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A NOVEL METHOD FOR INHIBITING TRANSCRIPTIONAL AUTOACTIVATION BY FUSION OF SRDX REPRESSION DOMAIN

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Abstract

Protein-protein interactions are fundamental components in the life activities of each cell. They play a pivotal role in various biological processes, including replication, transcription, translation, cell cycle regulation, and signal transduction. Distinct interaction networks are present in every species, individual, and cell. Various technical methods have been confirmed to map these interactions and to identify proteins that interact directly or indirectly. Yeast two-hybrid (Y2H) is an extensively employed system for determining the interaction sites or domains between two known proteins with physiological effects. However, the yeast dual hybrid method has certain limitations, as the autoactivation of bait proteins often leads to false positive outcomes. In this study, we optimized the assembly of bait proteins by introducing a transcriptional silencing motif (EAR inhibitory motif of SUPERMAN gene SRDX) to suppress the autoactivation. We selected five bait proteins with autoactivation activity, including ApGNAT12, ApCPP5, ApVOZ1, ApMYB2, and ApWRKY41. [Important findings] Notably, by introducing SRDX inhibitory motifs at the C-terminus of these proteins, the autoactivation activity of these proteins was effectively suppressed. In addition, we conducted a yeast two-hybrid library screening experiment coupled with high-throughput sequencing, using ApMYB2 as an example, and the outcomes revealed the reliability of this method. Together, our findings indicate that the inhibitory motif can effectively inhibit autoactivation in yeast two-hybrid systems, suggesting broad applications in the protein-protein interaction research.

Key words: Yeast two-hybrid; Autoactivation; SRDX; High-throughput sequencing

List of abbreviations and full name used in the article: BiFC: Bimolecular Fluorescence Complementation, TAP-MS: Tandem Affinity Purification-Mass Spectrometry, Y2H: Yeast two-Hybrid, BD: Binding Domain, 3-AT: 3-Aminotriazole EAR: Element Binding Factor-Associated Amphiphilic Repression, ERF: Ethylene-Responsive Element Binding Factors, C2H2: Cys2/His2-type, Aux/IAA: Auxin/Indoleacetic Acid, PEG: Polyethylene Glycol, AbA: Aureobasidin A, IPC: Inositol Phosphate Amide, PPI: Protein-protein interaction

Introduction

As the material basis of life, proteins play a critical role in all life activities (Liu *et al*., 2020). Proteins can operate alone or act as partners interacting with other proteins (Srihari *et al*., 2015; Alanis-Lobato *et al*., 2017). More commonly, proteins may function by forming functional complexes (Berggård *et al*., 2007; Wang *et al.*, 2020) to increase the variety of functional units present and enhance protein diversity (Miura, 2018). The interaction between proteins is essential for several cellular processes, encompassing signaling, molecular switching, ubiquitination, and so on (Kulandaisamy *et al.*, 2017). The protein-protein complexes are also vital for efficiently coordinating multiple enzymatic reactions into productive reaction pathways (Endutkin *et al.*, 2019). Consequently, alterations in protein-protein interactions can yield valuable insight into the cell biology principles and reveal the mechanisms of numerous fundamental biological processes, such as transcription, translation, and cell cycle (Low *et al.*, 2021). Therefore, understanding the interaction between proteins can help us better understand the life activity of cells and reveal the regulatory mechanism hidden beneath the surface.

Numerous methods have been proposed to detect the interactions between proteins, i.e., pull-down assays (Tinnefeld, 2011), co-immunoprecipitation (Das *et al.*, 2004), bimolecular fluorescence complementation (BiFC) (Miller *et al.*, 2015) and tandem affinity purification-mass spectrometry (TAP-MS) (Siva Sankar & Dengjel, 2021), and yeast two-hybrid (Y2H) (Paiano *et al.*, 2019). Among these various approaches, a two-hybrid assay is the most commonly used method to identify the interacting proteins *In vivo* (Gonzalez & Kann, 2012). Y2H screening is a well-established genetic approach, which has the advantage of being the most inexpensive, time-saving, and straightforward method (Berggård *et al.*, 2007; Paiano *et al.*, 2019). The establishment of an assay rests on the observation that eukaryotic transcription factors are modular in structure, often consisting of two or more

