International Journal on Science and Technology (IJSAT)
E-ISSN: 2229-7677 ● Website: www.ijsat.org ● Email: editor@ijsat.org

Cloning of PiEF hand Gene into in Indian mustard (Brassica juncea L.) plant for stress tolerance

Dr. Punam Kundu

Assistant Professor
Biotechnology
Chaudhary Ranbir Singh University Jind Haryana India

Abstract

Overexpression of abiotic stress tolerance genes, from algae and fungi develop symbiotic relationships with plants and help them survive in adverse conditions. Serendipita indica (Piriformospora indica) is a basidiomycete fungus that builds a mycorrhizal association with wide variety of plant species and protects the plant from plethora of environmental insults . Plants and fungus both mutually benefit each other as nutrient and carbon sources transported from plant to fungus; in turn, it increases nutrition and water uptake from soil. Fungal hyphae serve as a connection between a plant's root and the rhizosphere thus facilitates the H2O and inorganic nutrient supply. S.indica also protects plants to attack from other fungi.

INTRODUCTION

Overexpression of EF proteins in plants enhances tolerance of engineered plants under drought and salinity stress. In salinity stress, plant cells contain Na^+ ions in the cytoplasm and are more vulnerable under stress, while in transgenic plants, cytoplasmic Na^+ accumulation of cell enhances. GmCam4 overexpression in A. thaliana, up-regulate the gene P5CS which participate in proline biosynthesis (Yoo et al., 2005). Overexpression of MtCaMP1 (an EF-Hand family gene) in Arabidopsis confers osmotic and ionic stress tolerance (Wang et al., 2013). In rice, a C-terminal central-like domain OsCCD1 and in wheat TaCCD1 induced in response to osmotic and salt stress conditions. The overexpression of these genes in economically essential plants enhances tolerance against drought and salinity by activating the calcium-mediated ABA signaling pathway (Jing et al., 2016). In A. thaliana, AtSOS1 exporter passes Na^+ ion outside the cell while HKT antiporters family export Na^+ ions and import H^+ ions (Cao et al., 2020). Na^+ ions are a significant constraint factor that limits the growth and productivity of maize. ZmNSA1 comprises the EF-Hand domain with the calcium-binding property and promotes plants growth under salinity stress. This protein supports the transcription of H^+-ATPase by degrading proteasome, which negatively regulates H^+-ATPase transcription.

In addition to the overexpression of abiotic stress tolerance genes, some algae and fungi develop symbiotic relationships with plants and help them survive in adverse conditions. Serendipita indica (Piriformospora indica) is a basidiomycete fungus that builds a mycorrhizal association with wide variety of plant species and protects the plant from plethora of environmental insults (Verma et al., 1998; Singh et al., 2011; Gill et al., 2016). Plants and fungus both mutually benefit each other as nutrient and

carbon sources transported from plant to fungus; in turn, it increases nutrition and water uptake from soil (Bonfante and Genre, 2003; Gill et al., 2016). Fungal hyphae serve as a connection between a plant's root and the rhizosphere thus facilitates the H_2O and inorganic nutrient supply (Bucking and Heyser et al., 2008). S.indica also protects plants to attack from other fungi (Waller et al., 2005).

Keywords: Cloning vector, Fungal hyphae, Nutrient, Tolerance

MATERIALS AND METHODS and RESULTS

Isolation of EF-Hand gene from Piriformospora indica and cloning into pGMT vector

The complete sequence of the Piriformospora indica EF-Hand gene was reterived from gene bank with accession ID ACR40094.1. Gene specific forward and reverse primers were designed to cover full open reading frame with 521 base pair in order to amplify complete CDS sequence of PiEF-Hand gene. GeneJET extraction kit from thermoscientific was used for amplified product (Fig. 4.10 A) and cloned into the pGEMT easy vector (Promega). Ligation of PiEF-Hand was confirmed by restriction digestion of pGMT construct (Fig. 4.10 B). For primer designing the complete CDS sequence of PiEF-Hand was taken from NCBI Genebank as given below:

(A).

