Title

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Yeast Two-Hybrid Approaches to Dissecting the Plant Defense Response

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Summary

We describe a reliable GAL4-based yeast two-hybrid system for identifying and isolating clones encoding proteins interacting with a protein of interest. This two-hybrid system gives extremely low background and few false-positive clones, making it ideal for library screening purposes. We have successfully used it not only to isolate *Arabidopsis* NPR1-interactors from rice but also to pull out the rice NPR1 ortholog using one of the interactors as bait.

Key Words: Yeast two-hybrid; GAL4; NPR1; defense.

1. Introduction

The yeast two-hybrid approach has been widely used for library screening to identify and isolate genes encoding proteins that interact with a favorite protein. It has the capability of identifying and isolating desired clones in a relatively short time by allowing one to easily screen through tens of millions of clones. There are many two-hybrid systems available. Unfortunately, many of them may not be reliable; unacceptable backgrounds and troublesome false-positive results often are seen with many two-hybrid systems. Here, we describe a reliable GAL4-based yeast two-hybrid system that includes a bait plasmid vector derived from plasmid pPC86 ***(1)***, which is based on a *CEN/ARS* DNA replication system rather than the 2 μ replication origin (*see* **Note 1**). This two-hybrid system is compatible with most two-hybrid libraries and gives extremely low background and few false-positive clones, making it ideal for library screening purposes (*see* **Note 2**). We have used it not only to isolate *Arabidopsis* NPR1-interactors from rice but also to pull out the rice ortholog of NPR1 by using one of the NPR1-interactors as bait to back-screen the rice

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library *(2)*. The unique elements contributing to the successful use of this system are discussed.

2. Materials

1. Plasmid vector pMC86: The construction of this plasmid bas been described previously *(3)*.
2. Yeast strain HF7c.
3. YPD agar plates and liquid medium.
4. Synthetic dropout (SD) media. SD-Trp, SD-Trp-Leu, and SD-Trp-Leu-His + 10 m*M* 3-AT plates. SD-Trp liquid medium.
5. 1X TE/LiAc. Prepare from 10X LiAc (1N) and 10X TE (0.1 *M* Tris-HCl, 10 m*M* ethylene diamine tetraacetic acid, pH 7.5). For storage of yeast competent cells, add sterile glycerol to approx 25%.
6. Polyethylene glycol (PEG)/LiAc solution containing 40% PEG. Make fresh by mixing 1 v of 10X TE, 1 v of 10X LiAc, and 8 v of 50% PEG stock. Do not subject 50% PEG to prolonged autoclaving; autoclave briefly or filtrate.
7. Denatured, sheared herring testes carrier DNA (10 mg/mL).
8. Dimethyl sulfoxide.
9. Z buffer: $Na_2HPO_4 \cdot 7H_2O$ (16.1 g/L), $NaH_2PO_4 \cdot H_2O$ (5.5 g/L), KCl (0.75 g/L), $MgSO_4 \cdot 7H_2O$ (0.246 g/L), pH 7.0 and autoclave.
10. X-gal/Z buffer: Mix 66.8 µL X-gal stock solution (20 mg/mL in dimethylformamide) and 10.8 µL of β-mercaptoethanol with 4 mL of Z buffer.
11. Yeast lysis solution: 2% Triton X-100, 100 m*M* NaCl, 1% sodium dodecyl sulfate, 1 m*M* ethylene diamine tetraacetic acid, and 10 m*M* Tris-HCl, pH 8.0.

3. Methods

The following protocols are derived mainly from Clontech yeast protocols.

3.1. Preparation of the Bait Plasmid and Yeast Competent Cells

1. Construct bait plasmid coding for a fusion protein of the GAL4 DNA binding domain and your favorite protein (*see* **Note 3**) by using the multiple cloning sites available in pMC86. The pMC86 plasmid, carrying the GAL4 DNA binding domain and the *Trp1* selection marker, is compatible with vectors with the *Leu2* selection, such as pAD-GAL4 from Stratagene, for library construction. For library vectors that carry the *Trp1* selection, the pPC97 plasmid can be used for bait construction instead.
2. Inoculate a fresh colony of yeast HF7c in 3 mL of YPD liquid medium in the morning and grow the culture at 30°C with vigorous shaking. Inoculate 50 mL of YPD liquid medium with this 3 mL of HF7c culture at the end of the day and grow overnight.
3. Centrifuge yeast cells the next morning at 3000*g* at room temperature for 5 min, remove the supernatant, resuspend cells in 30 mL of sterile water, and spin down again. To prepare competent cells for storage, resuspend the yeast cells in sterile 1X TE/LiAc containing 25% glycerol. The competent cells can be stored

at –80°C for at least 1 yr; however, transformation efficiency will gradually decrease. As an alternative, the yeast transformation kit from Zymo Research (Orange, CA) works fairly well.

