

***StBEL5, a transcription factor in potato (*Solanum tuberosum* L.):
sequence analysis, predicting motifs, and molecular cloning***

A Dissertation submitted

In the partial fulfilment of the requirements
for the award degree of
Master of Science in Biochemistry



Submitted by

ANJALI

(Regd. No. 301507001)

Under the guidance of

Dr. N. Das

Professor

School of Chemistry and Biochemistry

Thapar University, Patiala

July 2017

CANDIDATE'S DECLARATION

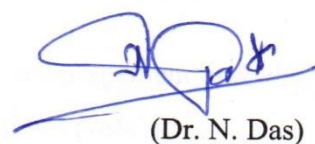
I, hereby declare that the work which is being presented in the dissertation entitled '**StBEL5, a transcription factor in potato (*Solanum tuberosum* L.): sequence analysis, predicting motifs, and molecular cloning**' in the partial fulfilment of the requirement for the award of degree of Master of Science in Biochemistry and being submitted to School of Chemistry and Biochemistry, Thapar University, Patiala, is my own research work of January to July 2017, under the supervision of Dr. Niranjana Das. The matter embodied in this dissertation has not been submitted to any other university or institute for award of other degree.

Anjali
(Anjali)

Thapar University, Patiala
July 17, 2017

CERTIFICATE

I hereby declare that the thesis entitled “*StBEL5*, a transcription factor in potato (*Solanum tuberosum* L.): sequence analysis, predicting motifs, and molecular cloning’ being submitted by Ms. Anjali in partial fulfilment of requirement for the award of the degree of **Masters of Science** in **School of Chemistry and Biochemistry, Thapar University, Patiala**, is a bonafied work carried out under the supervision of **Dr. N. Das** and no part of this project has been submitted for award of any degree by me.



(Dr. N. Das)

Professor & Supervisor
Department of biotechnology
Thapar University
Patiala

ACKNOWLEDGEMENT

To make a project successful, there are many helping hands. I would like to express my sincere gratitude and appreciation to many people who helped keep me on track toward the completion of my project.

Firstly, I express my sincere thanks to **Dr. Niranjana Das**, for providing exemplary guidance all necessary facilities, full co-operation, monitoring and constant encouragement throughout the course of this project work. The blessing, help and guidance given by him time to time shall carry me a long way in the journey of life on which I am about to embark.

I am grateful to all laboratory staff for their cooperation during the period of my assignment, which helped me in completing this task through various stages. In my daily work I have been blessed with a friendly and cheerful group of research scholars **Mrs. Gurpreet Kaur** and **Mr. Rajneesh**.

I owe the biggest thanks and sense of gratitude to **Dr. Amjad Ali** and other faculties of SCBC for guiding me and providing me support whenever I needed.

I wish to express my gratitude to my parents and friends for their encouragement and blessings which supported me throughout this, without which this project would not be possible.

Date: 17th July 2017

Place: Patiala

(Anjali)

List of contents

Title	Page No.
Declaration	i
Certificate	ii
Acknowledgement	iii
List of contents	Iv
List for tables	V
List of figures	Vi
Abbrevations	Vii
Abstract	Viii
Chapter -1 Introduction	1-3
1.1 Morphology of potato (<i>Solanum tuberosum</i> L)	1
1.2 Tuberization- a complex process	2-3
Chapter 2 Review of literature	4-6
2.1 Endogenous RNA constituents of Phloem and their possible role in long distance signalling.	4
2.2 Role of specific transcription factors during tuber development.	4-5
2.3 Work done so far in laboratory	5-6
2.4 Objectives of the study	6
Chapter 3: Material and methods	7-16
3.1 <i>In-silico</i> analysis of <i>StBEL5</i> sequences	7
3.2 Molecular cloning	7 -10
3.3 Methods	10-16
Chapter -4 Results and discussion	17- 32
4.1 <i>In silico</i> analysis of <i>StBEL5</i>	17
4.2 Prediction of salient attributes	18-26
4.3 Molecular cloning of <i>StBEL5</i>	27-31
Conclusion	32
References	33-34

List of Figures

Fig No.	Title	Page No.
1	Morphology of potato plant	2
2	Vector map of pUC19	14
3	Vector map of PMD-20	14
4	BLASTp analysis of StBEL5	17
5	Phylogenetic tree	23
6	Multiple sequence alignment of StBEL5	26
7	RNA isolation from different samples	27
8	Purification of Total RNA	28
9	RT-PCR of purified RNA samples	29
10	Eluted DNA bands from RT-PCR products	29
11	Checking of putative clones	30
12	RNase treatment	31
13	Restriction digestion analysis	31
14	PCR (Reamplification)	32

List of Tables

Table No.	Table name	Page No.
1	Chemical composition of Media	8
2	Composition of gel loading buffer	8
3	Composition of TBE(5X) buffer	8
4	Composition of TE buffer	9
5	Chemicals and composition For RNA solution	10
6	Thermal cycling conditions for PCR	12
7	Composition of linearized vector	13
8	Components for Ligation reaction by pUC19	14
9	Components for ligation reaction pMD20-T	15
10	Homologous BEL5 sequences	18
11	Representing biochemical attributes	18
12	Comparison of amino acid values	19
13	Representing secondary structure and solvent accessibility	19
14	Representing homeobox domain and signature	20
15	Representing protein and polynucleotide binding site	20
16	Representing motif sites and position	21
17	Comparison of BEL5 sequences on the basis of different chemical attributes.	24

List of Abbreviations

Bp	Base pairs
UDP	Uridine phosphate
ADP	Adenosine phosphate
INV	Invertase
kDa	Kilo Dalton
SUS	Sucrose synthase
Nts	Nucleotides
Aa	Amino acids
NCBI	National centre for biotechnology
UTR	Untranslated region
Kb	Kilo base pairs
cDNA	Complementary DNA
EDTA	Ethylene diamine – tetra acetic acid
TE	Tris EDTA
SD	Short day
H	Hours

ABSTRACT

StBEL5 is a long distance travelling RNA transcript which encodes a transcription factor involved in the complex signalling pathways during tuberization in potato. *StBEL5* is dimeric in nature with molecular weight ~76 kDa. It is a member of the TALE superfamily which contacts with KNOTTED1- type genes for the regulation of tuberization. During the last few decades, it has gained considerable importance with regard to its structure and function particularly in the regulation of gene expression. *StBEL5* proteins are found in some potato tissues. Several facile modern bioinformatics tools have been employed to generate data comprising of searching the sequence features and motifs, construction of phylogenetic tree. Moreover some other biochemical attributes were also predicted such as pI value, molecular weight, amino acid composition analysis. Total RNA was isolated and purified from the field-grown different potato tissues namely growing small tubers and leaves. RT-PCR approach was adopted using the total RNA preparations and oligo dT primer. The size of the amplicon was ~ 2.0 kb in each case. A number of putative *StBEL5* specific cDNA clones were obtained using pUC19 and pMD20-T vectors. The clones were partially characterized by restriction analysis and PCR. This study would help for further molecular studies on *BEL5* in potato and other *Solanaceae* family members.

Keywords: *Solanum tuberosum* L., *StBEL5*, Motif search, RT-PCR, Molecular cloning, *In silico* approaches.

Potato (*Solanum tuberosum* L.) is a major non-grain food crop that is grown all over the world. It ranks third, and comes only after wheat and rice (<http://faostat.fao.org/>). Potato belongs to the family Solanaceae which consists of more than 3,000 members. Apart from potato, tomato, eggplant, tobacco, pepper and capsicum are also economically important members of the Solanaceae family. Potato tubers are important dietary sources of starch, protein, antioxidants and vitamins. Currently, the major objectives of global potato research are optimization of production levels by producing resistant varieties towards various challenging biotic and abiotic stresses. Recent increases of potato production in the Asian continent have been noteworthy. The importance of this crop was realized soon after independence of our country in 1947. The Central Potato Research Institute (CPRI), Shimla, established in 1949, adopted conventional breeding programmes for the improvement of potato crop, and released a number of high-yielding commercially important cultivars including some processing varieties. Now India ranks third in terms of area of potato cultivation, and it is the second largest country (after China) in terms of annual production of potato (<http://www.fao.org>). Potato is regarded as a short-duration crop with an average maturation time of 90-100 days under Indian agro-climatic subcontinent.