ATGGATAACAACGCAGAATACAAGGAGGCCTTTGCGCTTTTTGACAAAAAGGAACGGGAACTGT
TCCACGAGAGACTCTTGGAGACTTGCTACGTGCTCTTGGGCAGAATCCAACACAGGCAGAAGTC
TCAGAGATTGTGAACAAGGCGCCGCGAGAAGTTGACTACAAGACCTTCCTCATATTCTGAACCG
TCCCGATGGTTTCAAACCAGCAGGAACCCCAGAGGATTCCAGGTCTTGAGTTTATTCGAGTGCA
AGGAAGGCAACGGCTATATCGGCGCAGGCGAGCTACTACGTTCTCACCCAGCTTGGAGAAAAGA
TGACAGATGAAGAAGTCGATGAGCTGCTCAAGGGTGTTCAGATTGGCGCCGATGGAAACGTAAA
CTATGAGAGCTTTGTACGAACCATCCTCAGCCAGTAG

(B).

MDNNAEYKEAFALFDKKGTGTVPRETLGDLLRALGQNPTQAEVSEIVNKAPREVDYKTFLSILNRP
DGFKPAGTPEEFIRGFQVFDKEGNGYIGAGELRYVLTQLGEKMTDEEVDELLKGVQIGADGNVNYE
SFVRTILSQ

Fig. (A) cDNA sequence of PiEF-Hand like gene from Piriformospora indica. Start and stop codon in the sequence are shown in green and red colour, respectively **(B)** Deduced amino acid sequence of the protein encoded by EF-Hand like gene cDNA.

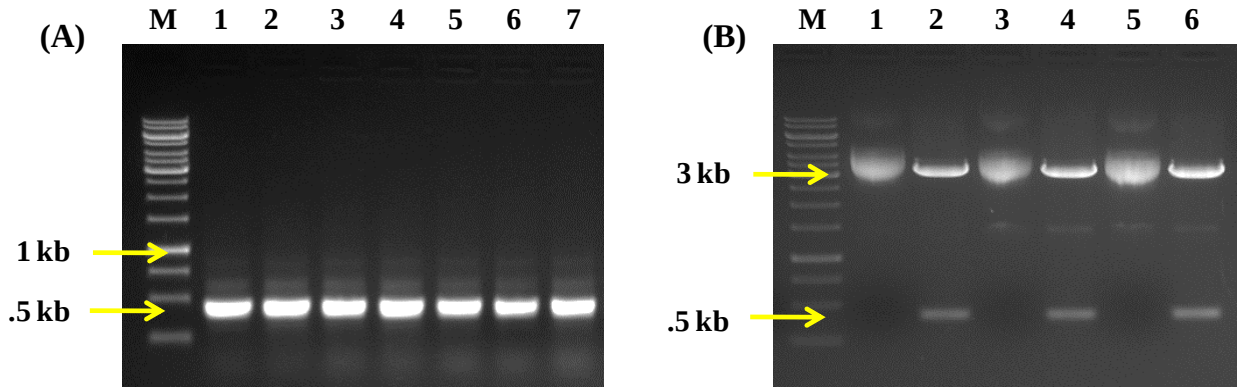

Fig. (A) Agarose gel electrophoresis of PCR amplified product (1 Kb ladder, Thermo), lane M, DNA ladder; Lane 1-7; amplified PCR product **(B)** Restriction digestion of pGEMT clones with EcoR1 and Pst1 releasing 500 bp fragment of PiEF-Hand. Lane M: DNA ladder, Lane 1,3,5 undigested pGEMT-PiEF-Hand construct; Lane 2, 4,6, digested pGEMT clones.

Cloning of PiEF-Hand like genes in to pET28a cloning vector

For expression analysis, PiEF-Hand gene was cloned into pET28a vector (Novagen, USA) under BamHI and HindIII sites. The recombinant plasmid carrying PiEF-Hand gene insert was transformed into freshly prepared competenant cells of E. coli BL21. ~5-10 µL of ligated productwas added to competent cells (200 µL). The transformed cells were incubated on ice for 30 min and then at 42°C for 2 min in dry bath and again on ice for 2 min.

