Sains Malaysiana 47(12)(2018): 2961–2968
http://dx.doi.org/10.17576/jsm-2018-4712-04

PEG-4000 Increased the Mating Efficiency of Yeast-Two Hybrid Screening Process using PmF-box1 as Bait

(PEG-4000 Meningkatkan Kecekapan Pengawanan bagi Proses Penyaringan Yis-Dua Hibrid menggunakan PmF-box1 sebagai Umpan)

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ABSTRACT

Protein degradation can occur through Ubiquitin 26S-Proteosome System (UPS). The degradation can be mediated by the SCF E3 ubiquitin ligase complex consisting of Skp1, Cullin, and F-box protein as the main components. The F-box protein at the C-terminal domain functions to recognize the targeted protein to be ubiquitinated and degraded via UPS. A stress-responsive F-box gene, PmF-box1 from Persicaria minor *was categorized in the F-box containing kelch repeat (FBK) family; a family that specific to plant kingdom. To identify the targeted protein of PmF-box1, yeast-two hybrid system (Y2H) was used. In the Y2H screening process, mating efficiency is very important to fish out the interacting proteins. Therefore, one modification was conducted to increase the mating efficiency. In this screening, PmF-box1 was used as a bait to screen for the Y2H library which was constructed using RNA from plant samples treated with abscisic acid (ABA) and polyethylene glycol (PEG)-8000 and control sample. Autoactivation and toxicity tests of bait were performed before the Y2H screening. Tests on PmF-box1 showed that it is not toxic to the yeast and cannot autoactivate the yeast reporter genes. Mating efficiency was improved from 2.07% to 9.15% after addition of PEG-4000 in the mating culture compared to the original protocol, which it also increased the colony number in the screening step afterward. Additionally, bands of gene with different sizes were observed on electrophoresis gel after colony PCR analysis from the improved technique. Those genes may code for potential interacting proteins that needs further identification and confirmation.*

Keywords: PmF-box1; polyethylene glycol-4000; yeast-two hybrid

ABSTRAK

Degradasi protein boleh berlaku melalui Sistem Ubikuitin 26S-Proteasome (UPS). Degradasi tersebut boleh berlaku berperantarakan kompleks SCF E3 ubikuitin ligase yang mengandungi Skp1, Cullin dan protein F-box sebagai komponen utama. Protein F-box pada domain C-terminal berfungsi untuk mengenal pasti protein yang disasarkan untuk diubikuitinasi dan didegradasi melalui UPS. Salah satu gen F-box yang bergerak balas terhadap tekanan, PmF-box1 daripada Persicaria minor *telah dikategorikan dalam famili F-box yang mengandungi ulangan kelch (FBK); merupakan famili yang khusus kepada alam tumbuhan. Untuk mengenal pasti protein yang disasarkan oleh PmF-box1, sistem yis-dua hibrid (Y2H) telah digunakan. Dalam proses penyaringan Y2H, kecekapan pengawanan adalah sangat penting untuk mencari protein-protein yang berinteraksi. Oleh itu, satu pengubahsuaian telah dijalankan untuk meningkatkan kecekapan pengawanan. Dalam penyaringan ini, PmF-box1 telah digunakan sebagai umpan untuk menyaring perpustakaan Y2H yang telah dibina menggunakan RNA daripada sampel tumbuhan yang dirawat dengan asid absisik (ABA) dan polietilena glikol (PEG)-8000 dan sampel kawalan. Ujian autopengaktifan dan ketoksikan umpan telah dijalankan sebelum penyaringan Y2H. Ujian tersebut menunjukkan bahawa PmF-box1 tidak toksik terhadap yis dan tidak dapat mengautoaktifkan gen-gen pelapor di dalam yis. Kecekapan pengawanan dapat ditingkatkan daripada 2.07% kepada 9.15% selepas PEG-4000 ditambahkan ke dalam kultur pengawanan berbanding dengan protokol asal dan ia juga telah meningkatkan bilangan koloni dalam langkah penyaringan selepas itu. Tambahan lagi, jalur-jalur gen dengan saiz yang berbeza telah diperhatikan di atas gel elektroforesis setelah analisis PCR koloni daripada teknik yang telah ditambah baik. Gen-gen tersebut mungkin mengekodkan protein-protein berinteraksi yang berpotensi yang memerlukan pengenalpastian dan pengesahan selanjutnya.*