4. Transform HF7c competent cells with the bait plasmid: Mix 1 μL of pMC86-derived bait plasmid DNA (approx 0.1 μg) and 5 μL of denatured herring testes carrier DNA (10 mg/mL) with 50 μL of the competent yeast cells. Add 300 μL of PEG/LiAc solution to the cells, mix, and incubate at 30°C with shaking for 30 to 60 min. Spread the mixture directly on a SD-Trp plate. Yeast colonies will appear in 2 to 3 d. Streak out individual colonies and make glycerol stocks in YPD medium plus 25% glycerol.

3.2. Library-Scale Transformation With Library DNA (Prey Plasmid)

1. Purify library DNA by using large-scale plasmid purification columns, such as Qiagen Maxi plasmid columns. Normally, at least several hundred micrograms of library DNA is needed.
2. On the morning of day 0, inoculate 3 mL of SD-Trp medium with several fresh colonies of HF7c containing the bait and grow at 30°C with vigorous shaking. At the end of the day, inoculate 100 mL of SD-Trp medium with this 3-mL seed culture and grow overnight (*see* **Notes 4** and **5**). The culture should almost reach stationary phase at the end of day one. Inoculate 1000 mL of SD-Trp medium with the whole 100-mL culture and grow overnight. In the morning of day 2, the culture should reach the late log phase. Spin down the yeast cells at 5000*g* for 5 min at room temperature. Remove the supernatant. Resuspend cells in 500 mL of sterile water and spin down cells again.
3. Resuspend the cells in 8 mL of 1X TE/LiAc. Mix 2 mL of denatured herring testes carrier DNA (10 mg/mL) and 100 to 500 μg of library DNA (prey plasmid) with the cells. Add this mixture to 60 mL of PEG/LiAc solution. Mix well.
4. Incubate at 30°C for 30 min with shaking.
5. Add 7 mL of dimethyl sulfoxide. Mix well with swirling.
6. Heat shock for 15 min at 42°C with occasional swirling. Chill cells on ice for 1 to 2 min (*see* **Note 5**).
7. Spin at 5000*g* for 5 min at room temperature to pellet cells. Remove the supernatant.
8. Resuspend the cells in 10 mL of 1X TE, pH 7.5.
9. Plate out cells on SD-Trp-Leu-His medium containing 10 m*M* 3-AT. This procedure requires approx 50 150 × 15-mm plates. Also plate out a small aliquot of cells on SD-Trp-Leu medium to estimate the total amount of yeast transformants. Incubate the plates at 30°C for up to 2 wk. Seal plates after a few days of incubation to slow down plate drying.
10. This protocol typically yields several to 20 million yeast transformants that grow on SD-Trp-Leu medium.

3.3. Perform β-Galactosidase Filter Assay to Select Positive Clones

1. Yeast colonies of putative interactors would start to appear in 5 d. Some yeast colonies may not show up until 2 wk after transformation.

2. Streak good colonies to new SD-Trp-Leu-His plates containing 10 m*M* 3-AT. The cells should grow into patches in 2 to 3 d.
3. Scrape up half of the cell mass of each clone from plate and patch on a sterile 3-mm filter circle with sterile toothpicks. Place the filter circle on a SD-Trp-Leu plate and incubate at 30°C overnight.
4. Lift the filter circle and air-dry it. Dip it in liquid N_2 for 10 s to permeabilize yeast cells. Thaw the filter at room temperature for 1 to 2 min.
5. At this time, add approx 1.9 mL of X-gal/Z buffer solution to a filter circle in a plate (100 × 15 mm) to prepare an X-gal-saturated filter circle.
6. Overlay the cells/filter on the X-gal-saturated filter in the plate.
7. Incubate at 30°C or room temperature until blue colors develop. This may take an hour to overnight incubation depending on the strength of interaction.

