Research Square

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Identification of genes directly regulated by a transcription factor in rice

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Method Article

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Abstract

A protocol is described for identification of gene \(promoter) targets regulated by an AP2/ERF transcription factor \(TF) in rice. A tandem affinity purification TAP-tagged construct of the AP2 TF transformed into rice is used in chromatin immunoprecipitation \(ChIP) assays to verify the putative AP2-regulated genes that are first identified from gene expression data and analysis of cis-regulatory elements in their promoters. Transcriptional activation/repression of the AP2 TF bound promoters is experimentally validated by a Dual Glo luciferase assay system \(firefly luciferase luminescence normalized to renilla luciferase) using putative regulated promoters cloned upstream of the luciferase and co-transformed with a construct expressing the AP2 TF in rice protoplasts. Finally, the protocol describes the identification of the direct targets identified from ChIP and luciferase assay using a steroid receptor based inducible system.

Introduction

Environmental stresses like drought and high temperature primarily affect the grain yield of cereals by reducing photosynthesis and related carbon metabolism[1]. A comprehensive analysis of the gene network regulating these complex processes will help in development of genetic tools for maintaining yield under stress. The expression of stress related genes is largely affected by transcription factors like AP2, MYB, NAC, bZIP etc. The APETALA2 \(AP2) family of transcription factors are associated with various developmental and stress related responses in plants and has a highly conserved DNA binding AP2/ERF domain that recognize conserved cis-regulatory elements \(CRE) of the GCC core[2]. The presence of conserved DNA binding domain in transcription factors therefore can be used for identification of potential regulated genes by both in vivo and in planta assays. Many DNA protein interaction studies have been previously done using in vitro assays such as electrophoretic mobility shift assay \(EMSA). However, chromatin immuno-precipitation \(ChIP) has now become a powerful tool to detect in vivo interactions between DNA-binding proteins and genomic DNA. ChIP DNA can be combined with RNA-seq or massively parallel sequencing to identify gene regulatory networks active during the biological processes of grain filling and/or in response to the environmental stresses. The ChIP procedure includes: \(1) Crosslinking of DNA with protein in vivo using formaldehyde; \(2) Sonication of the fixed chromatin to an average size of 500 bp ranging from 200 to 1000 bp; \(3) Extraction of chromatin using different buffers; \(4) Immunoprecipitation with specific antibodies; \(5) Removal of beads, reverse crosslinking, elution of DNA and purification; \(6) qPCR analysis of the bound DNA relative to positive and negative controls. However the major disadvantage of ChIP technology is the requirement of antibodies specific to the protein of interest. Here we report the use of TAP \(tandem affinity purification) tagged system which has previously been used for identification of protein interaction partners[3,4] for identification of targets of transcription factors \(DNA-protein interaction) in rice, making use of the commonly used His and myc antibodies. Furthermore, to elucidate the role of TFs in directly activating its targets in the transcription complex, we report here on the use of the steroid receptor-based inducible system. The TF is fused with a human estradiol receptor \(HER) which renders the transcription factor inactive in the cell as it is

sequestered in the cytoplasm via binding to a cytoplasmic complex. In presence of estrogen \(estradiol), the complex is disrupted owing to the binding of the hormone to the steroid-binding domain thereby allowing the transcription factor to enter the nucleus and regulate the expression of the downstream target genes[5]. In the presence of cycloheximide \(translation inhibitor) only the targeted transcription factor and its downstream target genes will be induced while the other downstream genes will not be induced owing to lack of protein synthesis, thereby making this steroid-inducible system a potential tool for identification direct targets of transcription factors[6,7]. The present study describes the protocol for identification of targets of an AP2 transcription factor HYR in rice followed by validation using a luciferase assay with the Dual Glo luciferase assay system \(firefly luciferase luminescence normalized to renilla luciferase). Finally, the protocol describes the identification of the direct targets identified from ChIP and luciferase assay using a steroid receptor based inducible system.