Kata kunci: PmF-box1; polietilena glikol-4000; yis-dua hibrid

INTRODUCTION

Protein degradation is one of the post-translational processes that relates to many important cellular functions in plants including the response of plants to stresses (Mazzucotelli et al. 2006). The degradation of protein via Ubiquitin 26S-Proteasome System (UPS) was mediated by an E3 ubiquitin ligase complex which have several classes such as HECT, RING, and U-box (Stefanowicz et al. 2015). The SCF complex is a type of E3 ubiquitin ligase that categorized under the RING class. This complex consists of three main components; Skp1, Cullin, and F-box protein which represent its name and this complex is the best-

known function of the F-box protein. The F-box protein has two motifs; an F-box motif is located at the N-terminal domain and another motif at the C-terminal domain which responsible for the recognition of the targeted protein to be ubiquitinated and degraded by UPS. Furthermore, motifs that were found at the C-terminal domain was used to categorize the F-box proteins (FBP) to different family such as WD-40, leucine rich repeat (LRR), kelch repeat and tubby (Gagne et al. 2002; Kipreos & Pagano 2000). The F-box containing kelch repeat (FBK) family is one of the protein family found in plants. This family is known to be specific to plant kingdom because of its rare occurrence in other kingdoms (Schumann et al. 2011).

In plants, F-box proteins become the protein of interest because of its involvement in many important biological processes including phytohormone signaling, plant development and morphogenesis, cell cycle, light signaling and circadian clock regulation, self-incompatibility, abiotic stress responses, plant-pathogen interaction and secondary metabolite regulation (Chen et al. 2014, 2013; Dharmasiri et al. 2005; Iantcheva et al. 2015; Shen et al. 2007; Stefanowicz et al. 2015; Wang et al. 2014, 2005; Yan et al. 2011; Zhang et al. 2015, 2013). One FBK member has been identified from *Persicaria minor* and designated as *PmF-box1* (Othman et al. 2017). *P. minor* is an aromatic herb that is high in secondary metabolites especially dodecanal and decanal (Baharum et al. 2010). From the previous gene expression analysis study on *PmF-box1*, it showed that *PmF-box1* is responsive to stress-related phytohormone such as jasmonic acid and salicylic acid (Gor et al. 2010; Othman et al. 2017).

To elucidate the role of PmF-box1 protein in plant stress response, it is important to identify the targeted proteins to further provide interesting functional insights. Yeast-two hybrid (Y2H) is one of the systems that can be used to study for protein-protein interaction. Y2H is a genetic tool that is used in identifying interacting protein *in vivo* which was first described by Fields and Song (1989). It is the most widely used method for protein-protein interaction studies and has been successfully used to map interaction networks on a large scale (Auerbach & Stagljar 2005; Lin & Lai 2017) such as in *Saccharomyces cerevisiae* (Uetz et al. 2000), *Helicobacter pylori* (Rain et al. 2001), *Caenorhabditis elegans* (Walhout et al. 2000), *Plasmodium falciparum* (Lacount et al. 2005) and *Arabidopsis thaliana* (Arabidopsis Interactome Mapping 2011; Hackbusch et al. 2005).

In this study, PmF-box1 was used as a bait to screen the Y2H library of *P. minor*. The Y2H system that was used is Matchmaker™ Gold Yeast Two-Hybrid System (Clontech, USA). According to the manual, mating efficiency that will be achieved by following the manufacturer's protocol is about 2-5% mating efficiency. Mating efficiency is very important for a successful determination of interacting protein through the Y2H screening besides of having a good Y2H library. Therefore, this paper discussed the improvement that has been performed to get a high mating efficiency for Y2H screening process using PmF-box1 as bait.