3.4. Isolation of Plasmid DNA From Yeast and Retransformation of Yeast Cells for Confirmation of Positive Interaction

1. Scrape up yeast cells from plates and resuspend them in 200 µL yeast lysis solution. Add 200 µL of phenol/chloroform and 200 mg of acid-washed glass beads.
2. Vortex for 2 min to break cells. Spin at 14,000 rpm for 5 min at room temperature.
3. Transfer the supernatant to a clean microcentrifuge tube. Add two volumes of ethanol and 1/10 volume of 3 *M* NaOAc to precipitate the DNA.
4. Spin down DNA and rinse the pellet with 70% ethanol. Dry the pellet.
5. Resuspend the DNA pellet in 20 µL of Tris-HCl or TE buffer, pH 8.0.
6. Transform *Escherichia coli* cells with 1 µL of the DNA by electroporation.
7. Pick two transformed *E. coli* colonies, grow in 2 mL of luria broth medium with carbenicillin or ampicillin, and extract plasmid DNA by miniprep.
8. Cut DNA with enzymes and run on a gel to confirm the presence of the prey plasmid and the insert. Based on the restriction patterns, the isolated clones can often be divided into groups.
9. Transform HF7c yeast cells with the isolated prey plasmid and the bait plasmid simultaneously. Plate out on SD-Trp-Leu medium.
10. Transfer several colonies for each putative clone to a SD-Trp-Leu-His plate containing 10 m*M* 3-AT to test for growth. Also perform β-galactosidase assay to confirm the interaction.

4. Notes

1. The pMC86 plasmid, derived from pPC86 and pPC97 ***(1)***, is based on the *CEN6/ARSH4* replication system, different from the 2-µ replication origin. In contrast to the high copy number of 2-µ-based plasmids, the *CEN6/ARSH4* replication system-based pMC86 has a low copy number. We have noticed that when a 2-µ-based plasmid is used as bait, the number of false-positive clones tends to be higher, possibly as a result of the higher DNA recombination events between the plasmid DNA and the genome. In addition to lowering false-positive clones, this feature of pMC86 also makes easier the recovery of the prey plasmid like pAD-

GAL4, carrying the 2-μ replication origin because most of the plasmid DNA recovered from yeast would be the prey plasmid.

2. The HF7c yeast strain, carrying *Trp1*, *Leu2*, and *His3* selectable markers and the *LacZ* reporter gene, has an extremely low background when plated on SD-Trp-Leu-His medium containing 10 m*M* 3-AT. When streaked directly on plate, HF7c does not require addition of 3-AT to suppress its growth. This feature is critical in library screening. On the contrary, many other commonly used yeast strains, such as PJ69-4A, carry significant leaky *His3* activity and require much higher 3-AT concentrations to suppress their growth, often give high backgrounds during library screening. It is possible to lower the concentration of 3-AT in medium for screening when using HF7c. In general, the level of background is proportional to the cell mass spread on each plate. The more cell mass on each plate, the higher concentration of 3-AT is needed.
3. We have noticed that the larger the protein encoded by the bait, the higher the number of false-positive clones, which may be attributable to the fact that yeast contains in its genome many protein sequences that can serve as a transcription activation domain and that DNA recombination rates are high in yeast. Any recombination event that creates a fusion protein between the bait and a transcription activation domain will generate a false-positive clone. In general, it is a good practice to keep the coding sequence of the bait less than 2 kb to avoid high number of false-positive clones.
4. However, HF7c does carry a disadvantageous feature; it often does not grow very vigorously in the SD-Trp medium and requires more time to grow to the needed cell mass compared to some other strains. This feature may contribute to lower transformation efficiency sometimes.
5. Some bait constructs may result in slower growth of the HF7c cells. This would usually give rise to lower transformation efficiency. To boost transformation efficiency, one can grow the HF7c cells in YPD medium for 2 h after spinning down cells from the 1000 mL SD-Trp culture before proceeding to transformation. After the heat shock treatment, the cells can also be cultured in YPD medium for a few hours before spun down for plating in order to help the yeast cells recover from the treatments.

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