Gold yeast one hybrid system for screening the stem-differentiation xylem expressed transcription factors and promoters of secondary cell wall biosynthesis genes

1.Introduction

Secondary cell walls, that usually are abundant in xylem, are composed of cellulose, hemicelluloses and lignin and account for the bulk of plant biomass. The coordination in transcriptional regulation of synthesis for each polymer is complex and vital to cell function. Recently, a root secondary cell wall network have been generated for elucidating the regulatory roles of root expressed transcription factors towards secondary cell wall genes (Taylor-Teeples et al., 2014). However, the xylem development and function are largely different between root and stem in woody plant (Schweingruber et al., 2007). Anatomical analyses also showed that the properties of root wood and stem wood in European black alder (*Alnus glutinosa L. Gaertn*.) are rather different (stem wood cell number percentage: fibers 58%, vessels 29%, rays 13%; root wood cell number percentage: fibers 72.5%, vessels 15.5%, rays 12%) (Vurdu., 1977). The analyses of phenotype, and anatomy in wood, combining with distinct transcriptome of root and stem in various plants, suggested that the regulatory network of wood formation in root and stem are largely different. Therefore, elucidating the regulatory network of transcription factors and secondary cell wall related genes in stem-differentiating xylem can provide us more information for wood formation, thus helping us modify the process for creating more biomass and high quality wood.

To analyze the interactions between transcription factors and regulatory genomic regions, several approaches have been developed. Transcription factor–centered (proteinto-DNA) methods such as chromatin immunoprecipitation (ChIP) target a transcription factor and determine the genomic regions with which it interacts. Gene-centered (DNA-to--protein) methods such as Y1H assays, in contrast, determine the repertoire of transcription factors that interact with a genomic region of interest. Y1H assays capture interactions in the yeast nucleus, which means that, in contrast to ChIP, interactions that occur in a few cells or under highly

specific conditions *in vivo* can be detected more readily. Both approaches have limitations, and a problem for these techniques is how achieve the interaction analysis in large-scale.

Y1H assays involve two components: ‘DNA baits’ and ‘protein ‘preys’. Our Y1H assays are based on Matchmaker Gold Systems that employ the strong selective power of Aureobasidin A resistance to produce screens with very low backgrounds for yeast one hybrid. In Matchmaker Gold Systems, the target DNA sequence, or bait sequence, is cloned into the pAbAi Vector as a single copy or tandem repeats. The pBait-AbAi construct is then efficiently integrated into the genome of the Y1HGold yeast strain by homologous recombination to generate a bait-specific reporter strain. The pAbAi integrants are able to grow because the plasmid encodes the wild-type URA3 biosynthesis gene, that is otherwise inactive in the parent strain, and allows the yeast to grow in the absence of uracil. Meanwhile, successful use of any yeast one-hybrid system depends upon no or low recognition of your target sequence by endogenous yeast transcription factors. Therefore, testing the Y1HGold[Bait-AbAi] strains for AbA^r expression (AbA resistance) need to be performed before the transformation of potential DNA-binding proteins. Then, prey proteins would be expressed in the strains whose genomes have been integrated by promoter of interest. For prey proteins, potential DNA-binding proteins, are expressed as fusion proteins containing the yeast GAL4 transcription activation domain (GAL4 AD). The coding sequences of potential DhaNA-binding proteins can be cloned into pGADT7 AD vector, and transcription of the GAL4 AD fusion protein is driven by constitutively active ADH1 promoter. Nuclear localization sequence (NLS) and GAL4 transcription activation domain sequence in vector are arranged before the sequence of cloned potential transcription factor, so in yeast the constructed vectors can be expressed into recombinant protein NLS-GAL4-TF that can come into nucleus and recognize the promoter of interest.

2.Development of the protocol

As introduced above, Matchmaker based Y1H assay are well-established for the general analysis for interaction of transcription factor and promoter. For our purpose that elucidating the regulatory network of secondary cell wall in stem-differentiating xylem, several modifications

have been performed for the Y1H system due to several special characteristic and requirement of our experiment.