1.1 Morphology of potato plant and tuber

Various parts of a potato plant is shown in **Fig. 1**. It is an herbaceous annual plant which can grow up to 20-40 inches (50 to 100 cm) high. Plant bears alternate and irregularly pinnate compound leaves. Flowers are pentamerous, actinomorphic, and perfect and have sympetalous coloured corollas (white, pink, red, blue, or purple). Usually, potato plant is propagated through tubers (vegetative or asexual propagation), known as “seed tubers” or “seed potatoes”. The tubers bear lateral buds (eyes) that grow into new plants when the conditions are favourable for growth. Under certain conditions, potato plants can produce small green fruits that contain seeds, also called "true seeds" or "botanical seeds". Potato plants can be propagated through botanical seeds, which are known as True Potato Seeds (TPS) which not so popular for usual cultivation but very useful in conventional breeding. Potato tubers are the storage tissues which grow by transformation of tips of the underground stems or stolons. Tuberization requires short days (small photoperiod) and long cool nights. In India, most of the cultivars refer to winter crop. Tuberization occurs by interaction of several processes, for example, stolon initiation and elongation etc. The potato skin colour can vary from brownish white to deep purple. In mature tubers, cortical and pith cells

accumulates large amount of starch and storage protein. Potato tubers also contain high-quality proteins, vitamins including vitamin C, minerals, and trace elements.

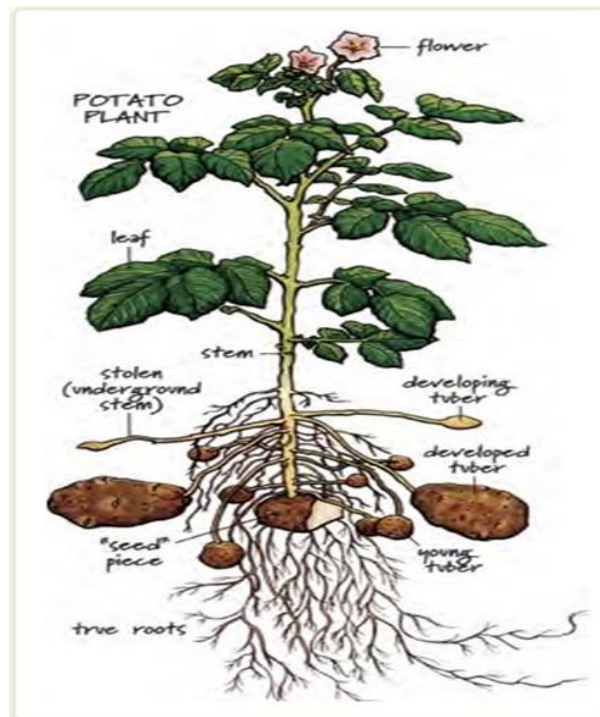


Fig.1: Diagram showing all the parts of potato plant (www. Vomiting chicken.com)

. The ploidy levels in potato varies from, haploid number of 12, ranging from diploid ($2n = 2x = 24$) to hexaploid ($2n = 6x = 72$) including triploids, tetraploids, and pentaploids. Improvement of these potato cultivars through classical breeding methods is an arduous task since they are autotetraploid ($2n = 4x = 48$) and highly heterozygous.

1.2 Tuberization - a complex process

Tuberization in potato is a complex developmental process influenced by some factors such as photoperiod, temperature, nitrogen nutrition, and endogenous levels of the phytohormones. Tuberization is an interactive process and important survival mechanism for the tuber formation in potato plant which occurs on the underground stolons (Van den Berg et al. 1996). This expanding process involves interaction of several extrinsic and intrinsic genetic, biochemical, and environmental factors. At morphological and biochemical level there are two distinct processes, associated with potato tuber formation. Stolon development and tuber induction are the processes which occur at the stolon tip. Biochemical processes involve starch synthesis (Tuberger et al. 2000) and storage of proteins (Taylor et al. 1992). Specific gene expression patterns are required to regulate both morphological and biochemical processes (Taylor et al. 1998; Bache metal. 2000; Verhees et al. 2002). Induction, initiation, and growth of an underground shoot are the crucial stages. Apical meristem is the origin, where a primary

developmental event of plant originates (Clark 1997) Transcription factors TFs promote development by binding to the DNA for changing the expression of target genes. *Knox* (knotted-like homeo box) is involved during tuberization in apical meristems (Reiser et al. 2000). TALE superclass is linked with *Knox* (Burglin 1997). It is easy to differentiate these factors by sequence conservation in the DNA-binding region composed of three α -helices (Kerstetter et al. 1994). KNOX and BEL types are the types of TALE superclass (Burglin 1997). *Knox* genes are accompanied by functional analysis and expression patterns of mutation, also it has been reported that KNOX genes are involved in regulating the level of gibberellins (GA). Gibberellins are is the important phytohormone involved during regulation of tuber formation. Regulation of GA levels is done by sucrose and abscisic acid (ABA) (Xu et al. 1998a). High level of GA promotes cell expansion for stolon elongation by altering orientation of microtubules and microfibrils, and also prevents tuber induction.

Isolation of POTH1 (Potato homeobox) was done from cDNA library of potato. POTH1 controls GA synthesis to regulate plant growth (Rosin et al. 2003a). BEL TFs have been incriminated in fruits and flower development (Reiser et al. 1995; Dong et al. 2000). Out of various BEL -like cDNAs have been identified in potato, hormone levels control *StBEL5* involvement in tuber development (Chen et al. 2003c). BEL1-like TFs interact with KNOTTED1- type for regulating many morphological processes (Bellaoui et al. 2001; Muller et al. 2001; Smith et al. 2002; Smith and Hake 2003). During tuber formation, various hormone levels are regulated in stolon tip by BEL1 TFs and POTH1 (Rosin et al. 2003; Chen et al. 2003; 2004). RNA detection method analysed that *StBEL5* transcripts are present in phloem cells and localized in stolon tips (Benerjee et al. 2006). Tuber production can be enhanced by short-day photoperiod, promote movement of RNA in *StBEL5* transcript (Benerjee et al. 2006).

In 19th century, importance of phloem transport was sighted by plant biologists in plant growth. Phloem mediated supply of various compounds are important for plant growth. Molecular biology techniques with genomics and real time microscopy regenerate plant physiology and helps in identifying and manipulating gene encoding phloem specific proteins. Translocation and high resolution visualization of phloem are measured by studying phloem physiology

2.1 Role in long distance signalling and endogenous RNA constituents of phloem

Communication networks are requisite for the appropriate coordination of biological activities in all living organisms. For coordinating cell differentiation and cell growth, messenger RNA transport is a crucial process of cellular communication. Short distances are covered by transcription factors such as the knotted 1- like homeobox and are thought to be in association with initiation and conservation of meristem (Lucas et al. 1995). Another fascinating observation was that many RNAs proteins and ribonucleo proteins complexes act as information transmitters and plays an indispensable role in long distance signalling (Opark and Cruz, 2000).

2.1.1 Import and transport mechanism of of phloem-mobile RNAs: Companion cells which are present adjacent to each other act as target sites for RNAs in sieve elements (Lough and Lucas, 2006) Some RNA- binding protein functions as chaperone and triggers the import mechanism. Macromolecular structures of RNAs could be rearranged for strengthening during translocation (Lough and Lucas, 2006; Sasaki et al. 1998). Complex of RNA, mRNAs and several interacting proteins help in facilitating transport. According to the metabolic flux rate, RNAs appeared to be translocated within sieve tube.

2.1.2 mRNA as a signal: In animals, to facilitate the function of mRNAs, their transport to the interior of cell occurs in a regulated manner. Direct localization involves various protein and RNA (Ferrandon et al. 1997) interactions during transport of RNA. Specific sites are present for the functioning of mobile mRNAs. Untranslated regions mediate transport of proteins and binding to proteins. Protection is required for NA- binding proteins which are actually cellular molecules as they are more prone to degradation (Muller et al. 2001; Chen et al. 2003). *StBEL5* RNA accumulation and tuber formation are correlated in an appropriate manner. Tuber production can be increased by over expression of *StBEL5* has consistently produced plant with enhanced strength (Chen et al. 2003; Banerjee et al. 2006).

