

## Yeast Two Hybrid cDNA Screening of *Arabidopsis thaliana* for SETH4 Protein Interaction

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### Abstract

SETH4 coding sequence with 2013 bp is a member of gene family expressed in gametophytic tissues of *Arabidopsis thaliana*. This fragment was PCR amplified using Kod Hi Fi DNA polymerase enzyme. This fragment was cloned into pGBKT7 bait vector and transformed *E. coli* DH5 $\alpha$  cells containing vector were selected on LB medium containing Kanamycin. Finally, pGBKT7-SETH4 bait was transformed into yeast strain Y187 and were selected on SD –Leu –Trp medium. After mating of this strain with yeast strain AH109 containing *Arabidopsis thaliana* cDNA library as prey vector, diploid cells were selected on SD –His –Leu –Trp medium. Screening of cDNA inserted into diploid cells indicated that, 35 colonies out of 240 showing X-gal staining. PCR amplified of cDNA insertion fragments in diploid cells showed some colonies have a chance for SETH4 protein interaction.

**Keywords:** Yeast two hybrid; SETH4; cDNA; *Arabidopsis thaliana*

### Introduction

SETH4 protein defines a small protein family in *Arabidopsis* that contains Armadillo (ARM) repeats as predicted protein-protein interactions domains. Gene sequences disrupted by *Ds* insertions encode proteins with diverse functions including protein anchoring (SETH1, SETH2), cell wall biosynthesis (SETH3), calcium sensing, signaling (SETH6, SETH7 and UNG6) and metabolism (SETH9, UNG10) [9]. *seth4* mutations completely block male transmission and pollen grains appear to be normally polarized but are unable to initiate pollen tube growth. Through a forward genetics approach for the identification of gametophytically expressed genes required during the progamic phase,

*SETH4* as a male-specific gametophytic gene has been identified. Analysis of *seth4* insertional mutants showed that the loss of *SETH4* function inhibited pollen germination using *in vitro* and *in vivo* assays. Pollen grains appeared to be normally polarized but were unable to initiate tube growth. [9]. SETH4 encodes a protein of 664 amino acids predicted to contain only Armadillo (ARM) repeats. ARM motifs are sequences of 40-42 amino acids, first identified in the *Drosophila melanogaster* segment polarity protein armadillo, the homolog of mammalian  $\beta$ -catenin [9]. Each ARM domain forms a 3  $\alpha$ -helical structure. Tandem ARM repeats interact to form a right-handed superhelix creating a site for protein-protein interactions [3,7].

Protein-protein interactions are intrinsic to virtually

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every cellular process ranging from DNA replication, transcription, splicing and translation, to secretion, cell cycle control, intermediary metabolism, formation of cellular macrostructures and enzymatic complexes [1,12,17].

The yeast two-hybrid system is proving itself to be a powerful tool for proteomic-based investigations. The technology has already been employed to investigate the protein-protein interactions between many of the full-length open reading frames predicted from the yeast (*Saccharomyces cerevisiae*) [10,11]. *Caenorhabditis elegans* [14], *Helicobacter pylori* [8] and *Arabidopsis thaliana* [5].

Classical Yeast Two-Hybrid System A protein of interest Y is expressed in yeast as a fusion to a DNA-binding domain (DBD, "bait"; circles denote expression plasmids). Another protein of interest X is fused to a transcriptional activation domain (AD, "prey"). The two yeast strains are mated to combine the two fusion proteins in one cell. Fields and Song (1989) [6] described the first yeast two-hybrid system. It was based on the fact that many eukaryotic transcription factors have discrete and separable DNA-binding and transcriptional activation domains. In their system, protein-protein interactions were tested by fusing one test protein to the DNA-binding domain of the yeast GAL4 transcription factor, and the second protein to the GAL4 activation domain. The fusion proteins were expressed in a suitable yeast strain and the interaction detected by assaying for expression of a GAL4 responsive reporter gene. [1-3,13].