MATERIALS AND METHODS

PREPARATION OF YEAST TWO HYBRID LIBRARY

Total RNA was isolated from leaves, stems and roots of control, ABA treated and PEG-8000 treated *P. minor* plants. *In vitro* plant culture of about 2 months old was used and the treatments were conducted hydroponically using Murashige and Skoog (MS) liquid medium as control treatment. For ABA treatment, 100 µM ABA was added into the MS liquid medium whereas for PEG treatment, 20% PEG-8000 was supplied into the MS liquid medium. The treatment was performed for 24 h under 16 h/8 h day/night regime at 24 ± 2°C. Before the treatment, the plants were acclimatized under the same photoperiod condition for 24 h using MS liquid as medium. For control, RNA was extracted directly after acclimatization, which indicates 0 h of treatment.

RNA extraction was performed using a modified López-Gómez and Gómez-Lim (1992) method in which every 10 mL of extraction buffer was added with 2 mL of 50% polyvinylpyrrolidone (PVP). To remove genomic DNA contamination, the RNA was treated with DNase using the Turbo DNA-Free kit (Ambion, USA). The same amount of total RNA from each sample were pooled together and 2 µg of RNA mixture was used to prepare the library using the Make Your Own "Mate & Plate™" Library System kit (Clontech, USA). CDSIII which is an Oligo-dT primer provided with the kit was used to generate single stranded cDNA (ss cDNA). Subsequently, the long-distance PCR (LD-PCR) was performed using Advantage 2 Polymerase Mix (Clontech, USA) to produce double stranded cDNA (ds cDNA) for the library. Amplification was performed with 5' and 3' PCR primers which were provided in the Make Your Own "Mate & Plate™" Library System kit. Next, the ds cDNA was purified using CHROMA SPIN+TE-400 Columns (Clontech, USA) to select ds cDNA with size greater than 200 bp.

The construction of the library was then conducted using *in vivo* recombination in yeast. Co-transformation of purified ds cDNA with 3 µg of prey vector pGADT7-Rec into yeast strain competent cell Y187 was performed by Yeastmaker™ Yeast Transformation System 2 (Clontech, USA) according to the large-scale transformation of manufacturer's protocol. To calculate the number of independent clones, 100 µL of 1/10 and 1/100 diluted transformed culture was spread on 100 mm SD/-Leu agar plates. The number of independent clones was calculated as in Table 1. The remainder of the transformed culture was then spread on 150 mm SD/-Leu plates. After 5 days, the plates were harvested following the 'Make Your Own "Mate & Plate" Library' kit procedure to produce library with cell density > 2×10^7 per mL. The number of independent clones should be more than one million before harvesting process. The types of medium used in this study was listed in Table 2.

TABLE 1. Formula used to count the number of independent clones and mating efficiency

Formula	Description
Number of independent clones	Resuspension volume is the volume of 0.9% NaCl solution used to resuspend the transformed yeast cells at the end of the large-scale yeast transformation protocol
Mating efficiency $\frac{\text{No. of cfu/mL of diploids}}{\text{No. of efu/mL of limiting factor}} \times 100$	No. of cfu/mL of diploids was determined from 100 mm DDO plate. Limiting partner was determined by comparing the no. of cfu/mL on 100 mm SDO plate and 100 mm SD/-Leu plate. The least calculated no. of cfu/mL is the limiting partner

TABLE 2. List of different media used in the experiment and the expected results after 3-5 days

Selective agar plate	Acronym	Functions	Distinct colonies	Color of colonies
SD/-Leu	-	Selection or growing medium for yeast carrying prey plasmid for Y2H library construction. Also used as medium to calculate the number of independent clones	Yes	White
SD/-Trp	SDO	Selection or growing medium for yeast carrying bait plasmid construct (pGBKT7)	Yes	White
		Toxicity test	Yes	White
		Bait autoactivation test	Yes	White
SD/-Trp/X-α-gal	SDO/X	Bait autoactivation test	Yes	White or very pale blue
SD/-Trp/X-α-gal/AbA	SDO/X/A	Bait autoactivation test	No	N/A
SD/-Leu/-Trp	DDO	Selection medium for diploid yeast (carrying bait and prey plasmid)	Yes	White
SD/-Leu/-Trp/X-α-Gal/AbA	DDO/X/A	Less stringent Y2H screening medium (selection based on 4 reporter genes)	Yes	White and blue
SD/-Leu/-Trp/-Ade/-His/AbA	QDO/A	Stringent Y2H screening medium (selection based on 3 reporter genes)	Yes	White
SD/-Leu/-Trp/-Ade/-His/X-α-Gal/AbA	QDO/X/A	Stringent Y2H screening medium (selection based on 4 reporter genes)	Yes	White and blue