2.2 Role of specific transcription factors during development

Interaction between the environmental, biochemical and genetic factors are important during tuberization. There are mainly 3 stages of tuberization: (a) tuber initiation, in which development of tuber primordium and differentiation occur (b) tuber enlargement, in which rapid visible swelling of stolon tip, cell division also starch accumulation and (c) tuber maturation, under this condition, dormant period arrives. Under the influence of environmental factors, the signal transmission process is switched on (Xu et al. 1998). All these changes are observed at both morphological and biochemical level during tuber formation whereas cell division and cell enlargement are observed at cellular level. Process of tuberization in potato is controlled by several multiple signalling pathways such as, phytohormone signalling, RNA signalling and Calcium ions signalling. Phytohormone signalling involves important environmental factors. Eventually tuberization occurs by the production of signal (Jackson et al. 1999). Two essential phytohormones involved in tuberization are gibberellin and cytokinins. It also influences the cell axis by transverse orientation of microtubules and microfibrils, which is important for stolon elongation. On other hand tuber induction is prevented by high concentration of GA. It has been found out that role of signal molecule in tuberization is played by mRNA. The transcription factors such as POTH1 and *StBEL5* influence tuberization. This *StBEL5* RNA regulates transcription of various genes which take part in tuber formation. In case of calcium signalling potato needs protein dephosphorylation. Three alpha helices are involved in the process and enhanced level of sequence conservation in DNA binding region differentiates these TF (Kerstetter et al. 1994). DNA-binding is controlled by recognition helix (Third helix) .Out of the six groups of RNAs, *StBEL5* is one of the essential TF for tuberization in potato. Among all the transcription factors, *StBEL5* RNA is the most crucial member. Tuber formation is activated by movement of *StBEL5* from leaf veins to stolon tip. Hormone levels are controlled by *StBEL5* and POTH1 enhancing tuber formation (Rosin et al. 2003; Chen et al. 2003). At the site of tuber induction, localization of *StBEL5* transcript occurs along with its movement across graft union. Sequence tags present in UTR of the *StBEL5* transcript is regulated by RNA. The movement of RNA enhances tuber formation which is regulated by SD photoperiod (Banerjee et al. 2006). *StBEL5* RNA is accumulated in stolon tip and moves from the transgenic scion into the wild type. There is a positive correlation between enhanced tuber production in the grafted plant and transport of transgenic *StBEL5* RNA (Banerjee et al. 2006).

2.3. Work done so far on *StBEL5* in our laboratory

Earlier, in our laboratory Garg (2016) made some progress on *StBEL5* in potato with regard to sequence analysis, amplicon profile and expression patterns covering some Indian potato cultivars namely Kufri-Chipsona (CS-1), Kufri Chandramukhi (KCM) and Kufri Jyoti (KJ). Since there was no report available on this transcription factor especially in these cultivars. Total RNA was isolated and purified from different tissues of potato. RT-PCR approach was adopted to know broadly the expression patterns of *StBEL5* in various tissues of potato namely stolon, tuberizing stolons, small tubers and leaves.

2.3.1 Objectives of this study

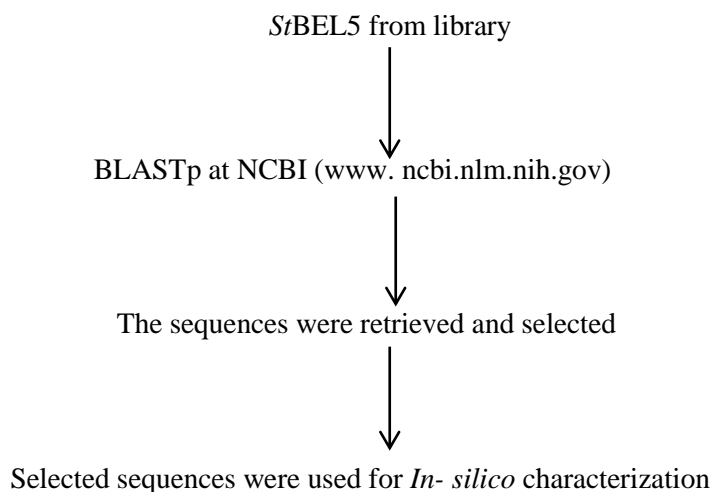
Keeping in view the importance of *StBEL5*, a major transcription factor in potato life cycle particularly during tuberization, and the work done so far in the laboratory, this thesis work focused on following objectives:

- ❖ *In silico* analysis based on the *StBEL5* sequence for predicting motifs, phylogenetic tree and other biochemical attributes sequences.

- ❖ RT- PCR approach for molecular cloning and partial characterization of potato *StBEL5* cDNA.

3.1 In silico analysis of *StBEL5* sequences

All available BEL5 protein sequences were retrieved from NCBI (National centre for biotechnology information). A flowchart elucidating strategies used in the study and identification of BEL5 sequences is as follows:



3.1.1 Biochemical characterization: Some important biochemical attributes such as molecular weight, theoretical (pI), amino acid composition of predicted protein, instability index and positively and negatively charged residues which refer to primary structure of protein were analysed by using ExPASy (<http://web.expasy.org/protparam/>) which is a proteomics server of Swiss institute of bioinformatics

3.1.2 Secondary and tertiary structure predictions: α helices, β helices and solvent accessibility were also predicted by using ExPASy server.

3.1.3 Identification of conserved domains: *StBEL5* sequences were uploaded on MY HITS tool which was used for predicting motifs and their position. For multiple sequence alignment, clustal omega tool was used.

3.2 Molecular cloning

3.2.1 Potato plant samples: For RNA isolation, leaf and tuber samples were collected from potato cultivar namely Kufri Chipsona -1(CS-1) maintained in the growth room, laboratory of Thapar University, Patiala.

3.2.2 Other materials: Various enzymes and primers used were bought from Bangalore Genei Pvt. Ltd, Bangalore. The chemicals were purchased from Sigma-Aldrich India Pvt. Ltd, and Himedia Pvt. Ltd, Mumbai.

3.2.3 Chemicals and their composition

Media : Composition and volume of media is given in **Table 1**.

Table 1: Composition and volume of media

Composition	Volume
Yeast extract	0.5% (w/v)
Tryptophan	1.0% (w/v)
NaCl	1.0% (w/v)
Agar	1.5% (w/v)

For preparing LA – Ampicillin medium, ampicillin was added to the LA medium at the working concentration of $50 \mu\text{g mL}^{-1}$. Media was autoclaved.

Gel loading buffer (5X) : Composition and volume of gel loading buffer is given in **Table 2**.

Table 2: Composition and volume of Gel loading buffer

Composition	Volume
Sucrose	35%
EDTA	50 mM
Tris	25 mM
Bromophenol blue	0.2% (w/v)

Solutions used for plasmid isolation by alkali lysis method

Solution I- 50mM glucose, 10mM EDTA, 25mM Tris HCL pH8.0

Solution II- 0.2M NAOH, 1.0%SDS (needs to be freshly prepared)

SolutionIII-3.0M with respect to potassium and 5.0M with respect to acetate .

TBE (5X) buffer : Composition and volume of TBE buffer is given in **Table 3**.

Table 3: Composition and volume of TBE (5X) buffer

Composition	Volume
Tris base	54 gl^{-1}
Boric acid	28 gl^{-1}
EDTA	3.8 gl^{-1}

The pH of the buffer was set at 8.0

TE buffer: Composition and volume of TE buffer is given in **Table 4** .

Table 4:Composition and volume of TE buffer

Composition	Volume
Tris HCL	10 mM (pH 8.0)
EDTA	1 mM (pH 8.0)

3.2.4 Enzymes used in molecular techniques

Restriction enzymes

EcoRI and SmaI were the restriction enzymes used in the digestion process. Reaction was performed at proper temperature. BSA was used according to the requirement as BSA is added during restriction digestion reaction. Buffer provided by manufacturer was used in which reaction was carried out.

Other enzymes

Lysozyme

Stock solution – 10 mg ml⁻¹

Working solution- 300 mg ml⁻¹

Lysozyme was freshly prepared during experiment.

T4 DNA ligase

Stock conc. – 400 U mL⁻¹

Working conc.- 40 U mL⁻¹

Enzyme was diluted using the dilution buffer as provided by manufacturer.