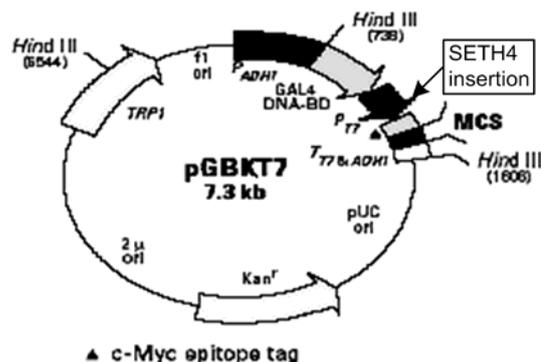
In this paper we are interested in to investigate any potential of *Arabidopsis thaliana* cDNA library expression and following interaction with SETH4 protein using Yeast two hybrid system

## Materials and Methods

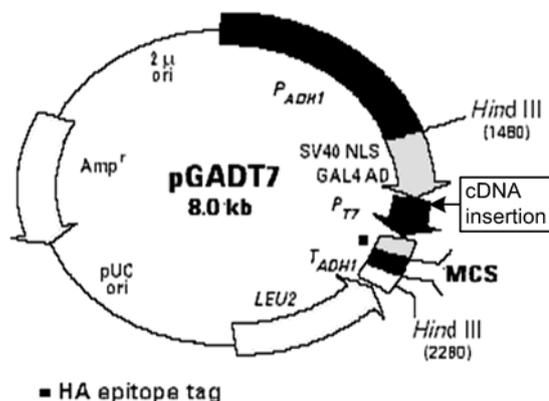
### PCR Amplification and Cloning

At the first step a construct containing Gal4-BD bait vector was made. For PCR amplification BAC (bacterial artificial chromosome) DNA containing SETH4 coding sequence (2013 bp) was amplified by primers containing *NcoI* and *SalI* sites. PCR reaction were carried out in a total volume of 25  $\mu$ l with final concentration of 1mM MgCl<sub>2</sub>, 2 mM dNTP, *Kod Hi Fi* DNA Polymerase enzyme (1 u/20  $\mu$ l reaction), with 100 ng BAC DNA as template. Thermocycling conditions were 94°C for 2 min for one cycle, 94°C for 15 s, 45°C for 5 s, 72°C for 20 s which repeated for 25 cycles followed by 5 min extension at 72°C. PCR products were gel-purified with PCR purification Kit (Qiagen

Gel Extraction Kit) according to manufacturer instruction. The purified product was digested with *NcoI* and *SalI* and ligated into pGBKT7 vector which digested with the same enzymes (Fig. 1) as bait vector to produce chimeric gene constructs in which the Gal4-binding domain region was fused in frame to the SETH4 coding sequence. Ligation was conducted according to commercial kit (Invitrogene). Bait plasmids were transferred into *E. coli* DH5 $\alpha$  and transformed cells were selected on LB medium supplemented with 50 mg/l Kanamycin. For testing of ligation, Bait plasmids were purified from selected Kanamycin resistance colonies by alkaline lyses method and were digested with *NcoI* and *SalI* enzymes. pGBK7 vector having cloned SETH4 coding region then was sequenced with SETHFW, SETHRV, T7 and BP primers. The homology of SETH4 fragment with original sequence was confirmed by alignment and comparison of original sequence and cloned fragment in vector pGBKT7.



**Figure 1.** Map of bait vector pGBKT7 used for cloning of SETH4 coding sequence.



**Figure 2.** Map of prey vector pGADT7 with *Arabidopsis thaliana* cDNA library insertion prepared by Simon G. Moller [1].

