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Construction of Yeast One-Hybird Library for Screening of *Eugenol Synthase Gene* Bait vectors in *Rosa chinensis*

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ABSTRACT

Roses are cultivated as ornamental plants in gardens, cut flowers and potted flowers, but also serviced in the perfume industry. Rose flowers product different volatile compounds including eugenol, being floral attractant for pollinators and used as an important composition of essential oil. Here, the eugenol synthase gene (*RcEGS1*) promoter was cloned from *R. chinensis* 'Old Blush'. The sequences of *RcEGS1* promoter were 1776bp, contained the conserved elements, and predicted to be with several cis-regulatory elements involved in secondary metabolism of plants. Yeast one-hybrid library of *R. chinensis* 'Old Blush' at flower opening was constructed with the three-frame reading. The library capacity was more than 1.12×10^7CFU. The length of fragments inserted was 1 Kb to 3 Kb, which is fully meeting the demand of selecting the yeast library. The bait yeast vectors with cis-transcription elements MBS-box, G-box and one fragment bait yeast vectors were constructed, respectively. Furthermore, the transcript factors R2R3-type MYB and bHLH3 were screened using the yeast one-hybrid system. The results suggested the two TFs might play an important role in the pathway of volatile biosynthesis in roses.

Introduction

Rose is not only one of the most important cut flowers, but also serviced economically value as a source of essential oil for their scent (Shalit et al., 2013, Roccia et al., 2019). However, the limit of traditional cross-breeding and complexity of scent-related metabolic pathway, fragrance seems to have been largely lost during the later stages of breeding process (Yan et al., 2011, Channelière et al., 2002). Most of garden roses lacking of fragrance, edible and oil roses cultivars lacking of diversity, and lagging behind in basic research problems of roses scent, are the "bottleneck" of limitation to rose industry (Yan et al., 2014).

Hundreds of different volatile compounds have been identified in roses, and these volatile molecules have been classified into mainly four groups including hydrocarbons, alcohols, esters,

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aromatic ethers (Weiss, 1997). Several of scent compounds, such as 2-phenylethanol (2PE), geraniol, 3,5-dimethoxytoluene (DMT), 1,3,5-trimethoxybenzene (TMB) have been shown to contribute greatly to the typical rose scent (Roccia et al., 2019, Scalliet et al., 2008). The volatile compound eugenol is one of important compounds phenylpropenes in many aromatic plants, serviced as a floral attractant for pollinators as well as essential oil (Grossman et al., 1993; Koeduka et al., 2008). Some rose cultivars also emit eugenol and its derivatives, especially in their petals and stamens (Yan et al., 2018; Rusanov et al., 2011).

The biosynthetic pathway of DMT and TMB, which are responsible for the "tea scent" of *R. chinensis* and some cultivars, involves in two closely related genes of orcinol methyltransferase (*OOMT1* and *OOMT2*) (Scalliet et al., 2006; Scalliet et al., 2008). The characteristic odor of rose 2PE, *RhPAAS* is involved in the biosynthesis pathway, which one allele was significantly expressed and was responsible for the production of 2PE (Roccia et al., 2019). Geraniol is one of the most important monoterpenes, used as constituents of essential oils of aromatics plants that are widely used in the cosmetic, perfume and pharmaceutical industries (Kozioł et al., 2014). A Nudix hydrolase, *RhNUDX1* was found to be responsible for the formation of geraniol (Magnard et al., 2015).