Ribonuclease A

Stock solution – 10 mg ml⁻¹

Working solution -10 mg ml⁻¹

DNase free ribonuclease A was prepared in a buffer containing 10 mM Tris (pH 8.0) and 15mM NaCl. The solution was boiled for 10 minutes, followed by slow cooling by placing at room for 2 hours, and then stored at -20°C for subsequent use.

Klenow fragment of DNA polymerase I

Stock conc. – 20 mg mL⁻¹

Working conc. – 20 µg mL⁻¹

It was prepared in fresh and sterile (autoclaved) water.

3.2.5 Chemicals/Biochemicals

X-gal(5- Bromo- 4 –chloro- 3- indoyl- β –D galactoside)

Stock conc. – 20 mg mL⁻¹

Working conc. – 20 μ g mL⁻¹

X-gal was prepared by dissolving in N, N – dimethyl formamide

IPTG (isopropyl thio β -D –galactoside)

Stock conc. -100 mg mL⁻¹

Working conc. – 100 μ g mL⁻¹

It was prepared in fresh and sterile (autoclaved) water.

3.3 Methods

3.3.1 Isolation of total RNA from potato tissues: Isolation of total RNA was done from various tissues such as tuber and leaves. Chemicals and composition required for RNA isolation is given in **Table 5**.

Materials required

- ❖ Lithium chloride
- ❖ RNA extraction buffer
- ❖ Tris –HCL (100mM) at pH 8.0
- ❖ EDTA (10mM) at pH8.0
- ❖ 1.0% SDS
- ❖ 0.2% (beta-mercaptaethanol)
- ❖ RNase-free DNase
- ❖ RNase-free deionized water

Table 5: Chemicals and composition for RNA isolation

Solution	Stock solution	Working solution
Tris buffer	0.5 M (pH 8.0)	100 mM (pH 8.0)
LiCl	8M	100 mM
EDTA	0.5 M (pH 8.0)	10 mM (pH 8.0)
SDS	10%	1%
β -mercaptaethanol	0.2%	0.1 ml

PROCEDURE

Isolation of RNA from plant material is cumbersome due to its inertness and quality. This is also because plant tissues contain nucleases, phenolics, high amount of polysaccharides. SDS-phenol method given by Gilman (1987) was adopted with some modifications depending upon plant material. The plant material (0.2 to 1.0g) were chilled and crushed with liquid nitrogen into fine powder. Buffer containing lithium chloride and SDS (RNA extraction buffer, 100mM tris –HCL pH 8.0, 10mM EDTA pH8.0, 10% SDS, 0.2% (beta-mercaptaethanol) was used in which all the contents were mixed. Phenol: chloroform extraction was done under ice-cold conditions, 8.0 LiCl (one third volume of aqueous solution) was poured to supernatant followed by incubation of 2 hours for precipitation of RNA. Purification of crude RNA was done by RNase-free DNase treatment followed by solvent extraction and ethanol precipitation. Dissolution of RNA in RNase-free deionized water was done and stored at -70 degree Celsius. Formaldehyde agarose gel electrophoresis was used for checking the quality of RNA samples using different potato gene specific primer. Quality was checked by spectrophotometric analysis also using A_{260}/A_{280} ratio of the RNA samples.

3.3.2 RNA purification

Materials required

- ❖ RNase
- ❖ Ethanol
- ❖ RNase free deionised water

Procedure

The crude RNA was purified with RNase free DNase followed by solvent extraction and ethanol precipitation. Sample was dissolved in RNase free deionised water and aliquots were stored at -70°C for further use.

3.3.3 Reverse transcription –polymerase chain reaction

Materials required

- ❖ DEPC water
- ❖ Oligo dT primer
- ❖ 10 mM DNTP
- ❖ RNase inhibitor
- ❖ Reaction buffer
- ❖ RNA sample

3.3.3.1 Reverse transcription: Revert Aid H minus M-MuLV reverse transcriptase was used for synthesis of first strand cDNA. During the synthesis, reduction of RNA does not occur as enzyme lacks ribonuclease H activity specific to RNA in RNA-DNA hybrids leading to higher yields of full length cDNA from long template upto 13kb. First strand cDNA synthesis kit from Fermentas life Sciences and oligo- dT primer was used for performing reverse transcription. 2.0 µg of total RNA either from tuber or leaf from Indian potato cultivars was used as template for each reaction mixture. RT product was taken as template for carrying out isolation of full length cDNA PCR. RNA sample was added after adding 6µl DEPC water. Oligo dT primer was then added followed by proper mixing and samples were then stored at 65 °C for 5 minutes and then kept for 5 minutes at room temperature. Reaction buffer (4µl), RNase inhibitor (1µl) and 10 mM DNTP were added. Incubation was done at 42 °C for 1 hour and at 65 °C for 5 minutes. The cDNA formed was used as template for PCR.

3.3.3.2 RT-PCR: For PCR initial denaturation was done at 94 °C for 1 m 30 s. Thermal cycling parameters during PCR were followed: denaturation at 94 °C for 1 min, annealing at 55 °C for 2 minutes; polymerization at 72 °C for 30 cycles followed by final extension at 72 °C for 5 min. Parameters of thermal are given in **Table 6**.

For RT-PCR cDNA was used as a template and thermal cycle parameters were followed.

Table 6: Thermal cycle parameters

Initial denaturing	94°C	1 min 30 s
Denaturing	94°C	1 min
Annealing	55°C	2 min
Polymerization	72°C	3 min
Final extension	72°C	5 min

3.3.4 PCR purification by elution of gel: Silica bead DNA extraction kit was used for the elution of DNA bands of Kufri-Chipsona-1 for cloning process. Gel slice containing DNA fragment was excised using a clean scalpel or razor blade. For minimising the gel volume, gel slice was cut as close as possible to DNA. Gel slice was then placed into pre- weighed 1.5 mL tube and then again weighed .Weight of gel slice was recorded. 3:1 volume of binding buffer was poured to gel slice. Incubation of gel mixture was done at 55°C for duration of 5 minutes For facilitating melting process, tube was mixed by inversion every few minutes. Colour of the solution was checked and yellow colour appearance indicated an optimal pH for DNA binding. Then resuspended silica powder suspension was added to DNA/binding buffer mixture. Mixture

was again incubated for 55°C for binding of DNA to silica matrix. Vortexing was done for keeping silica powder in suspension and mixing. Pellet was obtained after spinning silica powder/DNA mixture for 5 seconds and supernatant solution was carefully removed and discarded. 500 µL of ice cold washing buffer was added and the pellet obtained was resuspended followed by spinning. Supernatant was then discarded .procedure was repeated for 3 minutes. Remaining liquid was then removed by pipette. Air dried pellet for 15-20 minutes for avoiding the presence of residual ethanol in the purified DNA solution. Add 7µl of DNase free water and 7µl of TE buffer. Pellet was removed and incubation was done at 55°C for 5-7 minutes.

3.3.5 For cloning purpose two approaches were followed:

- ❖ pUC19 digested with SmaI as vector
- ❖ pMD20-T vector from TAKARA kit

3.3.5.1 Ligation reaction by pUC19 vector

3.3.5.1 (a) *Klenow fragment*: Klenow fragment is produced by the proteolytic cleavage of *E.coli* DNA polymerase I by protease subtilin. It retains the 5`-3` polymerase and 3`-5` exonuclease activity leads to processing of PCR products. This treatment makes the PCR products truly blunt ended called polishing step.

3.3.5.1 (b) *For preparation of linearized vector*: Restriction enzymes are the enzymes that cut or digest the DNA with in or adjacent to the specific recognition nucleotide sequences known as restriction sites. Restriction digestion is usually carried out in 15 µL volume for 2½ hours at optimum temperatures, depending on the nature of restriction enzymes used. Composition for preparation of linearized vector is shown in **Table 7**.

