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***Research Article** (Open Access)*

IDENTIFICATION OF INTERACTING PARTNER OF EF - HAND LIKE GENE IN *PIRIFORMOSPORA INDICA* BY YEAST TWO HYBRID METHOD

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ABSTRACT
To elucidate the function of an uncharacterized protein, Yeast two hybrid is a crucial and adopted system that recognize its interacting partner with known function. This system adopted modular nature of Gal 4 transcription factor with two domain, Gal4 DNA-binding domain (BD) and activation domain (AD). This transcription factor, Gal4 DNA-binding domain fused to a protein with unknown function called bait and activation domain fused to protein with known function prey. When both Protein interact in yeast AH109 it trigger the expression of downstream reporter genes, such as the β-galactosidase (β-gal), and selective reporter genes in charge of adenine or histidine biosynthesis, which complement the chromosomal mutation in a metabolic pathway. The interaction of individual bait protein with the prey protein triggers the reporter gene activation, enabling the yeast to grow on selective plates.

***Keywords**: Domain, Protein, Biosynthesis, Interaction Hybrid*

INTRODUCTION
Calcium is a vital element for plants' growth and development and imparts structural rigidity to the cells, and also involved in stress responses and tolerance. Myosins also possess EF-Hand like proteins. Myosin holoenzyme possesses heavy and light chains, myosin light chains are the member of CaM and CaM related gene family with EF-Hand domain while heavy chain possess three domain N-terminal motor, mid part neck and C-terminal tail domain. Myosin light chains non-covalently bind with heavy chain with 20-25 amino acids residue called IQ motif in neck domain. Myosins are crucially involved in many mechanoenzymatic functions in higher eukaryotes while in lower organism it involve in motility (Lin *et al.,* 2017) . EF-Hand motif comprises a structure of two a-helices with one loop, in which one a-helix E is connected with the second a-helix by a loop. EF-Hand mediated Ca^{2+} is involved in stress management and nutritional stress signaling in plants (Weagel *et al.,* 2022).

Protein–protein interaction networks reveal the information of metabolic pathways that takes place in eukaryotic cells (Baryshev *et al.,* 2004; Causier *et al.,* 2002). Yeast two hybrid screening are used to identify the interaction of one gene to other at expression level in various eukaryotic organisms including plants, animals, and fungi as it exploit the transcriptional activator comprising two separate domains one for binging for DNA while other for activation (Young *et al.,* 1998; Elhabashy *et al.,* 2022). In this assay EF-Hand gene was used as bait to elucidate interacting proteins in *Piriformospora indica.* Gal-4 based yeast two hybrid system was used for identification of interacting partner of EF hand like gene. Coding region of EF-hand like gene (approx. 495 bp) was is amplified from already cloned pGMT vector by PCR. Amplified gene was cloned into binding domain vector pGBKT7. The insertion of EF hand like gene was confirmed by Colony PCR. Restriction digestion of binding construct pGBKT7 with *EcoRI* and *PstI* result the presence of EF hand like gene in pGBKT7 vector (Lin *et al.,* 2023).

MATERIALS AND METHODS

Gal-4 based Yeast two hybrid system was used to identify the interacting partner of EF-hand like gene in *P.indica*. pGBKT7 was used as bait expression vector (Tabar *et al.,* 2022). To express as fusion protein EF-hand was fused DNA binding domain under T7 promotor. cDNA library was fused in pGADT7vector (Stynen *et al.,* 2012).Coding region of *Pi*EF-Hand like gene (~.5 kb) was amplified from already cloned pGEMT vector by PCR. Amplified gene was cloned into binding domain vector pGBKT7. Colony PCR was performed to confirm the presence of *Pi*EF-Hand like gene. Restriction digestions of binding construct pGBKT7 with *EcoRI* and *PstI* result in the presence of *Pi*EF-Hand like gene in pGBKT7. The pGBKT7 binding construct harbouring *Pi*EF-Hand like gene was co-transformed with pGAD7 vector harboring cDNA library into yeast strain AH109 by PEG or lithium acetate method. AH109 strain contains two reporter gene His3 and β-galactosidase (Mehla *et al.,* 2015). Leaky expression of $HIS3^+$ was inhibited by using 3-AT (3-Amino-1,2,4-triazole).