1. **Screening individual transcription factors directly for binding to DNA baits instead of using DNA baits to screen the cDNA library constructed for the expression of transcription factor protein.** 418 transcription factors used as prey proteins are identified based on cell-specific transcriptome, and many of these TFs are expressed in low level, whereas the frequency of a particular DNA sequence in a cDNA library depends on the abundance of the corresponding mRNA in the given tissue. Therefore, screening method by using cDNA library only provide low coverage, largely owing to the low abundance of some transcription factors in cDNA libraries and the difficulty of reaching saturation in library-based Y1H screens. Additionally, the library based method is also a time-consuming and high cost method because it is involved with many experiments, like extensive colony picking, retesting and sequencing. Based on these reasons, screening individual transcription factors directly for binding to DNA baits is more preferable. This can be done either by transforming plasmids encoding the prey proteins into a DNA bait strain or by mating a DNA bait strain with another yeast strain that expresses the prey protein. As described in previous studies (Reece-Hoyes et al., 2009), both approaches detect more interacting transcription factors, take less time and reduce cost and effort as compared to screening complex libraries. However, current configurations of mating assays detect only about half as many interactions compared to haploid transformation assays. Here, we employed haploid transformation method to express individual transcription factor into the genetic modified strain integrating with the promoter of interest.
2. **Adopt SD-ura/-leu medium to replace SD-leu medium in positive interaction assays.** Although integrated pAbi vectors are very stable in genome and overnight culture with uracil would not result in the loss of the integrant, our screening for positive interaction usually need to perform on plate for at least four days. Therefore, to avoid the loss of the integrant from the genome of the engineering strains, SD –ura/-leu mediums are used to replace SD-leu to keep the integrant in the genome. However, the growth rates of yeast on SD –ura/-leu would be lower than those on SD –leu medium.

3.Experiments used into test the feasibility of our modified systems

Previously, a secondary cell wall activator SND1-A2 have been demonstrate to directly activate MYB21 in poplar, and lignin pathway specific repressor MYB4 have been shown to bind with COMT2 promoter in vivo (Li et al., 2012; Shi et al., 2011). Therefore, now we use our system to analyze the interaction between PtrSND1-A2 and PtrMYB21 promoter (*MYB21P*), and between PtrMYB4 and PtrCOMT2 promoter (*COMT2P*). Full length (FL) of SND1-A2 and MYB4 were more preferable to be expressed in yeast strain as AD-protein because of they are native forms. Meanwhile, to guarantee that the success of experiment is not interfered by the toxicity of expression of these FL TFs, the DNA-binding domain (DBD) of SND1-A2 and MYB4 were also expressed in yeast strains for analyzing the interaction between these DBDs with promoters. Therefore, these interactions SND1-A2FL with *MYB21P*, SND1-A2DBD with *MYB21P*, MYB4FL with *COMT2P*, and MYB4DBD with *COMT2P* were tested by our modified system. The interaction between P53 protein and p53 promoter were also used to be analyzed by this system as positive control.

As instructed by Matchmaker yeast one hybrid manual, each of SND1-A2FL coding sequence (CDS), SND1-A2 DBD CDS, MYB4 FL CDS, and MYB4 DBD CDS was cloned into pGADT-AD vectors, and the genomic sequences of COMT2 promoter and MYB21 promoters were respectively cloned into pAbi vectors. The pGADT-AD P53 vector and pAbi-p53P vector were provided with Clontech Matchmaker® Gold Yeast One-Hybrid Library Screening System. With the preparation of these vectors, the analyses can be separated into two parts: 1. Established the bait-reporter strains and test the minimal inhibitory concentration of aureobasidin A for the bait used in Y1H assays. 2. Analysis the interaction between prey proteins (TFs) and bait sequence (Promoters) in bait-reporter strain.

