

Full Length Research Paper

Construction of gateway-compatible yeast two-hybrid vectors for high throughput analysis of protein interaction

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Yeast two-hybrid system combined with the gateway technology will greatly facilitate the cloning of interested DNA fragment into yeast two-hybrid vectors and therefore increase the efficiency of yeast two-hybrid analysis. In this study, we constructed a pair of Gateway-compatible yeast two-hybrid vectors pBTM116GW and pVP16GW by introducing the gateway cassette (*attR1-Cmr-ccdB-attR2*) into the multiple cloning sites (MCS) of the previously described vectors pBTM116SS and pVP16S1, respectively. The applicability of newly generated vectors was tested by assaying the interaction between the kinase domain XA21K of rice (*Oryza sativa*) receptor like kinase XA21 and its interactor XB3. Since both *Xa21K* and *Xb3* were cloned into a Gateway entry vector and then subcloned into pBTM116GW and pVP16GW by *in vitro* recombination with high efficiency, respectively, it demonstrated that the newly constructed gateway-compatible two-hybrid vectors will be useful in analysis of protein interactions in a high throughput way.

Key words: Yeast two-hybrid, gateway cloning technology, protein interaction.

INTRODUCTION

Protein-protein interactions play key roles in cells. Lots of experimental approaches and *in silico* methods have been developed to identify and predict large-scale protein-protein interactions. Yeast two-hybrid is one of the powerful systems (Fields and Song, 1989; Bartel and Fields, 1995). It offers a number of advantages over many of the biochemical procedures generally used in the analysis of protein-protein interactions. Yeast two-hybrid technology is relatively inexpensive since it avoids costly procedures such as antibody production and protein

purification. The yeast two-hybrid system has become a routine tool for the study of protein-protein interactions, for example cDNA expression libraries can easily be screened by yeast two-hybrid to isolate proteins interacted (Suter et al., 2008). Although the yeast two-hybrid system is proved to be an effective method to detect protein-protein interactions, the construction of yeast two-hybrid vectors for this purpose with the conventional restrictive endonuclease- and ligase-based method is laborious and often hampered by inappropriately positioned restriction enzyme sites. Therefore, the conventional two-hybrid method can not meet the need for study of protein interactions in a high-throughput way in the post-genomic era.

Gateway cloning technology is a powerful new methodology for DNA cloning by replacing restriction endonucleases and ligase with site-specific recombination (Hartley et al., 2000). The major techniques of gateway cloning system include LR and BP reactions. The LR reaction is mediated by the LR clonase, which is a recombination reaction between an Entry Clone containing

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Abbreviations: MCS, Multiple cloning sites; SD, synthetic medium; DB, DNA-binding domain; LB, Luria-Bertani; AD, activation domain; PCR, polymerase chain reaction.

the gene of interest flanked by Gateway *attL* recombination sites and a Destination Vector containing Gateway cassette including *attR* sites (*attR1-Cmr-ccdB-attR2*). This reaction transfers the gene of interest from the Entry Clone to the Destination Vector, creating an Expression Clone. The BP reaction, in essence, is the reverse of the LR reaction. The BP reaction transfers the gene of interest flanked by *attB* sites from an Expression clone into an Entry vector containing *attP* to produce the Entry Clone containing the gene of interest. Thus, Gateway cloning technology provides a powerful versatile system for transferring DNA segments between vectors. A number of Gateway compatible vectors have been developed and widely used for high-throughput study of gene functions in plants (Curtis and Grossniklaus, 2003; Estornell et al., 2009), yeast (Alberti et al., 2007), fungi (Shafran et al., 2008) and animals (Akbari et al., 2009). However, few Gateway-compatible yeast two-hybrid vectors are available for the study of protein interaction on a large scale.

Here, we report a new pair of Gateway compatible yeast two-hybrid vectors, bait vector pBTM116GW and prey vector pVP16GW, generated by introducing Gateway recombination cassette (*attR1-Cmr-ccdB-attR2*) into the multiple cloning sites (MCS) of the yeast two-hybrid vectors pBTM116SS and pVP16S1, respectively. The usefulness of newly generated Gateway-compatible yeast two-hybrid vectors was tested by analysis of interaction between the rice receptor like kinase domain XA21K and its interactor XB3.