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functionally independent domains, such as the DNA-binding domain (BD) and the activation domain (AD), which recruits the transcriptional machinery promoting transcription. These two domains still function separately when they are separated, but cannot activate transcription. Only when the two separated proteins are in close proximity does gene expression occur. In the two-hybrid system, two plasmids are constructed. The former encodes the cDNA encoding the specific protein of interest fused with the BD domain, defined as "bait." The latter encodes the cDNA encoding the given protein fused with the AD domain, defined as "prey." The two-hybrid system has a unique advantage in detecting and analyzing protein-protein interactions. The method was simple to handle without the steps of recombinant production and subsequent purification of proteins and possessed high sensitivity. Most importantly, an *In vivo* assay more closely resembles cellular conditions. However, this approach has some limitations including the false positive and false negative results. Furthermore, two general strategies have been employed to address these issues: remove the transcription activation domain or add the appropriate concentration of 3-AT (3-Aminotriazole) to the medium (Lathouwers *et al.*, 2018). However, these solutions have created new problems. For example, removing the transcription activation domain may affect the detection of protein interactions. Meanwhile, an elevated concentration of 3-AT will result in a false negative result.

The ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif is found in members of the ethylene-responsive element binding factor, such as ethylene-responsive element binding factors (ERF), Cys2/His2-type (C2H2) and auxin/indoleacetic acid (Aux/IAA) (Kazan, 2006). The EAR motif from these proteins has two distinct conserved amino acid sequences: L/FDLNL /F(x)P and LxLxL (where X can be any amino acid). Proteins containing these motifs are ubiquitously distributed across the plant kingdom and function as critical regulators by negatively regulating genes involved in various biological processes (Kagale *et al.*, 2010). SRDX, of just 12 amino acids (LDLDLELRLGFA), is a common EAR motif in plants and suppresses the expression of target genes, even in the presence of redundant transcription factors (Hiratsu *et al.*, 2003). Chimeric transcriptional repressors that included the strong repression domain SRDX could suppress the expression of their target genes and cause loss-of-function phenotypes (Heyl *et al.*, 2008; Hwang *et al.*, 2019; Cen *et al.*, 2020; Li *et al.*, 2020). As the SRDX could convert the transcriptional activator into a potent repressor, we attempted to suppress the autoactivation of the bait protein by fusing them with the SRDX in the Y2H system. In this study, we developed a novel method by fusing an SRDX at the C-terminus of the target protein, yielding a dominant negative mutant capable of inhibiting the self-activation of the bait protein. Using this method, we chose 5 transcription factors, namely ApGNAT12, ApCPP5, ApVOZ1, ApMYB2, and ApWRKY41, which are involved in different physiological processes. Our findings demonstrated the efficacy of SRDX motif in reducing the “autoactivation” of bait protein. Our experiment has proved that this new approach, which offers numerous advantages including simplicity, reliability and efficiency, has broad applicability for solving the problem of the bait protein, may independently result in reporter gene activation.

Material and Methods

Construction of Bait Vectors The full-length ApGNAT12, ApCPP5, ApVOZ1, ApMYB2, and ApWRKY41 (Chen *et al.*, 2025) coding regions were cloned into the pGBKT7 vector at the *Nde*I (CATATG)-*Bam*HI (GGATCC) restriction sites respectively to generate the GAL4 DNA-BD fusion and verified by sequencing. SRDX sequences were added to the 5' and 3' ends of the amplified DNA fragments, respectively. Finally, these recombinant plasmids were verified by restriction digestion and sequencing.

Evaluation of autoactivation: A series of combined bait constructs were transformed into the Y2HGold strain. To detect the bait expression in the Y2HGold strain, the pGBKT7+Bait construct was transformed into the yeast by the high-efficiency polyethylene glycol (PEG)/LiAc-based method following the manufacturer’s instruction in Yeast Transformation System 2 Kit (Clontech, CodeNo. 630439). The transformants were spread and incubated on plates as follows: DDO: SD/-Trp/-Leu; TDO: SD/-Trp/-Leu/-His; QDO: SD/-Trp/-Leu/-His/-Ade for 3- 5 days. Yeast strain containing pGBKT7-53 in combination with the empty pGADT7-T vector was used as the positive control. At the same time, the yeast strain containing pGBKT7-lam in combination with the empty pGADT7-T vector was used as the negative control.