RESULTS AND DISCUSSION

Detection of Auto Activation of Bait Vector

To reduce the false positive due to leaky expression of HIS3, recombinant bait pGBKT7- *Pi*EF-Hand was transformed into *E. coli* DH5α and the positive colonies were used to extract the plasmids. Purified plasmid was used to transform yeast AH109 without pGADT7 and tested for blue-white screening.

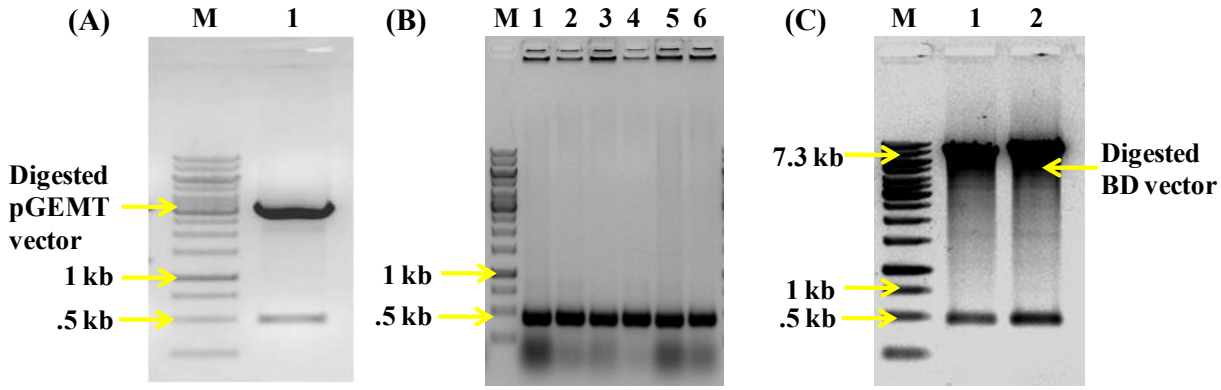

Figure 1: Cloning of EF-Hand gene into pGBKT7 vector. (A) Restriction digestion of *Pi*EF-Hand-pGEMT construct with *EcoR1* and *Pst 1* showing digested pGEMT construct and released *Pi*EF-Hand gene. **(B)** Colony PCR of *Pi*EF-Hand-pGBKT7 colonies with *Pi*EF-Hand gene specific primers. **(C)** Restriction digestion of *Pi*EF-Hand-pGBKT7 BD construct.

cDNA library construction

Total RNA extracted from *P. indica*, after EF-Hand inoculation was used for the construction of the Y2H library. The integrity for RNA after DNAase treatment was checked by gel electrophoresis. This RNA is used to obtain cDNA by using cDNA synthesis kit from clonetech. The cDNA was analysed by electrophoresis and purified and then transfered to pGADT7 vector for construction of library. The transformation reaction mixture is diluted to 10 to 10^4 times and then spread on plate to calculate transformation efficiency. Co-transformed yeast cells were grown on auxotropic synthetic media lacking leucine and tryptophan (Double drop out media) at 30°C for 16 to 18 hours. ~120 positive colonies were obtained, which randomly picked to grow on 3 Drop out synthetic dextrose media lacking histidine, leucine and tryptophan. 10 mM of 3-AT (3-Amino-1,2,4-triazole) also added into 3DO media to reduce false positive. Colonies grown on 3DO (SD + His^- Leu^- Trp^-) were transferred on quadruple dropout media containing 30 mM 3-AT for 5–7 days of incubation.

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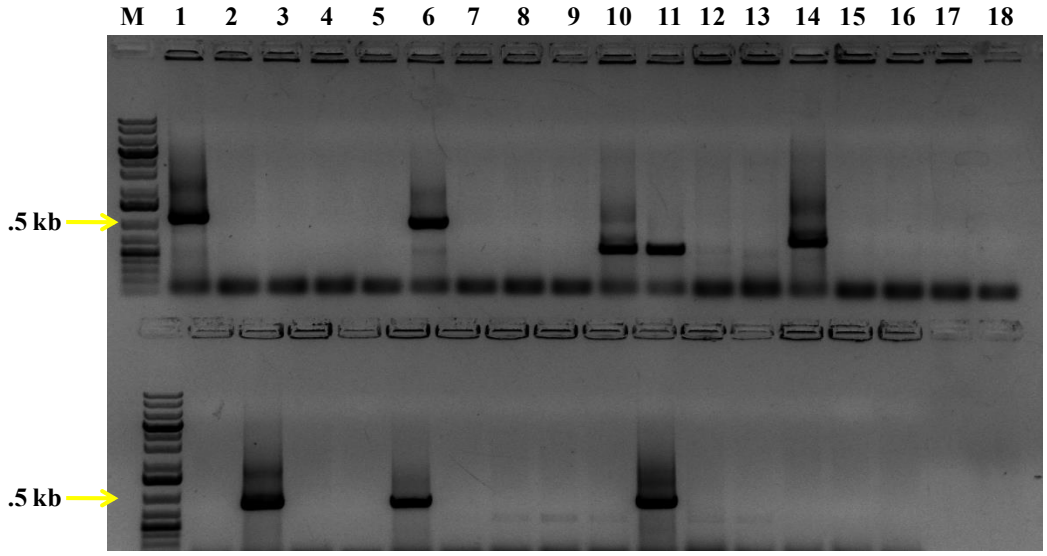

