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A Yeast Hybrid Split-Ubiquitin System for Plant Protein-Protein Interaction Research

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Abstract

Protein-protein interactions are essential for physiological activities in plant cells. To detect and determine the interactions between two proteins, numerous in vivo and in vitro methods have been published. The mating-based split-ubiquitin system (mbSUS) serves as an alternative to the classical yeast two-hybrid technique; it not only retains the rapid and convenient advantages of yeast two-hybrid, but also overcomes the limitations of conventional yeast two-hybrid methods regarding requirements for target proteins, false positive rates, and other aspects. Currently, the improved mbSUS method can be applied to interaction studies of both membrane proteins and soluble proteins, and has been utilized in plants to detect interactions between ion channels and membrane transporters. This article provides a detailed description of the technical principles, experimental protocols, and experimental considerations of the mbSUS method, establishing a foundation for plant protein interaction research.

Full Text

The Yeast Mating-Based Split-Ubiquitin System (mbSUS) for Plant Protein-Protein Interaction Studies

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Abstract

Protein-protein interactions (PPIs) are essential for plant cellular physiological activities. To detect and determine interactions between two proteins, numerous in vivo and in vitro methods have been developed. The yeast mating-based Split-Ubiquitin System (mbSUS) represents an alternative to the classical yeast two-hybrid (Y2H) technique. It not only retains the speed and convenience of Y2H but also overcomes limitations regarding protein requirements and false positive rates inherent to conventional Y2H methods. Recently improved versions of

mbSUS can now be applied to study interactions among both membrane and soluble proteins, and have been successfully used in plants to detect interactions between ion channels and membrane trafficking proteins. This article provides a detailed description of the technical principles, experimental protocols, and important considerations for mbSUS, establishing a foundation for plant PPI research.

Keywords: plant, protein-protein interaction, yeast, mbSUS

Introduction

Over the past several decades, a variety of techniques have been established for detecting protein interactions in vivo or in vitro, including the classic Yeast Two-Hybrid (Y2H) system, Förster Resonance Energy Transfer (FRET), Co-Immunoprecipitation (Co-IP), Bimolecular Fluorescence Complementation (BiFC), and Pull-down assays. These methods have been widely employed in plant PPI research (Xing et al., 2016). Among them, Y2H has become the preferred approach in most studies, particularly for large-scale screening of potential interacting proteins, due to its simple operation and low cost (Xing et al., 2016). However, because the transcriptional activation domain fused to the protein of interest must enter the nucleus to function, Y2H is unsuitable for detecting interactions involving large proteins and membrane proteins. Additionally, Y2H suffers from high false positive rates due to auto-activation effects. To address these limitations, the mating-based Split-Ubiquitin System (mbSUS) was developed as an extension of Y2H technology.

[Figure 1: see original paper]A illustrates the fundamental principle of mbSUS for protein interaction detection. Yeast ubiquitin is split into two fragments (Nub and Cub), with the C-terminal Cub fused to a reporter complex (ProteinA-LexA-VP16, PLV). To prevent spontaneous reassembly of the two ubiquitin fragments, the isoleucine at position 13 in the N-terminus is mutated from the wild-type (NubI) to glycine (NubG). The test proteins X and Y are then fused to NubG and Cub-PLV respectively, generating X-NubG as the prey and Y-Cub-PLV as the bait. If X and Y interact, the two ubiquitin fragments reconstitute and become functional, allowing ubiquitin-specific proteases to recognize the tag and cleave the PLV reporter from Cub. The released PLV enters the nucleus to activate reporter gene expression, thereby enabling screening for protein interactions. To clearly demonstrate the effect of interactions on yeast growth, bait protein expression is controlled by the met25 promoter, which is negatively regulated by methionine concentration in the culture medium. [Figure 1: see original paper]B shows the bait and prey vector structures used in mbSUS. All mbSUS vectors contain Gateway cassettes, allowing insertion of test protein genes via Gateway cloning. Prey vectors expressing only NubG or NubI serve as negative and positive controls, respectively.

