpful in screening for SL analogs and mimics with defined biological functions.

Recently, a genetically encoded, ratiometric sensor, StrigoQuant, was developed and allowed the quantification of the activity of a wide concentration range of SLs. StrigoQuant can be used for studies on the biosynthesis, function and signal transduction of this hormone class.

Here, we provide a comprehensive protocol for establishing the use of StrigoQuant in Arabidopsis protoplasts. We first describe the generation and transformation of the protoplasts with StrigoQuant and detail the application of the synthetic SL GR24. We then show the recording of the luminescence signal and how obtained data are processed and used to assess/determine SL perception.

Key words: Protoplasts, Arabidopsis, strigolactone, biosensor, synthetic biology, luminescence, firefly, StrigoQuant, D14, SMXL6, plant hormones, ratiometric sensor

1. Introduction

Strigolactones (SLs) play a multifaceted role in plant life, as they regulate different aspects of development and stress response, coordinate adaptation to nutrient availability, and mediate the communication with beneficial fungi and root parasitic plants [1, 2]. Distinguishable by their enol-ether bridge and their lactone ring (also called D-ring) – essential for biological activity --, SLs are classified into two categories (1) canonical, defined by the presence of an ABC-ring and (2) non-canonical [1]. SL biosynthesis is initiated by the isomerization of all-*trans*-β-carotene, followed by repeated oxidative cleavage steps and rearrangement, which yield carlactone (CL), the central precursor of SL biosynthesis. CL is further processed by different cytochrome P450 enzymes, which lead to different SLs according to the plant species, and are mostly responsible for the structural diversity of this plant hormone family [3, 4].

SL perception is initiated by the binding of an SL molecule to its receptor Dwarf14 (D14), an α/β-hydrolase superfamily protein [5]. D14 acts as a receptor and an enzyme that hydrolyzes the SL ligand and covalently binds to the released D-ring, which triggers SL signal transduction through a debated mechanism [6–8] The latter involves the degradation of repressors of SL-related genes [1]. Indeed, upon hydrolysis, changes in D14 conformation allow the recruitment of the central element of the Skp1-Cullin-F-box complex (SCF), a F-box protein called MAX2 in Arabidopsis. Skp1, Cullin and the ubiquitin ligase follow and bind to D14-MAX2. Simultaneously, Arabidopsis SMXL family proteins and repressors of SL-signaling transduction bind to the SCF complex [9]. Brought into vicinity of the ubiquitin ligase E2, the repressor SMXLs become poly-ubiquitinated and degraded through the 26S proteasome, initiating SL signal transduction [10, 11].

Based on this SL-mediated degradation mechanism, Samodelov et al. engineered a biologically encoded molecular sensor, named StrigoQuant [12]. Developed for quantitative SL studies, StrigoQuant incorporates the SL transcription repressor

AtSMXL6 [12]. This quantitative sensor monitors the SL dynamics in plant cells relying on two constitutively and stoichiometrically expressed modules, by the virtue of co-translation and subsequent cleavage of the co-encoded 2A peptide that separates the two modules [13]. This ratiometric sensor consists of: (1) a sensing part, *AtSMXL6* fused to the reporter *firefly luciferase* gene and (2) a normalization part, the reporter *renilla luciferase* gene (see Fig. 1 a). These two luciferase modules emit light in the presence of the respective, specific substrate D-luciferin and coelenterazine [14]. In the absence of SL, both luciferases emit light when provided with their substrates. However, SL application activates the signaling pathway, which leads to the degradation of the AtSMXL6-Firefly sensing module. This degradation can be monitored by determining the change in the firefly luminescent signal, which is inversely proportional to the SL concentration of the solution [12]. Inclusion of the steady renilla signal, which remains unchanged regardless of the presence/absence of SLs, as a normalization element, allows precise quantification of the AtSMXL6 degradation and thus SL activity (see Fig. 1 b).

Due to their versatile and biological functions, and large chemical diversity, SLs have increasingly attracted researchers' interest. To unravel the mechanisms underlying their regulatory roles, shed light on their structure-activity-relationships and to elucidate their biosynthesis, molecular and quantitative tools are sorely needed [15–17]. Our recently developed sensor StrigoQuant can provide quantitative insights in the structural specificity and sensitivity of the SL perception complex towards various natural and synthetic SLs, using Arabidopsis protoplasts as an investigation system [12]. StrigoQuant can be used to investigate the effect of other hormones on SL homeostasis and signaling and can provide new insights into the signaling processes mediated by natural and synthetic SLs.

Here we describe a comprehensive protocol for SL signaling studies in leaf protoplasts of Arabidopsis, using StrigoQuant. We exemplified our protocol with the study of GR24 application at different concentrations and describe the processing of the luminescence data.

2. Materials

Prepare all the solutions at room temperature, using sterilized double-distilled water. Use p.a. purity grade chemicals and plant cell culture tested reagents for media preparation. Adjust the pH of the solutions with HCl and KOH to avoid salt stress. Store all the solutions at 4 °C unless specified otherwise.

