Using the Split-Ubiquitin Yeast Two-Hybrid System to Test Protein-Protein Interactions of Transmembrane Proteins

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Summary

Proteins are responsible for many biological processes within living organisms. Many proteins have the ability to specifically interact with other proteins in order to function properly. The identification of protein-protein interactions (PPIs) can provide useful information about the function of a protein of interest. Historically, the properties of transmembrane proteins have caused difficulty in analyzing PPIs among transmembrane proteins. The development of an assay that is capable of analyzing PPIs involving transmembrane proteins, the split-ubiquitin yeast two-hybrid (SU-Y2H) assay, has provided a method to probe pairwise PPIs between two proteins of interest or to screen a single protein of interest for interaction partners. The following protocol explains how to use the SU-Y2H assay, which is compatible with the use of transmembrane proteins, to investigate PPIs between two proteins of interest and also briefly describes how to adjust the system to be used as a high-throughput screen for interaction partners of a particular protein of interest.

Key Words: split-ubiquitin, yeast two-hybrid, protein-protein interaction, transmembrane protein, yeast.

Abbreviations: 3-AT (3-amino-1,2,4-triazole), ADE2 (phosphoribosylaminoimidazole carboxylase gene in the adenine biosynthesis pathway), ADHp (promoter of yeast alcohol dehyrdrogenase 1), AmpR (ampicillin resistance gene), ccdb (gene encoding cytotoxic gene), CmR (chloramphenicol resistance gene) (Cub (C-terminal half of ubiquitin), ddH_2O (double distilled water), DMF (dimethylformamide), DMSO (dimethyl sulfoxide), H or His (Histidine), HIS3 (imidazoleglycerol-phosphate dehydratase gene in the histidine biosynthesis pathway), L or Leu (leucine), LacZ (β-galactosidase gene), LEU2 (Beta-isopropylmalate dehydrogenase gene in the leucine biosynthesis pathway), Met (Methionine), Met25p (methionine repressive promoter), NaCl (sodium chloride), Nub (N-terminal half of ubiquitin), NubG (mutated N-terminal half of ubiquitin with reduced Cub binding affinity), PEG (polyethylene glycol), PLV (ProteinA-LexA-VP16 reporter module), PPI (protein-protein interaction), SD (synthetic dropout), SpecR (spectinomycin resistance gene), ssDNA (salmon sperm DNA), SU-Y2H (split-ubiquitin yeast two-hybrid), TE (Tris and EDTA buffer), TOPO (topoisomerase), TRP (tryptophan), TRP1 (phosphoribosylanthranilate isomerase gene of the tryptophan biosynthesis pathway), USP (ubiquitin-specific protease), W (tryptophan), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), Y2H (yeast two-hybrid)

1. Introduction

The molecular machinery of living cells is predominantly comprised of proteins, which provide a majority of the

biological functions that are required to begin, develop, and sustain the life of cells and organisms. One quality possessed by proteins that is essential for proper function is the ability of certain proteins to specifically and coordinately form non-covalent interactions with other proteins. The identification of protein-protein interactions (PPIs) can significantly aid in the characterization of the function of a protein of interest, and the establishment of large-scale protein-protein interactomes can provide a more comprehensive understanding of complex biological processes.

On account of the importance of analyzing PPIs, several techniques have been developed to detect PPIs (*1*). One popular method by which PPIs have been tested or screened is the traditional yeast two-hybrid (Y2H) assay (*2*). In the traditional Y2H, one protein of interest, referred to as the bait protein, is fused to the DNA-binding domain of a transcription factor (GAL4) while a second protein of interest, referred to as the prey protein, is fused to the activation domain of GAL4. If an interaction occurs between the bait and prey proteins when co-expressed in yeast, the GAL4 transcription factor becomes reconstituted and triggers the expression of reporter genes that signify the bait and prey protein interaction in yeast (**Fig. 1A**). One limitation of the traditional Y2H is the requirement that the bait-prey protein interaction must occur in the yeast nucleus, the site of GAL4 function. Therefore, the traditional Y2H is incapable of analyzing PPIs of proteins that cannot gain access to the nucleus, such as transmembrane proteins. Likewise, many biochemical techniques for analyzing PPIs, such as co-immunoprecipitation or *in vitro* pull down assays, have difficulty in the analysis of transmembrane proteins due to the limited solubility exhibited by many transmembrane proteins. In order to circumvent the limitations of existing assays in analyzing PPIs of transmembrane proteins, a membrane-based variation of the Y2H assay, the split-ubiquitin yeast two-hybrid (SU-Y2H) assay, was developed (*3, 4*). In the SU-Y2H assay, the bait protein is fused to the C-terminal half of ubiquitin (Cub) that has an attached ProteinA-LexA-VP16 (PLV) reporter module to create a bait-CubPLV chimeric protein, and the prey protein is fused with a mutated version of the N-terminus of ubiquitin (NubG) to create a prey-NubG (XN21_GW) or NubG-prey (NX32_GW) chimeric protein (**Fig. 1B**). The mutation in NubG causes a significant reduction in the binding affinity between Nub and Cub compared to the wild type Nub (NubWT), thereby preventing spontaneous reconstitution of a functional ubiquitin molecule. However, if

the bait and prey proteins interact in yeast, the NubG of the prey construct and the Cub of the bait construct will reconstitute a functional ubiquitin protein, which is then recognized by ubiquitin specific proteases (USPs) that cleave and release the PLV module from the bait-CubPLV. The PLV module is then free to migrate to the nucleus to activate the transcription of reporter genes (**Fig. 1B**). The reporter genes in the SU-Y2H assay are LacZ, which is used for blue/white screening in the presence of X-Gal, HIS3, which is a histidine auxotrophy marker, and ADE2, which is an adenine auxotrophy marker.