Based on Matchmaker yeast one hybrid manual, Ura deficiency SD plate was used to test the success of integrating promoter into genomes of strains, and aureobasidin A were used to eliminate basal expression of the bait reporter stain in the absence of prey and check the interaction of promoter and transcription factors. Colony PCR was used to determine whether the integration of pAbi-promoter into genome is successful. By these experiments, *MYB21P*-reporter strain, *COMT2P*-reporter strain, and *p53P*-reporter strain were generated. Under the test using 2000 colonies plating, the minimal inhibitory concentration of aureobasidin A for

COMT2P-reporter strain and *p53P*-reporter strain are 100ng/ml and for *MYB21P*-reporter strain is 300 ng/ml.

To detect the interactions, prey proteins MYB4FL, and MYB4DBD were expressed in *COMT2P*-report strains, respectively, and SND1-A2FL,and SND1-A2DBD were expressed in *MYB21P*-reporter strain on SD –ura/-leu plate with each optimal concentration of aureobasidin A (Figure 1). As a positive control, we used the interaction between the TF p53 and its consensus DNA-binding site. The result showed that MYB4 proteins can recognize the *COMT2P*, and SND1-A2 proteins can recognize the *MYB21P*, whatever in form of DBD or FL. Therefore, our system can be used to analyze the interaction between TF and the promoter of its downstream genes involving in secondary cell wall formation.

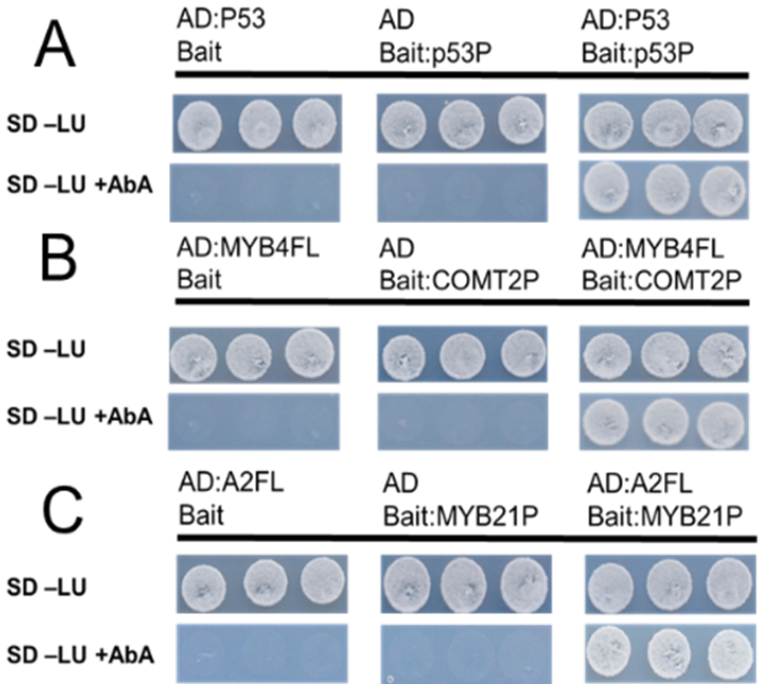

Figure 1. Yeast one-hybrid analyses. The p53P (p53-binding sites), MYB21P (2.1kb MYB21 promoter), COMT2P (3.8kb COMT2 promoter) were each fused into the genome of Y1H gold strain to generate strains Bait:p53P, Bait: COMT2P, and Bait: MYB21P, and negative control (Bait) is a strain generating by transforming empty pAbi vector into Y1H Gold strain. Each prey vectors were transformed corresponding into bait-reporter strains, as pGADT7-P53 (AD:P53) transformed into Bait:p53p, pGADT-MYB4 (AD:MYB4) transformed into Bait:COMT2P, and pGADT-SND1-A2 (AD:A2FL) transformed into Bait:MYB21P. Each analysis of interaction were combine with two type of negative control, one is the empty AD vector (AD) transformed in each bait-reporter strain, and second is each prey vector transformed in bait strain (A) Positive assays of the interaction between p53P (p53-interacting promoter site) and P53 TF. AD, Gal4 transcription activation domain; Bait, bait strain only transformed with pAbi-empty vector (B) Full length PtrMYB4 (MYB4FL) TF binds to the promoter of *PtrCOMT2* (*COMT2P*). (C) Full length PtrSND1-A2 (A2FL) TF binds to the promoter of *PtrMYB21* (*MYB21P*). Each interaction was tested in three biological replicates. All strains that show the interaction can grow on SD/-Leu/-Ura medium adding with aureobasidin A (SD-LU+AbA), and negative controls only grew on SD-Leu/-Ura medium (SD-LU) but not on SD-LU+AbA.