2.1 Biological Material

1. SCA (Seedling Culture Arabidopsis) [18]: 0.32% (w/v) Gamborg B5 basal salt powder with vitamins (bioWORLD), 4 mM $MgSO_4 \cdot 7H_2O$, 43.8 mM sucrose, and 0.8 % (w/v) phytoagar. Fill up with H_2O and adjust to pH 5.8. Autoclave, let it cool down a bit before adding 0.1 % (v/v) Gamborg B5 Vit Mix (bioWORLD) and 1:2000 ampicillin (100 mg/mL). Invert the bottle several times to ensure an appropriate mixing and pour 50 mL SCA medium into each square plate (see Note 1).
2. Seed sterilization solution for *A. thaliana* (modified from [18]): 5% (w/v) calcium hypochlorite, 0.02 % (v/v) Triton X-100 in 80 % (v/v) EtOH. Combine the chemicals in a bottle and mix for a few hours at room temperature. Check the formation of a precipitate. Prior to use, allow the precipitate to settle.
3. Sterile filter paper bands: cut Whatman filter paper (Merck KGaA, Darmstadt, Germany) into bands of ~1.5-2 cm large. Make stacks of 8-10, wrap the bands with aluminum foil and sterile autoclave.
4. Aluminum foil.
5. Square plates, 12 cm.
6. Parafilm.
7. Syringe and 22 µm filter.
8. Ampicillin stock solution: 100 g/mL in H_2O.
9. Growth chamber: Sanyo/Panasonic MLR-352-PE or Binder APT.line KBWF 240/ KBWF 720 (E5.3), with the settings 16h light – 8h dark cycles at 21°C.

2.2 **Protoplast Isolation and Transformation**

1. 10 mL, 20 mL and 50 mL stripettes.
2. Pipet boy.
3. Sterile 250 mL bottles.
4. Sterile 2 mL tubes.
5. Syringe and 22 µm filter.
6. MMC (MES, Mannitol, Calcium) solution [18]: we recommend preparing 1 L solution. Add 10 mM MES, 40 mM $CaCl_2 \cdot H_2O$, and 0.466 M mannitol. Adjust to pH 5.8, filter sterilize and aliquot into 250 mL sterile bottles.
7. Enzyme solution stock (10x concentrated): weigh 10 g cellulase Onozuka R10 and 10 g macerozyme R10 (SERVA Electrophoresis GmbH, Germany) in 20 mL MMC (preheated to 37 °C). Complete to 200 mL with H_20 (see Note 2). Sterilize the solution by passing it through a bottle-top 22 µm filter into a sterile bottle (see Note 3). Make 2 mL aliquots and store at −20 °C, avoid thaw–refreeze cycles.
8. MSC (MES, Sucrose, Calcium) solution [18]: we recommend preparing 1 L solution. Add 10 mM MES, 0.4 M sucrose, 20 mM $MgCl_2 \cdot 6H_2O$, and 0.466 M mannitol. Adjust to pH 5.8, filter sterilize, and aliquot into 250 mL sterile bottles.
9. W5 solution [18]: we recommend preparing 1 L solution. Add 2 mM MES, 154 mM NaCl, 125 mM $CaCl_2 \cdot 2H_2O$, 5 mM KCl, and 5 mM glucose. Adjust to pH 5.8, filter sterilize, and aliquot into 250 mL sterile bottles.
10. 3M (MES, Mannitol, Magnesium) solution [18]: 15 mM $MgCl_2$, 5 mM MES, 0.466 M mannitol. Adjust to pH 5.8, filter sterilize, and aliquot into 250 mL sterile bottles.
11. PEG solution [18]: made fresh for each experiment. Weigh 4 g polyethylene glycol 4000 (PEG_{4000}) and pour it in a 15 mL tube. Crush the PEG_{4000} flakes with a spatula into powder to facilitate its dissolution. Add 2.5 mL of 0.8 M mannitol, 1 mL of 1 M $CaCl_2$, and 3 mL H_2O to the PEG_{4000} powder. Vortex/mix thoroughly and place the tube in 37 °C water bath for full dissolution (see Note 4). Do not sterile filter.

12. Biotin stock solution: weigh 1 mg and in 5 mL H_2O. Warm it up to dissolve and make 1 mL aliquots.
13. Ampicillin stock solution: 100 g/mL in H_2O
14. PCA (Protoplast Culture Arabidopsis) solution [18]: we recommend preparing 1 L solution. Add 0.32 % (w/v) Gamborg B5 basal salt powder with vitamins (bioWORLD), 2 mM $MgSO_4 \cdot 7H_2O$, 3.4 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES, 0.342 mM L-glutamine, 58.4 mM sucrose, 0.444 M glucose, and 8.4 µM Ca-panthotenate. Add 2 % (v/v) biotin from biotin stock. Adjust to pH 5.8, filter sterilize, add 0.1 % (v/v) Gamborg B5 Vitamin Mix into the sterile solution (see Note 1), and aliquot into 250 mL sterile bottles. Prior to use, add 1:2000 ampicillin stock to the PCA working solution.
15. pGEN16 and StrigoQuant plasmids [12].
16. 50 mL Falcon tubes.
17. Sterile tweezers.
18. Sterile scalpel.
19. Disposable 70 µm pore size sieve (Greiner bio-one international, Germany).
20. Petri dish 94 × 16 mm.
21. Parafilm.
22. Aluminum foil.
23. 200 µl and 1 mL wide-open pipette tips (see Note 5).
24. Round-bottom 15 mL Falcon tubes.
25. Rosenthal cell counting chamber.
26. Nontreated 6-, and 12- or 24-well plates.
27. Centrifuge fitting 15 mL and 50mL tubes with adjustable acceleration and break speed.
28. Microscope.
29. Growth chamber: Sanyo/Panasonic MLR-352-PE, Binder APT.line KBWF 240 or KBWF 720 (E5.3) with the settings 16h light – 8h dark cycles at 21°C.