As with other types of Y2H assays, false positive and false negative results are possible, so proper positive and negative controls must be in place. As a positive control, bait-CubPLV constructs should be tested with NubWT to ensure that the CubPLV is properly translated and displayed in the yeast when fused with the bait protein of interest. As a negative control, bait-CubPLV constructs should also be tested with NubG that lacks a prey protein to ensure that non-specific activation of the reporter does not occur in the absence of a prey protein. Additional strategies can be taken to increase the stringency of the reporter system that is used to detect the PPIs. First, the transcription of bait-CubPLV is controlled by a Met25 promoter and can be repressed by the addition of methionine to the yeast growth medium (**Fig. 2**). Therefore, increasing the concentration of methionine suppresses the expression of bait-CubPLV and attenuates the reporter system. Secondly, 3-amino-1,2,4-triazole (3-AT) acts as a competitive inhibitor of imidazoleglycerol-phosphate dehydratase, the gene product of HIS3 (*5*). The addition of 3-AT can be used to increase the stringency of the HIS3 auxotrophy selection.

The SU-Y2H protocol highlighted below provides a detailed, step-by-step, robust procedure for testing the pairwise interaction between two proteins of interest via co-transformation of yeast with bait-CubPLV and prey-Nub vectors. The SU-Y2H bait and prey vectors have been modified for use with Gateway® recombination based cloning for rapid, easy, and high-throughput cloning (**Fig. 2**)(*6, 7*). Although this protocol focuses on testing for an interaction between two proteins of interest, high-throughput screening of putative interaction partners of a single protein of interest is also possible with this system by making a few adjustments (*see* **Note 31**)(*7*).

2. Materials

All stock solutions and media are prepared using double distilled water (ddH_2O) unless otherwise noted.

2.1 Vectors and Cloning Supplies

1. Entry vectors containing the genes of interest flanked by 5' attL1 and 3' attL2 Gateway® recombination sites (**Fig. 2**)(*see* **Note 1**).
2. SU-Y2H destination vectors: MetYC_GW (HQ646605), NWTX_GW (HQ700954), XN21_GW (HQ700951), NX32_GW (HQ700952)(**Fig. 2**).
3. Gateway® LR Clonase™ II enzyme mix kit (Invitrogen).
4. Chemically competent E. coli cells.
5. 1.5-mL microcentrifuge tubes, *autoclaved*.
6. 0.2-mL plastic PCR tubes.
7. Sterile petri dishes.
8. LB bacterial growth media: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCL, *autoclaved*.
9. LB bacterial growth plates: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCL, 15 g/L bacto agar, *autoclaved*.
10. Ampicillin (100 mg/mL stock solution).
11. Glass test tubes, *autoclaved.*
12. GeneJET Plasmid Miniprep Kit (Thermo) or the equivalent.

2.2 Yeast Transformation Reagents and Supplies

1. Yeast strain THY.AP4 [MATa *ura3⁻ leu2⁻ trp1⁻ his3⁻ ade2⁻* lexA::lacZ::trp1 lexA::HIS3 lexA::ADE2](*see* **Note 2**).
2. 40% glucose stock solution, *autoclaved.*
3. 2 g/L adenine hemisulfate stock solution, *autoclaved.*

4. YPAD yeast growth medium: 10 g/L yeast extract, 20 g/L peptone, 2% glucose (50 mL/L of 40% stock), 0.004% adenine (20 mL/L of 2 g/L stock), *autoclaved*.

5. 1M Tris HCl pH 7.5 stock solution, *autoclaved.*

6. 0.5M EDTA stock solution, *autoclaved.*

7. 10x TE buffer: 0.1M Tris HCl pH 7.5 (100 mL/L of 1M Tris HCl pH 7.5 stock), 0.01M EDTA (20 mL/L of 0.5M EDTA stock), *autoclaved*.

8. 1M lithium acetate (LiOAc) stock solution, *filter sterilized.*

9. 50% polyethylene glycol (PEG; average molecular weight 3,350) stock solution, *filter sterilized.*

10. Double distilled water (ddH_2O), *autoclaved*.

11. 50-mL sterile plastic conical tubes.

12. 15-mL sterile plastic conical tubes.

13. 250-mL and 500-mL Erlenmeyer flasks, *autoclaved.*

14. Spectrophotometer or cell density meter.

15. Centrifuge.

16. Salmon sperm DNA (ssDNA) type III sodium salt (Sigma).

17. Dimethyl Sulfoxide (DMSO).

18. 0.9% sodium chloride (NaCl) solution, *autoclaved*.

19. 10x phosphate buffer: 37 g/L sodium phosphate dibasic anhydrous (Na_2HPO_4), 30 g/L sodium phosphate monobasic anhydrous (NaH_2PO_4), *autoclaved*.

20. 10 g/L histidine stock solution, *autoclaved.*

21. Synthetic dropout (SD) yeast growth plates (-LW SD plates): 1.7 g/L yeast nitrogen base without amino acids or ammonium sulfate (Becton, Dickinson and Co.), 5 g/L ammonium sulfate, 0.6 g/L -Ade -Leu -Trp -His dropout supplement (Clontech), 20 g/L bacto agar, 100 mL/L of 10x phosphate buffer, 2% glucose (50 mL/L of 40% stock), 0.004% adenine (20 mL/L of 2 g/L stock), 0.002% histidine (2 mL/L of 10 g/L stock)(*see* **Note 3**).