Reagents

Enzymes used for cloning purposes XbaI, BamHI, KpnI, SacI, EcoRI BP and LR clonase \(Invitrogen) **ChIP Antibodies** anti-His \(R932-25, Invitrogen) anti-MYC \(AHO0062, Invitrogen) anti-Histone H3 conjugate \(nonspecific Ab, AHO1432, Invitrogen) Alkaline Phosphatase conjugated secondary Antibody \(WP20006, Invitrogen) **Protein purification/analysis** Ni-NTA agarose \(Invitrogen) Biotin 3’ End DNA Labeling Kit \(Pierce) Nylon membrane \(Biodyne) Light Shift Chemiluminescent EMSA kit **Luciferase analysis** Trizol \(Invitrogen) Reverse Trasncription System \(Promega, A3500) qPCR master mix \(A6001) Dual-Glo® Luciferase Assay System \(Promega, E2940) **Plasmids Used** pGL3-Basic \(Promega U47295) pGL4 70\[hRluc] Vector \(Promega, E688A) HER clone \(Fisher scientific)

Equipment

Bench top centrifuge \(Eppendorf 5430) Biorad Mini Protean System Cyclomixer GelDoc It^{TS2} Imager \(UVP) Incubator \(America Scientific Products, C144) Laminar Flow Hood \(Forma Scientific) Luminometer \(Promega, Glomax 20/20) Multiporator \(Eppendorf) PCR machine \(Biorad, C1000 touch thermal cycler) qPCR machine \(Biorad, CFX96) QSonica \(Sonicator) Refrigerated centrifuge \(Beckman, Allegra 25R) Tissue Lyser \(Retch, MM200) Water bath \(Precision) Water Purifying system \(Milipore, Synergy UV)

Procedure

A. Construct Preparation 1. A tandem affinity protein \(TAP) tagged HYR construct was made in pUC19 assembling the fragments of CaMV35S promoter[8] at XbaI and BamHI sites, gateway cassette \(for cloning gene of interest) is cloned between BamHI/KpnI sites, coding sequence for six His repeat \(6xHis), a 9x-myc peptide, a 3C protease cleavage site and two copies of the IgG binding domain \(2xIgG-BD) together known as TAP tag were amplified using pCTAPa \(CD3-679) as template at KpnI/ SacI sites[3,4] and the NOS terminator at KpnI/EcoRI sites. 2. The entire cassette was cloned between the Xba1-

