Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2020; 8(2): 1567-1571

Received: 20-01-2020
Accepted: 22-02-2020

Rahul Anand
Department of Agricultural Biotechnology and Molecular Biology, RPCAU, Pusa, Bihar, India

Sonali Kumari
Department of Biochemical Engineering, IIT BHU, Varanasi, Uttar Pradesh, India

Debasis Chattopadhyay
Staff Scientist VII, NIPGR (JNU), New Delhi, India

Mithilesh Kumar
Professor, Department of Agricultural Biotechnology and Molecular Biology, RPCAU, Pusa, Bihar, India

VK Sharma
Professor, Department of Agricultural Biotechnology and Molecular Biology, RPCAU, Pusa, Bihar, India

Satish Kumar Singh
Associate Professor, Department of Plant Breeding and Genetics, RPCAU, Pusa, Bihar, India

Somya Singh
Tirhut College of Agriculture, Dholi, (RPCAU, Pusa), Bihar, India

Sourav Panigrahi
Department of Agricultural Biotechnology and Molecular Biology, RPCAU, Pusa, Bihar, India

Mahi Baaniya
Department of Agricultural Biotechnology and Molecular Biology, RPCAU, Pusa, Bihar, India

Praphull Patel
Department of Agricultural Biotechnology and Molecular Biology, RPCAU, Pusa, Bihar, India

Arya Singh
Tirhut College of Agriculture, Dholi, (RPCAU, Pusa), Bihar, India

Madhuri Arya
Assistant Professor, Department of Plant Breeding and Genetics, TCA, Dholi, Bihar, India

Corresponding Author:
Rahul Anand
Department of Agricultural Biotechnology and Molecular Biology, RPCAU, Pusa, India

Cloning of *bHLH* promoter fragment in *pGEMT* vector using *E. coli* (Migula) strain for various stress tolerance in chickpea

Rahul Anand, Sonali Kumari, Debasis Chattopadhyay, Mithilesh Kumar, VK Sharma, Satish Kumar Singh, Somya Singh, Sourav Panigrahi, Mahi Baaniya, Praphull Patel, Arya Singh and Madhuri Arya

Abstract
Genomic DNA of chickpea was isolated for *bHLH* promoter and amplicon of size 1.7 kb was obtained by PCR of template DNA and its ligation with *pGEMT* vector was performed for cloning. *E. coli* strain DH5α was transformed with the ligated product. The colonies containing plasmids cloned with the gene of interest remains white while the other colonies turn blue. The white colonies were selected for further screening. Gene was correctly cloned in colony number 1,2, 3,4,5,6 and 8 whereas in colony number 7 and 9, the gene did not enter the plasmid. Hence, plasmid were isolated from the confirmed colonies and Plasmid PCR was performed with P_F/P_R and M13_F/P_R primer combinations and amplicon of size 1.85 kb was obtained validating the presence of insert. Digestion of *pGEMT-bHLH* promoter was performed with HindIII and SalI restriction enzymes to further confirm the presence of insert. After digestion, gel was run which showed two fragments, one of vector and the other corresponding to the size of insert (1.8 Kb).Thus, validating the presence of *bHLH* promoter in the vector.