2.3 Supplies for the Detection of Interactions

1. 20 mg/mL X-Gal stock solution in dimethylformamide (DMF).
2. 0.1M Methionine stock solution, *autoclaved.*
3. 1M 3-amino-1,2,4-triazole (3-AT), *filter sterilized.*

3. Methods

3.1 Cloning your genes of interest into the SU-Y2H destination vectors

1. Start by preparing entry vector constructs that contain the coding sequences of the genes of interest flanked by a 5' attL1 and a 3' attL2 Gateway® recombination site (**Fig. 1**)(*see* **Note 1**).
2. At room temperature, mix 50-150 ng of the attL1/gene-of-interest/attL2 entry vector and 150 ng of the desired SU-Y2H destination vector (**Fig. 2**) in a 0.2-mL PCR tube and adjust the volume of the mixture to 8 µL with 1x TE buffer or water (*see* **Note 4**).
3. Add 2 µL of LR Clonase™ II enzyme mix, vortex, and microcentrifuge briefly (*see* **Note 5**).
4. Incubate the reaction for 1 hour at room temperature (*see* **Note 6**).
5. Add 1 µL of Proteinase K solution to each reaction and incubate at 37°C for 10 minutes to terminate the reaction (*see* **Note 5**).
6. Prepare LB bacterial growth plates: 10g tryptone, 5g yeast extract, 10 g NaCL, 15 g bacto agar, *autoclaved*. After autoclaving, allow the media to cool to 45-55 °C before adding a 1:1000 dilution of the 100 mg/mL Ampicillin stock solution. Swirl the media to mix before pouring plates.
7. Transform chemically competent E. coli cells with the LR reaction mixture by (1) incubating the cells on ice in a 1.5-mL microfuge tube with 2-4 µL of each LR reaction for 30 minutes, (2) heat shocking the cells by incubating the mixture at 42°C for 30-45 seconds, (3) adding 450 µL of LB, and (4) incubating the cells at 37°C for 1 hour (*see* **Note 7**).

8. Spread 20-100 µL of the transformed E. coli cells on ampicillin-containing LB bacterial growth plates (*see* **Note 8**).

9. Incubate plates at 37 °C overnight.

10. Prepare LB bacterial growth media: 10g tryptone, 5g yeast extract, 10 g NaCL, *autoclaved*. After autoclaving, allow the media to cool to ≤ 55°C before adding a 1:1000 dilution of the 100 mg/mL Ampicillin stock solution.

11. Propagate several colonies from each plate in 2-3 mL of Ampicillin-containing liquid LB media at 37°C in autoclaved test tubes with agitation until a dense culture grows.

12. Isolate the plasmid DNA using a miniprep kit (*see* **Note 9**).

13. Verify that your genes-of-interest have been successfully inserted into the destination vectors using a diagnostic restriction enzyme digest and/or by sequencing the plasmids (*see* **Note 10**).

3.2 Co-transformation of yeast with bait and prey vectors for a pairwise protein-protein interaction assay

1. Prepare YPAD yeast growth medium: 10 g/L yeast extract, 20 g/L peptone, 2% glucose (50 mL/L of 40% stock), 0.002% adenine (20 mL/L of 2 g/L stock), *autoclaved* (*see* **Note 11**).

2. Prepare -LW SD plates: 1.7 g/L yeast nitrogen base without amino acids or ammonium sulfate (Becton, Dickinson and Co.), 5 g/L ammonium sulfate, 0.6 g/L -Ade -Leu -Trp -His dropout supplement (Clontech), 20 g/L bacto agar, 100 mL/L of 10x phosphate buffer, 2% glucose (50 mL/L of 40% stock), 0.004% adenine (20 mL/L of 2 g/L stock), 0.002% histidine (2 mL/L of 10 g/L stock)(*see* **Note 3**).

3. Prepare ssDNA solution by mixing 200 mg of ssDNA into 100 mL of autoclaved ddH_2O (*see* **Note 12**). Once dissolved, aliquot the ssDNA into autoclaved 1.5-mL microfuge tubes, boil for 5 minutes, and store at -20°C.

4. In an autoclaved 250-mL Erlenmeyer flask, inoculate 50 mL of YPAD medium with THY.AP4 yeast and incubate at

28-30°C overnight with shaking to propagate a dense culture (*see* **Note 2**).

5. Prepare LiOAc/TE solution by mixing 1.1 mL of 1M LiOAc stock solution, 1.1 mL of 10x TE pH 7.5 stock solution, and 7.8 mL of autoclaved ddH_2O in a sterile 15-mL plastic conical tube (*see* **Note 13**).

6. Prepare LiOAc/PEG solution by mixing 1.5 mL of 1M LiOAc stock solution, 1.5 mL of 10x TE pH 7.5 stock solution, and 12 mL of 50% PEG stock solution in a sterile 15-mL plastic conical tube (*see* **Note 13**).

7. In an autoclaved 500-mL Erlenmeyer flask, inoculate 150 mL of YPAD medium with 10-20 mL of the fresh overnight THY.AP4 culture (*see* **Notes 13 and 14**).