Figure 2: Construction of cDNA library and colony PCR of yeast colonies grown on YAPD medium plates with pGAD7-*Pi*EF-Hand (AD vector) specific primers.

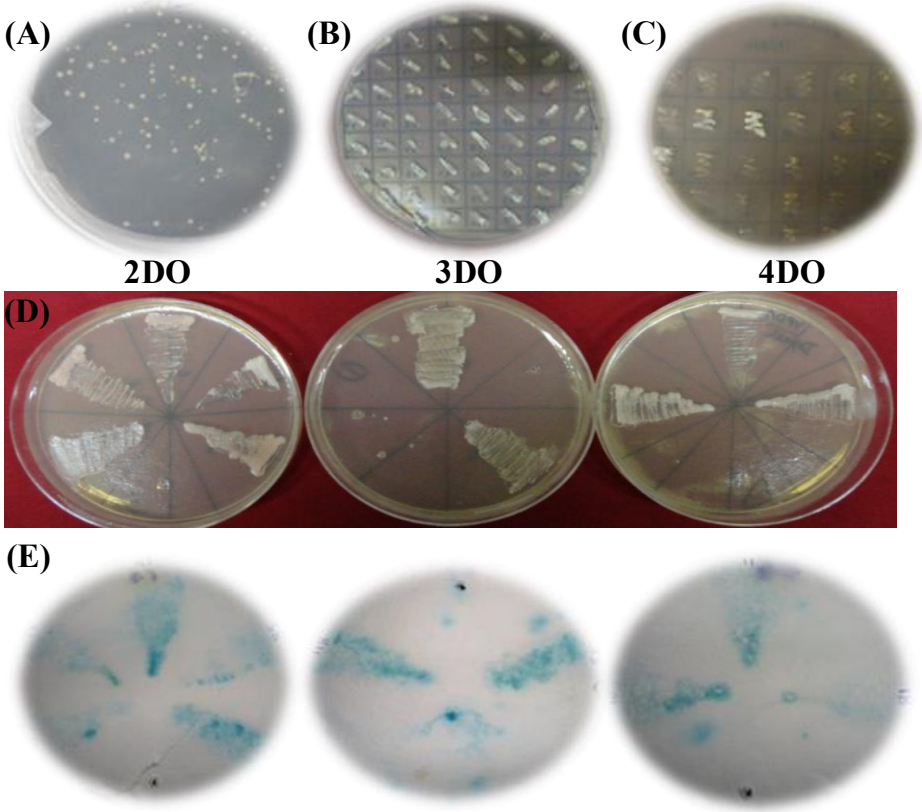

Figure 3: Yeast two hybrid system for isolation of interacting partner of *Pi*EF-Hand with *P. indica* cDNA library (A) Transformed yeast strain growth on SD media 2DO (lacking leucine and tryptophan) **(B)** 3DO (SD + His^- Leu^- Trp^-) **(C)** 4DO (SD + His^- Leu^- Trp Ade^-) **(D)** 4DO selected colonies grown on YAPD medium containing 10 mM 3-AT (3-Amino-1,2,4-triazole). **(E)** Same colonies which grow on 4DO media were analysed for X-gal filter lift assay

Colony Filter Lift Assay

The co-transformed yeast was analyzed for β-galactosidase expression by colony filter lift assay and ONPG/X-gal used as substrate. Yeast colonies that grown on SD-media were further tested for being true positives by blue-white screening. All positives colony screened gave a blue stain, indicating activation of the Lac Z reporter gene during protein–protein interaction event. The assay was repeated multiple times for confirmation but same results were obtained.

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Conflict of interest

The author declares no conflict of interest.

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