Keywords: PCR, cloning, plasmid DNA, DH5α , *pGEMT* vector

Introduction
Molecular Cloning refers to processes used to create copies of DNA fragments. It is done to amplify the DNA fragments containing the gene of interest and ensures purity of gene. When the concentration of the required gene products (proteins) is very low, the production of multiple clones is required to obtain large quantity of the gene products [10]. A DNA fragment containing the gene of interest is isolated by PCR amplification using primers with restriction enzyme sites (non-cutters of the gene of interest) at the ends. The amplified DNA fragments are then cleaved using the restriction enzymes and then ligated with a plasmid (cloning vector) that has been cleaved with the same restriction enzymes. The gene fragment of interest is joined with the cloning vector forms a recombinant DNA molecule. Following their introduction into suitable host cells through a process called transformation, the recombinant DNA can then be multiplied by multiplying the host organism [9].
Cloning vector includes a gene that the host cell requires for growth under specific conditions, such as a gene that confers resistance to an antibiotic. Only cells transformed by the recombinant plasmid can grow in the presence of that antibiotic, hence these genes which helps in selection of transformed cells are called selection markers [7, 8]. pGEMT – TA Cloning vectors are the plasmid vectors which are about 3 kilo base pairs in length and have ampicillin resistance as one of the selection markers. Blue white selection of recombinants – these have T7 and SP6 polymerase promoters flanking a multiple cloning site within alpha peptide coding region of the enzyme beta galactosidase (*i.e.*, the Lac Z gene). Insertional inactivation of lac Z gene allows identification of recombinants by blue white screening [11].

Materials and Methods
1. Amplification of the template DNA (Genomic DNA of *bHLH* promoter gene of *Cicer arietinum)* through polymerase chain reaction (Table 1 and 2) [4].
2. Ligation of PCR product in *pGEMT* vector was done by using various components (Table 3) [2].
3. Transformation of cloned gene into DH5α strain of *E. coli* cells was done in Laminar air flow chamber (Table 4) [3].

4. Colony PCR

A reaction mixture was set up to check whether the plasmid DNA has entered in the competent *E.coli* cells (DH5α strain). Two random white colonies were selected from the transformation plate and was set up for PCR amplification under two combinations-

a. Using plasmid-specific forward and gene-specific reverse primers [8]
b. Gene-specific forward and reverse primers [5].

The reaction mixture (Table 5) was given a quick spin to mix the components and was set up for thermocycling conditions (Table 6). The PCR was started with a heated lid condition in order to prevent evaporation of the sample mixtures. Once the process is completed, 2µl of samples and 2µl Loading buffer was mixed and was checked in agarose gel electrophoresis.

5. Plasmid isolation was done using various components (Table 7) followed by Plasmid PCR (Table 8 and 9) for the confirmation of cloning [6].
6. Digestion of *pGEMT-bHLH* promoter was performed with *HindIII* and *SalI* restriction enzymes to further confirm the presence of insert. After digestion, gel was run to validate presence of *bHLH* promoter in the vector [1].

Result and Discussion

Genomic DNA isolation of *bHLH* promoter was done and amplicon of size 1.7 kb was obtained (Fig. 1). Ligation of PCR product in *pGEMT* vector was done. In blue-white screening (Fig,2), the plasmid contains the lac Z gene which is disrupted by the entry of a gene of interest. Hence, β-galactosidase (product of the lac Z gene) cannot act on the substrate X- gal to give the characteristic blue colour. Hence, the colonies containing plasmids cloned with the gene of interest remains white while the other colonies turn blue. The white colonies were selected for further screening [10].

Lane 1-9 (Fig.3) in the gene forward and reverse primer combination shows distinct and prominent bands while in the plasmid forward and gene reverse primer combination, lanes 1,2,3,4,5,6 and 8 shows prominent bands with a slight increased shift [10]. It can be interpreted that my gene has been correctly cloned in colony number 1,2, 3,4,5,6 and 8 whereas in colony number 7 and 9, the gene did not enter the plasmid. Hence, plasmid will be isolated from the confirmed colonies (Fig.4) and plasmid PCR will be done to further validate the result [11].

Plasmid PCR (Fig.5) was performed with P_F/P_R and M13_F/P_R primer combinations and amplicon of size 1.85 kb was obtained validating the presence of insert. Digestion of *pGEMT-bHLH* promoter was performed with HindIII and SalI restriction enzymes (Fig.6) to further confirm the presence of insert [7]. After digestion, gel was run which showed two fragments; one of vector and the other corresponding to the size of insert (1.8 Kb). Thus, validating the presence of *bHLH* promoter in the vector.