Table 7: Composition for preparation of linearized vector

pUC19	1µl
Restriction enzyme	1ul
Buffer	Used at a conc.of 1x
Sterile deionized water	To make up the final volume

Phenol-chloroform extraction of linearized plasmid vector was done to remove the impurities such as restriction enzymes left behind after restriction digestion reaction which can inhibit the ligation. Ligation reaction was set up to ligate the insert into plasmid vector pUC19. The enzyme T4 DNA ligase was used in order to carry out ligation. The ends of vector DNA and the insert are covalently ligated by the formation of phosphodiester bonds between the 3'-hydroxyl group of one DNA terminus and 5'-phosphoryl of another. T4 DNA ligase has the ability to join

both blunt end and cohesive end termini. The reaction mixture was kept at 18°C for overnight. Ligation reaction was carried out in three different sets for 3 different inserts, two inserts were of KJ and third was of CS-2. Each ligation mix was used separately to transform competent *E.coli* DH5 α . Components and volume for ligation are given in **Table 8** and **Fig. 2** showing vector map of pUC19:-

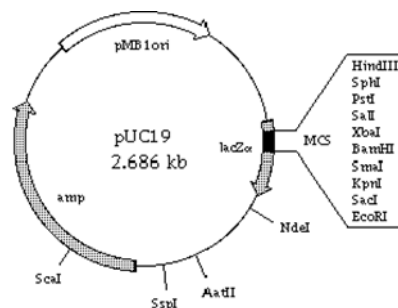


Fig. 2: Vector map of pUC19

Table 8: Components and volume required for ligation reaction

Components	Volume
Total reaction volume	15 μ l
Linearized vector	5 μ l
DNA ligase buffer	3 μ l
DNA ligase	1 μ l

The reaction mixture was kept 18°C for overnight .Ligation reaction was carried out for CS-1
3.3.5.2 (b) *Ligation reaction by pMD20-T* : Mighty TA-cloning kit (TAKARA) for ligation was used for the reaction. **Fig. 3** showing vector map of pMD20- T.

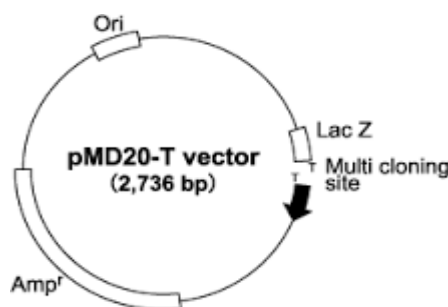


Fig. 3: Vector map of pMD20-T

Table 9: Components and volume required for ligation reaction by pMD20-T

Components	Volume
Linearized vector (pMD20-T)	1 μ L
PCR product (insert)	1 μ L
Sterilized water	3 μ L
Ligation Mighty Mix	5 μ L

Procedure

Added 1 μ L of the PCR product, 1 μ L pMD20-T vector and 3 μ L sterilized distilled water to a micro tube and mixed properly. Then added 5 μ L of ligation mixture from Mighty kit and mixed gently. Incubation was done at 16°C for one hour.

3.3.6 Genetic transformation of *E.coli* DH5 α with recombinant plasmid: Recombinant plasmids containing desired DNA inserts were used for transformation of *E.coli* from cultivar Kufri Chipsona -1 by standard CaCl₂ method.

Preparation of competent cells: Inoculation of single colony of *E.coli*DH5 α from a freshly grown plate was done in 25 mL of Luria broth and kept for overnight incubation at 37°C with vigorous shaking. Further inoculation with 200 μ L of the above grown culture in 20 mL of freshly prepared Luria broth in aseptic surroundings and again incubation was carried out at 37°C in rotary shaker for 2-3 hours. For slowing down metabolism of cells, culture was kept on ice for 5 minutes. Tubes were centrifuged for obtaining pellet at 8000 rpm for 10 min. 10mL ice cold 50 mM CaCl₂ was used for resuspending cell pellet. Centrifugation at 8000 rpm for 10 minutes at 4°C was carried out for recovering cell pellet. Resuspension of pellet was carried out in 1mL of pre-chilled 50 mM CaCl₂ and kept on ice for two and half hours.

Pre- chilled sterile microfuge tubes were used for dispensing 100 μ L of competent cells. Each microfuge tubes containing competent cell suspension was filled with 7 μ L of ligation mixture. Before keeping at 4°C for 30 minutes, tubes were mixed properly. Microfuge tubes were provided with heat shock treatment at 42°C for 2 minutes and then 1mL of Luria broth was added followed by incubation for one and half hour at 37°C. centrifugation of culture was done at 7000 rpm for 7 min. For making the cells concentrated, 600 μ L of supernatant was discarded. Then with the help of pipette, resuspension of pellet was done. Plating was done of 100 μ L of above transformed suspension in Luria–agar (LA) plates containing ampicillin (50 μ g mL⁻¹) with X-gal and IPTG. Incubation was done at 37°C. Blue –white screening was done for the selection of tranformants. Tooth picks were used for picking up each white colony

containing recombinant pUC19 and LA plates containing ampicillin with X-Gal and IPTG were used for making patches.

3.3.7 Plasmid DNA isolation by alkali lysis method: Alkali lysis method was used for plasmid isolation at mini scale. Inoculation was done of transformed *E.coli* colony in 25ml of Luria broth which contained ampicillin ($50\mu\text{g mL}^{-1}$). After inoculation of at 37°C , culture was kept overnight with vigorous shaking. Microfuge tubes were filled with 1.5mL of above grown culture and at 8000 rpm, tubes were centrifuged for 5 mins. Decantation of supernatant was done and in $150\mu\text{L}$ of ice cold solution I, pellet was resuspended with vigorous vortexing. Proper mixing was done after addition of $30\mu\text{L}$ lysozyme (10 mg mL^{-1}). With proper mixing, $225\mu\text{L}$ of freshly prepared solution II was added. Ice was used for storing tubes for 10 mins. Gentle inversion was done till white curdy precipitate is obtained after adding $300\mu\text{L}$ of Solution III and then stored for 12 mins on ice. Then centrifugation of tubes was done at 12,000 rpm for 15 minutes. Fresh microfuge tubes were used for transferring the lysate and equal volume of isopropanol was added to precipitate the DNA .Microfuge tubes were kept at 4°C for 30 mins. Microfuge tubes were centrifuged then at 12,000 rpm for 15 minutes at 4°C . Washing of pellet was done with 70% ethanol and kept tubes for some time. Supernatant was discarded air dried for 5 minutes. Again washing was done with $50\mu\text{L}$ of TE buffer and then after vortexing, tubes were kept on thermo mixer for 15 minutes at 37°C for carrying out proper mixing.

4.1 *In silico* analysis on StBEL5

Proteins are diverse macromolecules in terms of both structure and function and involved in various cellular metabolic activities. *StBEL5* is known to be one of the most important transcription factors involved in the complex tuberization process in potato. This transcription factor modulates the expression of some crucial genes associated with signalling pathways. Prior to in-depth biochemical and molecular studies, the available *StBEL5* sequence needs to be analysed for predicting salient sequence and structural features. Keeping in view, the amino acid sequence of this transcription factor was analysed using various reliable and useful software tools. The results are presented in the following sections:

4.2 Salient sequence features of *StBEL5* gene from potato

The transcription factor *StBEL5* consists of 688 amino acids (GenBank protein id: AAN03621) which is encoded by a 2735-bp gene from potato (GenBank Acc. No.: AF406697). The size of the ORF is 2067 bp (148-2214) which contains 689 codons including stop codon. *StBEL5* gene consists of 147-bp 5'- flanking region (including promoter), coding region and 521- bp 3'-UTR.

4.2.1 BLASTp analysis of *StBEL5*: BLASTp was performed using *StBEL5* protein (GenBank protein id: AAN03621) as a query. A number of homologous sequences were identified from the members of the *Solanaceae* family showing sequence identity ranging from 68-96% and amino acids of the *StBEL5* homologs range between 645-701 reflecting variations in terms of size. The data generated by the BLASTp analysis are shown in **Fig 4** and **Table 10**.