8. Using a spectrophotometer or cell density meter, record the cell density (OD_{600}) of the inoculated culture as the starting OD_{600}. A starting OD_{600} of 0.2-0.3 is desired (*see* **Note 14**).

9. Incubate the culture at 28-30°C with shaking until the OD_{600} approximately triples in value (*see* **Note 15**).

10. Boil ssDNA for 10 minutes and immediately transfer to ice. Keep on ice until needed.

11. Decant 50 mL of the culture into each of two sterile 50-mL plastic conical tubes and centrifuge at about 4000 x g for 1 minute to pellet the cells (*see* **Note 16**). Decant and discard supernatant.

12. Decant the remaining yeast culture evenly between the two 50-mL tubes that contain the yeast pellets from step 11. Centrifuge at about 4000 x g for 1 minute to pellet the cells. Decant and discard the supernatant.

13. Resuspend each of the pellets in 50 mL of autoclaved ddH_2O and vortex to wash the cells free of YPAD medium. Centrifuge at about 4000 x g for 1 minute to pellet the cells. Decant and discard the supernatant.

14. Resuspend each of the pellets in 2 mL of LiOAc/TE solution and transfer all of the resuspended yeast (approximately 4 mL) to a sterile 15-mL plastic conical tube. Centrifuge at about 4000 x g for 1 minute to pellet

the cells (*see* **Note 17**). Decant and discard the supernatant.

15. Resuspend the yeast pellet in 4 mL of LiOAc/TE solution (*see* **Note 18**).

16. For each co-transformation, gently mix the following components in the order listed in autoclaved 1.5-mL microcentrifuge tubes: 200 µL of LiOAc/TE/yeast solution, 200 ng of bait-CubPLV plasmid DNA, 200 ng of prey plasmid DNA (NubG-prey, prey-NubG, NWT_GW, or NX32_GW), 10 µL of ssDNA, 700 µL of LiOAc/PEG solution (*see* **Notes 19 and 20**).

17. Incubate the mixture at 28-30°C for 30 minutes.

18. Add 55 µL of DMSO to each transformation, gently mix, and heat shock at 42°C for 15 minutes (*see* **Note 20**).

19. Centrifuge each transformation tube for one minute to form a yeast pellet. Decant and discard the supernatant (*see* **Note 21**).

20. Resuspend each pellet with 500 µL of 0.9% NaCl solution. Centrifuge each tube for one minute to form a yeast pellet. Decant and discard the supernatant.

21. Resuspend each pellet with 300 µL of 0.9% NaCl solution (*see* **Note 22**).

22. Plate 100-200 µL of the yeast on -LW SD plates and allow to dry (*see* **Notes 23 and 24**).

23. Incubate plates at 28-30°C for several days (*see* **Note 25**).

3.3 Detection of interaction on growth plates

1. Prepare -LW +X-Gal SD plates: 1.7 g/L yeast nitrogen base without amino acids or ammonium sulfate (Becton, Dickinson and Co.), 5 g/L ammonium sulfate, 0.6 g/L -Ade -Leu -Trp -His dropout supplement (Clontech), 20 g/L bacto agar, 100 mL/L of 10x phosphate buffer, 2% glucose (50 mL/L of 40% stock), 0.004% adenine (20 mL/L of 2 g/L stock), 0.002% histidine (2 mL/L of 10 g/L stock), 0.008% X-Gal (4 mL/L of 20 mg/mL X-Gal stock solution)(*see* **Notes 3, 26, and 27**).

2. Prepare -LWH +Met SD plates with varying methionine concentrations: 1.7 g/L yeast nitrogen base without

amino acids or ammonium sulfate (Becton, Dickinson and Co.), 5 g/L ammonium sulfate, 0.6 g/L -Ade -Leu -Trp -His dropout supplement (Clontech), 20 g/L bacto agar, 100 mL/L of 10x phosphate buffer, 2% glucose (50 mL/L of 40% stock), 0.004% adenine (20 mL/L of 2 g/L stock), 0.15-1 mM methionine (0.15-1 mL/L of 1M stock)(*see* **Notes 3 and 26**).

3. Prepare -LWH +3-AT plates with varying 3-AT concentrations: 1.7 g/L yeast nitrogen base without amino acids or ammonium sulfate (Becton, Dickinson and Co.), 5 g/L ammonium sulfate, 0.6 g/L -Ade -Leu -Trp -His dropout supplement (Clontech), 20 g/L bacto agar, 100 mL/L of 10x phosphate buffer, 2% glucose (50 mL/L of 40% stock), 0.004% adenine (20 mL/L of 2 g/L stock), 5-50 mM 3-AT (5-50 mL/L of 1M stock)(*see* **Notes 3, 26, and 27**).

4. Pick a positive co-transformed yeast colony from a -LW SD plate using an autoclaved pipet tip and resuspend the yeast in 50-100 µL of autoclaved ddH_2O in an autoclaved 1.5-mL microcentrifuge tube.

5. Spot 5 µL of resuspended yeast onto each type of SD plate (-LW +X-Gal, -LWH +Met of each concentration, -LWH +3-AT of each concentration) and allow to dry (*see* **Note 28**).

6. Repeat steps 4 and 5 using several colonies from each transformation -LW SD plate. When dry, incubate plates at 28-30°C for 2-3 days until significant growth is apparent (*see* **Notes 29 and 30**).