Table 1: Components of DNA amplification PCR

S. No	Components	Volume	Concentration	Purpose
1.	10X Buffer	2µl		It provides the environment for the DNA polymerase for maximum activity.
2.	dNTP	2µl	2.5mM	Used by DNA Polymerase for extension of strands.
3.	Forward Primer	0.5µl	10 µM	Attaches to 5' initiation site of the gene of interest.
4.	Reverse Primer	0.5µl	10µM	Attaches to the 5' end of the complementary strand
5.	Taq polymerase	0.2µl		Polymerisation activity.
7.	Template	1 µl	20-50 ng	Provides the template having the gene of interest.
8.	MQ	13.8		

Table 2: Thermocycling conditions

Step	Temperature	Time	Purpose
Initial denaturation	95 ˚C	5 minutes	Lyse the bacteria and to turn dsDNA to ssDNA.
Melting	95 ˚C	30 seconds	Denatures template and amplicons.
Annealing	58 ˚C	1 minute	Attachment of primer to the template/amplicon.
Extension	72 ˚C	45 seconds	Strand extension by DNA Polymerase to form the amplicon.
Final extension	72 ˚C	10 minutes	Synthesis of incomplete amplicons and modification of 3' end if any (eg; Taq Polymerase).
Incubation	4 ˚C	∞ (for ever till samples are removed)	Storage

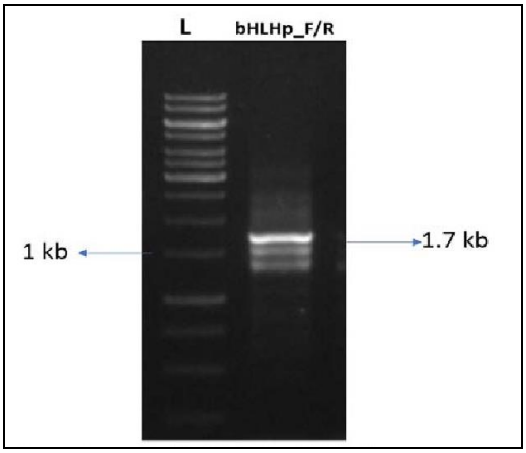

Fig 1: Genomic DNA PCR of bHLH promoter

Table 3: Components added to perform ligation of PCR product in pGEMT vector

S. No	Components	Concentration	VOLUME
1	PCR Product	32.1 ng	1.1µl
2	pGEMT Vector	50 ng	0.5µl
3	Ligase Buffer	2.5 ng	2.5µl
4	Ligase	3 Weiss units/µl	0.5µl
5	MQ H_2O		0.4µl
	Total		5µl

Table 4: Components for Transformation

Components/Reagents/Instruments		Purpose
DH5α		Competent cells with high transformation efficiency and provide blue/white selection.
Laminar Air Flow		Flows filtered air to prevent any contamination of the samples by bacteria.
Incubator shaker		Provides the environment for proper growth of the bacterial cells and homogenises the solution.
LB/agar IXA plate		
1	LB (Luria Bertani broth)	Provides the nutritive medium for bacterial growth.
2	Agar	Helps in solidification of the media on the plate.
3	I(IPTG)	Inducer of the lac operon and hence the reporter gene, lac Z.
4	X(X-Gal)	Substrate on which lac Z gene product (β- galactosidase) acts to help in blue white screening.
5	A(Ampicillin)	Selective marker.