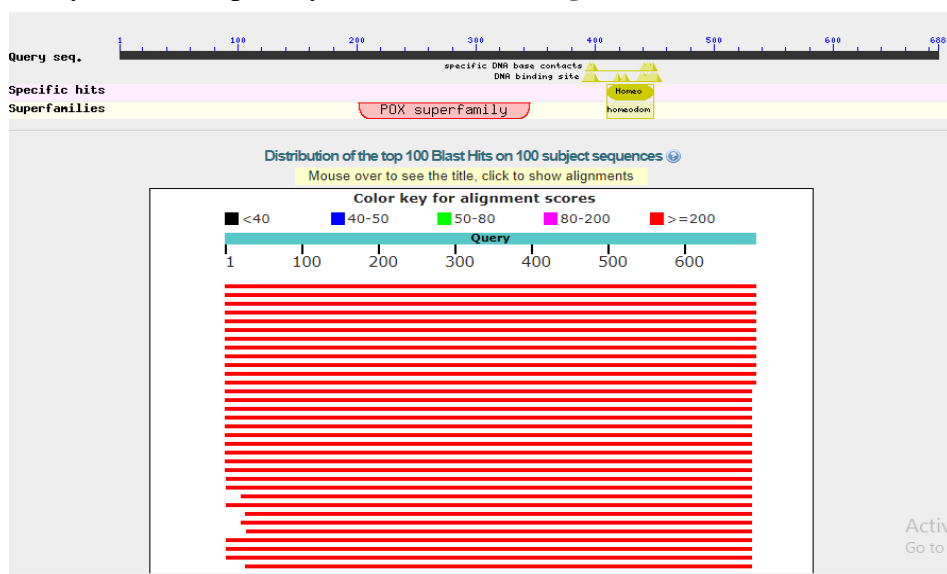


Fig. 4: BLASTp analysis using *StBEL5* sequence

Table 10: Some homologous sequences of *StBEL5* are given below

Species name	Accession no.	ORF length	Amino acids	score	Sequence identity (%)
<i>Solanum tuberosum 1</i>	NP_001274921 (AAN03621)	2067	688	1430	100
<i>Solanum tuberosum 2</i>	XP_006361091	2055	684	1354	96
<i>Solanum palustre</i>	ACD39464	2097	698	1175	93
<i>Solanum lycopersicm</i>	NP_00123599	2100	699	1181	91
<i>Solanum etuberosum</i>	ACD39468	2097	698	1175	93
<i>Solanum penelli</i>	XP_015079638	2106	701	1153	89
<i>Nicotiana sylvestris</i>	XP_009773899	127	645	791	68

4.2 Prediction of the salient attributes of *StBEL5*

Primary structure prediction: Primary structure analysis of protein was done using [ProtPram tool from ExPasy](#) (Expert protein analysis system). Various attributes such as isoelectric point, molecular weight, amino acid composition, instability index, total number of positively and negatively charged residues were analysed. **Table 11** shows various biochemical attributes:

Table 11: Prediction of biochemical attributes of *StBEL5*

Molecular weight	76 kDa
Isoelectric point	6.97
Instability index	52.12
Basic residues	61
Acidic residues	59

4.2.1 Analysis on the amino acid composition: There are total of 688 amino acids sequences of *StBEL5* protein which were analysed. Amongst 688 amino acids, 61 were strongly basic (+) (Arg +Lys), 59 were strongly acidic (-) (Asp + Glu). Instability index is obtained as 52.12 which suggests that protein is unstable. The amino acid composition data analysed that few of the amino acids such as serine (10.4%), glutamine (9.7%), asparagine (8.7%) occurred more and arginine (3.9%), tryptophan (0.7%) and cysteine (0.4%) occurred less frequently as compared to average occurrence. These data are shown in **Table 12**.

Table 12: Amino acids composition analysis (%)

Amino acids	BEL5	Average occurrence
Serine(S)	10.47	6.80
Glutamine(Q)	9.74	4.20
Asparagine(N)	8.28	4.30
Arginine (R)	3.92	5.10
Tryptophan(W)	0.73	1.40
Cysteine(C)	0.44	1.90

4.2.2 Secondary and tertiary structure prediction: The secondary and tertiary structures of protein are predicted by [ExPasy](#) tool which showed the occurrence of alpha helices, loops, strands and also solvent accessibility (**Table 13**).

Table 13: Representing secondary structure and solvent accessibility

Secondary structure	Occurrence	Solvent Accessibility	Occurrence
Loop	77.47	Exposed	75.15
Helix	22.49	Buried	20.64
Strand	2.03	Intermediate	4.22

Secondary structural features of proteins influence their overall three dimensional structures which is referred to as tertiary structure. Alpha helices which are referred to as right handed coiled strand and beta sheets are crucial secondary structural elements of secondary structure. Solvent accessibility tells about exposed, buried and intermediate surface area of biomolecule that is accessible to solvent.

Some homeobox and signature domains are also predicted as shown in **Table 14**. Homeobox domains are conserved having helix-turn-helix type of structures. Morphogenesis of many animals is regulated by this short sequence of DNA shared by many genes. Homeodomain is bound to DNA playing a role in transcription and is a folded domain of protein. Complete domain regulated by homeobox and homeodomain is 60 amino acids long.

Table 14: Homeobox domain and signature in *StBEL5*

Homeobox domain		Homeodomain signature	
Position	Sequence	Position	Sequence
390-453	PNAWRPQRGLPER AVSVLRAWLFEhfl HPYPKDSKIMLA KQTGLTRSQVSN WFINARVRLWKP	428-451	LAKqTgL TrsQVSN WFiNarvr IW

Protein and polynucleotide binding sites in *StBEL5* are shown in **Table 15**.

Table 15: Protein and polynucleotide binding sites of *StBEL5*

Subcellular localisation	Protein-protein binding sites		Polynucleotide binding sites	
	Length	Position	Length	Position
Nucleus	3	16, 18	2	397,397
	1	157,157		
	1	490,490		
	2	555,556		

4.2.3 Motif search significant protein motifs in *StBEL5* (GenBank protein id: AAN03621):

The amino acid sequence of *StBEL5* was used for searching the presence of motifs using the following link (http://myhits.isbsib.ch/cgi-bin/motif_scan) as given below:

```

MYYQGTSDNT NIQADHQQRH NHGNSNNNI QTLYLM]NPNN YMQGYTSDT QQQQQLLFLN 60
SSPAASNALC HANIQHAPLQ QQHFVGVPLP AVSLHDQINH HGLLQRMWNN QDQSQQVIVP 120
SSTGVSATSC GGITTDLASQ LAFQRPIPTP QHRQQQQQG GLSLSISPQL QQQISFNLI 180
SSSSPRTNVTIRGTLDGS SNMVLGSKYL KAAQELLDEV VNIVGKSIKG DDQKKDNSMN 240
KESMPLASDV NTNSGGGES SSRQKNEVAV ELTTAQRQEL QMKKAKLLAM LEEVEQRYRQ 300
YHHQMQUIIVL SFEQVAGIGS AKSYTQLALH AISKQFRCLK DAIAEQVKAT SKSLGEEEGL 360
GGKIEGSRLK FVDHHLRQQR ALQQIGMMQP NAWRPQRGLP ERAVSVLRAW LFEHFLHPYP 440
KDSKIMLAK QTGLTRSQVS NWFFINARVRL WKPMVEEMYL EEVKNQEQNS TNTSGDNKNK 500
ETNISAPNEE KHPIITSSLL QDGITTQAE ISTSTISTSP TAGASLHHAH NESLLGSEFM 560
DNTTTTVDHI ENNAKQQRND MHKFSPSSIL SSVDMEAKAR ESSNKGFTNP LMAAYAMGDF 620
GRFDPHDQQM TANFHGNNCV SLTLGLPPSE NLAMPVSQQN YLSNDLGSRS EMGSHYNRMG 660
YENIDFQSGN KRFPTQLLPD FVTGNLGT 690

```

Table 16: Protein motifs and their position in *SrBEL5*

Site	Motifs and position	Colour code
N-glycosylation site	NISS(179-182) NVTI(189-192) NSSG(253-256) NSTN(469-472) NISA(483-486) NFSF(531-534) NTTT(542-545)	Blue
Phosphorylation site	TTSD(46-49) SLHD(93-96) SLGE(353-356) TSGD(473-476) TQAE(507-510) TTVD(545-548) SSVD(571-574) SRSE(648-651)	Yellow
N- Myristoylation site	GTSDNT(5-10) GNSNNN(23-28) GVSATS(124-129) GGITTD(131-136) GLSLSL(161-166) GTLDGS(194-199) GGGESS(256-261) GIGSAK(317-322) GLTRSQ(433-438) GITTTQ(503-508) GVSLTL(619-624) GLPPSE(625-630) GSHYNR(653-658)	Sky blue
Protein kinase phosphorylation site	SPR(184-186) TIR(191-193) SIK(227-229) SSR(261-263) SAK(320-322) TSK(350-352) SDK(423-425) SNK(583-585)	Grey
Tyrosine kinase phosphorylation site	KPMvEEMY (452-459)	Dark green
Homeobox domain signature	LAKQTGLT RSQVSNWF INARVRLW (428-451)	Grey

N-glycosylation occurs at some specific amino acid residues. The target Asparagine residues are present in Asn/Xaa in which Xaa could be Ser/Thr. Phosphorylation occurs in Ser/Thr/Tyr residues which are involved in a number of cellular signalling processes. Casein kinase is involved in the phosphorylation of the proteins. Another process is membrane targeting and

leads to signal transduction in plant responsible to environmental stress is myristoylation. Protein kinase-C is also involved in the important signal transduction cascade. Tyrosin kinases catalyse phosphorylation process of tyrosine residues in proteins.