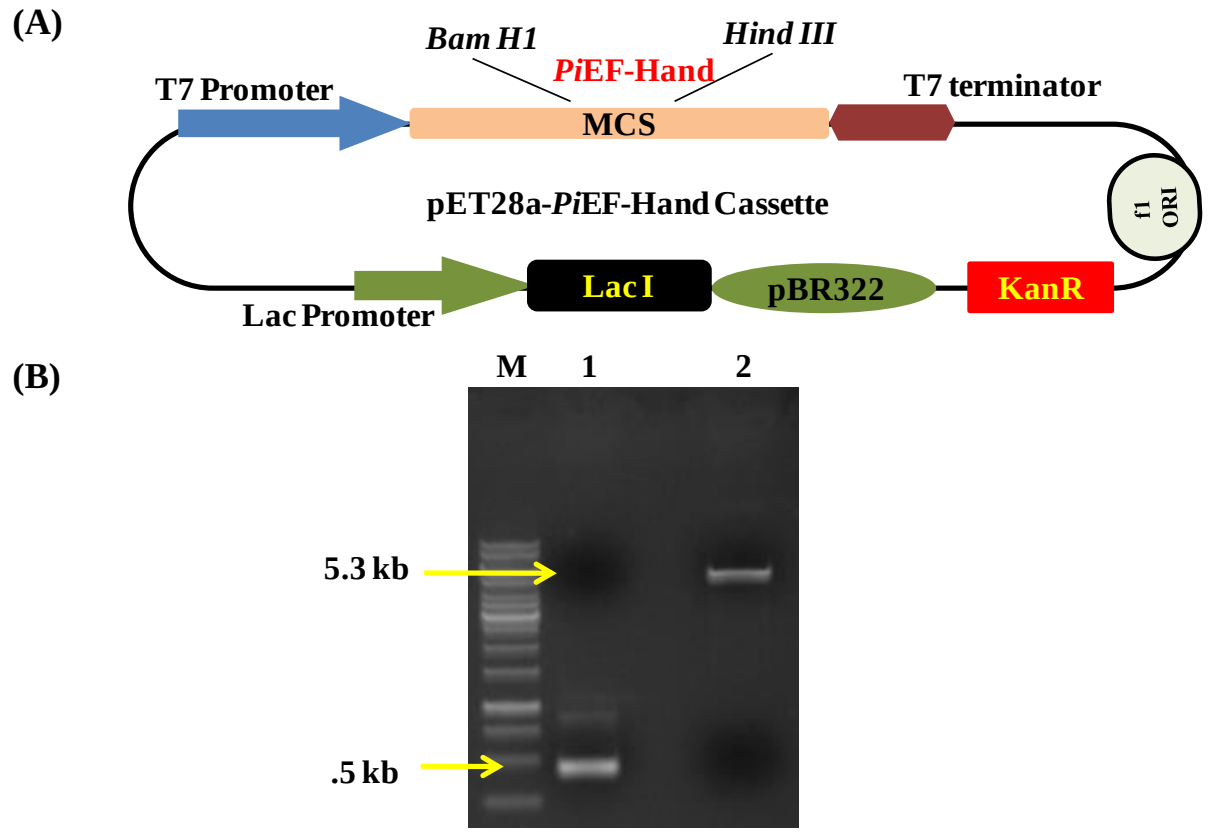

Fig. Cloning of EF-Hand gene into pET28a cloning vector (A) Schematic representation of pET28a vector harbouring PiEF-Hand like gene **(B)** Restriction digestion of pET28a clone with Bam H1 and Hind III for confirmation of insertion of PiEF-Hand like gene into the vector. Lane M, DNA marker; Lane 1 & 2, digested pET28a.