The eugenol synthase gene (*RcEGS1*) was firstly identified from *R. chinensis* 'Old Blush' (Wang et al., 2012). The expression of *RcEGS1* was mainly detected in stamens and petals by Western blot analysis. *RcEGS1* was significantly localized in the upper and lower epidermal layers. Down-regulation of *RcEGS1* expression in flowers reduced the relative content of eugenol in *RcEGS1*-silenced roses (Yan et al., 2018). These results demonstrated that *RcEGS1* participated in the biosynthesis of eugenol in roses. To understand the upstream gene involved in the regulation of eugenol, here *RcEGS1* promoter was cloned from *R. chinensis* 'Old Blush'. The *RcEGS1* promoter sequences contained the conserved elements, and several cis-regulatory elements related to secondary metabolism of plants. Yeast one-hybrid library of *R. chinensis* 'Old Blush' at flower opening was constructed with the three-frame reading. Furthermore, using yeast one-hybrid system, the transcript factor (TF), R2R3-type MYB and bHLH3 were respectively screened in this study, suggested the two TFs might regulate the pathway of volatile biosynthesis in roses.

Materials and Methods

Plant material

R. chinensis 'Old Blush' were grown at the rose germplasm garden of the Flower Research Institute, Yunnan Agriculture Academic Science, Kunming, China. Flowers at blooming stages were collected and frozen immediately in liquid nitrogen, then stored at -80 °C until use.

Cloning and analysis of RcEGS1 promoter

We searched the ATG upstream sequences of *RcEGS1* genes in the genome database of *R. chinensis* 'Old Blush' https://iris.angers.inra.fr/obh/ (Hibrand et al., 2018). The two primers were designed (Table 1).

Table 1. The sequences of element bait vectors

Element name	Element Sequence (5'-3')	Sequence of three tandem repeat elements (5'-3')	Transcription factor
MBS-Box-F	TAACTG	AGCTTCCTAATTAACTGTGGAAACCTAATTAACTGTGGAAACCTAATTAACTGTGGAAAGGTAC	MYB
MBS-Box-R		CTTTCCACAGTTAATTAGGTTTCCACAGTTAATTAGGTTTCCACAGTTAATTAGGA	
G-Box-F	CACGTG	AGCTTCCTGACCACGTGTCTTACCTGACCACGTGGTCTTACCTGACCACGTGGTCTTAGGTAC	bHLH
G-Box-R		CTAAGACGTCGTGGTCAGGTAAGACGTCGTGGTCAGGTAAGACGTCGTGGTCAGGA	

Using the *R. chinensis* 'Old Blush' genome as template, each PCR system contained the Phusion® High-Fidelity PCR Master Mix, 0.5 mM primer pairs and 1 ng/mL genomic DNA. The standard thermal profile was as follows: 94 °C for 4 min; 35 cycles of 94 °C for 10 s, 56 °C for 20 s, and 72 °C for 90 s; followed by a final extension for 10 min at 72 °C. The PCR products were cloned into the pMD19-T vector (Sangon Biotech, Shanghai China) for sequencing.

Construction of bait yeast vectors and determination of aureobasidin A (AbA) level basal expression

For elements bait vectors (64 bp MBS-box and 81 bp G-box), the tandem duplications of a dual repeat were obtained by the method of oligonucleotide synthesis with restriction endonuclease sites of Hind/ Kpn (Sangon Biotech, Shanghai, China). For fragment bait vectors, the 258 bp sequence of *RcEGS1* promoter was designed by gene synthesis that also introduced the Hind/ Kpn restriction sites (Sangon Biotech, Shanghai, China), then inserted into the pAbAi vector. The bait vector was linearized and transformed into the yeast cells Y1HGold, which were cultured on SD/−Ura media supplemented with AbA to determine the minimal suppressed concentration of AbA.

Healthy yeast colonies of bait vectors were picked and resuspended in 0.9% NaCl. After adjusting to obtain OD_{600} values of approximately 0.002, 100 uL yeast strain was transferred onto each of the following solid media: SD/Ura without AbA, and SD/-Ura with 200 ng/mL, 400 ng/mL, 600 ng/mL, 800 ng/mL, 1000 ng/mL AbA, respectively. The colonies were allowed to culture for 2-3 days at 30 °C. The minimum AbA concentration that completely suppressed colony growth was determined and used for subsequent library screening.