2.3 Hormone Induction and Bioluminescence Assay

1. Pre-frozen 1.5 mL and 15 mL black tubes at -80 °C.
2. Racemic (±)-GR24 (OlChemIm Ltd), 10 mM stock solution in methanol.
3. 50 mL PCA solution (see 2.2, step 12).
4. Firefly substrate [18] (*see* Note 6): Add 20 mM tricine, 2.67 mM $MgSO_4*7H_2O$, and 0.1 mM EDTA (*see* Note 7). Heat the solution to dissolve and move under the fume hood. Add 33.3 mM DTT, 524 μM ATP, 218 μg/mL acetyl-CoA. Switch off the light for the following steps. Add 131 μg/mL D-luciferin (Biosynth AG), 200 mL H_2O and stir the solution. By the addition of 5mM NaOH, the solution should turn yellow. Add 264 μM $MgCO_3 \cdot 5H_2O$ and adjust the solution to pH 8. Aliquot the substrate in precooled black 15 mL tubes and store them at -80 °C (see Note 8).
5. Renilla stock solution: for 12.5mL of 472 mM coelenterazine solution, dissolve 2.5 mg of coelenterazine (Roth 4094.4) in 12.5 mL of pre-cooled methanol. Aliquot 100 μL into black pre-frozen 1.5 mL tubes and store at -80 °C (see Note 8).
6. Sterile 200 μl and 1 mL wide-open pipette tips (see Note 5).
7. Multistep multichannel pipette
8. Multistep pipette and tips
9. 96-deepwell plates, non-treated (Merck).
10. Costar® 96-well flat-bottom white plate.

3. Methods

Carry out all procedures at room temperature.

3.1 Biological Material Preparation

1. Clean the seeds in a sterile working hood. Place the seeds in 1.5 mL tube(s), do not add more than the equivalent of 250 μl volume. We recommend increasing the

number of seed tubes used, instead of increasing the amount of seeds per tube, to ensure consistent sterilization.

2. Add 1 mL of 80 % (v/v) ethanol, invert several times and pipet out the liquid while trying to remove dirt and other plant debris.
3. Repeat step 2 until all the large pieces of debris are extracted from the seeds.
4. Surface sterilize the seeds with 1 mL sterilization solution and agitate for 10 min.
5. Pipet out the sterilization solution. Add 1 mL of 80 % (v/v) EtOH and agitate for 5 min.
6. Replace the solution with 1 mL sterilization solution and agitate for 2 min.
7. Replace the solution with 1 mL absolute ethanol (≥99.5 %) and agitate for 1 min.
8. Pipet out the ethanol and let the seeds dry completely under the sterile hood (see Note 9).
9. Organize two autoclaved filter paper strips per SCA plate. Place one at the top of the plate and the second one in the middle.
10. Add autoclaved water into the tube containing the sterilized Arabidopsis seeds, ~1.5-2x seed volume. Resuspend the seeds in the water and plate ~200-300 seeds/strip in a line following the boarder of the filter paper strip (pipet on top of the paper, not on the agar). Repeat the same procedure with the second filter paper strip of the same plate. Seal the plate tightly with parafilm (see Note 10).
11. Incubate the plates in a growth chamber set at 16h light – 8h dark cycles at 21°C. 1.5- to 2.5-week old plants can be used for protoplast isolation.

3.2 Protoplast Isolation and Transformation

Perform the following steps under a sterile working hood. Protoplasts isolation and polyethylene glycol-mediated transformation are performed as described by Ochoa et al. [18] and using a slightly modified version of Dovzhenko et al. [19]. Take extra care when manipulating protoplast-containing solutions and always pipet them with wide-orifice pipette tips. This reduces plant cell damage. Set the centrifuge parameters to the

equivalent of medium acceleration and low deceleration (or no break), depending to the centrifuge model.

1. Cut the Arabidopsis plants just above the roots with a scalpel. Transfer the material into a petri dish containing 2 mL MMC. Avoid transferring roots and seeds and make sure that the plant material is in contact with MMC. Finely slice or chop the green tissues into small pieces, do not crush them. One cutting petri dish usually fits the plant material harvested from two square plates, depending on seedling size/age.
2. Transfer the cut leaf material into a new petri dish containing 7 mL of MMC (see Note 11).
3. Add 1 mL enzyme solution stock (10x concentrated) to digest the plant material (each enzyme final concentration should be 0.5 %).
4. Carefully seal the dish with parafilm and cover it with aluminum foil. Incubate overnight (12–16 h) in the dark at 21 °C.
5. Take the plant lysate back under the sterile hood. The solution should have turned light green. Carefully extract the protoplasts from the cut leaf material by gently pipetting the solution up and down for 5 min. The solution should turn dark green.
6. Pass the lysate through a 70 µm pore size sieve, mounted on top of a 50 mL tube.
7. Wash the remaining enzyme-leaf material mixture in the petri dish with 5 mL MMC by pipetting up and down and filter it into the same 50 ml tube.
8. Repeat step 7 until the MMC solution has only a light green tint, indicating that most protoplasts have been released from plant material.
9. Fill up the 50 mL tube containing the filtered protoplasts with MMC (see Note 12). Centrifuge at 100 × *g* for 20 min, with a low deceleration setting (or break function turned off).
10. Transfer the filtered protoplast solution to 15 mL round bottom Falcon tubes. One tube should be used for each plate of digested leaf material. The remaining steps should be completed in these tubes.