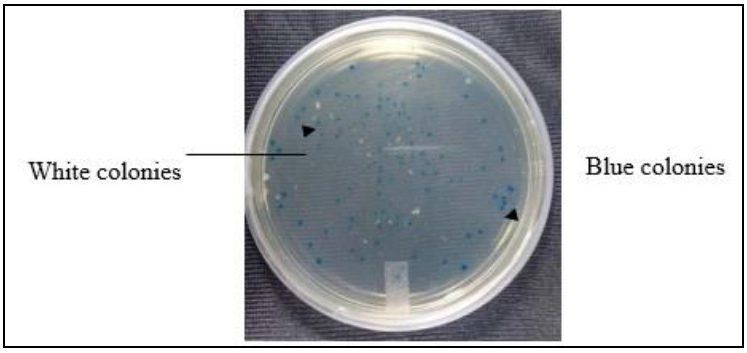

Fig 2: Blue and white colonies observed on IXA plate

Table 5: Reaction Components of Colony PCR

Component	Concentration		Volume	Purpose
	Initial conc.	Final conc.		
Standard Taq Reaction Buffer (NEB)	10X	1X	2µl	It provides the environment for the DNA polymerase for maximum activity.
dNTPs	10mM	200µM	2 µl	Used by DNA Polymerase for extension of strands.
Forward primer	10µM	0.2µM	0.5 µl	Attaches to 5' initiation site of the gene of interest.
Reverse primer	10µM	0.2µM	0.5 µl	Attaches to the 5' end of the complementary strand.
Template DNA	Bacterial colony		10µl	Provides the template having the gene of interest.
NEB Taq DNA Polymerase			0.20 µl	Polymerizations activity.
Nuclease free water			4.8µl (volume make up)	

Table 6: Thermocycling Condition for Colony PCR

Step	Temperature	Time	Purpose
Initial denaturation	95 ˚C	5 minutes	Lyse the bacteria and to turn dsDNA to ssDNA.
Melting	95 ˚C	30 seconds	Denatures template and amplicons.
Annealing	58 ˚C	1 minute	Attachment of primer to the template/amplicon.
Extension	72 ˚C	45 seconds	Strand extension by DNA Polymerase to form the amplicon.
Final extension	72 ˚C	10 minutes	Synthesis of incomplete amplicons and modification of 3' end if any (eg; Taq Polymerase).
Incubation	4 ˚C	∞ (for ever till samples are removed)	Storage

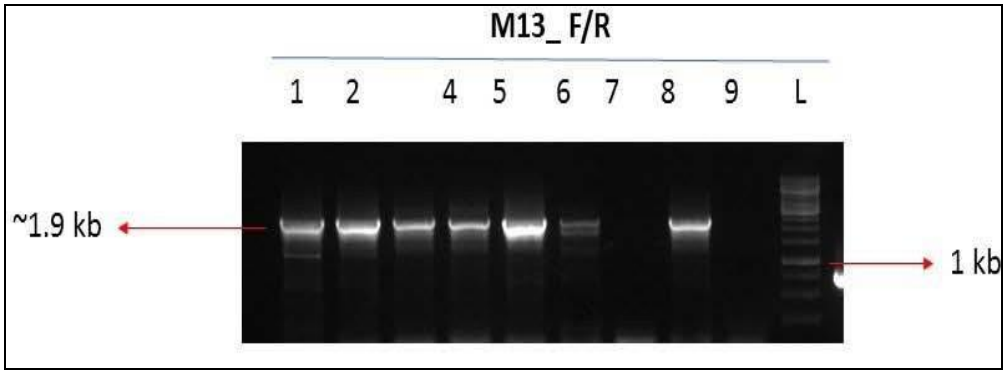

Fig 3: Colony PCR of pGEMT-bHLH -DH5α

Table 7: Components used in Plasmid Isolation

Solution	Concentration.		Volume/Amount	Purpose
	Stock conc.	Working conc.		
P1 Buffer			250µl	
Glucose	-	50mM		Maintains osmolarity so that the cells don't burst out its contents.
Tris-Cl	1M	25mM		Acts as the buffer and maintains the alkaline environment.
EDTA	1M	10mM		Chelates divalent ions to stop DNase activity.
Nuclease free water				
P2 Buffer				
Sodium hydroxide	1M	0.2N	250µl	Denatures the DNA and also breaks cell wall.
SDS	10%	1%		Solubilises membrane denatures proteins. cell and the
N3 Buffer				
Potassium acetate	-	3M	350µl	Decreases Alkalinity and the re- Establishes the dsDNA from ssDNA precipitating proteins,salts and other cell debris.