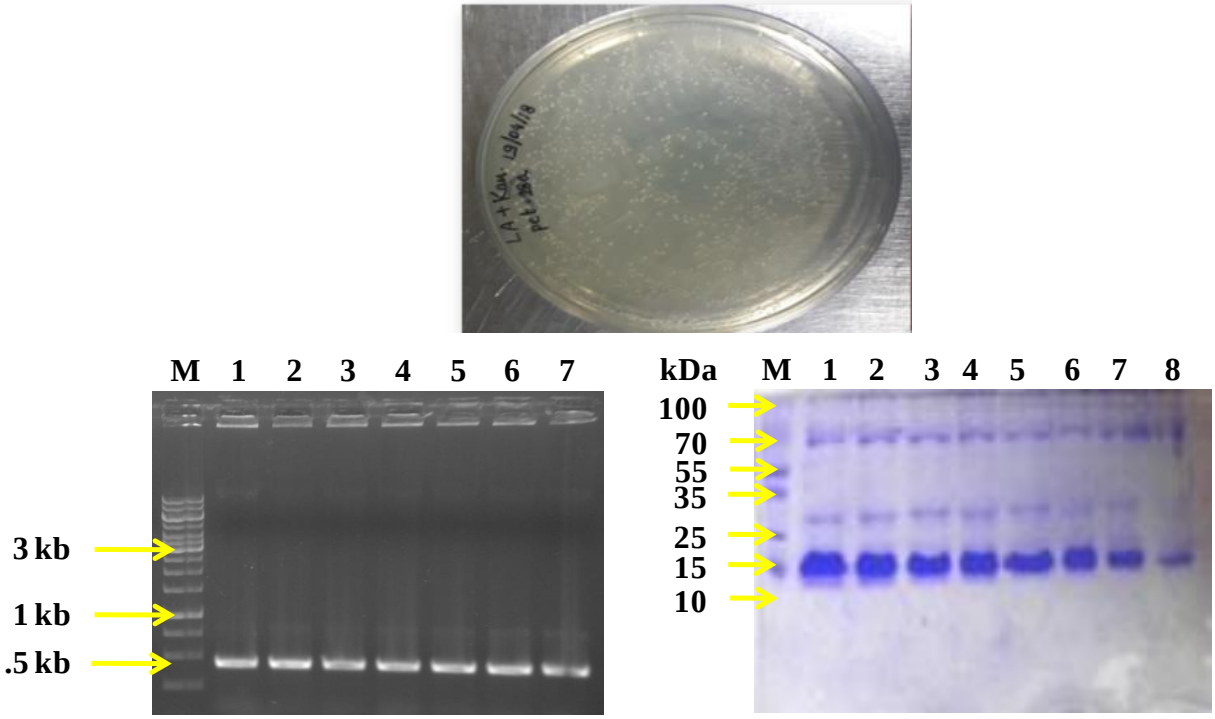

Fig.Transformation and expression of PiEF-Hand protein (A) Transformed cells of E. coli BL21 having pET28a-EF-Hand construct **(B)** PiEF-Hand protein cloned in pET28a vector confirmed by colony PCR with PiEF-Hand specific primers; Lanes: 1-7 colonies PCR; M, DNA ladder **(C)** SDS-PAGE analysis of PiEF-Hand stained with Coomassie Brilliant Blue, 15 kDa band confirm the expression of PiEF-Hand like gene in bacterial system. Lane M, ladder; Lanes: 1-8 IPTG induced expression of PiEF-Hand like gene.

Development of PiEF-Hand construct for transformation

In order to develop possible tolerance against salinity in Indian mustard, we targeted to clone PiEF-Hand gene into binary vector pCAMBIA 1301.

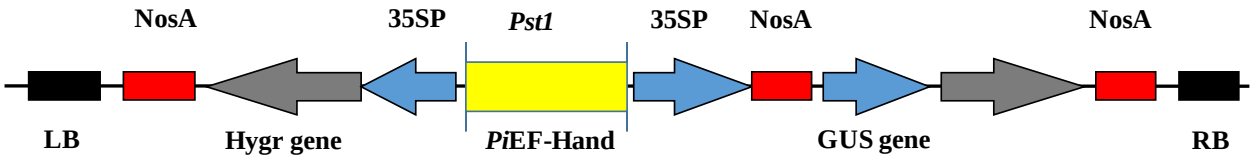

Fig. 4.14. T-DNA region of the pCAMBIA1301 plasmid containing PiEF-Hand was used for B. juncea transformation. The pCAMBIA-PiEF-Hand cassette was cloned at Pst1 site in the region of MCS in pCAMBIA1301 binary vector. This recombinant plasmid was mobilized in A. tumifacience

EHA105 and used for the transformation of B. juncea. Hygr, hygromycin phosphotransferase; GUS-gene, β -glucuronidase; 35SP, cauliflower mosaic virus 35 virus promotor; NosA, polyA nopaline synthase terminator; LB and RB, left and right border, respectively.