EcoR1 sites of pMOG22 \(Zeneca-Mogen), which contains a chimeric CaMV 35S hygromycin phosphotransferase-tNos for selection during rice transformation. **B. Promoter Analysis** 1. To identify potential cis-acting regulatory elements \(CAREs) involved in HYR \(target genes of interest) regulation of gene expression \(Fig. 1a), use FIRE [9] for de novo discovery of short DNA motifs specific to genes differentially regulated upon HYR \(target genes of interest) expression by comparing the motif content of 1 Kb upstream sequences of these genes to that of the rest of the genome, followed by comparison to known cis-elements[10]. 2. To identify the potential candidate genes regulated by HYR \(target genes of interest) for experimental validation, identify genes \(in HYR lines or transgenic or mutant plants with target genes of interest) that were also differentially expressed under drought[11] or high temperature[12] \(condition of interest). Analyze the promoter sequences \(1.0 kb) for these genes using Place database[10] and Plant Care[13] to look for the presence of cis elements. 3. HYR is an AP2 transcription factor that recognizes the GCC core \(GCCGCC), promoter sequences for the putative HYR-regulated genes were scanned by using FIRE and Place database to look for the presence of the GCC core, and similarly for canonical cis-elements recognized by the other TFs analyzed. Note: Recognition sequence is specific to the group or family of transcription factor being analyzed. **C. Chromatin Immunoprecipitation** **Crosslinking Buffer:** Prepare by mixing 3.4g of sucrose \(0.1M), 1 ml sodium phosphate buffer \(10 mM, pH7), 1 ml NaCl \(50 mM), volume made upto 100 ml. Autoclave and store on ice before use. **Buffer A:** Prepare by mixing 250 µl sodium phosphate buffer \(10 mM, pH7), 500 µl NaCl \(100 mM), 3.2 ml of 2-methyl 2.4 pentanediol and 17.7 µl of β-mercaptoethanol, volume made upto 25 ml. Add ½ tablet protease inhibitor cocktail and store at $4^{o}C$ for further use. **Buffer B:** Prepare by mixing 250 µl sodium phosphate buffer \(10 mM, pH7), 500 µl NaCl \(100 mM), 250 µl MgCl-2- \(10mM), 625 µl Triton-X-100 \(0.5%), 3.2 ml of 2-methyl 2.4 pentanediol and 17.7 µl of β-mercaptoethanol, volume made upto 25 ml. Add ½ tablet protease inhibitor cocktail and store at $4^{o}C$ for further use. **Buffer C:** Prepare by mixing 250 µl sodium phosphate buffer \(10 mM, pH7), 500 µl NaCl \(100 mM), and 17.7 µl of β-mercaptoethanol, volume made upto 25 ml. Add ½ tablet protease inhibitor cocktail and store at $4^{o}C$ for further use. **Sonic Buffer:** Prepare by mixing 250 µl sodium phosphate buffer \(10 mM, pH7), 500 µl NaCl \(100 mM), 50 µl EDTA \(10 mM) and 125 mg sarkosyl \(0.5%) volume made upto 25 ml. Filter sterilize. Add ½ tablet protease inhibitor cocktail \(fresh) and store at $4^{o}C$ for further use. **IP Buffer:** Prepare by mixing 2.5 ml HEPES buffer \(50 mM, pH 7.5), 750 µl NaCl \(150 mM), 125 µl MgCl-2- \(5 mM), 2.5 µl ZnSO-4- \(10 µM), 1.25 ml Triton-X-100 \(1%) and 125 µl SDS \(0.05%). Filter sterilize and store at $-2^{o}C$ in aliquots for further use. **Elution Buffer:** Prepare by mixing 2 ml glycine \(0.1M), 2.5 ml NaCl \(0.5M), 12.5 µl Tween 20 \(0.05%) volume made upto 25 ml. Filter sterilize and store at $4^{o}C$ for further use. **Chromatin crosslinking** **\(Fig 1b)** 1. Rice seedlings \(5g) from HYR:TAP tagged lines were placed in Falcon tubes \(50ml) and rinsed thoroughly with Millipore water. 2. Crosslink rice tissues in crosslinking buffer containing 2% formaldehyde on ice under vacuum in an exicator until air bubbles appear on the sides of the tubes. Release the vacuum after 15 min. Mix well and apply vacuum for another 25 min. 3. Stop fixation by adding 3 ml glycine \(2.5M) and apply vacuum for another 5 min. 4. Wash cross-linked leaf samples three times with buffer A \(30 ml) and then dry on filter paper followed by quick freeze in liquid nitrogen, Note: Pause point: Samples can be stored at $-80^{o}C$ for further use. **Nuclei