Compared with conventional Y2H, mbSUS exhibits significantly lower false positive rates because the PLV reporter released by cleavage enters the nucleus

rather than the full bait fusion protein. Moreover, mbSUS enables detection of interactions between large membrane proteins at the cell membrane. To date, mbSUS has been successfully applied to study plant membrane protein interactions, including discoveries of interactions between plasma membrane potassium channels KC1 and KAT1 with SNARE proteins and the membrane trafficking protein SEC11 (Grefen et al., 2010, 2015; Zhang et al., 2015; Karnik et al., 2015). Since mbSUS traditionally requires the bait to be a membrane protein, the GPS system (GPI signal peptide-anchored split-ubiquitin system) was developed to expand the method's applicability. As shown in [Figure 1: see original paper]B, this system tethers a GPI anchor peptide (Exg2GPI) to the N-terminus of the bait fusion protein, enabling small soluble proteins to serve as baits in mbSUS studies (Zhang et al., 2018). This system has been successfully used to screen for interactors of truncated SEC11 proteins in plants (Zhang et al., 2019). Thus, mbSUS can now be applied to study interactions among both membrane and soluble proteins, serving as a robust alternative to traditional Y2H technology. Below, we provide a detailed description of mbSUS experimental protocols and important considerations.

1.1 Vectors and Strains

Gateway entry vectors such as pDONR207 (Thermo Fisher Scientific) or other vectors containing attP1-attP2 Gateway cassettes are required for cloning. The structures of yeast expression vectors needed for experiments are shown in [Figure 1: see original paper]B and [Figure 1: see original paper]C. Vector information for pMetYC-Dest (Grefen et al., 2009), pMetExg2-Dest (Zhang et al., 2018), and pNX35-Dest (Grefen and Blatt, 2012) can be obtained from https://psrg.org.uk/resources. The required *Saccharomyces cerevisiae* strains are listed in .

1.2 Reagents and Formulations

Reagents: - CSM-ADE-HIS-LEU-MET-TRP-URA dropout media (MP Biomedicals, Cat#: 114560222) - Yeast Nitrogen Base without amino acids (YNB) (Beijing Solarbio Science & Technology, Cat#: Y8040) - Salmon sperm DNA (ssDNA) (Beijing Solarbio Science & Technology, Cat#: D8030) - Oxoid agar No.1 (Oxoid, Cat#: LP0011) - PEG3350 (Sigma-Aldrich, Cat#: P3640) - BP Clonase® II Enzyme Mix (Thermo Fisher Scientific, Invitrogen™, Cat#: 11789020) - LR Clonase® II Enzyme Mix (Thermo Fisher Scientific, Invitrogen™, Cat#: 11791020)

Antibodies for Western blot analysis: - Primary antibodies: anti-HA (1:20000, Anti-HA high-affinity Rat monoclonal antibody, Roche Diagnostics, Cat#: 11867423001); anti-VP16 (1:20000, Anti-VP16 tag antibody in Rabbit, Abcam, Cat#: ab4808) - Secondary antibodies: anti-rabbit HRP (1:20000, goat anti-rabbit IgG-HRP, Thermo Fisher Scientific, Invitrogen™, Cat#: G-21234); anti-rat HRP (1:20000, Rabbit anti-Rat IgG H&L [HRP], Abcam, Cat#: ab6734)

All other reagents were purchased from Sangon Biotech (Shanghai) Co., Ltd.

Reagent formulations are provided in , and yeast selection media recipes are shown in .

1.3 Equipment

Temperature-controlled shaker, incubator, benchtop refrigerated centrifuge, PCR thermal cycler, water bath, Western blot equipment, laminar flow hood, micro-spectrophotometer (Nano-300, Hangzhou Allsheng Instruments Co., Ltd.), scanner, and ultrasonic disruptor (UP-250, Ningbo Scientz Biotechnology Co., Ltd.).