4.Materials

4.1 For yeast culture

Yeast strain: the used strain is Y1HGold, and the genotype of this strain is MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met–, MEL1.

YPD: 2% (w/v) Bacto-peptone, 1% (w/v) yeast extract, and 2% (w/v) d-Glucose. YPD can also be obtained as a readyto-use powder mix (Duchefa, Haarlem, The Netherlands). For solid plates, 1.5% (w/v) Bacto agar or micro-agar (Duchefa, Haarlem, The Netherlands) is used (see Note 1). Yeast Peptone Dextrose complete medium (YPD) supplemented with adenine hemisulphate is named YAPD. The complete medium used in our experiments is YAPD. For antibiotic-containing medium, prepare YPD or YPDA as above. After autoclaved medium has cooled to 55°C, add antibiotics.

Minimal medium (SD): 0.17% (w/v) yeast nitrogen base without ammonium sulphate and aminoacids (Difco, Detroit, USA), 0.5% (w/v) ammonium sulphate, and 2% (w/v) D-glucose. For solid plates, 2% (w/v) Bacto agar or microagar (Duchefa, Haarlem, The Netherlands) is added.

10X Dropout (DO) Solution:

Nutrient	10X Concentration (mg/L)	Sigma Cat. No.
L-Adenine hemisulfate salt	200	A-9126
L-Arginine HCl	200	A-5131
L-Histidine HCl monohydrate	200	H-8125
L-Isoleucine	300	I-2752
L-Leucine	1000	L-8000
L-Lysine HCl	300	L-5626
L-Methionine	200	M-9625
L-Phenylalanine	500	P-2126
L-Threonine	2000	T-8625
L-Tryptophan	200	T-0254
L-Tyrosine	300	T-3754
L-Uracil	200	U-0750
L-Valine	1500	V-0500

Table 1. The concentration of all amino acid nutrients present in 10X Dropout Solution.

That’s all nutrients for complete Dropout solution. For example, if prepare SD/–Leu/–Trp plates, you only need to prepare a 10X Dropout Solution contains all with no leucine and tryptophan, and combine the prepared solution with minimal SD agar base.

SD/-Ura with Agar

SD/-Ura without Agar

SD/-Leu with Agar

SD/-Leu without Agar

SD/-Ura/-Leu with Agar

SD/-Ura/-Leu without Agar

Aureobasidin A (AbA) (Cat. Nos. 630466 & 630499, Clontech)

SD/-Ura/AbA agar plates containing 50–500 ng/ml AbA (The concentration of AbA is dependent on your minimal inhibitory concentration of aureobasidin A for the growth of bait-transformed strain**)**

SD/-Ura/-Leu agar plates containing specific AbA concentrations that have been tested for bait-transformed strain.

4.2 For yeast transformation

50% (w/v) polyethyleneglycol (PEG4000): stored at room temperature

10× LiAc stock: 1 M lithium acetate at pH 7.5 (HAc).

10× TE stock: 100 mM Tris-HCl pH 7.5, and 10 mM EDTA.

PEG/LiAc solution (polyethylene glycol/lithium acetate). Prepare fresh just prior to use. To prepare 10 ml of solution, 8 ml of 50% PEG4000, 1 ml of 10X TE buffer, and. 1 ml of 10X LiAc

100% DMSO (Dimethyl sulfoxide; Sigma Cat No. D-8779).