PiEF-Hand gene was obtained from pGEMT vector and cloned into pCAMBIA1301 at Pst1 site. Cloned product was transformed into competent E. coli DH5α and Agarobacterium. Positive colonies were grown on Kanamycin containing media were selected and confirmed by PCR. Recombinant strain of E.coli DH5α were used for preparation of glycerol stock to preserve recombinant pCAMBIA-PiEF-Hand construct, while recombinant strain of was used for transformation of B. juncea.

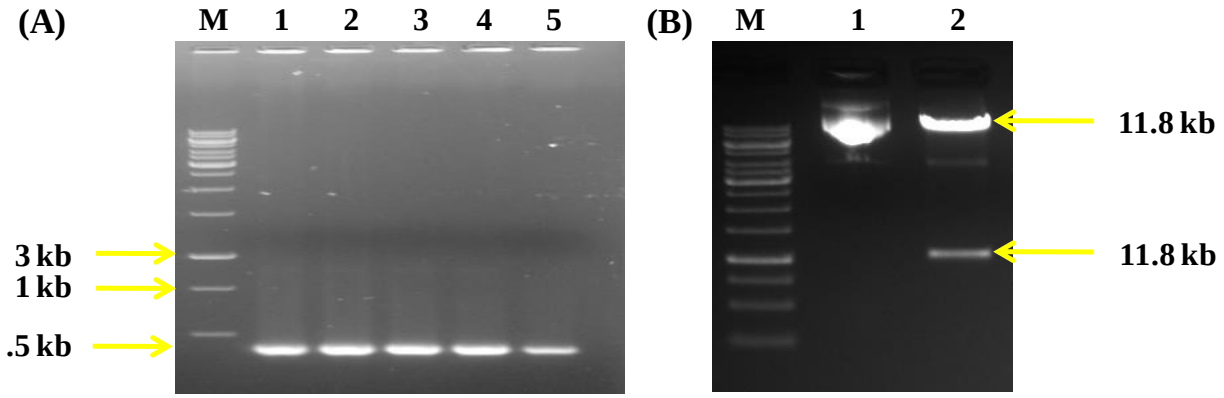

Fig. Restriction digestion and PCR analysis of the pCAMBIA1301 PiEF-Hand construct used for B. juncea (A) Colony PCR showing amplification of .500 kb insert from positive destination of E. coli DH5α. Lane M, DNA ladder; Lane 2 -7 amplification **(B)** Restriction digestion of destination clone with Pst1 resulting in .5 kb band. Lane M, DNA ladder; Lane 1 Empty clone and Lane 2 digested pCAMBIA1301-PiEF-Hand gene construct.

Mobilization of EF hand construct into A. tumifacien

Competant cell of Agarobacterium (Aliquot of 200µl in microfuge tube) were taken out from -80°C and incuabated at room temperature.5-10µl of plasmid DNA, having atleast 1µg of plasmid DNA, was added to competant cells. Tube covered with foil and freezed in liq. N_2 for 1 min followed by thaw at 37°C for 5 min. 1 mL YEP media was added and kept on shaker for 3 hours with genly shaking at 150rpm. After 3 hour centrifuged the tube and supernatent was disacarded and cells were suspended into 100 µL YEP broth. The cells were spread on YEP plate containing kanamycin, Rifampcin, Gentamycim and incubatedat 28 °C for 2 days. The positive colonies confirmed by colony PCR.