Plant materials and yeast library construction: Here, we take *Acer palmatum* as an example. The plant materials used in this study were collected in the test plots of Anhui Academy of Agricultural Sciences, Hefei City, Anhui Province, China (N 31.86°, E 117.27°). The leaves of *Acer palmatum* were put into liquid nitrogen (Yu *et al.*, 2025) for quick freezing and delivered to Zoonbio Biotechnology (Nanjing, China) to construct a Gateway system yeast library.

The transformation process of Y2H screening: To verify the effect of this method on the screening of interacting proteins, we chose to screen the interacting proteins of pGBKT7-ApMYB2 and pGBKT7-12AA-ApMYB2, respectively. pGBKT7-ApMYB2 (5 μg) and *Acer palmatum* yeast library (10 μg) were co-transformed into yeast strain Y2H using the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech, CodeNo.630489). Meanwhile, pGBKT7-12AA-ApMYB2 got the same treatment; the process details are described below.

The Y2H strain was spread on a solid YPDA culture medium and cultured at 30◦C for three days. One fresh, large (2–3 mm) colony was inoculated of the Y2H strain into 3 mL of YPDA liquid medium at 30℃ and 250 rpm. After eight hours, transfer 5 μL of the above culture

solution into 50 mL of liquid YPDA. Incubate shaking (250 rpm) at 30°C until the OD_{600} = 0.15-0.3. Centrifuge at 700×g for 5 min and discard the supernatant. The yeast was resuspended with 100 mL YPDA. Incubate shaking (250 rpm) at 30°C until the OD_{600} = 0.4-0.5. Centrifuge at 700×g for 5 min and discard the supernatant. The pellet was suspended in 60 mL of sterile water. Discard the supernatant after a third centrifugation (700 × g for 5 min). 3 mL of 1 × 1× TE/LiAc was added to each centrifuge tube. The solution was centrifuged at high speed (12,000 rpm) for 15 s, and the pellet was suspended in 600 µL 1× TE/LiAc. Combine 5 μg of pGBKT7-ApMYB2 plasmid, 10 μg of yeast pGADT7 library, and 5 μL of pre-denatured salmon sperm DNA in a sterile centrifuge tube and then add 300 µL 1× TE/LiAc and mix gently. The mixture was in a 30°C water bath for 30 min and vortexed gently every 10 min. Next, 20 μL of dimethyl sulfoxide (DMSO) was added, mixed, and heat-shocked with water bath heating (42℃ and 15 min) and vortexed gently every 5 min.

Selective medium screening by high-throughput sequencing: The transformed yeast solution was cultured in 0.2 mL YPD plus medium at 30°C for one hour, then the supernatant was discarded. Each precipitate was suspended in 100 µL 0.9% NaCl to achieve a final volume of 6 mL. From the mated culture, spread 100 µl dilutions on 100 mm SD/-Trp/-Leu agar plates. Plates were incubated at 30°C for 3–5 days. All colonies that grew on DDO were pooled into 3 mL of sterile water. PCR amplification was carried out with these samples as templates, and agarose gel electrophoresis was performed to detect the screening degree of bacterial liquids. PCR thermal cycling condition consisted of an initial denaturation step at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, extension at 72°C for 2 min, one cycle of final extension at 72°C for 5 min and then 4◦C indefinitely. Then the cDNA fragments were purified with MiniBEST DNA Fragment Purification Kit Ver. 4.0 (Takara), end-repaired, poly (A) added and ligated to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, PCR amplified, and sequenced using Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, CA, USA). The sequencing model X was paired-end (PE150), and the sequencing quantity was no less than 6 G.

Bioinformatics analysis: First, Cutadapt 2.10 was used to remove raw reads containing adapters or low-quality bases. The Trinity-v2.4.0 software package [10] with -min_glue 4 -min_kmer_cov 4 was used to de novo assemble the three sequencing libraries to obtain the gene sequence. The Trinity package align_and_estimate_abundance.pl was used to analyze the library's gene abundance with -est_method RSEM -aln_method bowtie2. GO enrichment analysis was performed (http://www.geneontology.org/). KEGG enrichment analysis was performed (http://www.genome.jp/kegg). The protein-protein interactions were visualized using Cytoscape (version 3.6.1).