11. We recommend preparing the fresh PEG solution during this centrifugation time and leave it in the 37 °C water bath until needed.
12. Separate the supernatant from the pellet (from step 10) by pipetting it out using a sterile serological pipette and an electronic pipettor set to low pipetting speed. Pay attention to not pipet too close to the pellet, as it is loose and protoplasts can easily be removed accidentally (see Note 13).
13. Gently resuspend the pellet in the remaining MMC within the tube, then adding 5 mL of fresh MMC (see Note 14). Transfer the filtered protoplast solution (dark green) into a 15 mL round-bottom tube.
14. Sediment the protoplasts by centrifugation (100 × *g* for 15 min). Remove the supernatant and resuspend the protoplasts in 10 mL MSC.
15. Carefully overlay the 10 mL protoplast solution with 2 mL 3M (see Note 15).
16. Centrifuge for 10 min at 80 × *g* (low deceleration or no break). Protoplasts will migrate upwards in the flotation tube and accumulate at the interphase of MSC and 3M, creating a 1-2 mm thick dark green layer.
17. Meanwhile, prepare 2 collection tubes (15 mL round bottom tube) with 7 mL W5 solution for each floatation tube.
18. Collect 750 µl of the protoplasts twice (1.5 ml total per centrifugation round), by pipetting the dark green layer at the interphase of MSC and 3M into each collection tube containing W5.
19. Carefully add additional 1.5 mL of 3M to the flotation tube, to compensate for the collected volume, and centrifuge again for 10 min at 80 × *g* (low deceleration or no break). Steps 18 and 19 can be repeated until no further protoplasts float to the interphase (no dark green ring is visible after centrifugation).
20. Centrifuge the collected protoplasts, the 2 collection tubes with W5, for 10 min at 100 × *g* to pellet the protoplasts. Discard the supernatant and resuspend the plant cells in 5 mL W5, combining the cells from the two collection tubes together. The

total volume of the cell solution should be noted, as it is necessary to determine total cell number after counting (see Note 16).

21. Determine the cell density using a Rosenthal cell counting chamber (see Note 17).
22. Centrifuge the protoplasts for 5 min at 80 × *g*. Discard the supernatant and resuspend the pellet to a density of $5x10^6$ cells/mL with 3M.
23. Use a 6-well plate for transformation and deposit 15-30 µg DNA in H_2O (see Note 18), adjusted to the maximum volume to 20 µL, on the rim of the well. For a total DNA volume lower than 20 µL, top up to 20 µL with 3M (see Note 19).
24. Mix 100 µL protoplast solution in each well with the DNA-3M drop, by using wide-orifice tips and gently pipetting up and down (see Note 20). Incubate for 5 min.
25. Gently shake the 6-well plate from side to side, to distribute the protoplasts and DNA along the rim and to avoid protoplast aggregation. Use the tip-in-tip method (see Note 15) to add 120 µl of PEG solution in a drop-wise manner, along the length of the protoplasts and DNA solution. Do not mix or move the plate after the addition of PEG to avoid shearing stress and protoplast rupture.
26. Incubate for 8 min exactly, to ensure high transformation efficiency. Then dilute the PEG by consecutively and quickly but carefully adding 120 µl of 3M and at least 1.2 mL of PCA with ampicillin (see Note 21). Each well contains around 1.6 mL of the transformed protoplast resuspension.
27. Gently mix by creating a rotation movement of the liquid in the wells. Carefully seal the plates with parafilm, cover them with aluminum foil and place them back into the growth chamber at 21 °C for ~18 h to allow exogenous gene expression.

3.3 Hormone Induction and Bioluminescence Assay

As an example, we treated protoplasts transformed with 15 µg StrigoQuant and 15 µg pGEN16 (plasmid encoding a constitutively expressed GFP) (see Note 22) with different concentrations of *rac*-GR24 (0 M, 1 pM, 10 pM, 100 pM, 1 nM and 10 nM) resuspended in PCA.

1. Before starting any experiment, check the transformed protoplasts using an inverted microscope (see Note 23). Quickly verify the transformation efficiency by testing firefly luciferase expression (see Note 24) or checking the fluorescence emission in the presence of a reporter plasmid.
2. Gently resuspend the protoplasts by pipetting up and down with a wide-orifice tip, and combine the transformations together in a 15 mL round-bottom tube. Adjust the volume with PCA if it is lower than the expected (1.6 mL x number of combined transformations).
3. Distribute 1 mL of protoplasts per treatment in a 96-deepwell plate following the final order of your measuring plate (see Fig. 2). One well is enough for 6 technical replicates for one treatment condition (this includes the determination of firefly and renilla luciferase activities).
4. In a separate 96-deepwell plate, prepare the serial dilution of the chemical treatment in PCA. The concentration of the solution in the 96-deepwell plate should be 11x more concentrated that the tested concentration (see Note 25). In our case, we prepared the following GR24 concentrations by serial dilution: 0M, 11 pM, 110 pM, 1.1 nM, 11 nM and 110 nM. We recommend to organize the chemical dilution in the 96-deepwell plate similarly to the final experiment (see Fig. 2). Store it at 4 °C if not directly used.
5. Apply 100 µL of each GR24 concentration to the 1 mL protoplast suspension using a multistep multichannel pipet. Mix well by gently pipetting up and down. Cover the 96-deepwell plate with a dark lid, wrap it in aluminum foil and incubate it for ~100 min into the growth chamber at 21 °C.
6. Gently mix the induced protoplast suspension with the multichannel pipette and transfer 80 µl (25,000 protoplasts) into two identical Costar® 96-well flat-bottom white plates, including 4–6 replicates for each condition (see Note 26). In our case, only 36 wells per measuring plate were used for each substrate (see Fig. 2).
7. Dim ambient lights before applying the luciferase substrates. Using a multistep pipette, supply 20 µL of Firefly substrate (thawed beforehand and kept on ice) to

each well in the first white plate (measurement plate). For renilla substrate, pipet 1.5 mL cold PBS directly in the aliquot, resuspend and supply 20 µL of diluted renilla substrate to each well in the second white plate.