CONSTRUCTION OF BAIT VECTOR CONTAINING *PMF-BOX1* INTO Y2HGOLD YEAST STRAIN

A pair of primer was designed to contain a 24 bp homology to *PmF-box1* (bait) and a 16 bp homology to the linear ends of pGBKT7 (the bait vector). Forward primer sequence (Y2H_PmF-box1_F) that homologous to bait was started from the start codon whereas the reverse primer (Y2H_PmF-box1_R) was started just before the stop codon (Table 3). cDNA synthesized from the total RNA that was isolated previously was used as a PCR template for bait amplification using the Y2H_PmF-box1_F and Y2H_PmF-box1_R primers. The PCR was performed using CloneAmp HiFi PCR Premix (Clontech, USA) based on the following PCR conditions: initial denaturation at 98°C for 30 s; 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 15 s and elongation at 72°C for 1.5 min; and final elongation at 72°C for 5 min.

TABLE 3. Primer sequences

Primers	Sequence 5' → 3'
Y2H_PmF-box1_F	[a] **CATGGAGGCCGAATTC**ATGTTGGAGGATCACTCTTGTCTG
Y2H_PmF-box1_R	[a] **GCAGGTCGACGGATCC**ACACCCCATCACCGCACAGTTATA
T7 promoter_F	AATACGACTCACTATAGGGCG
3'DNA-AD_R	AGATGGTGCACGATGCACAG

[a] Bold letters are sequences homology to pGBKT7 bait vector

Cloning of *PmF-box1* gene into pGBKT7 was conducted using In-Fusion HD cloning kit (Clontech, USA). The pGBKT7 vector was linearized using *Eco*RI and *Bam*HI. Following that, the constructed bait vector (Figure 1(A)) was transformed into *E. coli* Stellar competent cell (Clontech, USA). The transformed culture was spread onto LB medium supplemented with 50 mg/ L kanamycin and incubated overnight at 37°C. After that, plasmid was extracted from the colony and sent for sequencing to confirm the *PmF-box1* sequence.

AUTOACTIVATION AND TOXICITY TEST OF BAIT

After the *PmF-box1* sequence confirmation, the pGBKT7 plasmid containing *PmF-box1* was transformed into Y2HGold yeast competent cell using small-scale transformation procedure of Yeastmaker™ Yeast Transformation System 2 (Clontech, USA). Then, 100 µL of 1/10 and 1/100 diluted transformed culture were spread onto SD/-Trp (SDO), SD/-Trp/X-α-gal (SDO/X) and SD/-Trp/X-α-gal/AbA (SDO/X/A) for autoactivation test. Meanwhile, the empty pGBKT7 vector was transformed into Y2HGold using the same procedure mentioned above and the 1/10 and 1/100 diluted transformed culture were spread onto SDO. For toxicity test, empty vector (Y2HGold: pGBKT7_0) is used for the comparison with the Y2HGold with pGBKT7 containing *PmF-box1* (Y2HGold: pGBKT7_PmF-box1) by comparing the sizes of the colonies on the SDO plates. All plates were incubated at 30°C for 3-5 days.