Construction and screening of a yeast one-hybrid library

The yeast one-hybrid (Y1H) library of *R. chinensis* was performed using the Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech) (OE Biotech, Shanghai, China). The cDNA library was transformed into the bait strain and screened using synthetic dropout medium (SD) lacking (–Ura) with AbA. Yeast cell suspensions were diluted to 1/10, 1/100, 1/1000, and 1/10000, and 100 μL diluents were plated onto SD/-Ura plates with 600 ng/mL AbA, then screened the total number of colonies. The number of colonies on SD/-Ura was calculated 3-5 days later to count the total number of colonies (Yang et al., 2019).

Results

Cloning and analysis of RcEGS1 promoter

The promoter sequence of *R. chinensis* 'Old Blush' *RcEGS1* gene was cloned based on the two primers designed (Supplementary 1). The amplified products were detected by agarose gel electrophoresis, and the length was consistent with target length (Fig.1A). Sequencing results showed the sequence cloned is overlapped with *RcEGS1* 100 bp in length cloned, the length of *RcEGS1* gene promoter was 1776 bp in upstream region of start codon ATG (Fig.1B), indicated that *RcEGS1* promoter sequence was successfully cloned.

Sequencing analysis showed that the cloned promoter contained all of conserved motifs such as TATA box, CAAT box, and GATA box, which constituted the conservative elements of higher plant promoters. Moreover, the promoter contains some specific *cis* regulatory sequences related to secondary metabolism. For example, MBS-box (TAACTG), G-box (CACGTA), TGACG-motif (TGACG) and MYB core (CNGTTR) (Fig. 1B).