Isolation and shearing of chromatin** 5. Homogenize frozen tissue thoroughly in liquid Nitrogen in mortar and pestle and transferred to tubes with 10 ml buffer B. 6. Filter the slurry through three layers of cheese cloth and collect in 50 ml tube kept on ice followed by two washes with buffer A to collect all the nuclei. 7. Centrifuge the filtrate at 3500 rpm for 20 min at 4°C. 8. Wash nuclear pellet thrice with 5 ml of buffer C and centrifuge at 3500 rpm for 20 min at 4°C 9. Wash the pellet once with buffer D and centrifuge at 3500 rpm for 20 min at 4°C. 10. Resuspend the pellet in 1 ml sonic buffer and transfer to 2 ml safelock Eppendorf tubes. 11. Sonicate the chromatin for 5 cycles: 30 seconds ON and 1 minute OFF for each cycle on full power. Note: 15 µl for DNA is kept to check DNA extraction. 12. Centrifuge the suspension at 14000 rpm for 15 min at 4°C. Collect the supernatant and centrifuge again at top speed for 5 min. Collect the supernatant and transfer to new Eppendorf tubes. **Preclearing** 13. Transfer the supernatant to new tubes with equal volume of IP buffer. Keep 100 µl of the mix aside as control to check the degree of sonication. 14. Centrifuge the precleared chromatin at 14000 rpm for 15 min at 4°C. Collect the supernatant followed by incubation with 100 µl Protein A agarose for 1 hr 30 min \(prolonged incubation is required if pre-immune serum is not available). 15. Centrifuge at 4000 rpm for 5 min at 4°C. Collect the supernatant and centrifuge again at top speed for 10 min. 16. Divide supernatant into three parts : i) one that serves as negative control \(Histone Ab) ii) the other two are samples \(immunoprecipitated with 6X His and Myc antibody). 17. Incubate IP samples with 10 µl of antibody \(1.5-2µg) for 10 h at 4°C on a rotating wheel. 18. Centrifuge samples at 12000 rpm for 5 min at 4°C to remove the debris and collect supernatant into 2 ml safelock tubes. 19. Add 50 µl Protein A agarose to the IP samples and incubate at 4°C for 2hr on a rotating wheel. 20. Centrifuge at 5000 rpm for 5 min. Discard supernatant and retain pellet \(beads) for further use. 21. Wash the beads five times with 5 ml IP buffer at room temperature on a rotating wheel. 22. Elute Protein-DNA complexes by using 100 µl cold elution buffer, incubate for 5 min at 37°C. Centrifuge at top speed for 2 min. Collect supernatant and neutralize with 50 µl Tris-Cl \(pH-9) to neutralize. Repeat elution steps twice. **Reverse Crosslinking and DNA purification** 23. Add Proteinase K \(11.25 µl of 20 $mgml^{-1}$ stock) and incubate at 37°C for overnight with constant shaking on a rotating wheel. Proceed with Input DNA sample \(obtained from step 13) simultaneously from step 23 \(Add 330 µl of TE to reach the same volume as IP sample). 24. Add Proteinase K \(second aliquot) and incubate at 65°C overnight to allow reverse crosslinking. 25. Precipitate DNA by adding 1/10th volume sodium-acetate \(pH 5.2), 2.5 volume of ethanol \(100%) and 1 µl of glycogen overnight at -20°C. 26. Centrifuge at top speed for 15 min at 4°C. Resuspend DNA in 100 µl of Millipore water. 27. Purify DNA using PCR purification columns and elute in 35µl Millipore water. Note: Samples can be stored at -80°C for further use. 28. Carry out qPCR to test for enrichment of target and control primers for both positive and negative IP samples. 29. Calculate the fold enrichment for the His and MYC antibodies against the nonspecific antibody using the formula: Ct \(Target) - Ct \(Non Specific Ab) = dCt[16]. Normalize against the calibrator using the equation \($2^{-\Delta\Delta Ct}$)[11]. Represent fold enrichment of target genes as a mean of three replicates and the statistical significance of the variation over WT and transgenic can be tested by both sided t-test at $p \leq 0.01$. **D. HYR protein purification from bacteria** **PBS buffer** Prepare by mixing: 137 mM NaCl, 2.7mM KCl, 10 mM Na-2-HPO-4-, 2 mM KH-2-PO-4- and volume made upto 100 ml with dd H-2-O. Autoclaved and stored at room temperature for future use. 1.