MATERIALS AND METHODS

Strains and media

Escherichia coli DH5 α was used as the host for general cloning and *E. coli* DB3.1 (Invitrogen, CA, USA) was used for propagation of *ccdB* plasmids. *Saccharomyces cerevisiae* strain L40 was used as a host for two-hybrid protein interaction assay.

Luria-Bertani (LB) medium was used for *E. coli* culture. YPDA medium (2% Bacto-peptone, 1% Bacto-yeast extract, 1% glucose, 0.003% adenine) was used for general culture of yeast. For yeast transformants selection and protein interaction assay, synthetic medium (SD) supplemented with the corresponding amino acid mixture was used.

DNA cloning, primers synthesis and DNA sequencing

Plasmids were prepared using QIAprep Miniprep kit. PCR products were purified using QIAquick PCR Purification Kit. DNA recovery from agarose gel was performed using QIAEX II Gel Extraction Kit. These kits were purchased from QIAGEN (Hilden, Germany) and used according to the manual. Primers synthesis and DNA sequencing were done by the Sangon company (Shanghai, China).

Construction of gateway-compatible two-hybrid vectors

The yeast two-hybrid vectors pBTM116SS (bait vector) and pVP16S1 (prey vector) were used in this study as backbone. These two

vectors were derivatives of pBTM116 and pVP16 (Vojtek et al., 1993) and were developed by Yasunori Machida, Nagoya University, Japan. The pBTM116SS contains a selection marker for tryptophan auxotrophy, whereas pVP16S1 contains one for leucine auxotrophy (Figure 1A).

The new Gateway-compatible yeast two-hybrid vectors were generated by inserting the Gateway recombination cassette (*attR1-Cmr-ccdB-attR2*) into the pBTM116SS and pVP16S1 at *Bam* HI / *Not* I sites, respectively. Briefly, the Gateway recombination cassette flanked by the restriction endonuclease sites *Bcl* I and *Not* I was amplified with PCR from vector pGWB2 (GenBank accession number: AB289765), using upstream primer 5'-CGCAAGC TGATCAGTTATCAACAAGTTTGTACAAA-3' (the underlined is *Bcl* I) and downstream primer 5'-ATAAGAATGCGGCCGCGTTAT CAA CCACTTTGTACAAGAA-3' (the underlined is *Not* I). The PCR cycling parameters were as follows: an initial denaturation step at 95°C for 3 min followed by 28 cycles at 95°C for 45 s, 62°C for 30 s, 72°C for 2 min and a final extension at 72°C for 10 min. PCR product was purified and cut by *Bcl* I at 50°C for 3 h, followed by *Not* I at 37°C for 5 h. The reaction products were electrophoresed and recovered. The bait vector pBTM116SS and prey vector pVP16S1 were also cut by *Bam* HI and *Not* I, electrophoresed and recovered, respectively. The recovered fragments from vector and the Gateway recombination cassette were ligated overnight by T4 ligase (Promega, WI, USA), at 16°C and then transformed into *E. coli* strain DB3.1. The recombinant vectors were verified through restriction enzyme analysis and DNA sequencing and named as pBTM116GW and pVP16GW, respectively (Figure 1B).

Two-hybrid assay

To test the applicability of the new constructed Gateway-compatible two-hybrid vectors, the kinase domain XA21K of rice (*Oryza sativa*) receptor like kinase XA21 and its interactor XB3, an E3 ubiquitin ligase in rice, were chosen as bait and prey proteins. The interaction between XA21K and XB3 was previously described by Wang et al. (2006). The GenBank accession number for *Xa21* and *Xb3* cDNA sequence is U72723 and AF272860, respectively. The coding region for *Xa21K* and *Xb3* were amplified by RT-PCR with template of rice first-strand cDNA. The primer pairs used were 5'-CACCCACAAGAGAAGTAAAAGG-3' / 5'-TCAGAATTCAGGCTC CCACCTTCACA-3' for *Xa21K* and 5'-CACCATGGGTCACGG TGT CAGCTG-3' / 5'-TCATAGATCGTGCTCAGGCTTGT-3' for *Xb3*. The two PCR products were cloned into Gateway vector pENTR/D-TOPO (Invitrogen, CA, USA) to produce the entry clone pENTR-Xa21K and pENTR-Xb3 and the entry clones were confirmed by DNA sequencing. The destination bait vector pBTM116GW-Xa21K and prey vector pVP16GW-Xb3 were generated through LR recombination (Invitrogen, CA, USA) between the entry clone and the newly constructed vectors.