8. Run the luminescence determination for both plates in parallel, using two plate readers if necessary (dependent on sample number). Use a kinetics measurement to track the evolution of luminescence over 20 min at 480 nm for firefly and 570 nm for renilla (see Note 27).

3.4 Data Processing

The data from both plate readers are exported in .csv format. Here, we give guidelines to for data processing using the experiment with *rac*-GR24 treatment (0 M, 1 pM, 10 pM, 100 pM, 1 nM and 10 nM).

1. Plot the different luminescence values of each well over time. Firefly and renilla luminescence signals reach a luminescence plateau at different time points after substrate application: Firefly has constant RLU values in each individual well starting, in this case, from 8:17 min while renilla values plateau at 2:44 min (or 164 s, *see* Fig. 3a) (see Note 29).
2. Select the time frame where luminescence values are constant over time (yellow rectangle in Fig. 3a) for firefly and renilla measurement.
3. Average each RLU value over the selected time frame for each well of the measuring plate. We expect renilla signal to be constant throughout the GR24 concentrations as it is an internal standard for cell expression level. On the other hand, the firefly signal should decrease (inversely proportional to GR24 concentration increase), as the luciferase enzyme is fused to AtSMXL6, which is degraded in presence of SL.
4. Divide the firefly RLU by the renilla RLU for each well to obtain the firefly/renilla ratio and identify the outliers for each treatment (see Fig. 3b) [20].
5. Remove the outliers (see Fig. 3c) (see Note 30).

6. Normalize the signal ratio Fluc/Rluc to the 0 M GR24 value (see Fig. 3d). Statistical analysis, ordinary one-way ANOVAs and multiple comparisons, are performed with GraphPad Prism 8 for Mac OS version 8.3.0

4. Notes

1. Make sure to add the Gamborg B5 Vit Mix when the media is cooled down, reaching a milder temperature, i.e. when placing the inner part of the wrist on the bottle, you should not experience a burning but a warm feeling (ca. 30 °C).
2. Take special care, as the enzyme extracts must not be inhaled. We recommend dissolving the enzyme extracts under the fume hood by adding 10 mL MMC (prewarmed at 37 °C to facilitate the dissolution) directly in each bottle. Shake and pour into a beaker used for enzyme stock solution preparation. Rinse the bottles repeatedly with MMC, making sure to respect the 20 mL MMC final volume. Fill beaker to 200 mL with H_2O. The solution should be turbid and become clear brown after filtration. We recommend to use the stated brand to ensure the same digestion efficiency for the recommended plant material amount. If one resorts in using a different brand, an optimization step is necessary to adapt the quantities of macerozyme and cellulase.
3. Pay attention, the filter might clog. In this case, finish the filtration of the last 20 mL by hand with a syringe and a 22 µm filter.
4. Add the different solutions under the fume hood, as they should be sterile. We recommend preparing the PEG solution and incubating it prior to starting the transformation experiment. Vortexing for 10-15 s every 5 min can help to complete dissolving.
5. Wide-orifice pipette tips can be purchased or hand-made. For the latter, cut off the first 3 mm of the tip with scissors and autoclave them.
6. Be careful and fast, this preparation should be performed as quickly as possible, as luciferin is sensitive to light, oxygen, and high temperature. We recommend

purchasing small quantities of acetyl-CoA (50 mg for 200 mL firefly luciferase substrate), so that the content of the entire vial can then be used at once, avoiding loss of this expensive compound by a repeated weighing process.

7. Tricine, DTT, ATP, and acetyl-CoA stock solutions have to be freshly prepared. The other solutions can be made in advance.
8. Prevent coelenterazine degradation by pre-freezing 125 black Eppendorf tubes with the lid open at -80 °C in a closed Eppendorf tube rack with lid.
9. Spread the seeds on the tube wall with a pipette tip to facilitate ethanol evaporation.
10. We recommend two layers of Parafilm well-stretched and properly pressed against the plate, as the parafilm as a tendency to break after a week due to the growth chamber conditions. Improperly sealed plates will lead to varying growth conditions between plates and negatively impact seedling quality for protoplast extraction. Observe the seedlings over the incubation time and reseal any plates with additional parafilm strips, where the parafilm becomes porous. Seedlings receiving more oxygen through improper sealing will appear darker green than those in well-sealed plates and SCA culture media will show visual signs of drying.
11. The transfer of the cut leaf material into a new plate prevents the cells from getting stuck into the cutting marks imprinted on the cutting plate. It can be done by gently pushing and scrapping the material from the cutting plate to the new Petri dish.
12. The addition of a larger volume of MMC to digested plant material-extracted protoplasts facilitates pellet formation upon centrifugation.
13. Maximize the protoplast extraction by centrifuging the supernatant a second time, 20 min at 100 × *g* without brake. Then combine the resulting pellet with the pellet obtained from the previous centrifugation.
14. We recommend resuspending the pellet by gently rocking the tube horizontally. The liquid needs to wash over the pellet until complete resuspension. Slowly pipetting up and down with a wide-orifice tip also works.
15. Wet the wall of the tube by gently tilting and rolling the tube containing the protoplasts in MSC. It will facilitate the 2 layers formation. Add the 3M solution

using the tip-in-tip technique i.e. place a 10 µl tip on a 100-1000 µl tip attached to the pipette. Bend the extremity of the 10 µl tip against the tube wall and slowly pipet out 3M. A 3M layer, transparent color, should form on top of the green solution of protoplasts in MSC.