7. Blue color on -LW +X-Gal SD plates is indicative of a positive interaction. Growth on -LWH SD plates is also indicative of a positive interaction. Increasing concentrations of 3-AT or Met causes the interaction assay to be more stringent. In all cases, the interaction is only positive if the corresponding negative control remains white (-LW +X-Gal SD plates) or does not grow (-LWH SD plates) on plates that are under the equivalent conditions (**Fig. 3**). If neither the experimental nor the positive control colonies show indications of an interaction, the bait-CubPLV protein is not suitable for use in the SU-Y2H system.

4. Notes

1. The pCR®8/GW/TOPO® vector (Invitrogen) is a great choice for an entry vector in this system for multiple reasons. The pCR®8/GW/TOPO® vector allows for easy insertion of genes of interest through TOPO cloning techniques and easy sequencing with M13F/M13R sites. The attL1 and attL2 sites that flank the gene insertion site are compatible with the attR1 and attR2 sites of the destination vectors of the SU-Y2H system (**Fig. 2**). Lastly, pCR®8/GW/TOPO® contains SpecR for selection while the destination vectors contain AmpR, which prevents pCR®8/GW/TOPO®-gene-of-interest colonies from growing in later stages when screening for destination vector-gene-of-interest colonies.

2. The genetic background of THY.AP4 is specifically designed for this protocol. MATa *ura3*$^{-}$ *leu2*$^{-}$ *trp1*$^{-}$ *his3*$^{-}$ *ade2*$^{-}$ lexA::lacZ::trp1 lexA::HIS3 lexA::ADE2 The *leu2*$^{-}$ *trp1*$^{-}$ lesions are necessary for selection of positive Cub and Nub transformants. The *his3*$^{-}$ *ade2*$^{-}$ lexA::lacZ::trp1 lexA::HIS3 lexA::ADE2 modifications are required for the reporter system. The culture can be inoculated from a glycerol stock of THY.AP4 or by 2-3 fresh THY.AP4 colonies from a YPAD plate.

3. SD media is prone to boiling over during autoclaving. To avoid boiling over, it is recommended to add 10x phosphate buffer, glucose, adenine, and amino acid (histidine or methionine) stock solutions after autoclaving and to prepare the medium in a container that has a volume multiple times larger than the volume of medium being prepared (e.g. 500 mL of media in a 2L flask).

4. Genes to be used as bait should be cloned into the MetYC_GW vector and genes to be used as prey should be cloned into the NX32_GW and the XN21_GW vector. It is recommended to use both vectors because detection of some interactions have been observed to depend on whether NubG is fused to the N-terminus or C-terminus of the prey protein.

5. Efficient LR reactions have been performed using half of the suggested amount of LR Clonase™ II enzyme mix (1 µL) and Proteinase K (0.5 µL). LR Clonase™ II enzyme mix should be kept at -80°C and distributed into multiple aliquots to avoid excessive freeze/thaw cycles.

6. In our hands, the efficiency of the LR reaction has been increased significantly by incubating at room temperature overnight instead of for 1 hour.

7. If using commercially purchased competent cells, refer to the instruction manual. If using competent cells that were prepared on site, familiar heat shock procedures should be successful.

8. The desired amount of bacteria to plate is contingent on the transformation efficiency. It is desirable to plate a volume that provides a large number of well-spaced colonies. Left over transformed bacteria can be stored at 4°C and plated several days later if required to provide a suitable number of colonies.

9. Several plasmid miniprep kits are commercially available (e.g. GeneJET Plasmid Miniprep Kit by Thermo Scientific) and can be used to isolate and purify the plasmid DNA.

10. The complete SU-Y2H destination vector sequences are available through GenBank [MetYC_GW (HQ646605), NWTX_GW (HQ700954), XN21_GW (HQ700951), NX32_GW (HQ700952)] and can be used to locate restriction enzyme sites and/or to design sequencing primers that can be used to determine that the genes-of-interest have been properly cloned into the SU-Y2H vectors. Met25pF primer (TCTATTACCCCCATCCATAC) can be used to sequence the 5' end of genes that are inserted in MetYC_GW and ADHpF primer (CAAGCTATACCAAGCATAC) can be used to sequence the 5' end of genes that are inserted in NWTX_GW, XN21_GW, or NX32_GW.

11. Adding glucose stock solution and adenine stock solution to the medium after autoclaving is recommended to prevent the medium from becoming discolored or boiling over during autoclaving.

12. ssDNA may be difficult to dissolve into solution. It may be necessary to leave on a stir plate or shaker overnight to completely dissolve the ssDNA.

13. This protocol prepares enough competent yeast cells, LiOAc/TE solution, and LiOAc/PEG solution for approximately 20 transformations, but can be scaled up to accommodate additional transformations.

14. The target OD_{600} cell density at the time of inoculation is 0.2-0.3. Since the density of the overnight culture can vary, the amount of inoculum required to obtain the target cell density can also vary. 10-15 mL of inoculum is usually sufficient to obtain the target cell density, but it is possible that more or less inoculum is required to match the target cell density.

15. It usually takes the culture 2-4 hours to triple in density (e.g. if the starting OD_{600} is 0.25, the final OD_{600} should be approximately 0.75).