The two-hybrid assay was performed as described in protocols (Vojtek et al., 1993; Hollenberg et al., 1995) with some modification. Briefly, yeast L40 competent cells were cotransformed with the bait pBTM116GW-Xa21K and prey pVP16GW-Xb3 using Frozen-EZ Yeast Transformation II kit (Zymo Research, CA, USA). Positive transformants were selected by plating on minimal media lacking leucine and tryptophan. The interaction of XA21K and XB3 was assayed based on the ability to activate transcription of the HIS3 reporter gene, e.g. yeast colonies grown on the plates lacking histidine and supplemented with 2.5 mM 3-aminotriazole (3AT) after 3 - 5 days at 30°C were scored positive. The 3AT inhibits the activity of the His reporter enzyme and thus it is used to test the strength of the interaction and avoid the autoactivation. As a control, the pair of pBTM116GW-Xa21K/ pVP16GW and the pair of pBTM116GW /pVP16GW-Xb3 were also tested to determine whether it could activate the transcription of the reporter gene non-specifically.

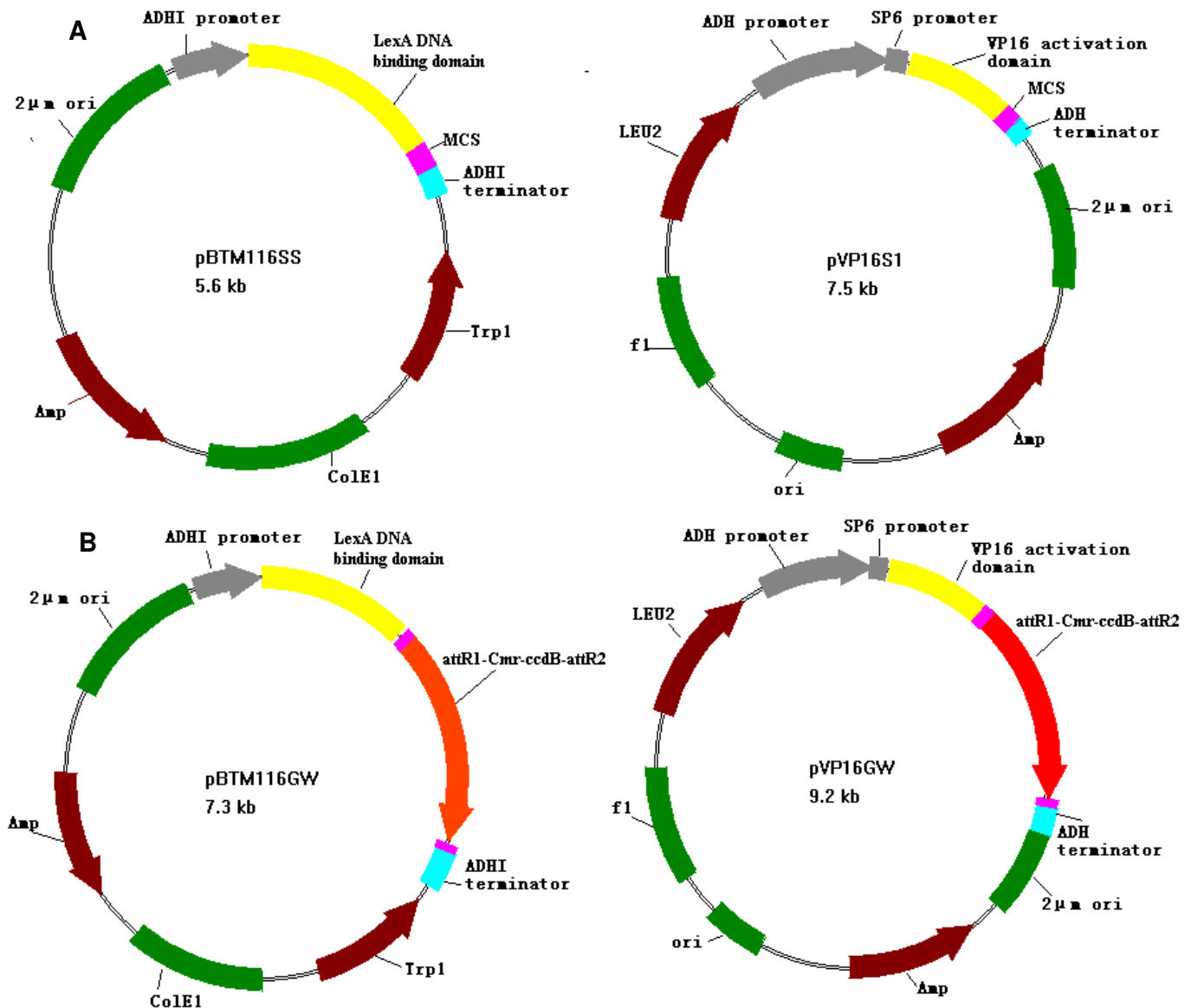


Figure 1. Schematic map of the vectors. **A)** The map of yeast two-hybrid vectors pBTM116SS and pVP16S1, the MCS of pBTM116SS are *Sal I/Bam HI/Nco I/Spe I/Not I/Pst I* and the MCS of pVP16S1 are *Bam HI/Sal I/Sma I/Kpn I/Sac I/Not I*; **B)** The map of newly constructed gateway-compatible yeast two-hybrid vectors pBTMGW and pVP16GW, in which the Gateway cassette of *attR1-Cmr-ccdB-attR2* was inserted into the *Bam HI* and *Not I* sites of pBTM116SS and pVP16S1, respectively.

RESULTS

Gateway-compatible two-hybrid vectors pBTM116GW and pVP16GW

