**Exploiting the Gal4/UAS system as plant orthogonal molecular toolbox to control reporter expression in Arabidopsis protoplasts.**

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**Abstract**

The ability of protein domains to fold independently from the rest of the polypeptide is the principle governing the generation of fusion proteins with customized functions. A clear example is the split transcription factor system based on the yeast GAL4 protein and its cognate UAS enhancer. The rare occurrence of the UAS element in the transcriptionally sensitive regions of the Arabidopsis genome makes this transcription factor an ideal orthogonal platform to control reporter induction. Moreover, heterodimeric transcriptional complexes can be generated by exploiting post-translational modifications hampering or promoting the interaction between GAL4-fused transcriptional partners, whenever this leads to the reconstitution of a fully functional GAL4 factor.

The assembly of multiple engineered proteins into a synthetic transcriptional complex requires preliminary testing, before its components can be stably introduced into the plant genome. Mesophyll protoplast transformation represents a fast and reliable technique to test and optimize synthetic regulatory modules. Remarkable properties are the possibility to transform different combinations of plasmids (co-transformation) and the physiological resemblance of these isolated cells with the original tissue.

Here we describe an extensive protocol to produce and exploit Arabidopsis mesophyll protoplasts to investigate the transcriptional output of GAL4/UAS-based complexes that are sensitive to post-translational protein modifications.

**Key words**: Arabidopsis mesophyll protoplasts, GAL4, regulatory complex, Maxiprep

## 1 INTRODUCTION

Transcription factors (TFs) are defined as proteins that are able to affect the expression of genes by binding to their promoter regions [1]. They generally contain a characteristic domain conferring selective DNA binding properties and a domain that, acting independently from the previous one, mediates or prevents the recruitment of the transcriptional apparatus. Moreover, most TFs contain regulatory regions subjected to post-translational modifications mediating their stability, activity and interaction with transcriptional partners. Notably, such domains can be separated from the full proteins without losing their structure and function. Thanks to these properties, DNA binding domains (DBD), transcriptional activation domains (AD) and even regulatory domains coming from different sources can be mixed to generate novel chimeric TFs with customized functions and regulatory properties [2]. The GAL4/UAS system [3, 4] represents a clear paradigm of such principle: GAL4 is a modular yeast TF [5] whose DBD and AD have both been fully characterized [6], as well as the DNA sequence recognized by its DBD on *cis*-acting promoter regions [7]. According to this knowledge, a GAL4/UAS system has been originally developed to investigate protein-protein interactions and identify the responsible domains [8]. Adapted variants have been further exploited for gene activation-tagging in Drosophila [9], zebrafish [10] and Arabidopsis [11]. Apart from these applications, GAL4/UAS represents a powerful tool to control target gene expression. In plants, such goal is facilitated by the absence of detectable off-target effects on transcription by ectopically expressed GAL4 transcriptional complexes, as ascertained in Arabidopsis plants stably transformed with  [12]. This qualifies GAL4/UAS as an orthogonal reporter expression system for plants.

Any couple of interacting peptides respectively equipped with the GAL4-DBD and GAL4-AD (and conveniently targeted to the nucleus) can in principle generate a heterodimeric transcription factor that will induce the expression of a target gene driven by a UAS-containing promoter. This

constitutive mechanism, however, is amenable to regulation and can be further turned into a variety of switchable configurations. Among other possibilities, the existence (or imposition) of inducible post-translational modifications on one partner can be exploited to achieve conditional activation or inactivation of the GAL4/UAS system, depending on their positive or negative impact on the interaction between the GAL4 fused proteins (**Fig.1**).