### Yeast Transformation

Yeast strains AH109 and Y187 used as mating strains in yeast two hybrid experiments were kindly supplied by Dr. Simon G. Moller (Department of Biology, Leicester University). pGBKT7-SETH4 Containing cDNA library from *Arabidopsis thaliana* as bait plasmids (Fig. 2) were transformed into *E. coli* DH5 $\alpha$  and transformed colonies were then selected on SD medium [1] lacking tryptophan (-Trp) and Histidine (-His). We also transformed a mixed pGBKT7-SETH4 vector and pGADT7 vector (as empty vector) into AH109 and Y187 yeast strains and were selected on SD lacking Leucine (-Leu), -His, -Trp. All yeast cultures were incubated at 28°C. In this experiment in order to reduce false positives in transformed yeasts we tested autoactivation reaction by adding 3-amino-1,2,4-triazole (3-AT) at concentration of 0, 0.25, 0.5, 1.0, and 2.0 mM to YPDA -Leu -His -Trp medium [10]. 3-AT is a competitive inhibitor of His gene product and reduces the background due to basal His expression. According to the results of autoactivation test we used 0.5 mM 3-AT for selection of positive yeast colonies. For determination of OD selected yeasts Y187 containing pGBKT7-SETH4 were cultured on liquid SD-Trp medium and after overnight incubation OD was 2.03-2.06 by spectrophotometer at 600nm. The plating efficiency was calculated by spread culture of 1/00, 1/1000/1/10000 dilution of Y187 bait on SD -Leu -Trp. Colony efficiency was approx. 5%.

### Yeast Mating

Selected Y187 having pGBKT7-SETH4 as bait vector were transferred to liquid SD -Leu -Trp and were incubated over night in shaker at 28° with 250 rpm. Then, 9 ml of liquid YPDA, 1 ml of well grown Y187 pGBKT7-SETH4 bait, 100  $\mu$ l of AH109 cDNA library of *Arabidopsis thaliana* (obtained from Jodi Maple and Simon G. Moller, Leicester University, UK) and 50 mg/l Kanamycin were mixed gently in a 100 ml flask and incubated in shaker incubator at 28° with 50 rpm for 26 h. After mating yeast cells were plated on SD -His -Leu -Trp medium containing 0.5 mM 3-AT for selection of diploids containing both BD (binding domain) and AD (activated domain) plasmids. Positive colonies were scored after 5 h, 18 h, and 2, 3, 4, 5 days and were replica streaked on the same SD medium without 3-AT. Selected colonies then were spotted onto SD -His -Leu -Trp plates and were assayed with  $\beta$ -galactosidase (X-gal) for Gal4 reporter gene activation as described by Sims and Ordanic (2001) [13]. On X-gal positive colonies, insert cDNA size determined by PCR amplification using T7 and Gal4-AD primers.

### Results

Amplified PCR of SETH4 coding region with about 2013 bp is shown in Figure 3. Since, *Kod Hi Fi* enzyme has exonuclease proof reading the amplification condition was optimized for PCR to reduce any risk of mutation in frame.

For testing the accuracy of SETH4 inserted DNA fragment into pGBKT7 vector transformed vector digested with *Nco*I and *Sal*I enzymes. As positive control pGBKT7 containing 3 kb fragment supplied kindly by Andrew Johnson (Biology lab. Leicester University, UK). Result of digestion is shown in Figure 4.

After transformation of yeast strain Y187 containing bait vector with SETH4 coding region, yeasts colonies were selected on SD -Leu -Trp medium (Fig. 5). After mating Y187 bait vector with AH109 containing cDNA library from *Arabidopsis thaliana*, 240 colonies were detected on SD -His -Leu -Trp medium these colonies were then tested for  $\beta$  galactosidase activity. 35 colonies out of 240 were showed blue color with some variation in color density (Fig. 6).

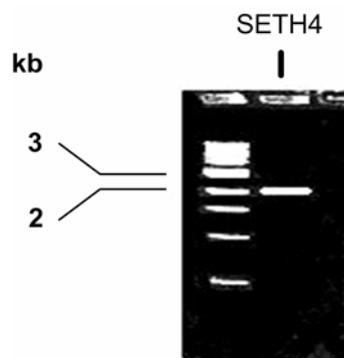
According to data obtained from cDNA insertion size in Figure 7, we can line up similar DNA size in 3 different groups of yeast colonies:

Group 1: colony No. 48 and 117 in Figure 7B, colony No 180 in Figure 7D with approx. size 400 bp;

Group 2: colony No. 3, 7, 37 in Figure 7A, 106 in Figure 7C, 268, 298 in Figure 7E, 308 in Figure 7F, 34, 89, 97 in Figure 7B with approx. size of 600 bp;

Group 3: colony No. 9, 18, 26 in Figure 7A, 120, 178, 201, 212 in Figure 7D with approx. size 550 bp.