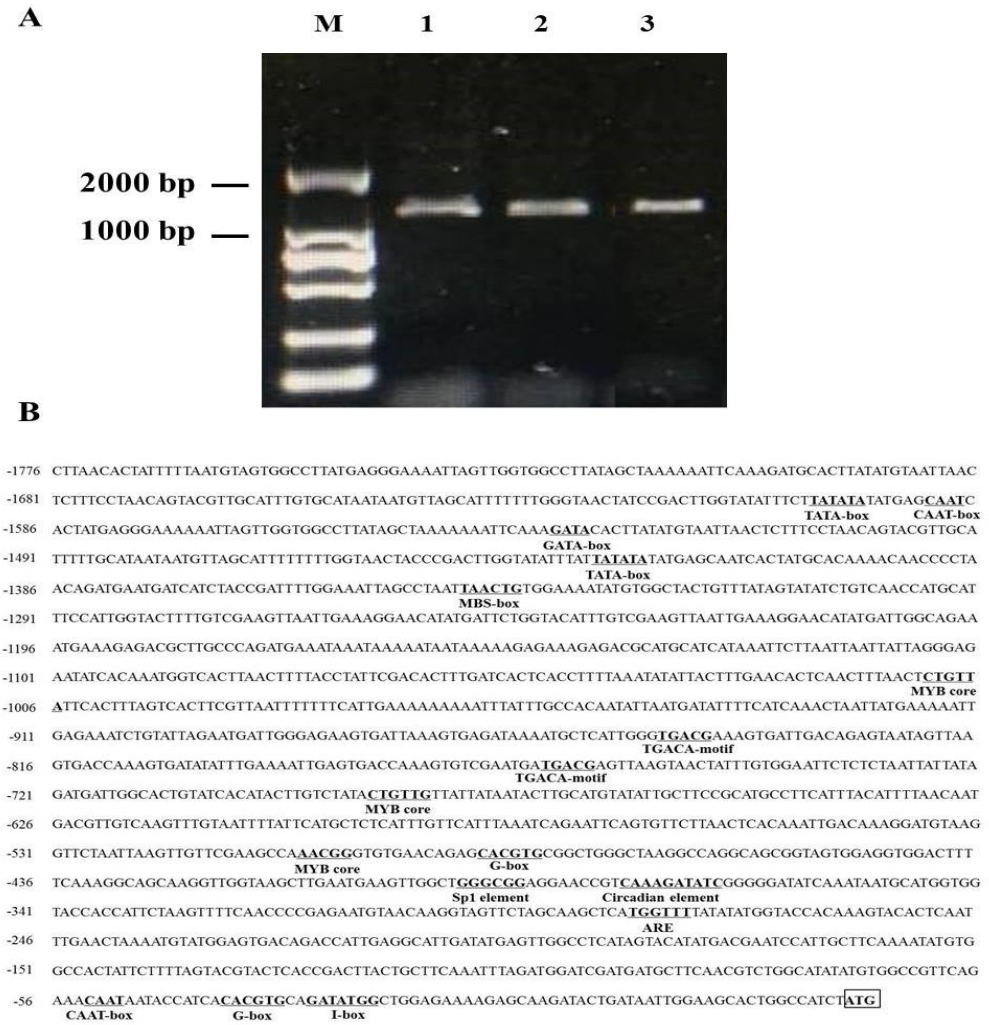

Fig. 1. Cloning and sequence analysis of *eugenol synthase gene* (*RcEGS1*) promoter from 'Old Blush'

A. Cloning *RcEGS1* promoter by PCR

B. Nucleic acid sequence and structure of *RcEGS1* promoter

M: 2000bp Marker; 1-3: the bands of *RcEGS1* promoter in three independent samples

Construction of bait vectors and identification of AbA level basal expression

Based on the regulatory elements of transcription factors MYB and bHLH, the two elements bait vectors of MBS-box (TAACTG) and G-box (CACGTG) were designed (Table 1). The bait elements were three tandem deplications of a dual repeat unit, and obtained by the method of oligonucleotide synthesis with restriction sites of HindIII/ KpnI (Sangon Biotech, Shanghai, China). Moreover, a 258 bp fragment bait vector was designed based on the site of MYB-core and G-box in the *RcEGS1* promoter sequence by gene synthesis that also introduced the HindIII/ KpnI restriction sites (Fig. 2A) (Sangon Biotech, Shanghai, China).

Three bait recombinant vectors (pMBS-Box-AbAi, pG-Box-AbAi and one fragment-AbAi with pMYB-G-Box) were transformed into yeast competent cell Y1HGold. The results of colony PCR showed that the obtained fragments length were consistent with the expected size of these bait vectors (Fig.2B), indicating that the three bait recombinant vectors were successfully transformed into the yeast cells.

To eliminate the influence of target sequences recognition by transcription factors of endogenous yeast, the minimum inhibitory concentration of Aureobasidin A (AbA, basal expression level of reporter gene) was performed. In this study, the minimum concentration of AbA inhibited basal expression of the fragment bait strains were 600 ng/mL (Fig.3). However, pMBS-Box-AbAi and pG-Box-AbAi of the two element bait strains were still grown in higher concentration on solid media SD/-Ura with 1000 ng/mL AbA, suggested the two elements baits possessed autoactivation and toxicity, and cannot be used for subsequent yeast screening.