16. Make sure to have more W5 than 3M in the collection tube, to ensure the correct sedimentation of the protoplasts.
17. Use that time to also verify the quality of the protoplast isolation. Protoplasts are usually easily visible at x40 magnification with a microscope. They should appear as rounded cells with green colored organelles that are the chloroplasts. If a cell is not fully round, it will burst in the next minutes. When burst, the chloroplasts are released in the media, that can be a clue for the quality of the extraction.
18. 15-30 µg DNA account for the total amount of DNA to be transformed. In the case of more than one plasmid, each DNA plasmid must be added in the same amount, conserving the 15-30 µg total DNA. DNA should be prepared using midiprep or maxiprep kits (eluted in sterile ddH_2O) to ensure high concentration and quality of the DNA preparations. We recommend checking its quality by agarose gel electrophoresis, by checking RNA content at the bottom of the running lane or potential plasmid degradation by the presence of extra DNA bands.
19. The transfection of a plasmid containing a reporter gene can be useful later on to check rapidly the transformation efficacy (see Subheading 3.3, step 1).
20. Protoplasts precipitate quite rapidly. Make sure to resuspend the cells every few minutes, by gently pipetting up and down using wide-orifice tips. This will ensure consistent cell concentration throughout the pipetting of the different transformations.
21. We recommend using a 20-200 µL and a 100-1000 µL pipettes simultaneously, one in each hand, set on 120 µL for 3M and 600 µL for PCA that will be used twice in a row to deliver 1.2 mL PCA to the well. If it is too complex, sequential addition also works.

22. In the case of the co-transformation of StrigoQuant with one or more plasmids, the control condition (meaning StrigoQuant alone) requires the co-transformation of a stuffer. This stuffer plasmid, usually p35S-driven YFP expression cassette, allows reflecting the exogenous transcription and translation demand that would come from 2 or more co-expressed plasmids.
23. Successfully transformed and healthy protoplasts tend to aggregate in groups of 3 or more plant cells.
24. Use a wide-orifice tip to place 80 µL protoplasts into a measuring 96-well plate. Under dimmed ambient light conditions, add 20 µL firefly substrate and read the maximum emission signal at 480 nm after 10-15 min incubation.
25. 100 µL chemical diluted in PCA will then be added to the 1 mL protoplast-containing 96-deepwell plate described in the previous step (3.3 step 3). The applied chemical solution must be 11x concentrated prior induction, to be at a 1x concentration in the protoplast solution.
26. Each 96-well flat-bottom white plate will be used for either firefly or renilla luminescence measurement. We insist here on the necessity to create two identical plates, as it will facilitate the data processing post measurement. In the case of large experiments, different conditions lead to a high number of measurement plates. Once the samples are induced, protoplasts can directly be transferred to the different white plates and covered to avoid light exposure and media evaporation. They are then ready for measurement and can be taken out of the growth chamber 2 min before the measuring time.
27. For larger experiments, when several plates have to be measured, do not induce all the samples at the same time. Make sure that the time during the induction and the measurement is consistent throughout the measuring plates. Thus, we recommend inducing two plates (for firefly and renilla) every 25-30 min, which should give one enough time for measurement (~20 min) and substrate pipetting.

28. Pipetting the protoplasts in one plate first and then in the other can create a variation of protoplast concentration between the two plates: one plate will most probably contain more protoplasts than the other.
29. Pipet the substrate and start the measurement quickly, especially for renilla, to fully record the negative exponential curve. It is important to collect data during the increase of luminescence before it reaches the plateau, to have more accurate values.
30. The luminescence measurement is delicate, and several variations can be introduced along the process. We recommend extra caution and careful resuspension of the plant cells during the protoplasts pipetting into the 96-deepwell plate, their transfer to the measuring plates after induction, and the fast pipetting of substrate prior luminescence measurement. Due to these 3 critical steps, we encourage doing 6 replicates for each luciferase measurement when possible.

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Figures

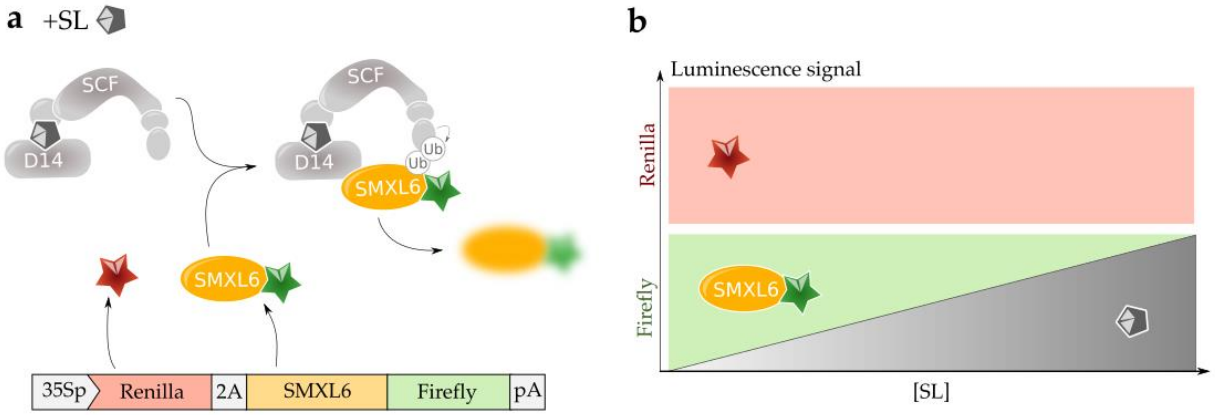

Figure 1: **Schematic diagram of StrigoQuant function in Arabidopsis.** (**a**) StrigoQuant expression is driven by the Cauliflower mosaic virus 35S promoter (35Sp), ensuring a constitutive expression of the sensor. It is composed of two parts -- (1) renilla luciferase (red star) and (2) *Arabidopsis thaliana* SMXL6 fused to firefly luciferase (green star) – linked by the self-cleavable 2A peptide. This design allows stoichiometric expression of the two modules, enabling the use of renilla luminescence as a normalization factor. The sensing part, which includes SMXL6, is degraded in the presence of strigolactone (SL) after its interaction with Dwarf14 (D14) and the Skp1-Cullin-F-box complex (SCF). (**b**) Expected output luminescence signals measured with a spectrophotometer. The renilla

signal is constant, as this module part is insensitive to SL. However, SMXL6 degradation and the firefly signal decrease are proportional to cellular SL concentration. Abbreviations: poly-A (pA), ubiquitin (Ubi).