2 Experimental Methods

All yeast experiments must be performed under sterile conditions.

2.1 Yeast Transformation

1. Bait or prey clones for yeast transformation are generated via Gateway cloning (Thermo Fisher Scientific). Follow the manufacturer's instructions for detailed procedures.
2. Using a sterile toothpick, pick THY.AP4 and THY.AP5 yeast from -80°C stocks or pre-grown plates and inoculate into 5 mL YPD medium. Incubate at 28°C with shaking at 200 rpm overnight.
3. Combine overnight culture with 45 mL YPD medium and incubate at 28°C with shaking at 200 rpm for 3-5 hours until OD600 reaches 0.6-0.8.
4. Harvest cells by centrifugation (2000 g, 4°C, 10 min) and discard supernatant.
5. Wash pellet with 20 mL sterile water, centrifuge again (2000 g, 4°C, 10 min), and discard supernatant.
6. Resuspend cells in 1.8 mL 0.1 $mol \cdot L^{-1}$ LiAc, transfer to 2 mL microcentrifuge tube, centrifuge (2000 g, 4°C, 30 s), and discard supernatant.
7. Add appropriate volume of 0.1 $mol \cdot L^{-1}$ LiAc (20 L per transformation sample), incubate at room temperature for 30 min to prepare competent cells.
8. In a sterile PCR tube, add 10 L ssDNA solution and 5 L sample DNA (>100 $ng \cdot \ L^{-1}$).
9. Prepare mixture for each transformation: 70 L PEG3350 solution, 10 L 1 $mol \cdot L^{-1}$ LiAc, and 20 L competent yeast (from step 6).
10. Combine mixture with DNA from step 8.
11. Incubate in PCR thermal cycler at 30°C for 30 min, then heat shock at 43°C for 20 min.
12. Centrifuge yeast at 2000 g for 1 min and discard supernatant.
13. Wash yeast with 100 L sterile water, centrifuge and discard supernatant.
14. Resuspend yeast in 50 L sterile water and plate on appropriate selection media (THY.AP4 on CSM-LM solid medium, THY.AP5 on CSM-TUM

solid medium as shown in [Figure 1: see original paper]C).

15. Incubate at 28°C for 48-72 hours.

2.2 Yeast Mating and Selective Media Culture

1. Pick 5-15 THY.AP5 yeast colonies transformed with prey plasmid and inoculate into 3 mL CSM-TUM liquid medium. Grow overnight at 28°C with shaking at 200 rpm.
2. Pick 5-15 THY.AP4 yeast colonies transformed with bait plasmid and inoculate into 3 mL CSM-LM liquid medium. Grow overnight at 28°C with shaking at 200 rpm.
3. Reserve 750 L of overnight yeast culture, mix with 250 L 60% glycerol, snap-freeze in liquid nitrogen, and store at -80°C.
4. Harvest yeast cells by centrifugation at 2000 g for 1 min at room temperature and resuspend in YPD liquid medium (20 L per mating pair).
5. Mix 20 L of bait-containing and prey-containing yeast in a sterile PCR tube, spot 5 L of mixed yeast onto YPD plate, and incubate at 28°C overnight for mating.
6. Transfer mated yeast from YPD plate to CSM-LTUM plate (as shown in [Figure 1: see original paper]C) using a sterile toothpick and grow overnight at 28°C.
7. Pick mated yeast colonies from CSM-LTUM plate for each sample and inoculate into 3 mL CSM-LTUM liquid medium. Grow overnight at 28°C with shaking at 200 rpm.
8. Inoculate 1 mL of overnight mated yeast culture into 4 mL fresh CSM-LTUM liquid medium and grow at 28°C with shaking at 200 rpm for 4-6 hours before harvesting for Western blot analysis.
9. Measure OD600 of yeast cells from step 7.
10. Harvest 200 L of yeast culture from step 7 by centrifugation at 2000 g for 1 min at room temperature and dilute to OD600 1.0 and 0.1 in sterile water.
11. Spot 4 L of diluted yeast from step 10 onto CSM-LTUM and CSM-LTUMAH plates containing different methionine concentrations (0.5, 5, 50, 500 $\mathrm{mol} \cdot \mathrm{L}^{-1}$). Incubate at 28°C for 24-72 hours. Remove CSM-LTUM plates after 24 hours and CSM-LTUMAH plates after 72 hours to check yeast growth and document by scanner or camera.