16. Any speed that efficiently causes the cells the pellet is sufficient.

17. Always be sure to balance the centrifuge with a counterbalancing tube of water when needed.

18. The cells are stable at this step for several hours if required.

19. For each protein-protein interaction test with the bait-CubPLV and NubG-prey or prey-NubG, two control co-transformations must be done in addition to the experimental co-transformations (**Fig. 3**). In this case, the experimental co-transformations are bait-CubPLV plus NubG-prey and bait-CubPLV plus prey-NubG. As a positive control, the yeast should be co-transformed with bait-CubPLV and NWTX_GW. As a negative control, the yeast should be co-transformed with bait-CubPLV and NX32_GW.

20. Do not mix by pipetting. Mix by gently tapping/flicking the tube. DMSO has been shown to improve the transformation efficiency but it can be skipped if transformation efficiency is not a concern.

21. The addition of an optional step between step 19 and 20 in section 3.2 might improve transformation efficiency, which may be especially helpful if performing high-throughput screening (*see* **Note 31**). *Optional*: Resuspend each pellet in 1 mL of YPAD medium and incubate at 28-30°C for 90 minutes. Centrifuge each tube for one minute to form a yeast pellet. Decant and discard the supernatant.

22. Resuspending twice in 0.9% NaCl solution has a dual role. First, these steps act as a washing step. The washes are

intended to rid the yeast of potentially harmful reagents from the transformation protocol and to remove any contaminating nutrients (e.g. YPAD medium from **Note 21**) from interfering with auxotrophy selection. Secondly, the 0.9% NaCl solution acts as a favorable osmotic environment for the yeast cells to recuperate from the transformation procedure.

23. The amount of yeast to plate varies depending on the transformation efficiency. If the efficiency is low, all of the yeast can be plated on a single plate. Any yeast remaining after plating can be stored at 4°C and plated days later if required.

24. -LW SD plates will be selective for colonies that were successfully co-transformed with at least one copy of each of the bait plasmid and the prey plasmid.

25. Colonies are typically visible after 2-3 days. Plates can be incubated until colonies reach a desirable size (2-3 mm diameter).

26. Other ingredients may be supplemented to or retracted from the -LW SD media recipe to make different varieties of SD media. -LW +X-Gal SD media includes the addition of 0.008% X-Gal (4 mL/L of 20 mg/mL X-Gal stock solution), which allows for blue/white screening of colonies to assay protein-protein interactions. -LWH SD media, which is selective for colonies exhibiting a positive protein-protein interaction, is made by withholding histidine from the recipe. Either 5-50 mM 3-Amino-1,2,4,-triazole (3-AT) or 0.15-1 mM methionine (Met) should be added to -LWH SD media in order to increase the stringency of selection.

27. X-Gal and 3-AT stock solutions are heat labile and should only be added to medium after autoclaving and after cooling to ≤ 55°C.

28. To organize and label spotted colonies, draw a grid pattern on the underside of all SD plates and properly label each cell prior to the spotting of colonies.

29. It is common for only a certain percentage of co-transformed colonies to show indications of a positive interaction. Therefore, it is recommended to test many colonies from each co-transformation.

30. Alternatively, yeast from the co-transformation procedure can be plated directly on -LW +X-Gal SD plates in step 22 of section 3.2 to select for positive co-transformants and to screen for positive interacting colonies in a single step. If using this approach, colonies may take longer to show blue color. Store plates at 4°C after they grow to a desirable size. Blue color may not develop significantly until several days after being placed at 4°C.

31. A protocol for the high-throughput screening for putative interaction partners of a protein of interest is beyond the scope of this protocol, but with the construction of a cDNA prey library and a slight modification of the protocol above, high-throughput screening becomes possible. First, the protein of interest (bait protein) should be cloned into the pMetCY_GW vector and transformed into yeast as described in sections 3.1 and 3.2 to create a stable yeast strain that contains the bait-CubPLV vector. This yeast strain should be grown on -L SD plates [-LW SD medium with the addition of 0.002% tryptophan (2 mL/L of 10 g/L stock)]. A prey library can be prepared from the organism of interest by isolating mRNA, performing reverse transcription to convert the mRNA into complementary DNA (cDNA), and by inserting the cDNA clones into an entry vector that has attL1 and attL2 sites as in step 1 of section 3.1 (*see* **Note 1**). This attL1-cDNA-attL2 entry vector library can then be cloned into the XN21_GW or NX32_GW vector as described in section 3.1 and transformed into the yeast that already contains the bait-CubPLV and plated on -LW SD plates as described in section 3.2. Colonies can be tested for positive interactions as described in section 3.3. For each positive colony, propagate the yeast in liquid -LW SD medium, isolate the plasmid DNA from the yeast, and sequence the plasmids using ADHpF (CAAGCTATACCAAGCATAC), and BLAST the obtained sequence to identify the putative interaction partner of your bait protein of interest. The cDNA clone likely only contains a piece of a prey gene and should be checked to ensure that the sequence is in frame with NubG. As a follow-up to the high-throughput screen, the full-length sequence of each positive cDNA prey should be tested pairwise with the bait-CubPLV in the SU-Y2H as described in sections 3.1-3.3 above.

5. Concluding Remarks and Applications

The pairwise SU-Y2H assay described above has been integral to the investigation of several PPIs in plants (**Fig. 3**) (*6-12*) and the high-throughput SU-Y2H screen (*see* **Note 31**) has provided several leads for future research endeavors. The SU-Y2H is a useful tool in investigating the biological role of proteins by examining the PPIs shared among proteins that work cooperatively in biological processes. The SU-Y2H has several advantages over alternative PPI techniques including: the ability to investigate PPIs among transmembrane proteins, the capability to test PPIs in both a pairwise and a high-throughput manner, cloning procedures that are quick and easy via Gateway® recombination, and easy accessibility of the vectors through the Arabidopsis Biological Resource Center (ABRC).