When theorized, a regulatory module of this kind needs to be quickly tested and verified. For plant biology, many transient transformation systems are available. Naked cells (protoplasts) isolated from leaf mesophyll have become some classical material for high throughput evaluation of gene constructs in plant cells. Indeed, in Arabidopsis research, mesophyll protoplasts provide important advantages that make them suitable for routine screening of specific effector proteins and entire synthetic modules: transient experiments can be carried out in non-sterile conditions [13, 14], with low-input technologies, and cells can be isolated from multiple genotypes for comparative studies. Most importantly, the isolated protoplasts overall retain most leaf physiological responses, thereby providing a valuable tool to monitor molecular regulatory mechanisms under different biotic [15, 16] and abiotic stresses [17–19].

The preparation of good quality, high yield plasmid DNA is a sensitive, though sometimes underestimated, aspect for success when testing synthetic molecular pathways in Arabidopsis protoplasts. Several commercial kits facilitate plasmid DNA purification from large cultured (also indicated as maxi-preparation). However, for routine tests, cheaper extraction protocols conjugating speed and reliability are desirable. A kit-free maxi-preparation protocol in use in our laboratory enables the recovery of 200-400 μg plasmid DNA from 100 ml bacterial culture (scalable to larger volumes) in roughly 4 hours, with a quality that is adequate to protoplasts transformation. Our method is adapted from the one presented by Sambrook in 2001, [20], based on sequential steps of Alkaline Lysis, Isopropanol and PEG precipitation, phenol-chloroform purification and finally ethanol precipitation.

In this chapter, we present a detailed protocol covering the main steps required for the transient test of orthogonal regulatory modules in Arabidopsis protoplasts. We will focus on the generation and maxi-preparation of GAL-DBD and GAL4-AD fused effector plasmids, on protoplasts isolation and on their transformation.

## 2 MATERIALS

### 2.1 REPORTER AND EFFECTOR PLASMIDS

1. Reporter plasmid 4xUAS:FLuc pGreenII 0800-LUC [12, 21] (*see* **Note 1**).
2. Gateway destination plasmid p35S-GAD-GW [22] for effector modules.
3. Gateway destination plasmid p35S-GBD-GW [22] for effector modules.
4. Gateway destination plasmid p2gw7 [23] for the normalization module.
5. Gateway LR ClonaseII Mix (Thermo-Fisher Scientific).
6. *E. coli* Mach1 competent cells (*see* **Note 2**).
7. Controlled temperature orbital shaker.

### 2.2 MAXI-PREPARATION OF PLASMID DNA

#### 2.2.1 Equipment and materials

1. Autoclaved 300 ml Erlenmeyer flasks.
2. 50 ml Falcon tubes.
3. 2 ml Eppendorf tubes.
4. Refrigerated centrifuge with interchangeable rotor for 50 ml tubes and 2 ml vials tubes.

#### 2.2.2 Solutions and reagents

1. Lysogeny broth (LB) medium: 10 g $l^{-1}$ triptone, 10 g $l^{-1}$ NaCl, 5 g $l^{-1}$ yeast extract. Autoclave for 15 minutes at 120°C and 15 psi.
2. Solution I: 50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA pH 8. Autoclave for 15 minutes at 120°C and 15 psi. Store the solution at 4°C.

3. Solution II: 200 mM NaOH, 1% SDS. Prepare fresh.
4. Solution III: 3M KOAc, 5.75 ml glacial acetic acid (Sigma-Aldrich 33209), $H_2O$ up to 50 ml.
5. Isopropanol.
6. TE buffer: 10 mM Tris-HCl pH 8, 1 mM EDTA.
7. RNaseA solution, 20 mg $ml^{-1}$ (Sigma Aldrich R6148).
8. Polyethylene glycol (PEG) solution: 13% PEG 8000 (w/v) (Fluka 81272) dissolved in 1.6 M NaCl. Prepare fresh.
9. Phenol:chloroform:isoamyl alcohol = 25:24:1 mixture pH 7.7-8.3 (Sigma-Aldrich 77617). Store at 4°C.
10. Chloroform (Sigma-Aldrich 34854).
11. 10 M ammonium acetate (Sigma-Aldrich A1542). Store at room temperature.
12. Absolute ethanol.