YEAST MATING FOR Y2H SCREENING

The mating procedure for Y2H screening was conducted following Matchmaker™ Gold Yeast Two-Hybrid System (Clontech, USA). Y2HGold containing bait vector,

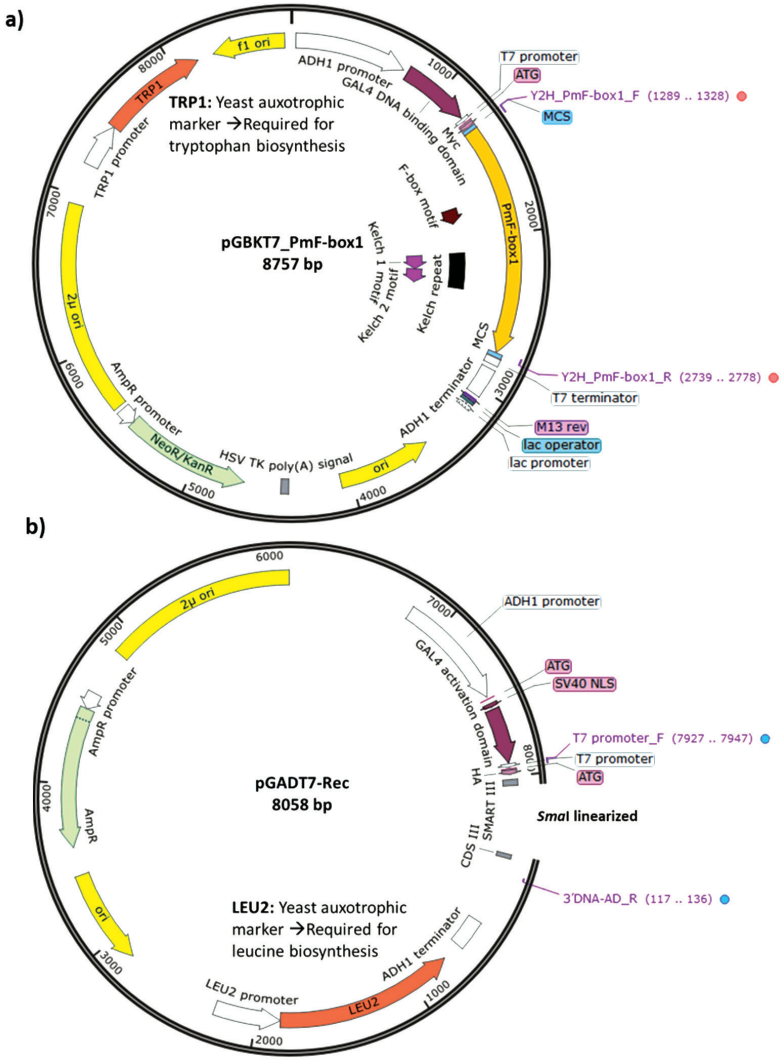

FIGURE 1. Vectors used in the Y2H. a) Constructed bait vector, pGBKT7 containing *PmF-box1*. Y2H_PmF-box1_F and Y2H_PmF-box1_R are the position of the primers used for the bait vector construction. b) *Sma*I linearized prey vector pGADT7-Rec used in the construction of Y2H prey library. T7 promoter_F and 3'DNA-AD_R are the position of primers used for the colony PCR

Y2HGold: pGBKT7_PmF-box1 culture was combined with 1 mL of Mate & Plate library in a sterile 2 L flask to allow mating to occur at 30°C for 24 h with 40 rpm shaking. After washing and resuspension of the mated culture with 10 mL of 0.5X YPDA/Kan liquid medium, 100 µL of 1/10, 1/100, 1/1000 and 1/10000 diluted mated culture was spread onto 100 mm SDO, SD/-Leu and SD/-Leu/-Trp (DDO) plates for determination of mating efficiency (Table 1). The remaining suspension of mated culture was spread onto 150 mm SD/-Leu/-Trp/X-α-Gal/AbA (DDO/X/A) plates with 200 μL for each plate about 50-60 plates for the screening of interacting proteins. When blue colonies appear on the DDO/X/A plate, all the colonies were transferred onto stringent screening medium plates, SD/-Leu/-Trp/-Ade/-His/X-α-Gal/AbA (QDO/X/A).

In the second experiment, the mating and screening procedures were repeated with some modification by adding polyethylene glycol-4000 (PEG-4000) in the combined culture to a final concentration 10% PEG-4000. The mated culture was washed using 0.9% NaCl and resuspended with 10 mL of 0.9% NaCl before 200 µL culture is spread onto each of 150 mm SD/-Leu/-Trp/-Ade/-His/AbA (QDO/A) medium plates (50-60 plates). Mating efficiency was determined by spreading 100 µL of 1/10, 1/100, 1/1000 and 1/10000 diluted mated culture onto SDO, SD/-Leu and DDO media (Table 1). After colonies appeared on the QDO/A, the colonies were transferred onto more stringent screening medium plates, QDO/X/A.