However, according to the PCR results in Figure 7, colonies, No. 120,178,179, 201, 212 and 114 with approx. size of 550 bp are the most repeatable size which means they are more likely to be a potential of interactor with SETH4 protein.



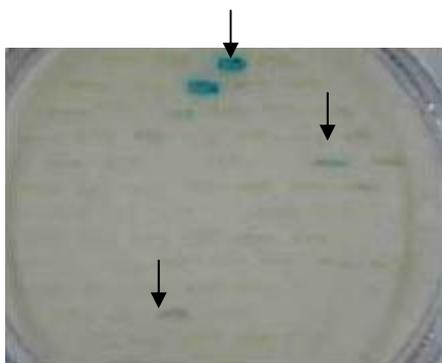
**Figure 3.** Amplified PCR of coding region of SETH4 fragment with 2013 bp, (1 kb ladder used as Marker).



**Figure 4.** Digestion of SETH4 DNA fragment. Lanes 1, 2, 3, 4, digestion with *NcoI* and *SalI*, Lanes 5, 6, 7, 8 digestions with *NcoI* enzyme. C: positive control 9, 10, 11 and 12 undigested.



**Figure 5.** Selected yeast Y187 colonies on medium SD –Leu –Trp.



**Figure 6.**  $\beta$ -galactosidase activity (X-gal) of selected colonies on SD –His –Leu –Trp.

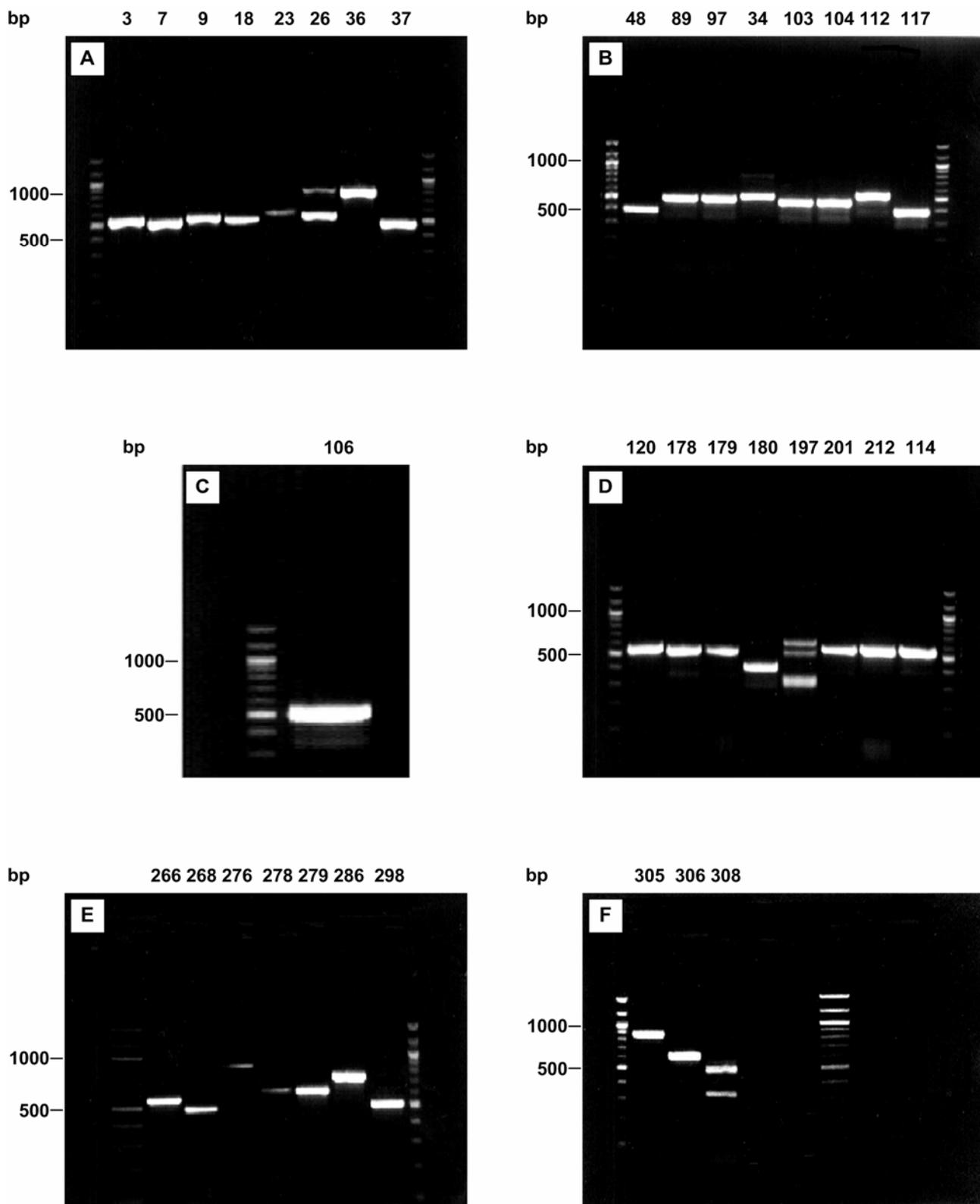
### Discussion

The yeast two hybrid system is used for the analysis of protein-protein interaction between two protein or for determining protein domains or it can be used to identify unknown proteins, encoded by a cDNA library, that interact with a protein of interest. In the latter case the yeast two hybrid systems becomes a powerful tool

for investigation the network of interaction that form between proteins involved in particular processes [16,17]. Although the system offers many advantages over biochemical methods, such as cost, convince and sensitivity, it still has several associated problems. False-positives are sometime generated in two hybrid library screens. For example, in our study we detected over 240 colonies were able to grow on SD –His –Leu –Trp medium indicating possible potential for interaction between SETH4 protein and proteins expressed from *Arabidopsis* cDNA library insertion, but only 35 colonies out of that were able to show blue staining in X-gal test. It is predicted that, not all of those positive colonies are able to interact with SETH4 protein. In fact, because of low level of cDNA expression it might be concluded