## 2.3 PROTOPLAST ISOLATION AND TRANSFORMATION

### 2.2.1 Equipment and materials

1. Paper tape.
2. Scissors.
3. Three to four-week-old *Arabidopsis thaliana* plants (*see* **Note 3**).
4. Centrifuge with interchangeable rotor for 50 ml tubes and 2 ml vials tubes.
5. 80 μm nylon mesh (optional).
6. Six-well plates (*see* **Note 4**).

### 2.3.2 Reagents and solutions

Prepare all solutions fresh starting from appropriate, filter sterilized stock solutions. Use bi-distilled water. For protoplast incubation up to 48 hour long, strictly sterile working conditions do not need to be maintained, but become necessary for more prolonged incubation time.

1. Enzymatic solution: 20 mM MES pH 5.7, 0.4 M mannitol, 20 mM KCl, 1.5% Cellulase R-10 (w/v) (Duchefa, from *Trichoderma viride* ca. 1 U/mg), 0.4% Macerozyme R-10 (w/v) (Duchefa da *Rhizopus* sp.), 10 mM $CaCl_2$, 0.1% bovine serum albumin (w/v) (BSA, A9576, Sigma-Aldrich).
2. W5 solution: 2 mM MES pH 5.7, 154mM NaCl, 125mM $CaCl_2$, 5mM KCl.
3. PEG 4000 solution: 40% (w/v) PEG 4000 (#81240, Fluka), 200 mM mannitol, 100 mM $CaCl_2$.
4. MMG solution: 4 mM MES pH 5.7, 0.4 M mannitol, 15 mM $MgCl_2$.
5. WI solution: 4 mM MES pH 5.7, 0.5 M mannitol, 20 mM KCl, 10 mM glucose.

### 2.4 DUAL LUCIFERASE REPORTER ASSAY (*see* Note 1)

#### 2.2.1 Equipment and materials

Firefly and Renilla luciferase activities are measured using the Dual-luciferase® Reporter Assay System (Promega).

1. Luminometer (Lumat LB 9507, Berthold Technologies, or equivalent).
2. Polystyrene test tubes, 12x75 mm.

## 3 METHODS

### 3.1 CLONING OF REPORTER AND EFFECTOR PLASMIDS

For routine cloning we use the directional Gateway cloning system. To this purpose, both promoter and coding sequences are firstly cloned into pENTR/D-TOPO® vector (Thermo Fisher Scientific) and subsequently recombined into the appropriate destination vector. Moreover, mammalian or prokaryotic protein domains are usually expressed in Arabidopsis after codon optimization of the coding sequences, which is preferentially achieved through direct synthesis (see below).

#### 3.1.1 Cloning of chimeric effector modules into Gateway™ entry vectors

1. Design the synthetic coding sequences by selection of the protein domains of interest (*see* **Note 5**).
2. Use online tools to optimize the codon sequence (*see* **Note 6**). Add a 5'-CACC extension to satisfy the requirements for directional cloning in the pENTR/D-TOPO® vector [24].
3. Perform the cloning reaction into the pENTR/D-TOPO® vector, following the manufacturer's recommendations.
8. Transform *E. coli* Mach1$^{TM}$ (Thermo Fisher Scientific) competent cells.
4. Grow transformed cells on LB plates with the appropriate antibiotic (kanamycin).
5. Inoculate three to four colonies in 5 ml liquid LB medium supplemented with kanamycin.
6. Perform plasmid DNA purification at the mini-prep scale, following a manual extraction protocol or a commercial kit of choice.
7. Check the entry vectors by enzymatic restriction and Sanger sequencing.