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Fig. 1. Schematic representations of the traditional yeast two-hybrid (Y2H) assay (A) and the split-ubiquitin yeast two-hybrid (SU-Y2H) assay (B). (A) In the traditional Y2H, the bait protein is fused with the DNA-binding domain (DBD) of the GAL4 transcription factor and the prey protein is fused to the activation domain (AD) of GAL4. A positive bait-prey interaction in the nucleus of the yeast reconstitutes a functional GAL4 transcription factor, which triggers the expression of UAS_G regulated reporter genes (Experimental #1). If the bait and prey do not interact, no reporter expression occurs (Experimental #2). If either the bait protein (bait #2) or the prey protein (prey #3) is unable to enter the nucleus, the traditional Y2H assay is not suitable for detecting PPIs. (B) In the SU-Y2H, the bait protein is fused with the C-terminal half of ubiquitin (Cub), which is in turn fused with a Protein A-LexA-VP16 (PLV) reporter module to create bait-CubPLV. The prey protein is fused with a mutated N-terminal half of ubiquitin (NubG), which has a reduced binding affinity with Cub, to create NubG-prey or prey-NubG. NubG and Cub will reconstitute a functional ubiquitin molecule only if the bait and prey proteins interact (Experimental #1 and #2). The reconstituted ubiquitin is recognized by ubiquitin specific proteases (USPs) in the yeast that release the PLV reporter

module, which activates the transcription of LexA driven reporter genes. As a positive control, NubWT binds to Cub of the bait-CubPLV chimeric protein to mimic a positive interaction in the system. As a negative control, NubG lacking a prey protein is unable to bind to bait-CubPLV in the absence of a prey protein and should not elicit a reporter response. Experimental #1 shows an interaction between a transmembrane bait protein and a cytoplasmic prey protein. Experimental #2 shows an interaction between two transmembrane proteins. Experimental #3 shows a negative result in which the bait and prey proteins do not interact and therefore do not trigger reporter expression.

Fig. 2. Vector information for the recommended entry vector, pCR®8/GW/TOPO® vector (Invitrogen), and the SU-Y2H destination vectors, MetYC_GW (GenBank #HQ646605 & ABRC stock # CD3-1740), XN21_GW (GenBank #HQ700951 & ABRC stock # CD3-1734), NX32_GW (GenBank #HQ700952 & ABRC stock # CD3-1737), NWTX_GW (GenBank #HQ700954 & ABRC stock # CD3-1739).

Fig. 3. A representative SU-Y2H assay modified from (*8*) (www.plantphysiol.org, Copyright American Society of Plant Biologists). The positive control (Row #1) shows a positive interaction between bait-CubPLV and NubWT. The negative control (Row #2) has no indication of reporter gene expression, which indicates that there is not an interaction between bait-CubPLV and NubG lacking a fused prey protein. The experimental interaction test between bait-CubPLV and prey-NubG (Row #3) shows reporter gene expression, which is indicative of a positive interaction. In this assay, -LW +X-Gal SD plates were used for blue/white screening and -LWH SD plates were used for histidine auxotrophy selection. Either 30 mM 3-AT or 1mM Met was added to the -LWH SD plates to increase the stringency of the assay.

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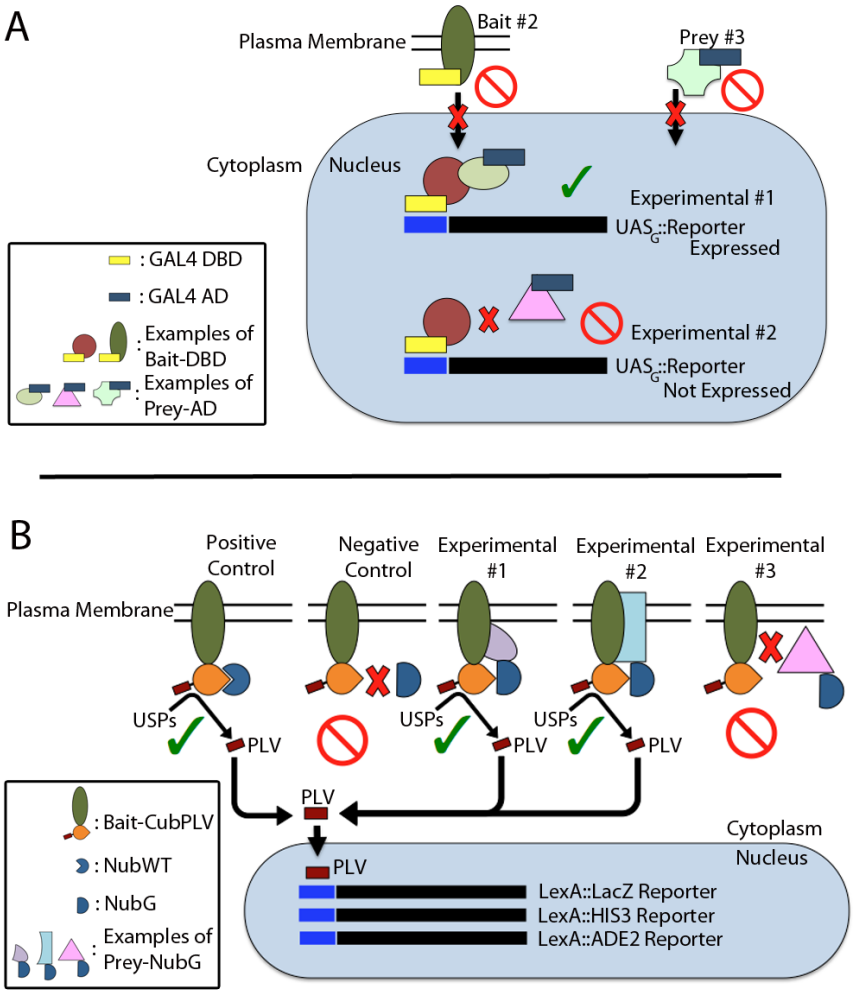
A
Plasma Membrane
Bait #2
Prey #3
Cytoplasm
Nucleus
Experimental #1
UAS$_G$::Reporter
Expressed
Experimental #2
UAS$_G$::Reporter
Not Expressed
: GAL4 DBD
: GAL4 AD
: Examples of Bait-DBD
: Examples of Prey-AD
B
Positive Control
Negative Control
Experimental #1
Experimental #2
Experimental #3
Plasma Membrane
USPs
PLV
: Bait-CubPLV
: NubWT
: NubG
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Cytoplasm
Nucleus
LexA::LacZ Reporter
LexA::HIS3 Reporter
LexA::ADE2 Reporter