that a few proteins have a potential for true interaction with SETH4 protein. The results of PCR insertion screening showed that a few colonies have a better chance for interaction and most of them may consider as false positive or very weak interactors. However, use of two or more reporter genes to assay for an interaction has been reported by Fashena *et al.* (2000) [7]. In our study, some of the cDNA inserted with higher number of repetitive DNA inserted fragment were able to express and interact with SETH4 protein. For these clones there is a good chance for rolling consider as a transcription factor. A similar observation has already been reported for bZIP transcription factor in *Arabidopsis thaliana* [5]. The classical yeast two hybrid systems relies on the transcriptional activation of reporter genes and so may not be suited to the identification of proteins such as transcriptional repressors [4]. As a general idea, if cDNA inserted fragments are repetitive after PCR amplification, then there is a good chance for protein-protein interaction. However, protein SETH4 in bate vector interacts with a protein expressed in colonies No. 120, 178, 179, 201, 212 in the resulting diploids cells with approx size of 550 bp. They may reconstitute a transcription factor which activates a reporter gene (here: HIS3) and therefore allows the cell to growth on selective media (here: media lacking Histidine). We are speculating that X-gal staining has approved SETH4 protein interacting with another protein resulting from cDNA expression with 550 bp length. Identification of these proteins interacting in yeast cells needs to be studied in details in the future.

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**Figure 7.** A, B, C, D, E, and F, PCR amplification of cDNA insertion screening in selected diploid yeast colonies showing X-gal activity (100 bp ladder used as Marker).

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