Restriction enzymes BstBI or BbsI, for linearizing your pBait-AbAi plasmid prior to transforming it into Y1HGold for integration.

The yeast transformation method we used is small-scale LiAc Method, following the instruction of Yeast protocol book, Clontech.

4.3 For vector construction and PCR analyses

***E.coli* strain: TOP10.** We constructed our plasmids by using the strain for cloning and plasmid propagation. Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG

Matchmaker Insert Check PCR Mix 1 (Cat. No. 630496, Clontech). This complete 2X mix contains PCR enzyme, specific primers, dNTPs, and buffer for performing rapid yeast colony PCR for confirming that your pBait-AbA plasmid containing your target sequence has integrated into the Y1HGold genome.

Matchmaker Insert Check PCR Mix 2 (Cat. No. 630497, Clontech) for amplifying and characterizing the cDNA inserts from the library that are contained in the positive clones that emerge from your screening.

pGADT7 AD Vector (Supplemental Figure 1) as AD vector. Here we used is pGADT7 AD vector, not pGADT7 AD Rec vector. Therefore, we constructed our AD vector according to enzyme-digestion-ligation method.

pAbAi Vector (Supplemental Figure 2) as Prey vector. We also constructed our AD vector according to enzyme-digestion-ligation method.

Plasmid extraction: All plasmids from *E.coli*, can be extracted by using phenol chloroform extraction protocol (http://ko.cwru.edu/protocols/davesnotes3.html), or using QIAprep Spin Miniprep Kit. Plasmid extraction from yeast is following the instruction of Yeast protocol book, Clontech.

GoTaq DNA polymerase (cat. no. M3005, Promega). All PCR examination during vector construction was performed using engineering *E.coli.*

Phusion DNA Polymerases (F530S, Life technology). Promoter sequences are cloned from genomic DNA of *P.trichocarpa* and coding sequences are cloned from cDNA of *P.trichocarp,* using this enzyme.

Restriction enzymes (New England lab**).** Enzymes are used based on your demand in constructing vectors.

QIAquick PCR Purification Kit (cat. no. 28104, Qiagen**).** The kit Is used to purify the DNA.

5.Methods

Here, we used the interaction between secondary cell wall master regulator full sized PtrSND1-A2 and the promoter of MYB21 as example for introducing the detailed method of yeast one hybrid method. The interaction have been demonstrated by our Y1H method, and other methods as EMSA (Electrophoretic mobility shift assay), and promoter-reporter. Therefore, we just take it as example for introducing our methods, and don't mention negative control and positive control in our description of methods. The data of coding sequence of PtrSND1-A2, and the promoter sequence of PtrMYB21 were obtained from *Li et al., 2012*. Our basic strategy was shown in Figure 2.