Results

Construction of bait plasmid and testing its autoactivation: The expression plasmids designed for recombinant proteins (ApGNAT12, ApCPP5, ApVOZ1, ApMYB2, and ApWRKY41) were subjected to PCR amplification and subsequent sequencing to validate the outcomes. To test the auto-activation activity of these series of bait proteins, the pGADT7 and pGBKT7-bait plasmids were introduced into Y2HGold strain, and subsequently, the transformants were grown on SD/-Trp/-Leu (DDO), SD/-Trp/-Leu/-His (TDO) and SD/-Trp/-Leu/-His/-Ade (QDO) plates (Fig. 1). As a positive control, transformants containing pGBKT7-53 and pGADT7-T were grown on SD/-Trp/-Leu (DDO) and SD/-Trp/-Leu/-His/-Ade (QDO) plates. Transformants containing pGBKT7-lam and pGADT7-T were used as negative control. The results showed that the colonies containing pGADT7 and pGBKT7-bait plasmids were white on DDO, TDO, and QDO plates, which indicated that these recombinant bait plasmids had auto-activation activity. In light of these findings, these recombinant bait plasmids were suitable for the subsequent experiments.

SRDX-fusion method validation: Validation of the SRDX fusion system was performed using the five bait vectors (ApGNAT12, ApCPP5, ApVOZ1, ApMYB2, and ApWRKY41) described above. SRDX fragments were introduced to the N-terminus and C-terminus of the bait proteins, respectively, and then the auto-activation of these bait proteins was detected. The results showed that for ApGNAT12 and ApCPP5, the autoactivation of the recombinant bait plasmid was significantly inhibited by inserting SRDX fragments at both the C and N-terminus (Figs. 1A and 1B). For the ApVOZ1, ApMYB2, and ApWRKY41, inserting SRDX fragments at the C-terminus can reduce the dosage of 3-AT inhibitors and further eliminate false positives (Figs. 1C, 1D, and 1E). These findings collectively indicated that the C-terminal insertion of the SRDX fragment could effectively inhibit the auto-activation activity of the bait protein and SRDX fusion system can be used to eliminate the self-activation activity of bait proteins.

High-throughput sequencing and bioinformatics analysis: *Acer palmatum* ApMYB2 functions as a transcription factor with regulatory control over numerous target proteins. Here, to validate the efficacy of the novel method, we chose to screen the interacting proteins of pGBKT7-ApMYB2 (~with 40 mmol/L 3-AT) and pGBKT7-ApMYB2-12AA, respectively. Following an initial cultivation and nutrition-deficient selective culture, the interacting proteins were enriched by a liquid screening medium. Subsequently, high-throughput sequencing was employed to capture the gene sequences and abundance. The results unveiled that 213 pairs of high-confidence pGBKT7-ApMYB2 protein interactions were identified, while the pGBKT7-ApMYB2-12AA exhibited 187 pairs of high-confidence protein interactions. After comparing the high-confidence protein interaction results of these two pairs of interaction data sets, the 166 pairs of interaction were detected in both datasets (Fig. 2). Our findings further demonstrate that the validity and feasibility of our novel method.