4.2.4 Phylogenetic tree: Phylogenetic analysis was done with neighbour-joining method. It can predict the evolutionary consequences. Its branching patterns tell about the evolutionary relationship between various species. The joined taxa indicate that all the species are descended from common ancestor. Phylogenetic tree is shown in **Fig. 5**.

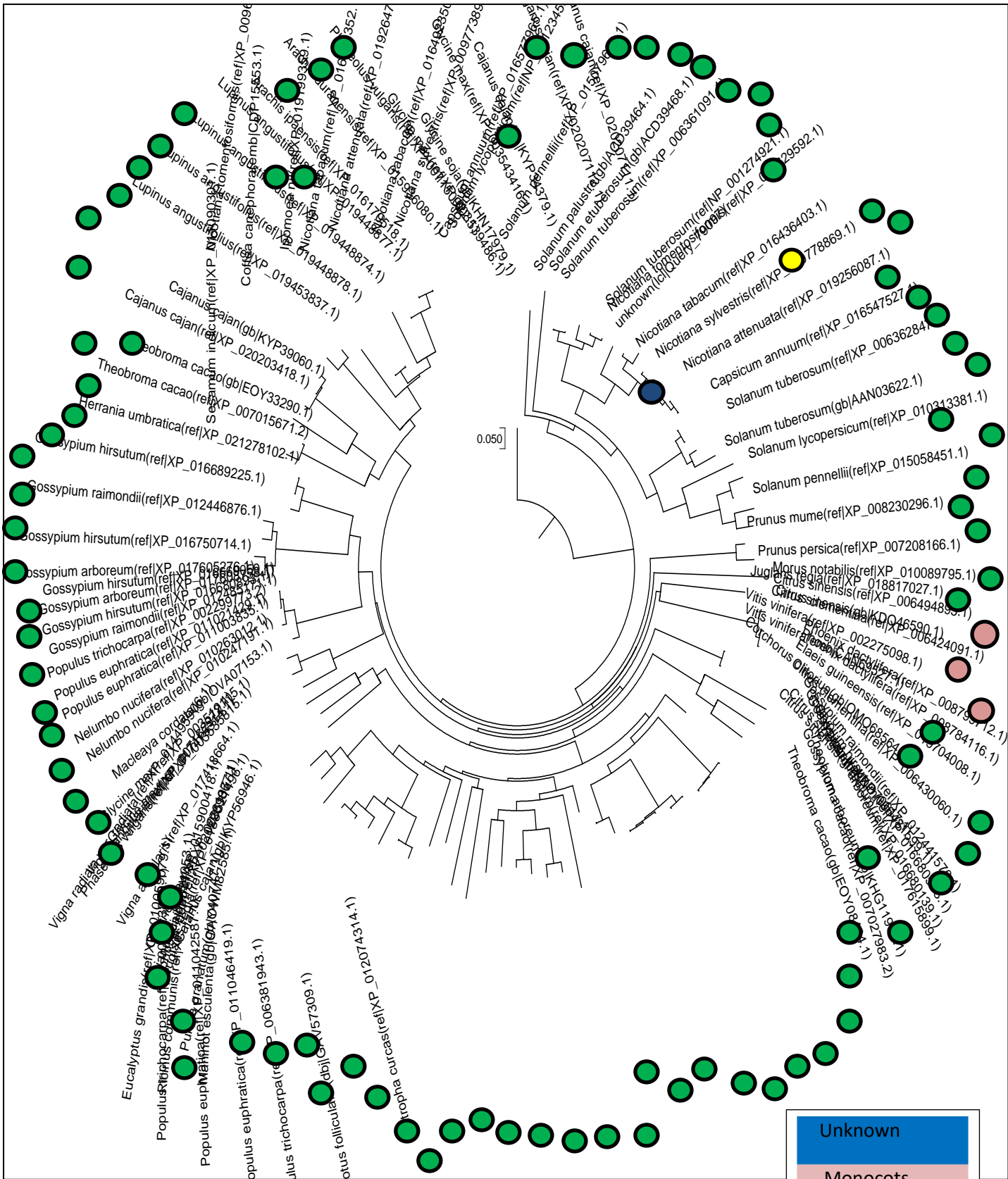
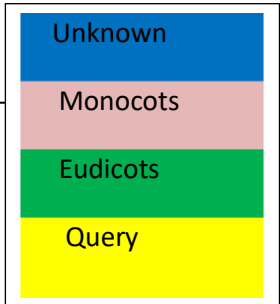


Fig. 5: Phylogenetic tree based on the *StBEL5* homologous sequences



A number of *StBEL5* homologs were identified by BLASTp search. The salient biochemical attributes of the individual homologs were predicted and presented in **Table 17** for comparison. The biochemical attributes mainly include molecular weight, isoelectric point, instability index, acidic and basic residues.

Table 17: Comparison of *StBEL5* sequences on the basis of different biochemical attributes

Species name	Accession no.	Molecular weight (kDa)	pI	Positively charged residues	Negatively Charged residues	Instability index
<i>Solanum tuberosum1</i>	NP_001274921	22	4.90	61	59	52.12
<i>Solanum tuberosum 2</i>	XP_006361091	22	4.90	60	57	52.84
<i>Solanum palustre</i>	ACD39464	22	4.89	60	60	50.88
<i>Solanum lycopersicum</i>	NP_001234599	22	4.90	61	58	52.62
<i>Solanum etuberosum</i>	ACD39468	22	4.90	61	59	50.69
<i>Solanum penelli</i>	XP_015079638	22	4.90	60	60	53.19
<i>Nicotiana sylvestris</i>	XP_009773899	20	4.90	55	57	51.53

Multiple Sequence Alignment (MSA): Multiple sequence alignment is a program that uses seeded guide trees and HMM profile-profile techniques and all the above information was generated by performing multiple sequence alignment using clustal omega for generation of alignments between three or more protein sequences. Seven different forms of *StBEL5* were used for obtaining multiple sequence alignment of deduced amino acid sequences from potato (*Solanum tuberosum*) cultivars (GenBank protein id: AAN03621). Conserved amino acid between all seven forms of *StBEL5* proteins were denoted by star symbol. Divergence was shown in alignment of *BEL5* protein with respect to other six forms of *BEL5* which are highlighted with blue colour.

Some other observations were revealed after segment wise comparison between different forms of *BEL5*. 25 semi conserved mutations, 39 conserved mutations, 14 non conserved mutations highlighted by blue colour were observed out of 96 variant regions.