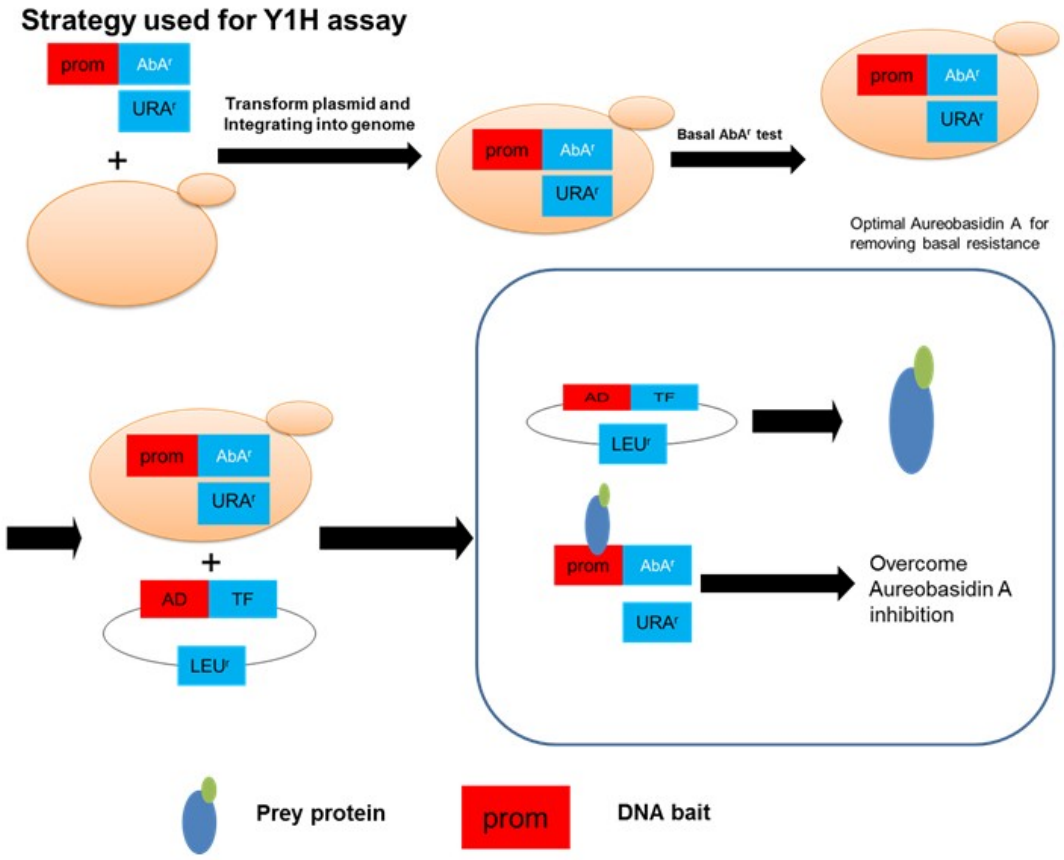

Figure 2. Schematic of Y1H assays by transformation. prom, promoter (or DNA bait); AD, Gal4 transcription activation domain; TF, transcription factor; LEU^r, the gene that enable yeast to grow without leucine in culture; URA^r, the gene that enable yeast to grow without uracil; AbA^r, AbA resistance gene

5.1. Construction of prey vector and bait vector used in Y1H assays. (Time needed: 7 days)

PtrSND1-A2 coding sequence was cloned from *P.trichocarpa* xylem cDNAs by using SND1A2F and SND1A2FLR, and then was digested by *NdeI* and *XhoI*. The digested fragments were ligated into pGADT7 AD Vector to generate AD-SND1-A2 vector. The promoter sequence of PtrMYB21 was cloned from *P. trichocarpa* genomic DNA using PtrMYB21proF and PtrMYB21proR, and then was digested by *HindIII* and *XhoI*. This promoter with digested sites was inserted into pAbi vector before AbA reporter to generate bait vector pAbiProMYB21. The constructed vectors were propagated, and extracted to prepare for transformation in to Y1H strain. All primers used in the vector construction were listed in Table 2.

Primer name	Primer sequences
PtrMYB21proF	GGGAAGCTTCTTCCCCACTTAATTGATGTTTGGC
PtrMYB21proR	CCGCTCGAGCTAGAAAGGTGATCTATATCTCTTCCC
SND1A2F	GATCCATATGATGCCTGAGGATATGATGAAT
SND1A2FLR	CCGCTCGAGTTATACCGATAAGTGGCATAATG

Table 2. Primers used to clone PtrMYB21 promoter sequence and SND1-A2 coding sequence.