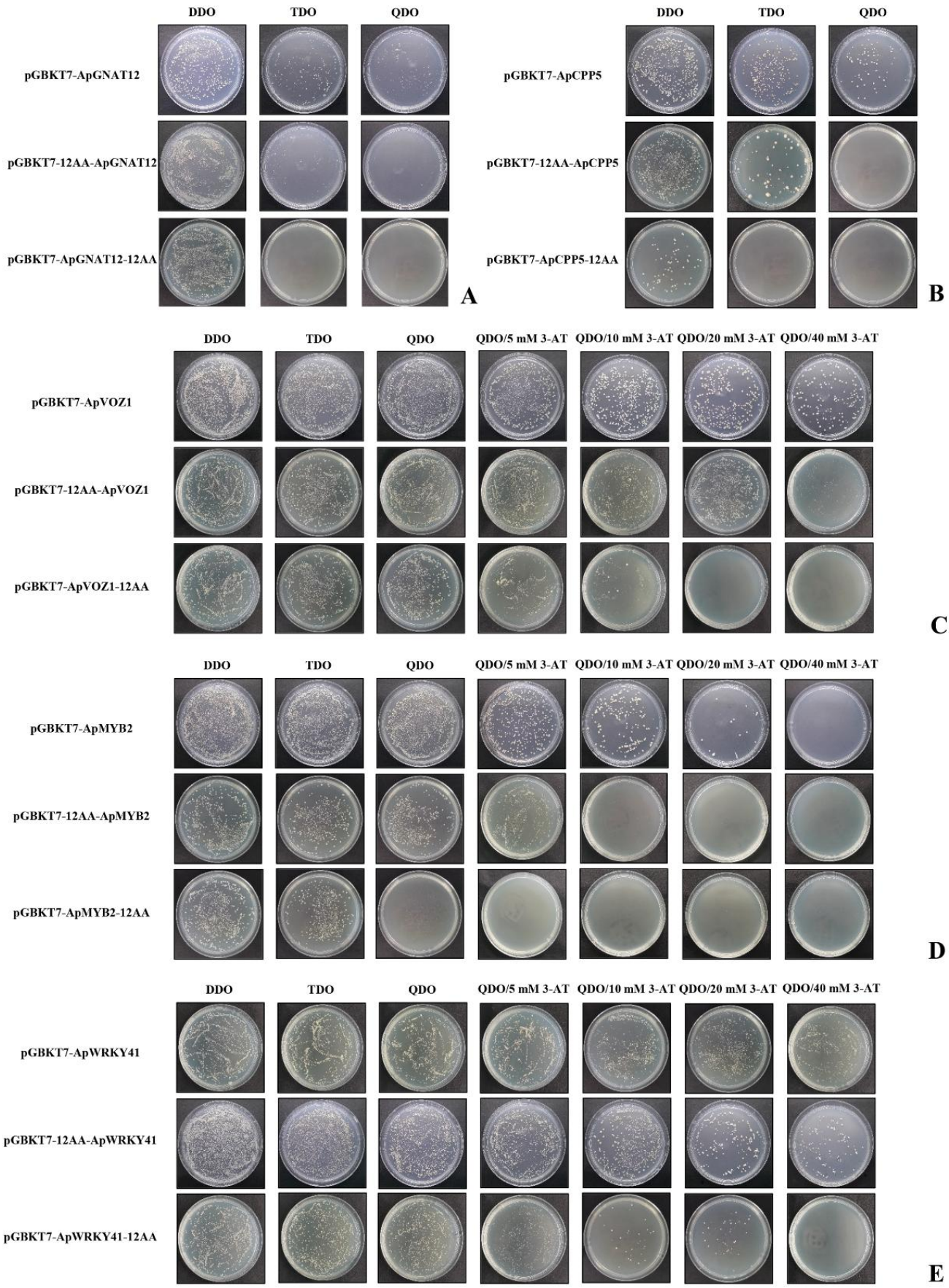

Fig. 1. Autoactivation detection for five transcription factors. (A). pGBKT7-ApGNAT12, pGBKT7-12AA-ApGNAT12, pGBKT7-ApGNAT12-12AA; (B). pGBKT7-ApCPP5, pGBKT7-12AA-ApCPP5, pGBKT7-ApCPP5-12AA; (C). pGBKT7-ApVOZ1, pGBKT7-12AA-ApVOZ1, pGBKT7-ApVOZ1-12AA; (D). pGBKT7-ApMYB2, pGBKT7-12AA-ApMYB2, pGBKT7-ApMYB2-12AA; (E). pGBKT7-ApWRKY41, pGBKT7-12AA-ApWRKY41, pGBKT7-ApWRKY41-12AA. DDO: SD/-Trp/-Leu; TDO: SD/-Trp/-Leu/-His; QDO: SD/-Trp/-Leu/-His/-Ade; 3-AT: 3-Aminotriazole.