The Gateway-compatible two-hybrid vectors pBTM116GW and pVP16GW were generated by inserting the gateway cassette (*attR1-Cmr-ccdB-attR*) into MCS of the previously constructed yeast two hybrid vectors pBTM116SS and pVP16S1, respectively (Figure 1B). Similar to its backbone vectors, the bait vector pBTM116GW contains *TRP1* gene as a selection marker for tryptophan auxotrophy, whereas the prey vector pVP16GW contains

LEU2 gene for leucine auxotrophy. In this two-hybrid system, a bait protein, for which one wants to find interacting partners, can be fused to the DNA-binding domain (DB) of the bacterial LexA protein in pBTM116GW by selection of tryptophan auxotrophy. Then one or more prey proteins can be fused to the transcriptional activation domain (AD) of VP16 in pVP16GW by selection of leucine auxotrophy. Since host strain yeast L40 also lacks an endogenous copy of *HIS3*, its growth depends on the interaction between a prey protein fused to the AD domain and the bait protein fused to the BD domain. When the *HIS3* reporter gene was activated through the protein interaction, L40 cells will grow in the medium

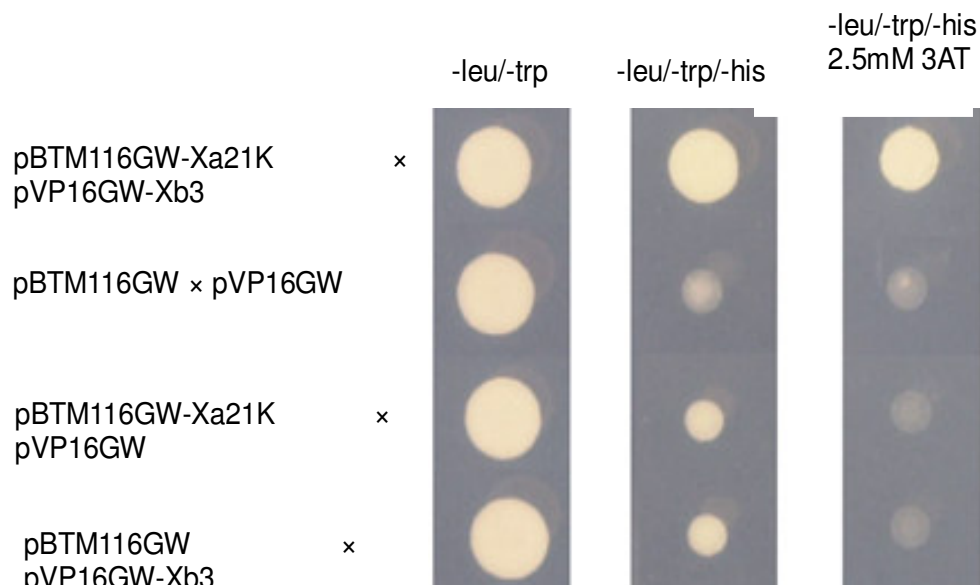


Figure 2. Results of yeast two-hybrid assay. Yeast was transformed with pairwise combinations of empty vectors, bait and prey fused vectors as described in Materials and methods. Positive transformants were screened on the SD plate lacking leucine and tryptophan. Colonies from each transformation were grown overnight in YPD and dropped onto fresh SD plates. Cells grown on the SD/-leu-trp-his plate supplemented with 2.5 mM 3-aminotriazole (3-AT) indicate the interaction between Xa21K and Xb3 proteins

absence of histidine.