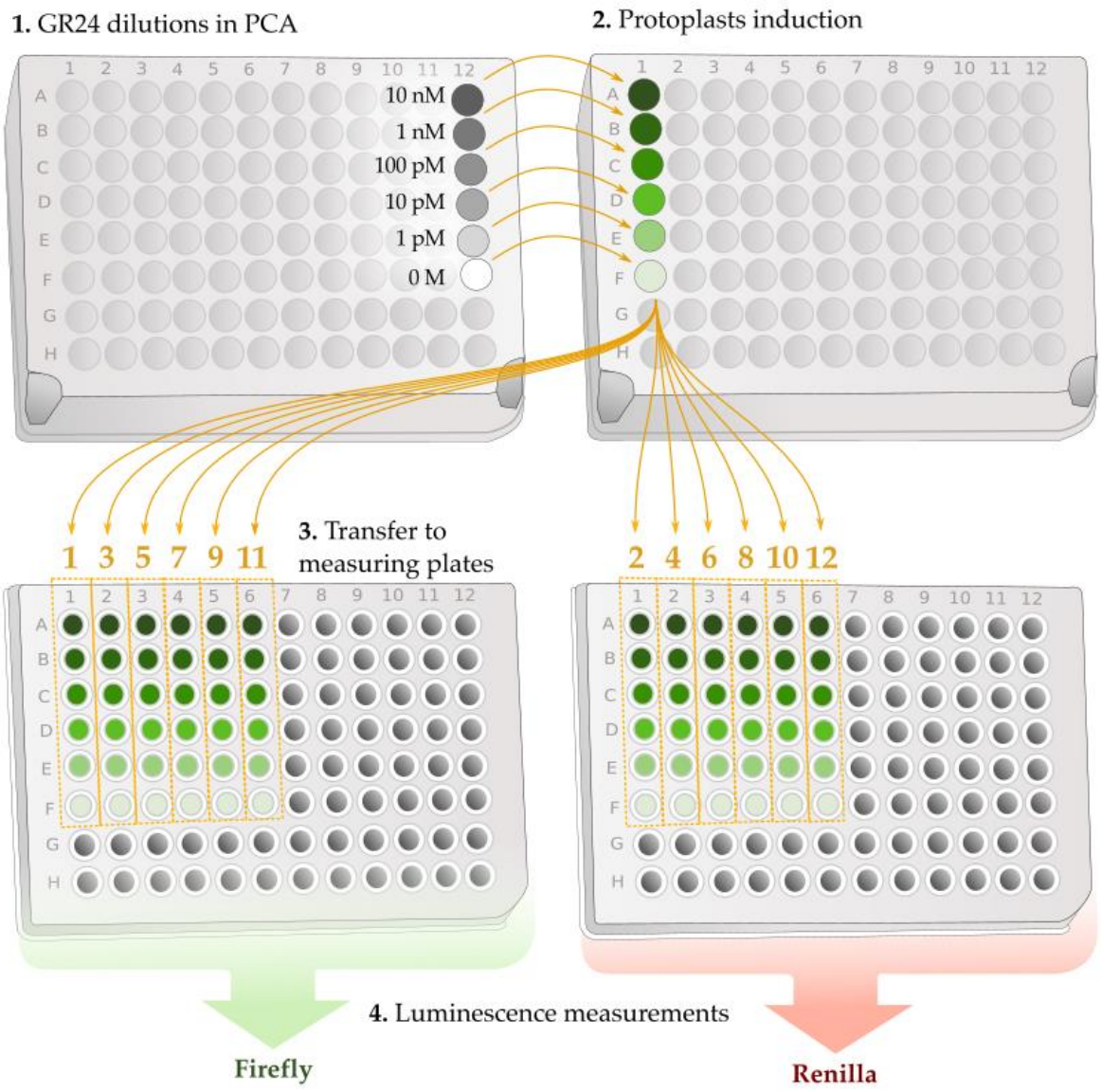

Figure 2: Scheme of the **Experimental set-up for protoplast induction and preparation of the measuring plate.** The figure shows the experimental workflow for the induction of sensor degradation with the strigolactone analog *rac*-GR24. (**1**) Prepare the different GR24 dilutions (0 M, 1 pM, 10 pM, 100 pM, 1 nM and 10 nM) and pipet ≥ 150 µL in a 96-deepwell plate. Cover the plate and keep it at 4 °C until the induction time. (**2**)

Transfer 1 mL of the Arabidopsis protoplasts, transformed 18 h before with StrigoQuant, into a different 96-deepwell plate. Induce the protoplasts with 100 µL of the GR24 dilutions. We recommend using a multichannel pipette with wide-orifice tips for the induction step. Mix the protoplasts gently with the applied chemical by pipetting up and down. Cover the induced plate and place it back at 22 °C. (**3**) After 2 h of induction, transfer 80 µL protoplasts per replicate to two measuring plates, for a total of 6 replicates per condition per plate. Use a multichannel pipette with wide-orifice tips and make sure to frequently resuspend the protoplast suspension, as they rapidly aggregate at the bottom of the well. We recommend following our pipetting scheme as well as the order of pipetting (indicated from 1 to 12), to ensure consistency between plates (see Note 30). (**4**) Add 20 µL of substrates for each luciferase and measure the luminescence signal for both firefly and renilla.