Amplify the HYR coding region by PCR using forward and reverse primer containing attB1 and attB2 sites respectively. 2. Clone PCR purified HYR \(target genes) and clone into pDEST42 \(C-6X-His tag Gateway expression vector of choice, 12276-010) vector using the Gateway cloning strategy \(BP and LR reaction system, Invitrogen) and transform into E. coli strain BL-21. 3. Induce recombinant protein for 4 hr using 1mM IPTG . 4. Pellet down cells and resuspend in PBS buffer. Add lysozyme \(4mg/ml) and incubate for 30 min in ice. Sonicate for 3 min \(50% duty cycle) with pulses of 15s. Note: Care should be taken that the solution is very clean and not gelatinous. 4. Pellet down cells by centrifugation at 12,000 rpm, 10 min 4°C. Collect the supernatant and use for protein purification using Ni-NTA agarose \(Invitrogen). 5. Confirm identity of the proteins by immunoblotting using His-tag antibody or any antibody of choice \(1:1000). **E. Electrophoretic Mobility Shift Assay \(EMSA)** 1. Design specific sets of primers for 250 bp spanning the binding sites in the promoter region of target genes of HYR and its downstream genes \(**Fig 1a**), and amplify the fragments using genomic DNA of Nipponbare rice as template. 2. Gel purify the amplified promoter fragments and label using Biotin 3’ End DNA Labeling Kit \(Pierce). 3. Perform the binding reactions and the electrophoretic mobility shift assay according to the manufacturer’s instructions \(Light Shift Chemiluminescent EMSA kit) 4. Incubate the binding reactions for 10 min. on ice after adding 100 fold excess of unlabeled competitor DNA followed by the addition of labeled DNA and further incubate for 20 min. on ice before loading on to 5% native polyacrylamide gel. 5. Electroblot the resolved DNA protein complexes onto Nylon membrane \(Biodyne) and detect gel-shift using the chemiluminescent nucleic acid detection kit. **F. Transactivation Analysis using Dual Glo Luciferase Assay in Protoplasts** **Buffer A** Prepare by mixing 0.6M mannitol, 10 mM MES \[pH 5.7], 10 mM CaCl2 and the volume made upto 100 ml with water. Autoclave and store at room temperature. **Buffer B** Prepare by mixing 0.6 M mannitol, 10 mM MES \(pH 5.7), 1.5% Cellulase \(C1794, Sigma) 0.75% Pectinase \(P2401, Sigma) and warm to 55°C to inactivate DNAse and proteases to enhance solubility. Cooled to room temperature, then 10mM CaCl2, 2.5 mM β-mercaptoethanol and add 0.1 % BSA and store for further use. **Buffer C** Prepare by mixing 154 mM NaCl, 125 mM CaCl2, 5 mM KCl and 2 mM MES \(pH 5.7) and volume made upto 100ml with Millipore water. Autoclave and stored for further use. **MMG solution** Prepare by mixing 0.4 M mannitol, 15 mM MgCl2 and 4 mM MES \(pH 5.7) and volume made upto 25ml with Millipore water. Autoclave and stored for further use. **Buffer D** Prepare by mixing 0.5 M mannitol, 20 mM KCl and 4 mM MES \(pH 5.7) and volume made upto 25ml with Millipore water. Autoclave and store for further use. **Isolation of Protoplasts from rice seedlings** Protoplasts were isolated from rice cultivar Nipponbare[17,18] with modifications. 1. Dehull rice seeds \(Nipponbare) and sterilize with 75% ethanol for 30s followed by sterilization in 75% bleach for 30 min with continuous shaking. 2. Wash Sterilized seeds three times with sterile water followed by one wash with MS liquid medium. 3. Incubate the seeds in ¼th MS medium \(0.8% Gellan gum) at 28°C for 15 days with a photoperiod of 12 h light \(about 150 μmol m^{-2} s^{-1}) and 12 h dark. 4. Rice plants \(40-50 seedlings) were cut together into strips of 0.5-1mm length using a sharp razor blade and immediately put into 20ml buffer A for 10 min at 28˚C with shaking \(60-80 rpm) in dark. Note: Care should be taken that leaf tips are not damaged during cutting. 5. Discard mannitol and immediately incubate the rice samples in 20ml; buffer B for 6 hr in the dark at 28°C with constant shaking \(60-80 rpm). Note: The enzyme solution should turn green indicating the release of protoplasts. 6. Dilute the enzyme solution with equal volume of buffer C to