Protein interaction between rice proteins XA21K and its interactor XB3

To test the usefulness of newly generated Gateway-compatible yeast two-hybrid vectors, the rice kinase domain XA21K of receptor like kinase XA21 and its interactor XB3 were chosen for illustration. The interaction between XA21K and XB3 in yeast has been described previously by Wang et al. (2006). In this study, the coding regions of *Xa21K* and *Xb3* were amplified by RT-PCR and cloned into the entry vector, respectively. And then the bait pBTM116GW-Xa21K and prey pVP16GW-Xb3 were generated through the LR recombination with high efficiency as described in materials and methods. After co-transformation of the yeast L40 with these two vectors, the protein interaction was assayed (Figure 2). As expected, the protein interaction was demonstrated only in the pair of bait pBTM116GW-Xa21K and prey pVP16GW-Xb3. To assess the impact of introduced Gateway cassette (*attR1-Cmr-ccdB-attR*) on the constructed vectors, the background control was designed by pair-wise combination of fused bait and prey protein or empty vectors (Figure 2). No obvious background was detected in the selection medium. The above results indicated that the newly generated Gateway-compatible yeast two-hybrid vectors will be useful in analysis of protein interaction in a high throughput way.

DISCUSSION

Protein-protein interactions play important roles in cells for a variety of biological processes. Studies of protein interaction are crucial for understanding the functions of a large number of genes predicted by genomic study (Rual et al., 2005). Therefore, the method for large-scale study of protein interaction is urgently needed. Yeast two-hybrid assay is a simple and powerful tool for protein interaction assay. Several excellent yeast two-hybrid systems have been developed. For example, the Hollenberg system including the bait vector pBTM116 and prey vector pVP16 is one of the most extensively used system to study the interaction between proteins (Vojtek et al., 1993; Hollenberg et al., 1995; Vojtek et al., 1995). The bait vector pBTM116SS and prey vector pVP16S1 were derivatives from pBTM116 and pVP16 by adding restriction endonuclease sites in the MCS. However, the cloning of interested DNA fragment into these yeast two-hybrid systems is still based on the general restrictive endonuclease- and ligase-based DNA cloning technology and therefore is laborious and inefficient.

The Gateway technology provides a simple and highly efficient gene cloning and target gene transfer method between vectors. By introducing Gateway cassette into the conventional vector is a good way to create Gateway-based vector. Through modification of the existing vectors, a new Gateway yeast two-hybrid system, including bait vector pEZY202 and prey vector pEZY45, was constructed for protein interaction study (Guo et al., 2007).

Another Gateway-compatible two-hybrid system based on GAL4 DNA-binding (BD) and activation domains (AD) were established for high-throughput screening of protein-protein interactions especially useful in living plant cells (Ehlert et al., 2006). However, Gateway-compatible yeast two-hybrid vectors for wide and universal use for the study of protein interaction are still needed.

To improve the efficiency of Hollenberg system of yeast two-hybrid vector for high throughput analysis of protein interaction, we modified this widely used system. By introducing the gateway recombinant cassette (*attR1-Cmr-ccdB-attR*) into pBTM116SS and pVP16S1, we constructed the gateway-compatible yeast two-hybrid vectors pBTM116GW and pVP16GW. To test this system, the kinase domain XA21K of rice receptor like kinase XA21 and its interactor XB3, was cloned into the newly generated vectors, respectively, with gateway technology and the interaction between these two proteins was examined by two-hybrid assay. Our results indicated that this newly generated gateway-compatible yeast two-hybrid vectors is more efficient than the pBTM116SS and pVP16S1 in the study of protein interaction. Since the newly generated two-hybrid system combined with the advantage of gateway technology, the desired DNA fragment between entry vector and gateway-compatible two-hybrid vectors can be transferred easily and efficiently. Therefore, the application of pBTM116GW and pVP16GW will greatly facilitate the construction of the bait and prey vectors used in high throughput analysis of protein-interactions.

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