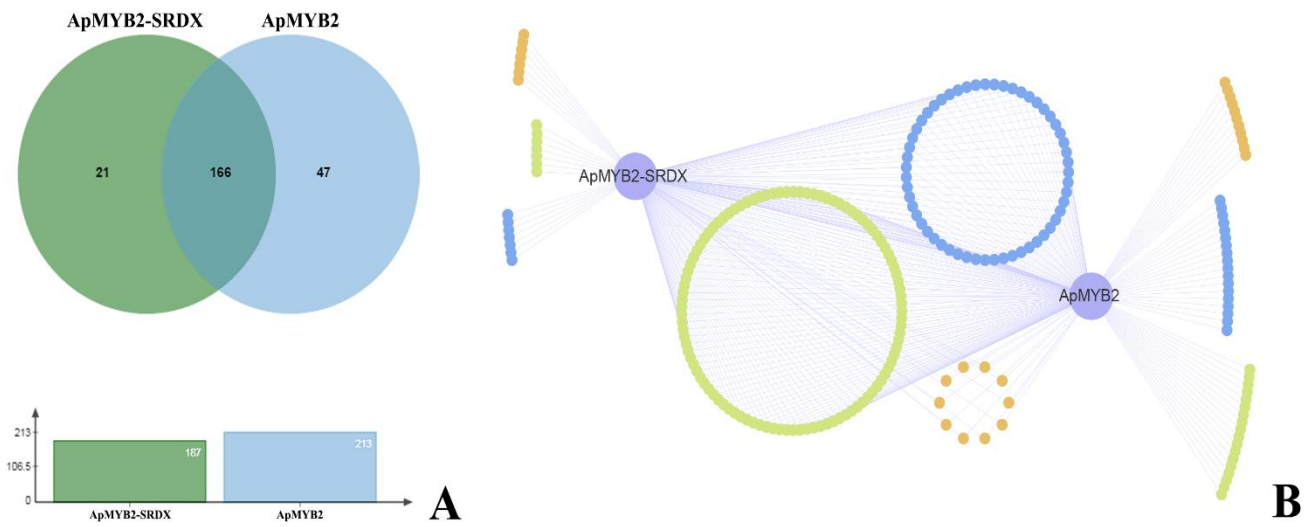

Fig. 2. The number of high-throughput sequencing for PCR amplification products of pGBKT7-ApMYB2 (~with 40 mmol/L 3-AT) and pGBKT7-ApMYB2-12AA (SRDX), respectively. (A) Venn diagram showing the number of enriched protein-coding genes of pGBKT7-ApMYB2 and pGBKT7-ApMYB2-12AA (SRDX). (B). The PPI network analysis. Purple nodes represent pGBKT7-ApMYB2 and pGBKT7-ApMYB2-12AA (SRDX); orange nodes represent cellular component proteins; green nodes represent biological process proteins; blue nodes represent molecular function proteins.

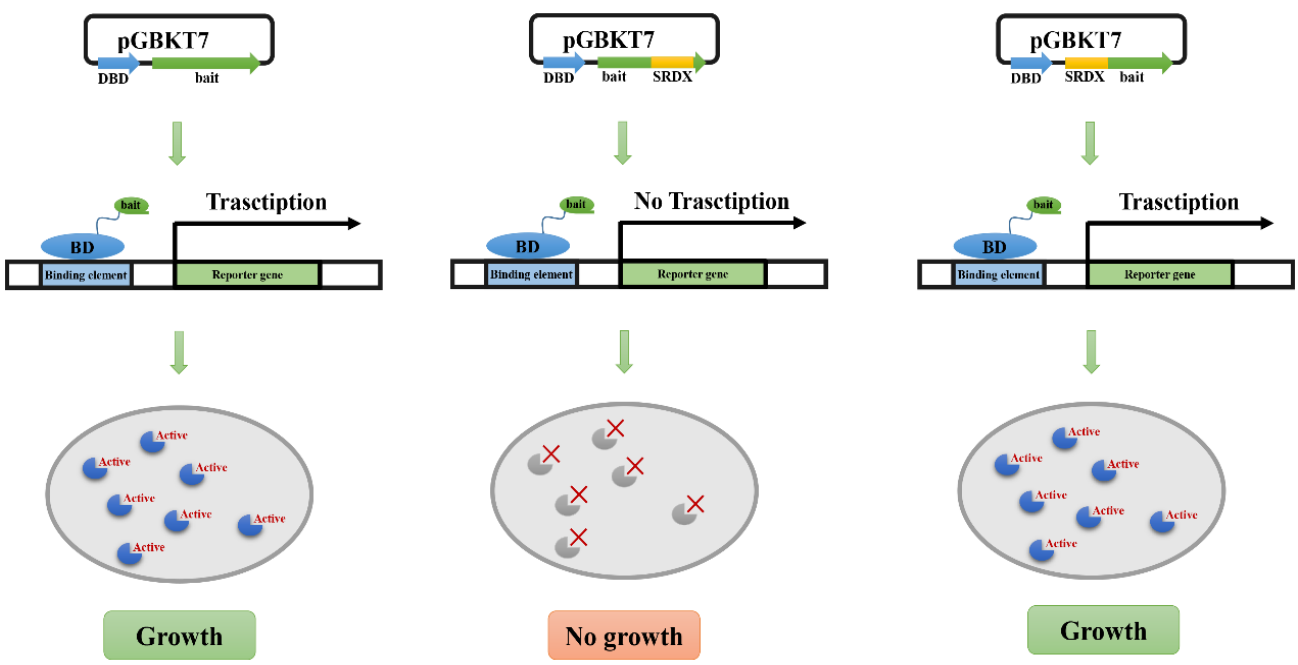

Fig. 3. The flow chart of the protocol.