5.2. Create a bait/reporter strain by integrating the pBait-AbAi plasmid into the Y1HGold yeast genome (Time needed: 5 or 6 days)

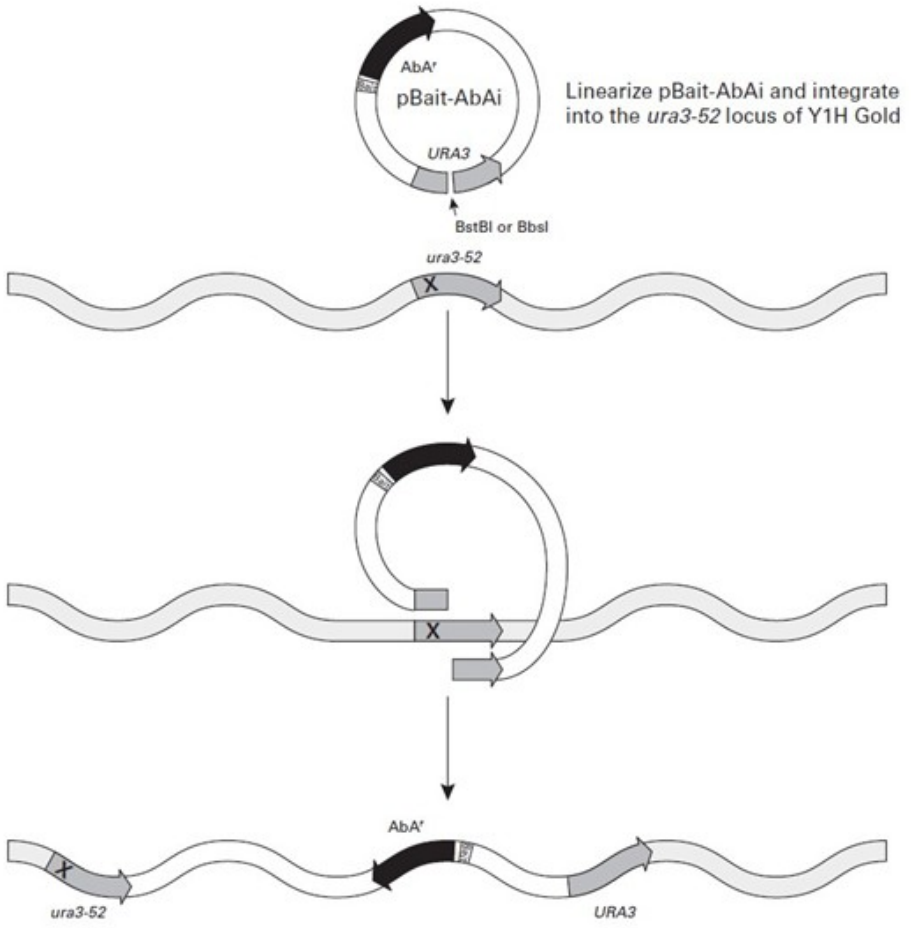

Figure 3. Create your bait reporter strain by homologous recombination into the genome of Y1HGold (The picture is referred from Matchmaker Gold Yeast One-Hybrid Library Screening System User Manual). The inactive ura3-52 locus of Y1HGold consists of an irreversible transposon disruption that can only be repaired by homologous recombination with the wild type URA3 gene, which is provided by the pBait-AbAi vector. Transformation of Y1HGold with a pBait-AbAi vector linearized with BstBI or BbsI, results in colonies that can grow in the absence of uracil on SD/-Ura agar plates. These colonies also contain a stable Bait-AbAi reporter that can be used to screen for protein-DNA interactions.

As shown in Figure 3, the bait-reporter strain is generated by homologous integration into Y1HGold strain. 2 μg pAbiProMYB21vectors were digested with BstBI within the URA3 gene of the vectors, and then the products of digested vectors were purified by QIAquick PCR Purification Kit. 1ug of linearized bait vectors were transformed in Y1H Gold strain using small-scale LiAc Method instructed by Yeast protocol book, Clontech. Each transformation reaction was diluted as 1/10, 1/100, and 1/1000. 100 μl from each dilution was plated on SD/-Ura plate,

respectively. After 3 days culture, 5 colonies were picked and analyzed by colony PCR using the Matchmaker Insert Check PCR Mix. The expected colony PCR analysis results of pAbiProMYB21integrated strain will get the products with about 3.5kb in size. The strategy how to identify correctly integrated clones was shown in Figure 4.