Table 8: Components of Plasmid PCR

S. No	Components	Volume	Concentration	Purpose
1.	10X Buffer	2µl		It provides the environment for the DNA polymerase for maximum activity.
2.	dNTP	2µl		Used by DNA Polymerase for extension of strands.
3.	Forward Primer	0.5µl	10µM	Attaches to 5' initiation site of the gene of interest.
4.	Reverse Primer	0.5µl	10µM	Attaches to the 5' end of the complementary strand
5.	Taq polymerase	0.2µl		Polymerisation activity.
7.	Template	1 µl	20-50ng	Provides the template having the gene of interest.
8.	MQ	13.8		

Table 9: Thermocycling Conditions for Plasmid PCR

Step	Temperature	Time	Purpose
Initial denaturation	95 ˚C	5 minutes	Lyse the bacteria and to turn dsDNA to ssDNA.
Melting	95 ˚C	30 seconds	Denatures template and amplicons.
Annealing	50 ˚C	1 minute	Attachment of primer to the template/amplicon.
Extension	72 ˚C	45 seconds	Strand extension by DNA Polymerase to form the amplicon.
Final extension	72 ˚C	10 minutes	Synthesis of incomplete amplicons and modification of 3' end if any (eg; Taq Polymerase).
Incubation	4˚C	∞(forevertill samples are removed)	Storage

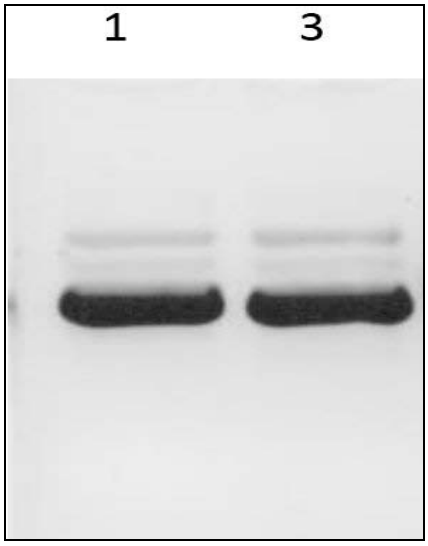

Fig 4: pGEMT-bHLH promoter Plasmid (DH5α)

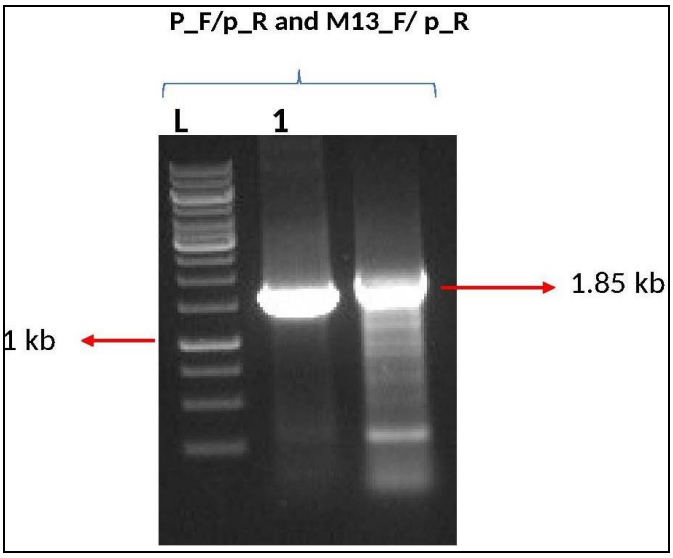

Fig 5: Plasmid PCR of pGEMT-bHLH isolated from E. coli (DH5α)