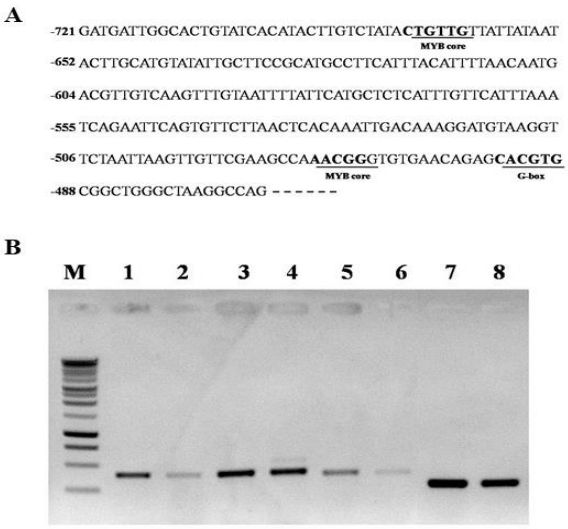

Fig. 2. Construction of yeast bait vectors

A. Identification of target elements cloned to pAbAi

B. The sequence of bait fragment

M: Marker, 1-3: pMBS-Box-AbAi, 4-6: pG-Box- Box-AbAi, 7-8: pAbAi

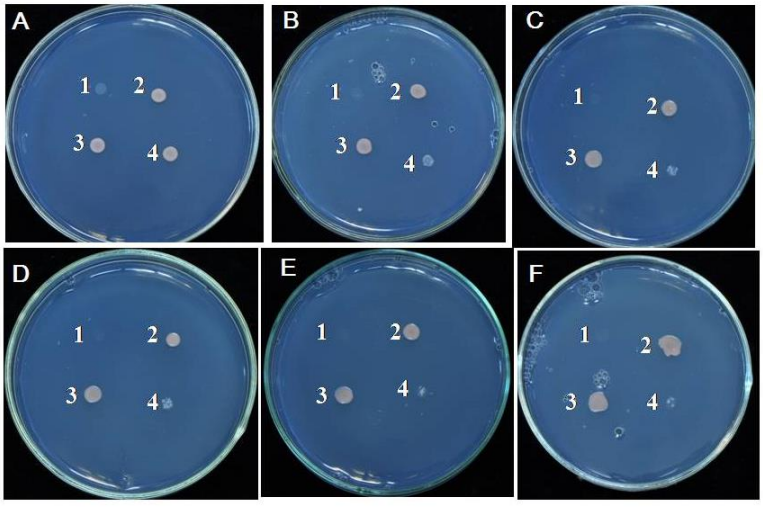

Fig. 3. Identification of bait yeast stain for AbA^r expression

A. 100 ng/mL AbA B. 200 ng/mL AbA, C. 400 ng/mL AbA, D. 600 ng/mL AbA, E. 800 ng/mL AbA, F. 1000 ng/mL AbA; 1. Negative control, 2. Y1H[pMBS-Box-AbAi], 3. Y1H [pG-Box-Box-AbAi], 4. Y1H of fragment bait vector

Construction of the yeast one-hybrid library in R. chinensis

Blooming flowers including petals and stamens of *R. chinensis* 'Old Blush' were extracted for total RNA extraction. Then quality of the total RNA samples was examined, the results obviously showed the bands corresponding to the intact 28S and 18S mRNA (Supplementary 2). The all RNA samples had an A260/A280 ratio between 1.8~2.0 and concentration of 2.5 μg/μL, which meant the qualified for library construction.

The mRNA was purified from total RNA using magnetic beads adsorption (Oligotex mRNA Midi Kit). The length of collected mRNAs ranged from 100 to 3000 bp (Supplementary 2). Then, the mRNA was transcribed reverse to first-strand cDNA, and synthesized the double-stranded cDNA by LD-PCR. The normalization showed that cDNAs were uniformly dispersed, without the disproportionate enrichment of specific fragments (Takara, Shiga, Japan).

The Y1H three-frame cDNA libraries were constructed in collaboration with Shanghai OE Biotech using the Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech). Furthermore, the plasmids were extracted and identified by electrophoresis. The inserted fragments were 750 bp to 3000 bp in length, with a recombination efficiency of 96% (Fig. 4A). The transformed yeast plasmids were diluted for plate counting, and the result showed that the yeast library capacity was 1.12×10^7 CFU/μg of cDNA inserted (Fig.4B), suggested the yeast one-hybrid library was successfully constructed.