### 3.1.2 Generation of expression vectors for Arabidopsis protoplast transformation

1. Select the proper destination vectors, in light of the best expression level of each protein module in protoplasts. Plant compatible Gateway destination vectors have been for instance developed by Karimi et al. [25, 26] and Grefen et al. [27]. To improve and equalize the co-transfection efficiency of separate protein-coding effector modules, it is recommended to choose destination vectors with small backbones: non binary plasmids are generally the preferable option, due to their minimum size.
2. Promote the recombination reaction between the att sites present on the entry (attL sites) and destination vector (attR sites) using the Gateway LR Clonase II Mix (Thermo-Fisher Scientific). Combine 75 ng of entry and 75 ng of destination vector in a 5 µl reaction and follow the manufacturer's recommendations.
3. Incubate the mixture for 1 h at room temperature.
4. Transform *E. coli* Mach1$^{TM}$ (Thermo Fisher Scientific) competent cells.
5. Select the transformed cells on LB plates containing the appropriate antibiotic.

6. Inoculate two colonies in 5 ml liquid LB medium supplemented with the selective agent.
7. Perform plasmid DNA purification at the mini-prep scale.
8. Check the plasmids by enzymatic restriction.
9. Store the positive clones in the form of bacterial glycerol stocks, at -80°C. If correctly maintained, stocks can be repeatedly inoculated into mid- or large-scale cultures, speeding up the extraction process of large plasmid amounts needed for protoplast experiments.

## 3.2 MAXI-PREPARATION OF HIGH-QUALITY PLASMID DNA FOR PROTOPLAST TRANSFORMATION

1. By scraping the frozen bacterial stock, inoculate the cells carrying the plasmid of interest into 100 ml liquid LB medium, containing the appropriate antibiotic, dispensed in a 300 ml Erlenmeyer flask.
1. Grow the culture over-night, at 37°C and 180 rpm shaking.
2. Harvest cells by centrifugation at 5000 g for 10 minutes in 50 ml tubes.
3. Resuspend cell pellets in 2 ml ice-cold Solution I.
4. Add 4 ml of freshly made, room temperature Solution II, and mix by inverting four times.
5. Incubate on ice for 5 minutes.
6. Add 3 ml Solution III and mix by inversion. Do not vortex.
7. Incubate on ice for 5 minutes.
8. Centrifuge at maximum speed for 10 minutes (*see* **Note 7**).
9. Transfer the supernatant into a new 50 ml tube.
10. Add an equal amount of isopropanol, mix and incubate at room temperature for 5 minutes.
11. Centrifuge at maximum speed for 15 minutes at 4°C.
12. Remove the supernatant and let the pellet dry (*see* **Note 8**).
13. Resuspend the pellet with 500 µl TE buffer and transfer the solution into a clean 2 ml vial.
14. Add 2.5 µl RnaseA solution, mix and incubate at 37°C for 30 minutes.
15. Add 500 µl PEG 8000 solution and mix thoroughly to homogenize the solutions.

16. Incubate the mixture on ice for 15 minutes.
17. Centrifuge at top speed for 15 minutes at 4°C.
18. Remove the supernatant and resuspend the pellet in 400 µl nuclease-free water.
19. Add 400 µl phenol:chloroform:isoamyl alcohol solution and vortex for 5 seconds.
20. Centrifuge at maximum speed for 2 minutes at 4°C.
21. Move the aqueous phase into a new 2 ml vial.
22. Add 400 µl chloroform and vortex for 5 seconds.
23. Centrifuge at maximum speed for 2 minutes at 4°C.
24. Move the aqueous phase into a new 2 ml vial.
25. Add 100 µl 10M ammonium acetate and mix.
26. Add a double volume of ice-cold absolute ethanol and mix by inversion several times.
27. Incubate at room temperature for 10 minutes.
28. Centrifuge at maximum speed for 10 minutes at 4°C.
29. Remove the supernatant and let the pellet dry.
30. Resuspend the pellet in 100 µl nuclease-free water.
31. Measure the DNA concentration and check the plasmid by restriction.