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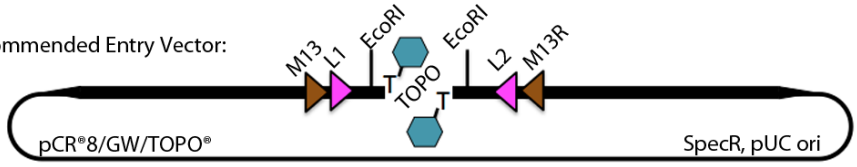

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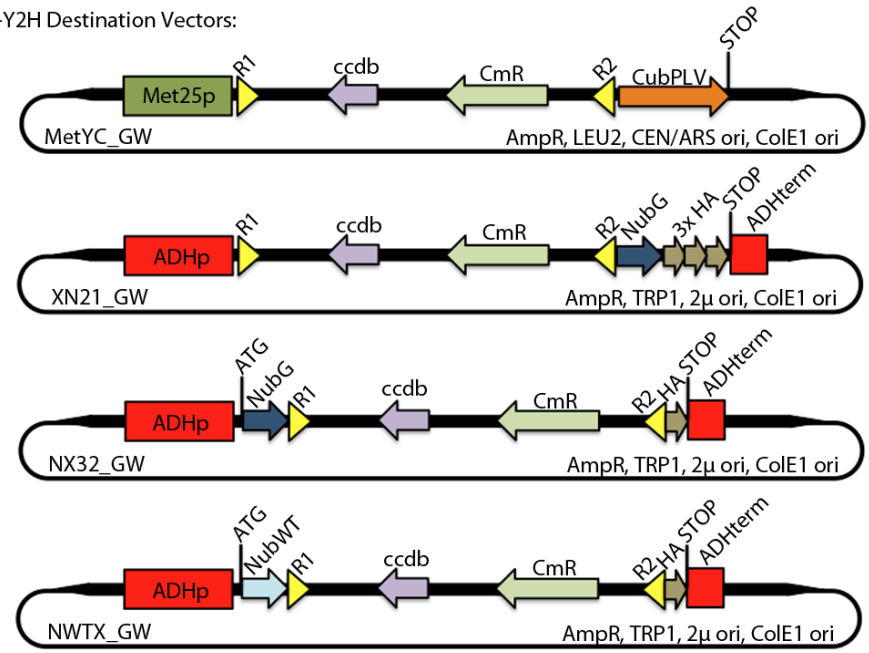

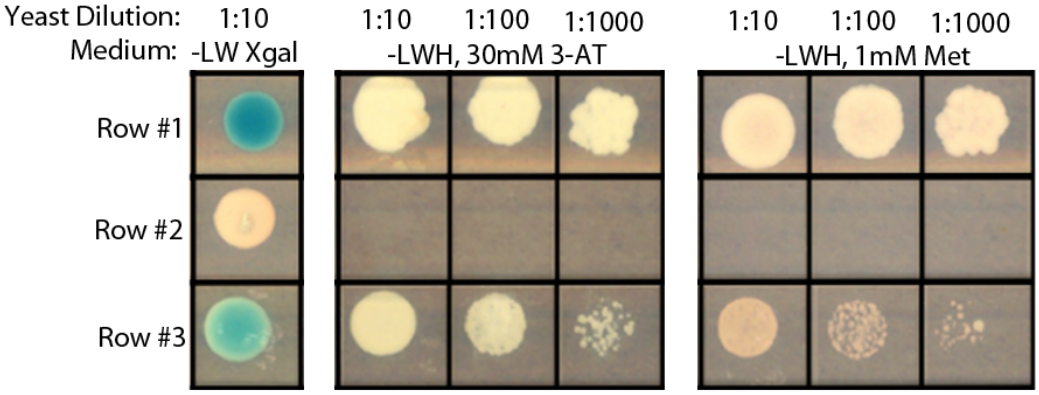
Yeast Dilution:
1:10
1:10
1:100
1:1000
1:10
1:100
1:1000
Medium:
-LW Xgal
-LWH, 30mM 3-AT
-LWH, 1mM Met
Row #1
Row #2
Row #3