YEAST COLONY PCR

Yeast colony PCR was conducted to analyze the presence of inserts carried by the yeast in the vector. Primers that were used are T7 promoter_F and 3'DNA-AD_R as listed in Table 3. The primer sequences were designed based on the pGADT7 vector sequence that flank the insert. GoTaq® Green Master Mix (Promega, USA) was used to perform the PCR. The denaturation temperature used was 95°C for 30 s, annealing temperature used was 55°C for 30 s and elongation at 72°C for 1.5 min. This cycle was repeated 30 times. The PCR products were analyzed by electrophoresis on 1 % Agarose/EtBr gel.

Results and Discussion

LIBRARY CONSTRUCTION AND BAIT TESTING

The Y2H library was constructed using ds cDNA that was synthesized from pooled RNA isolated from leaves, stems and roots of control (0 h) and samples treated with ABA (24 h) which is a stress-related phytohormone and PEG-8000 (24 h) which caused osmotic stress to enrich the library. Therefore, the Y2H library that was produced is a representative of temporal and spatial expression of stress-induced genes. ABA and PEG-8000 were chosen because, there is homolog of *PmF-box1* from *Arabidopsis thaliana* which is *AT2G02870* also known as *SKIP11* is highly responsive to osmotic stress at 12 h and 24 h after treatment and slightly responsive to ABA at 3 h after treatment according to Arabidopsis eFP (Electronic Fluorescent Pictographic) which can be retrieved at The Arabidopsis Information Resource (TAIR): https://www.arabidopsis.org/servlets/TairObject?type=locus&name=At2g02870. Li et al. (2016) also showed that *AT2G02870* named as *ARKP1* is responsive to 50 µM ABA at 6 h after treatment. Regarding the osmotic stress, in this study 20% PEG-8000 was applied to *P. minor* while according to the TAIR the *AT2G02870* expression was induced by 300 mM mannitol. Figure 2 shows the ds cDNA that was synthesized and purified using CHROMA SPIN+TE-400 column. The purified ds cDNA showed very little smear with size less than 500 bp. This indicates that smaller fragments of ds cDNA were successfully discarded.

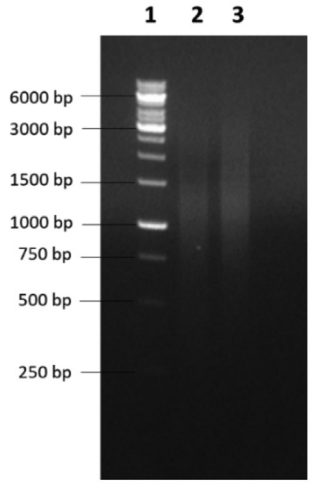

FIGURE 2. Analysis of ds cDNA of *P. minor* on 1.5 % agarose gel. Lane 1, O'GeneRuler 1 kb DNA Ladder (Thermo Scientific™ SM1163); Lane 2, 7 µL of unpurified ds cDNA; Lane 3, 1 µL of purified ds cDNA

After co-transformation of the ds cDNA with 3 µg of pGADT7-Rec (Figure 1(B)) into Y187 yeast competent cells for library preparation, the number of independent clones was calculated. The requirement of the number of independent clones should be more than one million. It is important to get high number of independent clones because it shows the complexity of the library and can maximize the chances to get the genuine interacting protein. In this study, the number of independent clones was 3.30×10^6. The yeast cells were harvested after the number of independent clones had been determine. The calculated concentration of the Y2H library using hemocytometer was 5.05×10^8 cells/mL. This number is more than the minimum cell density required for the construction of Y2H library, which is 2×10^7 cells/mL.