### 3.3 PROTOPLAST ISOLATION AND TRANSFORMATION

The protocol described here is suited for a maximum incubation time of 48 hours before cell collection. If longer time is used, all steps should be performed under sterile conditions and all solutions should be filter-sterilized before use.

1. Prepare fresh enzymatic solution (*see* **Note 9**), adding MES, mannitol, KCl, cellulase and macerozyme and adjusting the volume with bi-distilled water. Heat the solution at 60°C for 5 minutes to solubilize the enzymes completely. After cooling down the solution to room temperature, add $CaCl_2$ and BSA. Pour the enzymatic solution into a petri dish.
2. With scissors, cut small portions of paper tape.

3. Detach one leaf from Arabidopsis plants choosing among fully expanded leaves starting from the second pair (*see* **Notes 3 and 9**).
4. Place the leaf between two pieces of paper tape and remove the lower epidermal layer by slowly pulling away the tape portion contacting it.
5. Remove the extra portion of paper tape and immerse the leaf in the enzymatic solution with the exposed mesophyll facing downwards, making sure that it fully contacts the solution.
6. Repeat steps 4 and 5 until the petri dish is fully covered by leaves (*see* **Note 9**).
7. Cover the petri dish containing the enzymatic solutions and pealed leaves with aluminum foil and incubate at 23°C for 2 to 4 hours.
8. Swirl the petri dish to release protoplasts.
9. Wash the 80 μm Nylon Mesh with W5 solution.
10. Harvest protoplasts in a 50ml Falcon tube by filter the solution using the 80 μm Nylon Mesh.
11. Harvest the remaining protoplasts by adding one volume of W5 inside the petri dish equivalent to that of the enzymatic solution used and repeat step 10.
12. Centrifuge the Falcon tube containing the protoplasts at 100g for 1 minute.
13. Remove supernatant with a pipette and resuspend the protoplasts in W5.
14. Cover the falcon with aluminum foil and wait until all protoplasts sediment.
15. Remove supernatant with a pipette.
16. Add MMG solution until reaching a protoplast density of $5 \times 10^5$ cells $ml^{-1}$.
17. Pipette 3 μg of each plasmid into a 2ml Eppendorf tube (*see* **Note 10**).
18. Add 100 μl of protoplasts inside the 2ml Eppendorf tube containing the combination of plasmids.
19. Mix the protoplasts/plasmid mixture gently (*see* **Note 11**).
20. Add 110 μl of PEG 4000 40% and mix gently until the mixture is properly homogenized (*see* **Note 11**).

21. Incubate the transformed protoplasts in the dark for 20 minutes.

22. Add 440 µl of W5 and mix gently by inversion.

23. Centrifuge the protoplasts at 100g for 1 minute.

24. Remove supernatant and resuspend with 1 ml of WI solution.

25. Transfer the transformed protoplasts in 6 wells plates.

26. Incubate the protoplasts in dark at 23°C (Note 12).

### 3.4 DUAL LUCIFERASE ASSAY ACTIVITY

1. Harvest protoplasts into 2ml Eppendorf tubes using a pipette.
2. Centrifuge protoplasts at 750g for 2 minutes.
3. Remove supernatant and freeze the protoplasts in liquid nitrogen (Nota).
4. Prepare reagents working solution as specified by manufacturer instructions.
5. Measure FLuc and RLuc protein activity using the luminometer.
6. Plot FLuc/Ren ratios as Fluc activity relative to reporter plasmid control (Fig.1).