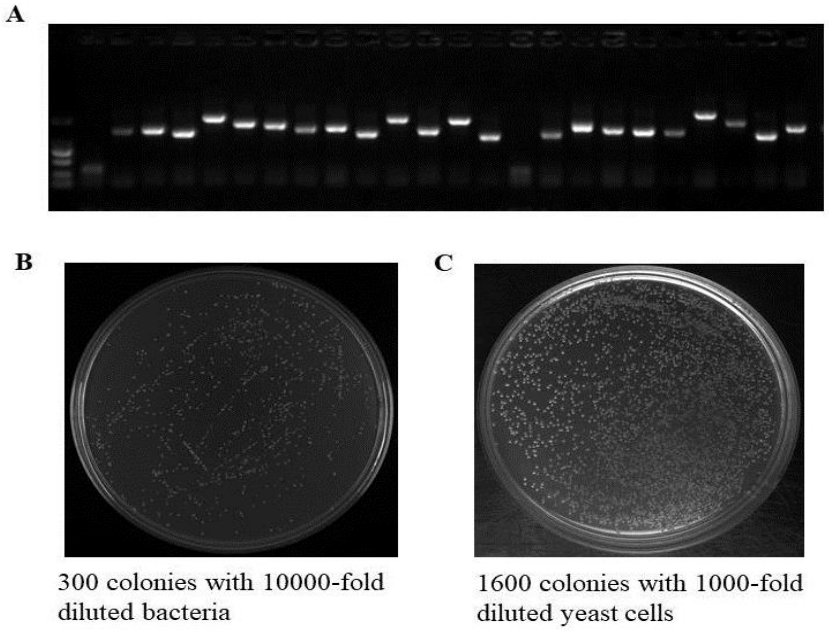

Fig. 4. The quantification of the library by sequencing of positive colonies and plate counting

A. Agarose gel electrophoresis of PCR products from randomly selected 24 colonies. B. Plate counting of 10000-fold diluted bacteria from the *E. coli* library

C. Plate counting of 1000-fold diluted yeast cells from the yeast library

M: DL 2000 DNA Marker; 1-24: PCR products of 20 colonies; +: pGADT7-T vector as positive control; −: ddH2O as negative control

Screening of yeast one-hybrid library

The plasmid of yeast one-hybrid library was transferred into the bait yeast strain Y187 with the bait fragment-AbAi. If the protein synthesized by the library plasmid can be interacted with the bait sequence, it can grow normally on the medium with SD / -Ura + 600 ng/mL AbA.

The cell suspensions of fragment bait vector yeast were diluted to 1/10, 1/100, 1/1000, and 100 μL diluents were coated in the medium with SD/-Ura + 600 ng/mL AbA. 24 colonies were obtained on SD/-Ura plates (Fig. 5). The results showed that fragments inserted were 500 bp to 2000 bp in length, indicating that the capacity and quality of the yeast library constructed were adequate.

The identification with PCR assay was performed on a total of 26 positive colonies with *RcEGS1* promoter sequences, and 11 PCR products with single bands and long fragments were sequenced and analyzed. Finally, 11 sequences of *R. chinensis* 'Old Blush' were selected, two transcript factors were identified, including MYB with specifically R2R3 conserved domain, and 67.65% amino acids homologous with *RhMYBs4-1*(KJ664810), which is involved in regulation of anthocyanin level in rose. The other transcript factor is the basic helix-loop-helix (bHLH) transcript factor family, which has shared high homologous with transcription factor bHLH3 in *Fragaria* x *ananassa* (strawberry). The results indicated the transcript factors MYB and bHLH might play a central regulatory role in rose scent biosynthesis.