Discussion

In the fundamental exploration and research of life sciences, Y2H technology has played a significant role in determining protein interactions and analyzing various mechanisms of life phenomena. It has always been highly favored as a classic protein interaction technology. Compared to conventional protein interaction detection techniques, the yeast two-hybrid system does not require antibodies, protein extraction and purification, and can detect protein interactions directly in living cells.

However, the yeast two-hybrid system also has certain limitations. The most common issue is false positives, possibly due to the autoactivation phenomenon of bait proteins (Yuan *et al.*, 2021). Some methods have been used to suppress the "autoactivation", for instance, adding Aureobasidin A (AbA) (Yu *et al.*, 2016) or 3-Amino-1,2,4-triazole (3-AT) (Zhou *et al.*, 2016; Gu *et al.*, 2019). The mechanism of action of AbA is to inhibit the activity of inositol phosphate amide (IPC) synthase encoded by the *AUR1* gene (1, 2) in yeast, interfering with sphingolipid synthesis and thereby preventing cell survival. Meanwhile, using AbA can inhibit the "autoactivation" of reporter gene AbAr within a specific range. 3-AT is a competitive inhibition of histidine, inhibiting the leakage expression and slight "autoactivation" of HIS3. However, both need to explore appropriate concentrations.

Transcription factors play a crucial role in regulating gene expression (Deng *et al.*, 2022). Transcription factors generally consist of 4 functional regions: DNA binding

region, transcriptional regulation region (including activation region or inhibition region), oligomerization site, and nuclear localization signal (Liu *et al.*, 1999). Two types of transcriptional suppressors are present in eukaryotes: active suppressor proteins and passive suppressor proteins (Thiel *et al.*, 2004). How active suppressor proteins contribute to the complex process of regulation of transcription generally involves the interaction of the activation 'domain'. Multiple inhibitory domains have been discovered in plants, among which the most common is the EAR domain (Dong & Liu, 2010; Liu *et al.*, 2021). The EAR motif has highly conserved amino acid sequences, namely L/FDLNL/F(x)P and LxLxL (Chow *et al.*, 2023). Transcription factors containing EAR motifs, such as members of the ERF, C2H2 (Xu *et al.*, 2020), and AUX/IAA (Choi, *et al.*, 2018; Tao & Estelle, 2018) transcription factor family, can negatively regulate the expression of genes related to plant growth, development, and stress response, enabling plants to maintain stable physiological activity in different environments. The plant-specific, ERF-related repressor domain SRDX can convert transcription activating factors into strong repressors. In Arabidopsis, the chimeric protein resulting from fusion of SRDX and GAL4-BD exhibited repressive activity (Yamaguchi *et al.*, 2010). In maize, the use of transcriptional inhibition domain SRDX fusion to inhibit ZmMs7 transcription can significantly inhibit the development of maize anthers and pollen, leading to complete male infertility (An *et al.*, 2020). The expression of the SCR-SRDX gene in *M. truncatula*, *L. japonicus,* and *G.* max can reduce the formation of root nodules (Dong *et al.*, 2021).

The function of the ERF domain in active inhibition inspired us to incorporate a common EAR motif, SRDX, into the construction of bait proteins to suppress “autoactivation.” Therefore, we fused the SRDX domain (LDLDLELRLGFA) into 5 distinct transcription factors with “autoactivation”. Our findings confirmed that the "autoactivation" phenomenon could be significantly inhibited, and the amount of 3-AT used was significantly reduced, proving that the fusion of this domain at the C-terminus of the target protein could play an "autoactivation" inhibitory function (Fig. 3). Subsequently, the screening results of ApMYB2 screening were confirmed through high-throughput sequencing, and the reliability of the method was validated. The outcomes indicate that our approach can obtain the most potential Protein-protein interaction (PPI) in the target library compared to conventional techniques. This approach can effectively reduce experimental steps and workload, which has significant scientific and practical benefits for investigating transcription factor function and gene expression regulation.

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Conflict of Interests: The authors declare that they have no conflict of interest.

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