remove undigested protoplasts before filtration. 7. Filter the enzyme/protoplast solution through three layers of cheese cloth pre-wetted with buffer C. Wash leaf tissues twice with buffer C and then pool the entire filtrate. 8. Centrifuge the filtered solution at 2500 rpm for 5 min in a swing bucket rotor to collect the protoplast at the bottom of the tube. Wash the protoplast after centrifugation once with buffer C. 9. Resuspend the protoplast in MMG solution at a concentration of 2×10^{6} cells mL^{-1} determined by using a haemocytometer. **Transfection of protoplast by electroporation** **\(Fig 1c)** 10. Incubate 300μl of freshly prepared protoplast with 15μg of plasmid DNA in microcentifuge tube. Mix gently by tapping and incubate for 10 min at room temperature. 11. Carry out electroporation using Multiporator \(Eppendorf) with conditions: 5 msec, 400 V and 200 μF and 2 pulses. Carrier DNA is not required. Note 2-3 pulses are required if a high transformation efficiency is required. 12. Incubate the protoplast-DNA solution after electroporation in ice for 10 min. Dilute the protoplast-DNA solution with buffer C and then pellet by centrifugation at 3500 rpm for 5 min. 13. Resuspend the protoplasts gently in 1 mL buffer D \(0.5 M mannitol, 20 mM KCl and 4 mM MES at pH 5.7) and incubate O/N at 28°C **Protoplast Culture and Harvest** 14. After the desired period of incubation, harvest protoplasts by centrifugation at 2000 rpm for 5 min. 15. Discard the supernatant and store the pelleted protoplasts at -80°C for further use. **Transactivation Analysis by using Dual Glo Luciferase Assay** 16. Incubate 300μl of protoplast with reporter and effector constructs respectively \(15μg of plasmid DNA) along with control plasmid \(Renilla luciferase driven by constitutive promoter). Reporter Plasmid: Promoter of interest driving firefly luciferase gene. Effector Plasmid: Constitutive promoter driving the gene of interest. Note: One tube containing only protoplast will serve as control. Only reporter and control to show that there is no luciferase activity coming from reporter construct. 17. Continue from step 11. 18. Incubate the pelleted protoplasts with 100μl of protoplast lysis buffer and mix thoroughly by vortexing for 5s and then incubate on ice for 5 min. 19. Pellet cells by centrifugation at 12,000 rpm for 5 min. Collect supernatant for luciferase assay. 20. Mix 75μl of supernatant with equal volume of LAR reagent \(prepared again to Promega technical manual). Measure luminescence after 10 min in Glomax 20/20 luminometer \(Promega). Stop reaction by adding Dual Glo Stop and Glo solution and measure luminescence due to Renilla luciferase same order as firefly luminescence was measured[20]. 21. Results are expressed normalized to the Renilla luminescence. **Identification of direct targets using steroid receptor-based inducible system** 22. Mix freshly prepared protoplast \(step 9) with 15μg plasmid \(construct containing the gene of interest fused with the HER[21, 22] \[Human estrogen receptor]). 23. After electroporation incubate protoplasts with 2 μM estradiol for 6 h to induce the activity of the respective genes. The same concentration of ethanol used to dissolve estradiol was applied to the control protoplasts as the mock treatment. Where indicated, protoplasts were treated with cycloheximide \(2 μM) 30 min before addition of estradiol to inhibit new protein synthesis **\(Fig 1d)**. 24. Continue to step 12 25. Resuspend pelleted protoplasts in buffer D and incubated for 6 h. Following incubation, harvest the cells and stored at -80°C for further analysis. 26. Isolate total RNA using Trizol method \(Invitrogen, technical manual) 27. Synthesize cDNA using 1μg of total RNA \(DNase treated) and analyze expression of downstream target genes by qRT-PCR. 28. The gene expression is represented as fold change after estradiol treatments over control \(mock treatment with ethanol) while for cycloheximide and estradiol \(CHX+E) it is represented as fold change over

protoplasts treated with CHX alone. **Statistical Analysis of data** 29. Express fold change of target genes as a mean of three replicates and the statistical significance of the variation over WT and transgenic \(control protoplasts and electroporated) can be tested by the 2-sided t-test at p≤0.01.
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Timing