Based on the bait testing, we found that *PmF-box1* gene is not toxic to the yeast Y2HGold because the colony of Y2HGold carrying pGBKT7_PmF-box1 has almost the same size with the Y2HGold carrying empty vector (Figure 3(A)). Additionally, PmF-box1 did not autonomously

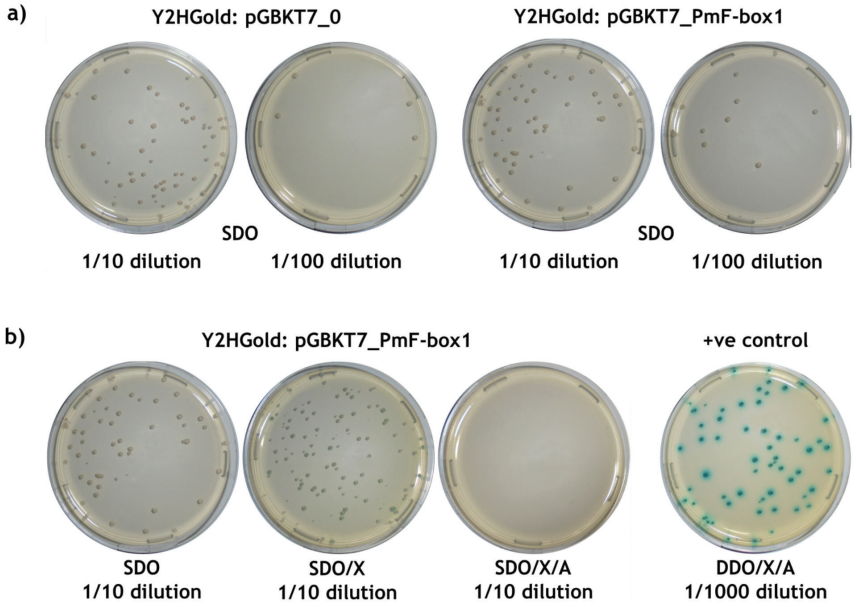

FIGURE 3. Yeast Y2HGold cells carrying empty bait vector, pGBKT7_0 and Y2HGold carrying pGBKT7 containing PmF-box1. a) Plates comparison of toxicity test. b) Plates comparison of autoactivation with positive diploid control of mated culture between Y2HGold containing pGBKT7-53 and Y187 containing pGADT7-T

activate the reporter genes (Figure 3(B)). Although there were very pale blue color colonies appeared on the SDO/X plate, there was no single colony grew on the SDO/X/A plate. This confirmed that PmF-box1 cannot autoactivate the reporter genes.

YEAST-TWO HYBRID SCREENING

In the Y2H screening process of the first experiment, about 40 colonies with blue color were observed on less stringent screening medium DDO/X/A plates. All the colonies were then transferred onto more stringent medium, QDO/X/A plates. However, none of the colonies grew with blue color. That indicates that the preys caused the autoactivation of the reporter genes on the DDO/X/A plates. Thus, in the second experiment, one modification was performed to increase the mating efficiency of Y2H. By increasing the mating efficiency, the chances to find the interacting prey proteins will be high. The addition of PEG-4000, a high molecular weight compound to the mating culture will facilitate the yeast of different mating type to increase physical contact because the cells became aggregated (Albers et al. 2002). This is proven, because after the addition of PEG-4000, the calculated mating efficiency was increased to 9.15% in comparison to the first experiment which was only 2.07 % (Figure 4(A)). Moreover, with the addition of PEG-4000, the number of screened clones was increased to 1.01×10^7 (Figure 4(B)). By using the modified protocol, colonies with blue color were observed on the final stringent screening medium QDO/X/A. The blue colonies that grew on the QDO/X/A medium were then used for yeast colony PCR. Varying size of bands was observed on the gel electrophoresis (Figure 4(C)), which may encode the potential interacting proteins which need further confirmation of positive interaction, as well as sequencing to identify the genes that might be targeted by PmF-box1.