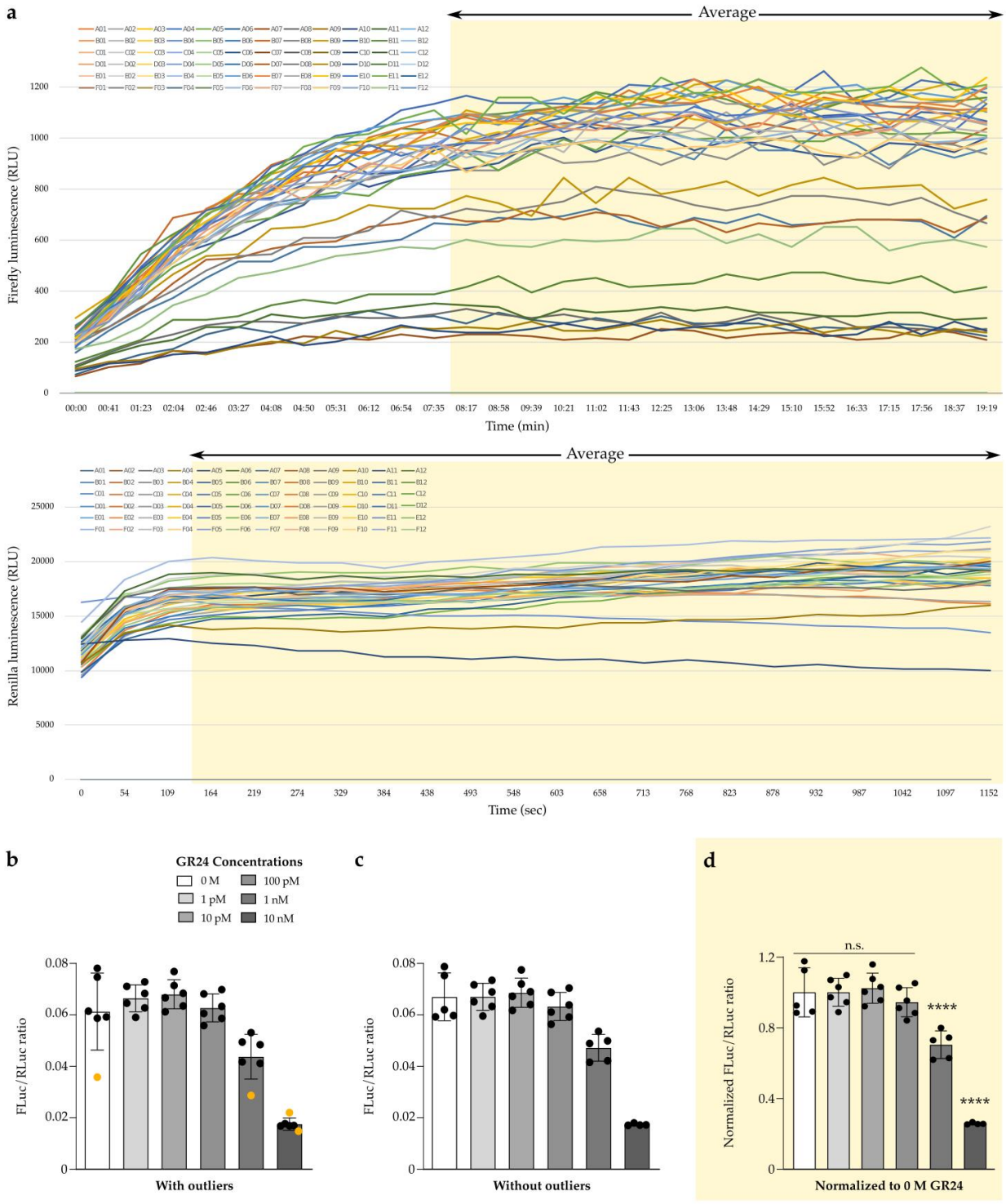

Figure 3: **Raw data curation of luciferase-based luminescence assays after 2 hour-induction with *rac*-GR24.** (**a**) Representation of the luminescence signal of each well measured every 0.46 s for 20 min, for both measuring plates: firefly (up) and renilla

(down). Exported data from the plate reader corresponding to the quantified luminescence values for each measuring plate (every 0.46 s for 20 min) were represented. A time frame is selected (represented in yellow), where all the curves are reaching a plateau (this time can be different between the two measuring plates) and calculated the average luminescence value for each well of each plate over this time period. (**b**) Representation of the average ratio of the luminescence signal for each treatment conditions. The ratio for each measuring well was calculated by dividing the firefly luminescence value by its equivalent in the renilla plate (A1 FLuc/A1 RLuc). The luminescence values for each GR24 concentration correspond to the average of the 6 replicates. Outliers were then identified following the calculation of Jacobs et al. [20] (represented as orange dots). (**c**) Representation of mean FLuc/RLuc ratio for each GR24 treatment after data curation (outlier exclusion). (**d**) Representation of the normalized average FLuc/RLuc ratio for each GR24 treatment after outlier exclusion. The statistical significance is determined by one-way ANOVA and Dunnett's multiple comparison test, $n \geq 4$ (**$P < 0.01$, ***$P < 0.001$, ****$P \leq 0.0001$, n.s. not significant). The ratio represented is normalized to the control condition (0 M GR24). (b-d) Means are depicted as bars with error bars that indicate standard error.