2 weeks

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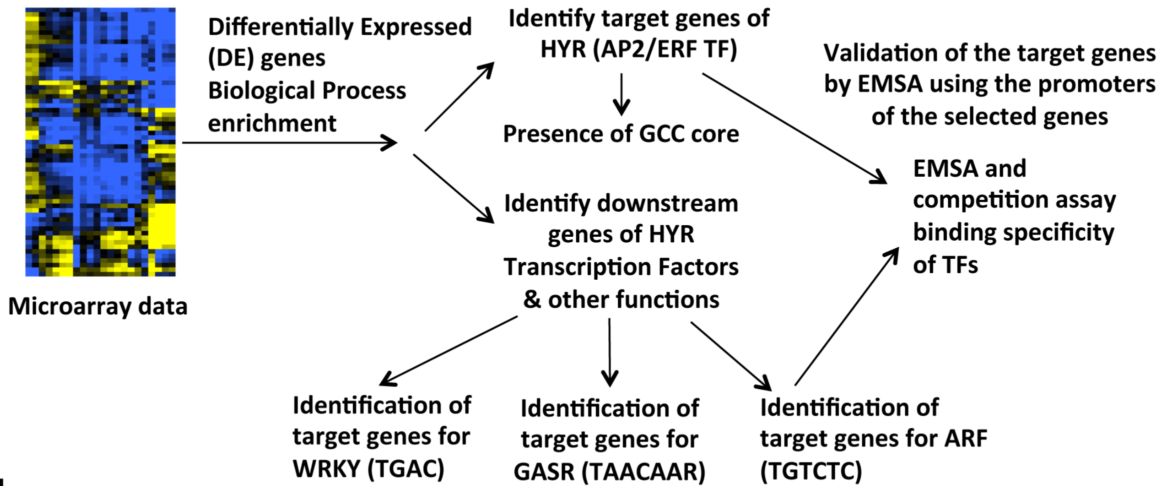
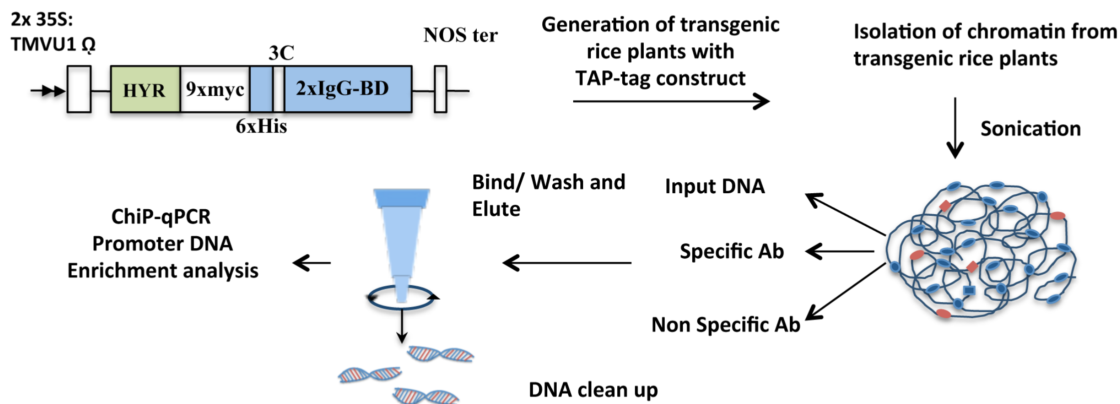
Figures

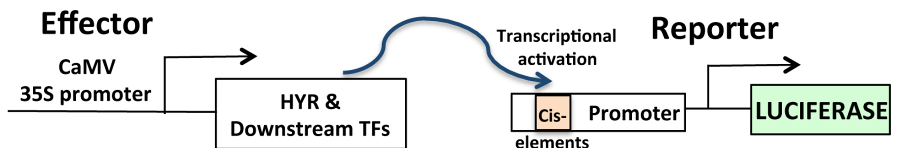
a Selection of HYR target genes based on differential expression in microarray and promoter sequences



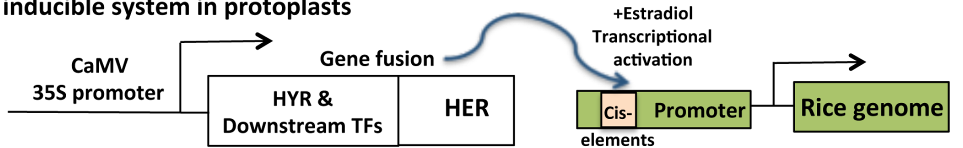
b HYR Chromatin Immunoprecipitation HYR ChIP to prove binding to specific promoters



c Transactivation assays based on the binding of TFs to the cis-element recognition sequence in promoters of downstream target genes and regulating their expression



d Direct activation of genes in rice genome employing estrogen receptor based steroid inducible system in protoplasts



HER – Human Estrogen Receptor + steroid Estradiol -> enters nucleus and activates transcription
In presence of cycloheximide shows no other protein synthesis required,
only steroid induced HER fusion transfer to nucleus and transcriptional activation of plant gene promoter

Figure 1

Strategy to show transcriptional regulatory functions of HYR and other TFs.