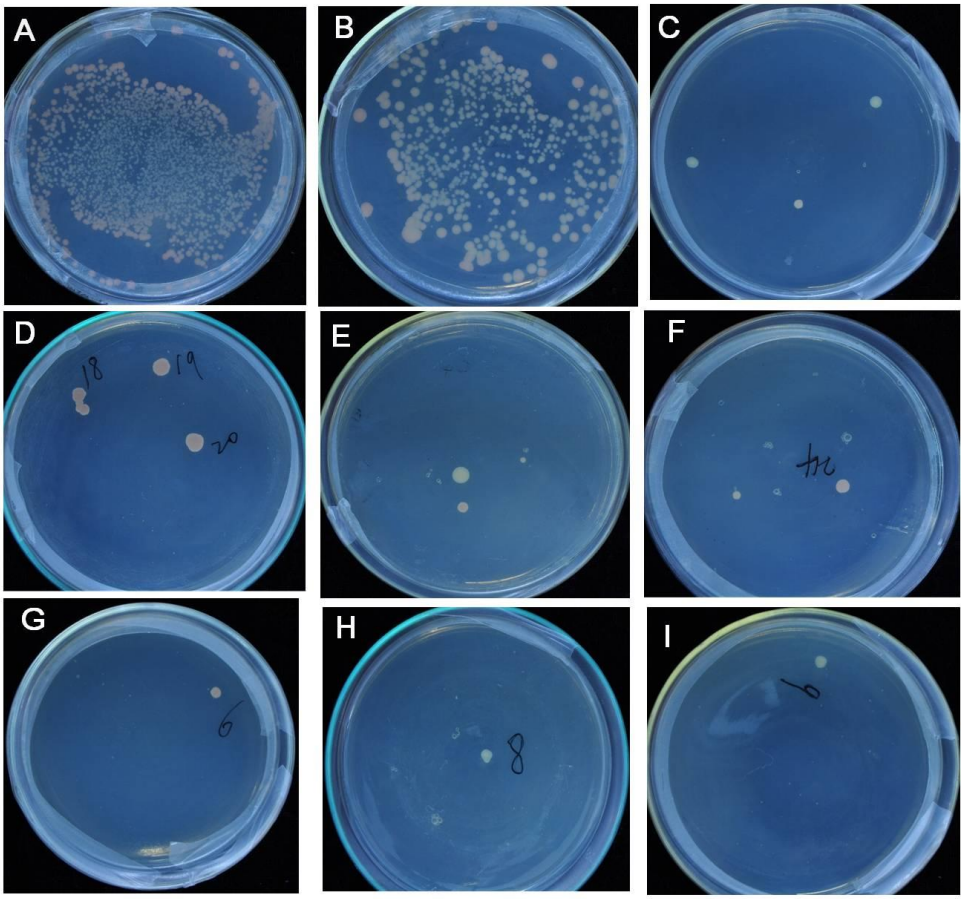

Fig. 5. The screening of cDNA transformed bait yeast strains with SD /-Ura + 600 ng/mL aureobasidin A (AbA)
A-B: Positive control; C-I: Positive clones

Discussion

R. chinensis 'Old Blush' (*Rosa* L.) is one of the most important ancient rose cultivars originating in China (Yan et al., 2014). In recent years, 'Old Blush' was used as a model to develop tools for genomics and genetic transformation, which has contributed to recurrent flowering and tea scent characters (Dubois et al., 2012). Furthermore, the high-quality genome sequence for 'Old Blush' has been obtained (Hibrand et al., 2018; Raymond et al., 2018). In the present study, the yeast one-hybrid library of 'Old Blush' was successfully constructed, which provides a significant foundation for functional identification between DNA and DNA-binding proteins, such as transcript factors (Luo et al., 1996). The high-quality yeast library was decided the crucial elements including the recombination and transformation efficiency, the library capacity and the lengths of inserted fragments. Generally, a cDNA yeast library that includes the integral expression information must contain 1×10^6 CFU at least (Ohara et al., 2001). In this study, the three main indices of the yeast library of *R. chinensis* 'Old Blush' were 95%, 1.12×10^7CFU/μg library capacity and above 1000 bp of cDNA insert, respectively, which is fully meeting the demand of subsequent screening library.

In our previous study, the function of eugenol synthase gene (*RcEGS1*) was functionally characterized in *R. chinensis* 'Old Blush' (Yan et al., 2018). In order to identify the transcriptional regulation of eugenol pathway, here the promoter of *RcEGS1* was isolated and analyzed via bioinformatics, and several putative secondary metabolism cis-elements such as MBS-box, G-box, TGACG-motif, MYB core were identified. MBS-box is a protein site located between MYB transcription factor and targeting genes (Borevitz et al., 2000), TGACG-motif might be activating the domain with bZIP protein family (Menkens et al., 1995), G-box (CACGTG) might be the domain interacted with bHLH transcription factor (Quattrocchio et al., 1998). The presence of above-mentioned regulatory elements indicated that *RcEGS1* gene expression may be regulated by the TFs including MYB, bZIP and bHLH.