Then one colony for each confirmed bait was picked and streak them onto SD/-Ura agar medium. After 3 days at 30 ℃, store at 4℃f
MYB21P-reporter bait strain.

Figure 4. Confirming pBait-AbAi integration by colony PCR. The primers in the Matchmaker Insert Check PCR Mix 1 are located in the AbA^r gene and in the Y1Hgold genome, downstream of the URA3 locus.

5.3. Determining the Minimal Inhibitory Concentration of Aureobasidin A for MYB21P-reporter Bait Strain (5 or 6 days)

A large healthy colony from Y1HGoldMYB21Pro strains was resuspended in 0.9% NaCl and the OD600 of cultured strain was adjusted to ~0.002 (for approximately 2000 cells per 100 µl). 100 µl of adjusted yeast culture was plated on each of the following mediums: SD/-Ura, SD/-Ura with AbA (50 ng/ml),SD/-Ura with AbA (100 ng/ml), SD/-Ura with AbA (150 ng/ml),SD/-Ura with AbA (200 ng/ml), SD/-Ura with AbA (300 ng/ml), SD/-Ura with AbA (400 ng/ml), SD/-Ura with AbA (500 ng/ml). Allow colonies to grow for 2–3 days at 30°C.

The test results for the Minimal inhibitory concentration of aureobasidin A for your bait strain is shown in Table 3. It suggested that the minimal inhibitory concentration of aureobasidin A for MYB21P-reporter strain is 300 ng/ml.

Culture medium	Number of growed colony
SD/-Ura	about 2000 colonies
SD/-Ura with AbA (50 ng/ml)	about 800 colonies
SD/-Ura with AbA (100 ng/ml)	about 300 colonies
SD/-Ura with AbA (150 ng/ml)	97 colonies
SD/-Ura with AbA (200 ng/ml)	24 colonies
SD/-Ura with AbA (300 ng/ml)	0 colonies
SD/-Ura with AbA (400 ng/ml)	0 colonies
SD/-Ura with AbA (500 ng/ml).	0 colonies

Table 3. Number of colonies on SD/-Ura plates with various concentration of AbA.

5.4. Transforming AD-SND1-A2 vector into MYB21P-reporter strain and analyze the interaction under the minimal inhibitory concentration of aureobasidin A

0.1g AD-SND1-A2 vectors were transformed into MYB21P-reporter strain by using LiAC/PEG method as described in Yeast protocol Handbook. From each of the transformation reactions, spread 100 µ l of 1/10, 1/100, 1/1,000, and 1/10,000 dilutions on one of each of the following 100 mm agar plates as SD/-Ura/-Leu, and SD/-Ura/-Leu/AbA^{300}. Meanwhile, MYB21P-reporter strain were also plated on plates as SD/-Ura/-Leu, and SD/-Ura/-Leu/AbA^{300}. The plates were incubated (colony side down) for 3–5 days. The number of screened clones were caculated by counting the number of colonies on these plates after 3–5 days. As for detecting for the interaction between SND1-A2 and MYB21 promoters, no colonies were grown on SD/-Ura/-Leu/AbA^{300} medium that only plated MYB21P-reporter, and the number of colonies grown on SD/-Ura/-Leu, and SD/-Ura/-Leu/AbA300 medium that plated MYB21P-reporter strain with transforming AD-SND1-A2 are listed in Table 4.

Dilution	SD/-Ura/-Leu with AbA (300 ng/ml)	SD/-Ura/-Leu without AbA
1/10	97	113
1/100	11	15
1/1000	1	2
1/10000	0	1

Table 4. The number of colonies grown on SD/-Ura/-Leu, and SD/-Ura/-Leu/AbA300 medium that plated MYB21P-reporter strain with transforming AD-SND1-A2.

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Yeast protocol Handbook

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