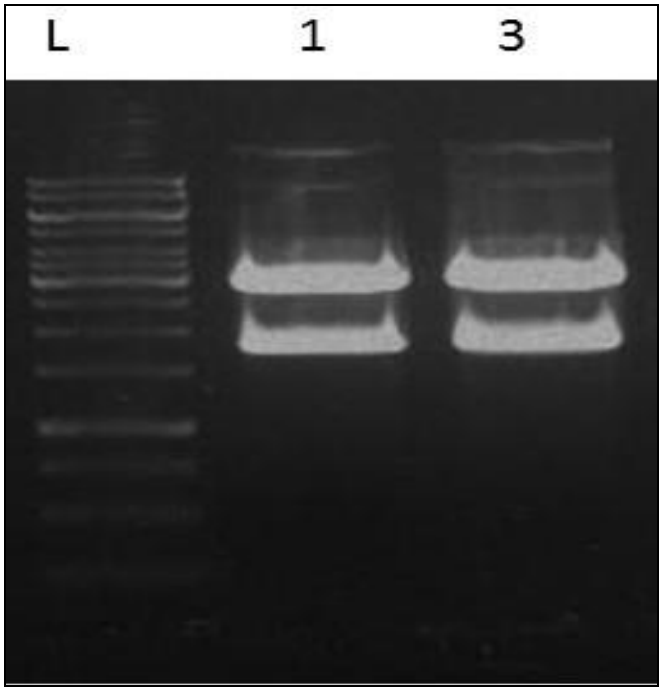

Fig 6: Digestion with HindIII and SalI restriction enzymes

Conclusion

TA Cloning of *bHLH* promoter gene in *pGEMT* vector using DH5α strain was done in desi cultivar of chickpea (*Cicer arietinum*) for various stress tolerance. This will help in developing varieties by working at molecular level for resistance against several biotic factors (various insect pests). This will decrease insect infestation leading to better yielding in crop.

Acknowledgement

I hereby take this opportunity to thank INSA-IAS-NASI for providing me fellowship for the completion of my work and Dr. Debasis Chattopadhyay NIPGR (JNU) for providing me proper guidance and much needed suggestions.

References

1. Bloch KD, Grossmann B. Digestion of DNA with restriction endonucleases. Current protocols in molecular biology. 1995; 31(1):3-1.
2. Haqqi TM. Direct ligation of PCR products for cloning and sequencing. Nucleic acids research. 1992; 20(23):6427.
3. Mello C, Fire A. DNA transformation. Methods in Cell Biology. 1995; 48(48):451-482
4. Mullis K, Faloona F, Scharf S, Saiki RK, Horn GT, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. In Cold Spring Harbor symposia on quantitative biology Cold Spring Harbor Laboratory Press. 1986; 51:263-273.
5. Packeiser H, Lim C, Balagurunathan B, Wu J, Zhao H. An extremely simple and effective colony PCR procedure for bacteria, yeasts, and microalgae. Applied biochemistry and biotechnology. 2013; 169(2):695-700.
6. Parikh A, Peter Guengerich F. Random mutagenesis by whole-plasmid PCR amplification. Biotechniques. 1998; 24(3):428-431.
7. Rodriguez PL, Leube MP, Grill E. Molecular cloning in Arabidopsis thaliana of a new protein phosphatase 2C (PP2C) with homology to ABI1 and ABI2. Plant molecular biology. 1998; 38(5):879-883
8. Scharf SJ. Cloning with PCR. PCR Protocols: A guide to methods and applications. 1990, 84-91.
9. Trinh H, Barker DRP *et al*. Regeneration and transformation methods. Manual of the EMBO Practical Course on the New Plant Model System *Medicago truncatula* at http://www. isv. cnrs-gif. fr/embo01/manuels/index. Html, 2001.
10. Yamaguchi-Shinozaki K, Koizumi M, Urao S, Shinozaki K. Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in Arabidopsis thaliana: Sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant and Cell Physiology. 1992; 33(3):217-224.
11. Zhou MY, Gomez-Sanchez CE. Universal TA cloning. Current issues in molecular biology. 2000; 2:1-8.