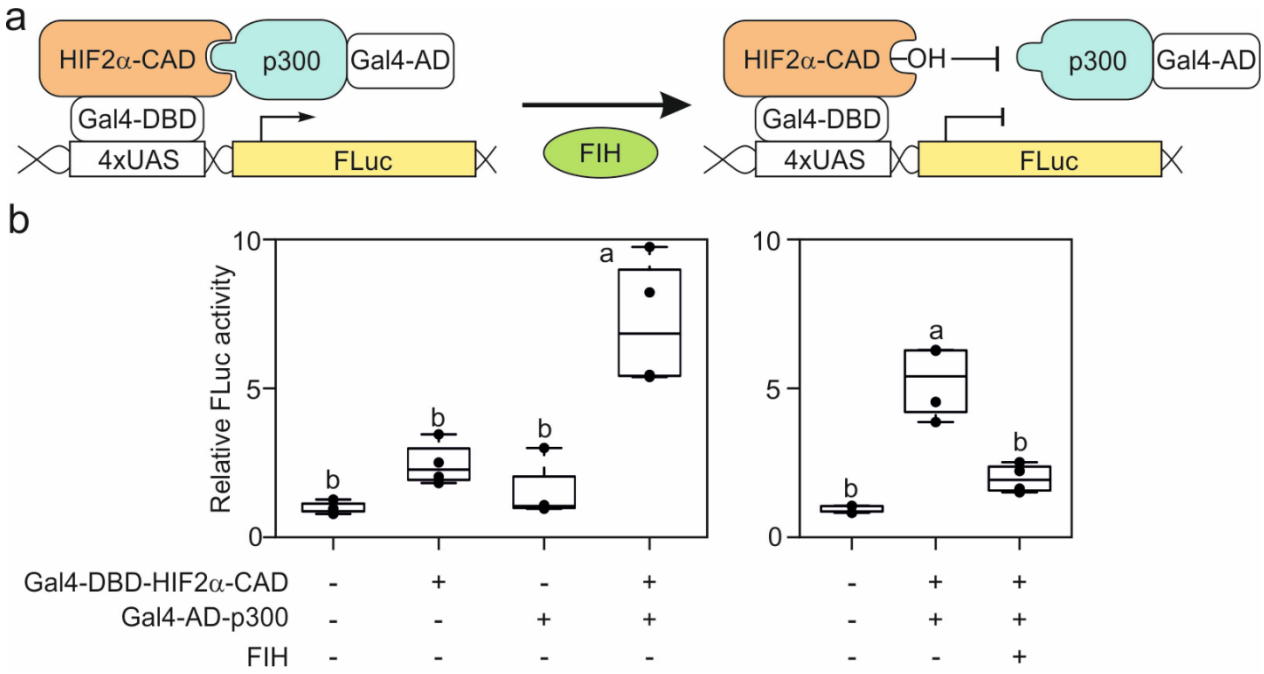

**Fig. 1 Example of synthetic regulatory modules based on the GAL4/UAS system**. **(a)** The C-terminal activation domain of HIF2α protein isolated from *Mus musculus* (aa 774-874) is fused with Gal4-DBD. The interaction with the TAZ type 1 domain of the human p300 protein (aa 300-528), in turn fused to Gal4-AD, triggers the formation of a heterodimeric transcription factor able to induce the expression of reporter genes located downstream of a promoter containing 4 repeats of the UAS sequence. Hydroxylation of Asparagine 851 by FIH hydroxylases hinders the interaction. **(b)** Comparison of FLuc activity among protoplasts transformed with different combinations of effector plasmids depicted in **(a)**. The data, relative to the subgroup of protoplasts transformed only with the reporter plasmid, report an increase of FLuc activity exclusively when both Gal4 fused proteins are co-transformed together, in the absence of FIH.