Plating method of the mated culture of the modified protocol was slightly different from the original protocol. Instead of DDO/X/A plates, the mated culture was spread on QDO/A plates. The use of QDO/A plates without X-α-gal for initial screening saved the cost of screening process and also decreased the selection of false positive colonies. By using the QDO/A plates for initial screening process, only white colonies grew on the medium. Colonies with fast growth were selected to be transferred onto the QDO/X/A stringent medium plates. From the Y2H experiment carried out by Cao and Yan (2013), they used QDO plates for initial screening process and for the stringent selection, QDO/X plates were used. By using that method, proteins that interact with the bait were successfully screened. Compared to our study, Aureobasidin A (AbA) was not used in their study. In our opinion, if there is slightly pale blue color of the bait in the autoactivation test, it is better to include AbA in the selection plates to reduce the number of false positive interaction and also to reduce the background during screening process. These will make screening process easier. AbA is an antifungal antibiotic produced by *Aureobasidium pullulans* R106.2 (Takesako et al. 1991) which inhibits a wide range of pathogenic fungi (Takesako et al. 1993) including *Saccharomyces cerevisiae*, by inhibiting the normal budding process and leading to cell death (Endo et al. 1997). Other than that, the use of 0.9% NaCl in resuspending the mated culture in the second experiment in this study has reduced the background formed on the screening plates.

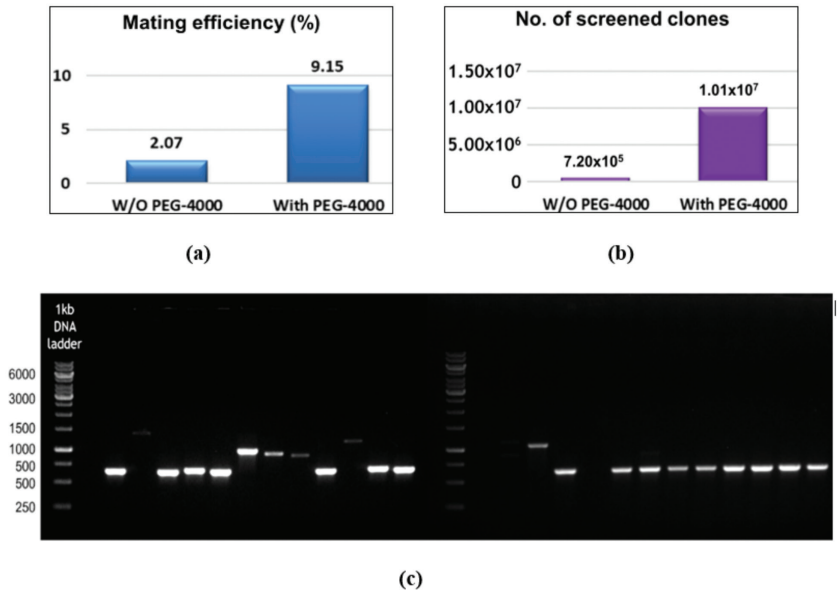

FIGURE 4. Comparison of mating efficiency (a) and no. of screened clones (b) of the mating culture without PEG-4000 and with PEG-4000. Analysis of colony PCR product for prey protein screening from the improved technique; mating culture with PEG-4000 (c)

CONCLUSION

Y2H is a powerful tool genomic approach to study protein-protein interactions *in vivo*. Through Y2H we are able to map interaction networks from any organisms. Preliminary bait toxicity and autoactivation tests are very important to be determined before starting the Y2H screening procedure. Toxic bait will decrease the efficiency of the Y2H screening while autoactivation of reporters by the bait will cause the inability to differentiate a true protein-protein interaction. In addition, the accessibility of a high-quality and rich Y2H library can facilitates in determining the potential interactors. Mating efficiency is another important factor that also contributes to the success of the Y2H screening. In this study, the addition of PEG-4000 caused the aggregation of yeast cells to occur. This condition facilitates the yeast of different mating type to increase physical contact and caused the increment of the mating efficiency between Y2HGold carrying *PmF-box1* and Y187 (carrying the prey library). Consequently, it also intensified the number of screened clones that leads to the higher chances of capturing the genuine interacting proteins.

ACKNOWLEDGEMENTS

This research was supported by FRGS/1/2015/SG03/UKM/01/1 grant from Ministry of Higher Education, Malaysia.

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Received: 30 May 2018
Accepted: 19 September 2018