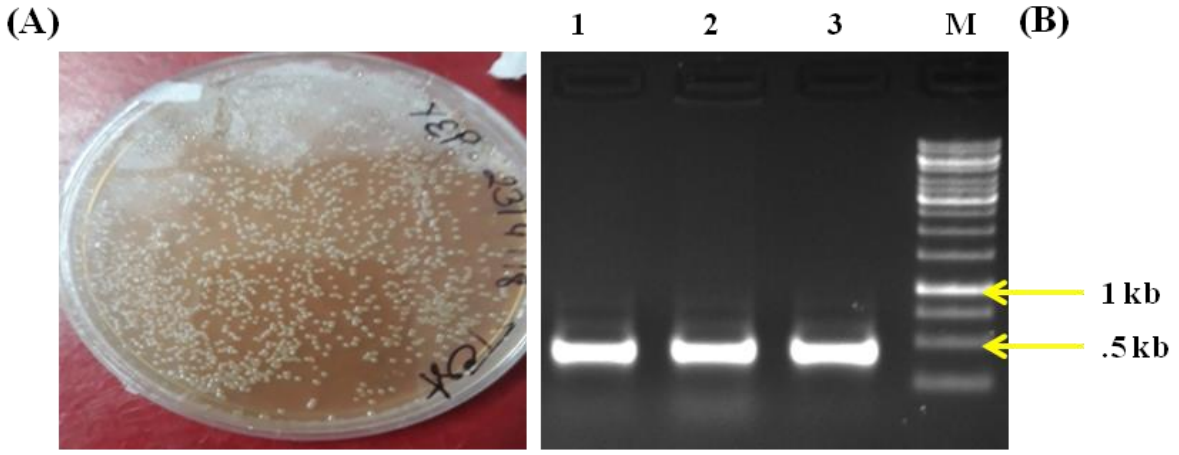

Fig. Transformation and Colony PCR of Agrobacterium pCAMBIA1301-PiEF-Hand (A) Transformed colonies of Agrobacterium strain harbouring pCAMBIA-PiEF-Hand gene construct (B) Colony PCR of transformed colonies showing amplification of PiEF-Hand gene insert. Lane M, DNA ladder; Lane 1-3, transformed colonies used for colony PCR.

DISCUSSION

Vectors are important but tightly regulated gene expression are necessary tools in research on extremely useful signalling genes. CDS sequence of the EF-hand gene was obtained from GenBank by searching with the GenBank accession (ACR40094.1). A pair of gene-specific primers was designed to amplify the full-length EF-hand cDNA sequence. The PCR products were purified using geneJET gel extraction kit and cloned into the pRT101and pGMT vector under XhoI and BamHI and EcoR1and Pst1 site respectively.For expression and screening, EF hand gene in E. coli, it was subcloned into pET 28a vector between BamH1 and Hind III restriction enzyme sites and transformed into E. coli DH5α competent cells for maintenance and E. coli BL21 cells for protein expression. Transformants were selected on LB agar plates containing kanamycin for selection. Gene specific primers were used for screening by direct colony PCR and SDS PAGE was performed to check the expression of EF Hand gene.

Declaration of competing interest

There is no conflict of interest.

Acknowledgments

Work on plant abiotic stress tolerance in laboratory was partially supported by University Grants Commission (UGC), Department of Science and Technology (DST), Council of Scientific & Industrial Research (CSIR), Govt. of India. PK acknowledges the receipt of Junior Research Fellow scholarship (CSIR File No. 09/382(0174)-2015 EMR-I) from CSIR, Govt. of India and CRSU Jind Haryana.

References

1. Sezonov, G., Joseleau-Petit, D., & d'Ari, R. (2007). Escherichia coli physiology in Luria-Bertani broth. Journal of bacteriology, 189(23), 8746-8749.
2. Pope, B., & Kent, H. M. (1996). High efficiency 5 min transformation of Escherichia coli. Nucleic acids research, 24(3), 536-537.
3. Rosano, G. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in Escherichia coli: advances and challenges. Frontiers in microbiology, 5, 172.
4. Deng, T., Noel, J. P., & Tsai, M. D. (1990). A novel expression vector for high-level synthesis and secretion of foreign proteins in Escherichia coli: overproduction of bovine pancreatic phospholipase A2. Gene, 93(2), 229-234.
5. Kim, H. M., Hong, Y., & Chen, J. (2022). A decade of CRISPR-Cas gnome editing in C. elegans. International Journal of Molecular Sciences, 23(24), 15863.
6. Li, H., Hao, C., & Xu, D. (2017). Development of a novel vector for cloning and expressing extremely toxic genes in Escherichia coli. Electronic Journal of Biotechnology, 30, 88-94.
7. Romeo, L., Esposito, A., Bernacchi, A., Colazzo, D., Vassallo, A., Zaccaroni, M., ... & Del Duca, S. (2023). Application of cloning-free genome engineering to Escherichia coli. Microorganisms, 11(1), 215.
8. Kim, H. M., Hong, Y., & Chen, J. (2022). A decade of CRISPR-Cas gnome editing in C. elegans. International Journal of Molecular Sciences, 23(24), 15863.