The bait yeast vectors with cis-transcription elements MBS-box, G-box and one fragment bait yeast vectors were constructed and co-transformed into competent cell of yeast stain Y1HGold, respectively. The library screening of yeast one-hybrid was based on the activation of the aureobasidin resistance gene in the pAbAi vector, and positive colonies were found on plates with a gradient of aureobasidin concentrations (Yang et al., 2019). In this study, the minimum concentration of AbA needed to suppress basal expression of the fragment bait strain was 600 ng/mL (Fig.3). However, the other two bait yeasts Y1H [pG-Box-AbAi] and Y1H [pMBS-Box-AbAi] were still grown in high concentration on solid media SD/-Ura with 1000 ng/mL AbA, and possessed autoactivation and toxicity. The results suggested three tandem iterated with elements of G-Box and MBS-Box might be activated by the endogenous TFs of yeast Y1HGold. In the AbA basal expression, autoactivation is likely to be caused by the endogenous yeast TF binding the DNA bait (Deplancke et al., 2004). Regarding some baits sequences (10%-20%), the autoactivity levels for all integrants were too high, making Y1H screens impossible (Deplancke et al., 2004). For the highly autoactive baits vectors, they might be desirable to confer lower autoactivity using smaller fragments of the DNA bait (Reece-Hoyes et al., 2013).

The transcriptional regulation of structural genes involved in the pathway of scent biosynthesis represents one of the main mechanisms determining volatile biosynthesis and emission (Zvi et al., 2012 ; Sun et al., 2016). In recent years, R2R3-MYB transcription factors *ODO1*, *EOBI*, *EOBII* and *MYB4* were identified involved in scent production in petunia (*Petunia hybrida*). *ODO1* regulated transcript levels of genes encoding shikimate pathway enzymes, and affected metabolic flow toward phenylpropanoid biosynthesis (Verdonk et al., 2005). EOBI transcriptional expression regulates scent production by activating *ODO1* and scent-related structural genes in petunia (Spitzer-Rimon et al., 2010; Spitzer-Rimon et al., 2012). *PhMYB4* suppresses production of eugenol and isoeugenol (Colquhoun et al., 2012). In rose, *PAP1* is a MYB transcription factor from *Arabidopsis thaliana* has been shown previously to exert broad activation of the phenylpropanoid pathway in roses (Zvi et al., 2012). *RhMYB1* was specifically expressed in the petals of scented roses (Yan et al., 2011). In this study, one R2R3-type MYB TF has been screened by yeast one-hybrid system. Amino acids homology analysis revealed that the R2R3 MYB shared a high degree of homology with MYBs4-1 (KJ664810) from modern rose, which has been demonstrated related to anthocyanin biosynthesis in cut rose (Lin-Wang et al., 2010; Han et al., 2017). Furthermore, the bHLH has been screened with high homology with bHLH3 in strawberry, which was identified to control pro-anthocyanidin biosynthesis in fruits of *Fragaria* x *ananassa* (Schaart et al., 2013).

The protein complex consisting of MYB factors, bHLH, and WD40 protein, regulates the production of anthocyanidin and scent compounds in rose (Raymond et al., 2018).

In this study, the results provide an important foundation for future analysis of the regulation and functions of the R2R3 MYB and bHLH3 gene family in flower color and scent biosynthesis.

Conclusion

In this study, eugenol synthase gene (*RcEGS1*) promoter was cloned from *R. chinensis* 'Old Blush'. Three bait yeast vectors and yeast one-hybrid library of *R. chinensis* 'Old Blush' at flower opening was constructed with the three-frame reading, respectively. Furthermore, the transcript factors R2R3-type MYB and bHLH3 were screened. The results suggested the two TFs might play an important role in the pathway of volatile biosynthesis eugenol in roses.

Acknowledgments

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Conflict of interest

The authors indicate no conflict of interest for this study.

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