2.3 Western Blot Analysis of Yeast Protein Expression

1. Measure OD600 of yeast culture obtained from step 8 in section 2.2.
2. Harvest 2 mL yeast culture by centrifugation and resuspend in 50-100 L sterile water based on OD600 measurement to normalize yeast concentration across samples (calculation: if yeast culture OD600 is 5 after 10-fold dilution, then original OD600 is 50; 2 mL culture yields a value of 100; to obtain final concentration of 100, resuspend pellet in 100 L sterile water).
3. Add equal volume of Lyse & Load buffer to yeast samples from step 2.

4. Lyse yeast thoroughly using ultrasonic disruptor (10% amplitude for 20 s, keep samples on ice).
5. Incubate at 65°C water bath for 30 min.
6. Centrifuge at 4000 g for 1 min at room temperature; supernatant can be used for Western blot analysis or stored at -20°C for 4-6 weeks.
7. Perform Western blot analysis following standard procedures.

3 Experimental Considerations

1. As shown in [Figure 1: see original paper], when constructing yeast bait or prey expression clones, note that Cub-PLV is fused to the C-terminus while NubG is fused to the N-terminus of the test proteins. Both Cub-PLV and NubG must reside in the cytoplasm.
2. Proteins smaller than 40 kDa can passively diffuse into the nucleus through nuclear pores, and some larger proteins may also enter the nucleus (Marfori et al., 2011). Therefore, as illustrated in [Figure 1: see original paper]A, to minimize background activation from bait fusion proteins entering the nucleus, the bait protein should ideally be a membrane protein. As shown in [Figure 1: see original paper]C, to enable soluble proteins as baits in mbSUS, the GPS system includes a GPI anchor peptide fused to the 5' end of the Gateway cassette in bait vectors, which effectively prevents bait proteins from entering the nucleus (Zhang et al., 2018). Although we have not observed non-specific interactions due to the GPI anchor in our experiments, this possibility cannot be completely excluded. Therefore, for membrane protein baits, we recommend using bait vectors without the GPI anchor peptide.
3. In the mbSUS system, bait protein expression is controlled by the met25 promoter, which is suppressed by methionine addition (Grefen et al., 2009). However, we have found that bait expression is also severely affected when methionine is completely omitted. Therefore, we recommend adding at least 0.5 $\mathrm{mol \cdot L^{-1}}$ methionine when preparing CSM-LTUMAH plates.
4. Before spotting yeast cultures onto plates, dry the plates in a laminar flow hood for 30 minutes to obtain circular yeast colonies.
5. We recommend pre-printing spotting patterns on paper (as shown in [Figure 1: see original paper]C) and placing it under the plate before spotting yeast cultures.
6. For Western blot analysis, overnight culture is not recommended. As described in step 8 of section 2.2, inoculating 1 mL culture into 4 mL fresh CSM-LTUM liquid medium and growing for 4-6 hours yields optimal results.
7. Yeast cultures collected according to step 3 in section 2.2 can be stored long-term at -80°C. Based on our experience, we recommend reactivating frozen cells once per year.
8. Based on our experience, when preparing yeast samples containing ion channel membrane proteins for Western blot analysis, avoid heat treat-

ment above 80°C.

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