## 4 NOTES

1. One of the main aspects that operator should take care performing the protoplast transient transformation is its efficiency. It depends in good part on plant age and quality, protoplasts handlings and DNA quality [28]. The Dual Luciferase assay provides an optimal tool to monitor transformation efficiency and normalize the reporter values in each sample accordingly. The principle of the method is the possibility to distinguish the sequential light emission by two distinct luciferase enzymes that use different substrates. The *Photinus pyralis* luciferase (FLuc) is used as the preferential reporter to monitor UAS promoter activity, while the *Renilla reniformis* luciferase (RLuc) can be placed under the control of a constitutive 35S promoter [29]. Under the assumption that the 35S promoter is not regulated in the experimental conditions used, RLuc activity can be considered as proportional to the number of transfected protoplasts and therefore exploited to monitor transformation efficiency. The FLuc and RLuc transcriptional units can be either carried by independent plasmids, or incorporated in the same vector. Adopting the second strategy, in our lab we deploy a Gateway-compatible version of the pGREEN 0800-LUC vector [21], which

harbours a 35S:RLuc transcriptional cassette in its backbone. Fluc fusions of the investigated promoters, including UAS [12], can then be generated through att site recombination.

2. Mach-1 T1R cells (Thermo Fisher Scientific) are preferred to other *E. coli* strains for their fast growth, however DH10B, DH5α, TOP10$^{TM}$ and Stbl2 stains can be used without modifying the protocols described here. High efficiency home-made Mach-1 competent cells can be prepared using the Inoue protocol [30].
3. We obtain highest transformation efficiency using Arabidopsis plants at the end of their third week of growth. However, plants ranging from three to four weeks of age can be efficiently used as good sources of mesophyll protoplasts, provided that fully expanded and undamaged leaves are sampled.
4. For promoter investigation using the FLuc reporter, 5 x $10^4$ cells concentrated into 0,1 ml are generally sufficient to detection a luminescent signal. At the end of the protocol, such amount of cells is resuspended in 1 ml of WI solution. We use 6 well plates to easily handle many transformations at the same time. However, if more material is needed for other purposes, such as protein extraction and subsequent immunoblotting, or RNA extraction for gene expression measurements, larger suspension volumes and larger plates can be used.
5. The recent advances in *de novo* DNA synthesis have made it possible to customize almost any desired DNA sequence, independently from its length and complexity. When designing a coding sequence, it is essential to identify exclusively the domains needed to achieve the desired output. Protein domain selection should thereby be based on previously published papers and should avoid the introduction of additional regulatory domains that could interfere with the final output.
6. Different organisms use specific codons at higher frequency than other synonymous codons [31]. Therefore, when expressing heterologous proteins inside plants, a codon optimization step is appropriate to ensure efficient translation of the transgene in the host organism [32,

33]. Different software [34] and online tools platforms [35, 36] allow the optimization of the DNA sequence starting from the desired amino acid sequence.

7. Centrifugation at top speed for 10 minutes is usually enough for the complete pelleting of cell wall and membranes. However, if the soluble phase appears not to be clear at the end, additional centrifugation step is needed, following transfer of the soluble phase to a new tube. This step is crucial to ensure complete removal of the bacterial genomic DNA anchored to the membrane.
8. After centrifugation a white pellet is visible. The supernatant can be removed directly by inverting the tube. However, to speed up the drying process, an additional spin down will allow removal of the remaining of the solvent with a pipette tip. As soon as the pellet begins to dry, its color will shift from white to transparent.
9. Usually, enough protoplasts can be released and recovered from 10 ml enzyme solution for roughly 50 individual transfections, following the protocol outlined in Section 3.3, points 17-24.
10. When performing transactivation assays, it is necessary to introduce the proper controls in the experimental setup (Figure 1). These can be represented by single effector constructs used or unrelated proteins equipped with the GAL4-DBD and GAL4-AD. Four to six biological replicates are sufficient for this kind of transactivation experiments, given the low variability and high reproducibility associated to this experimental system.
11. A single protoplast transformation reaction can require from 10 to 15 seconds . In this frame, 30 reaction can be followed and handled together. If working with higher numbers, it is recommendable to split the tubes into different sets to be handled sequentially.
12. We wait a minimum of 12 hours prior to quantify reporter activity or to carry out any treatment. If the treatment should induce a reduction of reporter induction levels, additional 12 hours are required to detect any difference due to the relative long half-life of the FLuc reporter protein [12].

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