St1	MYYQGTSDN-NIQADHQRHNNHGNS-NNNIQTLYLMNPNSYMQGYTTTDTQQHQHQNQNH	60
St2	MYYQGTSDN-NIQADHQRHNNHGNS-NNNIQTLYLMNPNSYMQGYTTTDTQQHQHQNQNH	60
Sp	MYYQGTSDNTNIQADHQRHNNHGNS-NNNNIQTLYLMNPNNYMQGYTTSDTQQ-----	60
Sl	MYYQGTSDN-NIQADHQRHNNHGNSNNNIQTLYLMNPNNYMQGYTTSDTQQ----- *****:*****:*****:*****:*****:*****:*****:*****:*****	60
St1	QQQLLFLNSSPAGSNALGHANIHPHAPLQQQHfVGVPLPAVSLHDQINHHGLLQRMWNNQD	120
St2	QQQLLFLNSSPAGSNALGHANIHPHAPLQQQHfVGVPLPAVSLHDQINHHGLLQRMWNNQD	120
Sp	QQQLLFLNSSPAASNALCHANIQHAPLQQQHfVGVPLPAVSLHDQINHHGLLQRMWNNQD	120
Sl	QQQLLFLNSSPAGSNALCHANIQHAPLQQQHfVGVPLPAVSLHDQINHHGLLQRMWNNQD *****:*****:*****:*****:*****:*****:*****:*****:*****	120
St1	QSQQVIVPSSTVVSATSCGGTTTDLASQLAFQRPIVVSPTPQHRQQ-QQQQGGLSLSLSP	180
St2	QSQQVIVPSSTVVSATSCGGTTTDLASQLAFQRPIVVSPTPQHRQQ-QQQQGGLSLSLSP	180
Sp	QSQQVIVPSSTVVSATSCGGTTTDLASQLAFQRPI---PTPQHRQQQ-QQQQGGLSLSLSP	180
Sl	QSQQVIVPSSTVVSATSCGGTTTDLASQLAFQRPI---PTPQHRQQQQQQQQGGLSLSLSP *****:*****:*****:*****:*****:*****:*****:*****:*****	180
St1	QQQQQISFNNNISSSSPRTNNVTIRGTLDGSSSNMVLGSKYLKAAQELLDEVVNIIVGKSI	240
St2	QQQQQISFNNNISSSSPRTNNVTIRGTLDGSSSNMVLGSKYLKAAQELLDEVVNIIVGKSI	240
Sp	QQQQQISFNNNISSSSPRTNNVTIRGTLDGSSSNMVLGSKYLKAAQELLDEVVNIIVGKSI	240
Sl	QQQQQISFNNNISSSSPRTNNVTIRGTLDGSSSNMVLGSKYLKAAQELLDEVVNIIVGKSI * *****:*****:*****:*****:*****:*****:*****:*****:*****	240
St1	KGDDQKKENSMNKESMPLASDVNTNSSGG--GGESSRQKNEVAVELTTAQRQELQMKA	300
St2	KGDDQKKENSMNKESMPLASDVNTNSSGG--GGESSRQKNEVAVELTTAQRQELQMKA	300
Sp	KGDDQKKDNSMKNKESMPLASDVNTNSSG---GGESSRQKNEVAVELTTAQRQELQMKA	300
Sl	KGDDQKKDNS----MPLASDVNTNSSGGGGGGESSRQKNEVAVELTTAQRQELQMKA *****:*** *****:*****:*****:*****:*****:*****:*****:*****	300
St1	KLLAMLEEVEQRYRQYHHQMQUIVSSFEQVAGIGSAKSYAQLALHAISKQFRCLKDAIAE	360
St2	KLLAMLEEVEQRYRQYHHQMQUIVSSFEQVAGIGSAKSYTQLALHAISKQFRCLKDAIAE	360
Sp	KLLAMLEEVEQRYRQYHHQMQUIVLSFEQVAGIGSAKSYTQLALHAISKQFRCLKDAIAE	360
Sl	KLLAMLEEVEQRYRQYHHQMQUIVSSFEQVAGIGSAKSYTQLALHAISKQFRCLKDAIAE *****:*****:*****:*****:*****:*****:*****:*****:*****	360
St1	QVKATSKSLGEEGLGGKIEGSRLKFVDHHLRQQRALQQIGMMQPNAWRPQRGLPERAVS	420
St2	QVKATSKSLGEEGLGGKIEGSRLKFVDHHLRQQRALQQIGMMQPNAWRPQRGLPERAVS	420
Sp	QVKATSKSLGEEGLGGKIEGSRLKFVDHHLRQQRALQQIGMMQPNAWRPQRGLPERAVS	420
Sl	QVKATSKSLGEEGLGGKIEGSRLKFVDHHLRQQRALQQIGMMQPNAWRPQRGLPERAVS *****:*****:*****:*****:*****:*****:*****:*****:*****	420
St1	VLRAWLFEHFLHPYPKDSKIMLAKQTGLTRSQVSNWFINARVRLWKPMEEMYLEEVKN	480
St2	VLRAWLFEHFLHPYPKDSKIMLAKQTGLTRSQVSNWFINARVRLWKPMEEMYLEEVKN	480
Sp	VLRAWLFEHFLHPYPKDSKIMLAKQTGLTRSQVSNWFINARVRLWKPMEEMYLEEVKN	480
Sl	VLRAWLFEHFLHPYPKDSKIMLAKQTGLTRSQVSNWFINARVRLWKPMEEMYLEEVKN *****:*****:*****:*****:*****:*****:*****:*****:*****	480
St1	QEQNST-TSGDNKKNKTNISAPNEEKQPIITSSLLQDGTITTTQAEISTSTISTSPTAGAS	540
St2	QEQNST-TSGDNKKNKTNISAPNEEKQPIITSSLLQDGTITTTQAEISTSTISTSPTAGAS	540
Sp	QEQNSTNTSGDNKKNKTNISAPNEEKHPIITSSLLQDGTITTTQAEISTSTISTSPTAGAS	540
Sl	QEQNSTNTSGDNKKNKE---APNEEKHPIITSSLLQDGTITTTQAEISTSTISTSPTAGAS ***** ***** *****:*****:*****:*****:*****:*****:*****:*****	540
St1	LHHAHNFSLGFSFNMKNTTTTNTVDHIENNAKQPRNDMOKFSPSSILSLVDMEAKARESS	600
St2	LHHAHNFSLGFSFNMKNTTTTNTVDHIENNAKQPRNDMOKFSPSSILSLVDMEAKARESS	600
Sp	LHHAHNFSLGFSFNMKNTT--TVDHIENNAKQPRNDMOKFSPSSILSSVDMEAKARESS	600
Sl	LHHAHNFSLGFSFNMKNTT-TTVDHIENNAKQPRNDMOKFSPSSILSSVDMEAKARESS *****:*** *****:*****:*****:*****:*****:*****:*****:*****	600
St1	NKGFTNPLMAAYAMGDFGRFDPHDQQTANFHGNNGVSLTLGLPPSENLAMPVQQNYLS	660
St2	NKGFTNPLMAAYAMGDFGRFDPHDQQTANFHGNNGVSLTLGLPPSENLAMPVQQNYLS	660
Sp	NKGFTNPLMAAYAMGDFGRFDPHDQQTANFHGNNGVSLTLGLPPSENLAMPVQQNYLS	660
Sl	NKGFTNPLMAAYAMGDFGRFDPHDQQTANFHGNNGVSLTLGLPPSENLAMPVQQNYLS *****:*****:*****:*****:*****:*****:*****:*****:*****	660

```

St1      NELGSRPEMGSHYNRMGYENIEFQSGNKRFPTRLLPDFVTGNLGT      705
St2      NELGSRPEMGSHYNRMGYENIEFQSGNKRFPTRLLPDFVTGNLGT      705
Sp       NDLGSRSEMGSHYNRMGYENIDFQSGNKRFPTRLLPDFVTGNLGT      705
Sl       NDLGSRPEMGSHYNRMGYENIDFQSGNKRFPTRLLPDFVTGNLGT      705
*:*:*:*:*: *****: *****: *****: *****

```

Fig. 6: Multiple sequence alignment of BEL5 sequences from potato. Clustal omega tool was used for performing sequence alignment along with some adjustments. Dashes indicate gaps that arise during alignment .Astericks (*) indicate identical amino acids between all the BEL5 sequences presented here. The bullet denotes identical amino acid residues between BEL family members along with semiconservative substitution where St1 stands for *Solanum tuberosum* 1, St 2 stands for *Solanum tuberosum* 2, Sp stands for *Solanum palustre*, Sl stands for *Solanum Lycopersicum*.

All the data based on the *StBEL5* sequence as generated by various bioinformatics tools could be quite useful during further in-depth biochemical and molecular studies.

4.3 Molecular cloning and partial characterization of *StBEL5*

StBEL5 has a great importance in gene expression. Therefore, molecular cloning of *StBEL5* cDNA is prerequisite to carry out recombinant expression of proteins. Keeping in view, this study focussed on *StBEL5* cDNA cloning studies and described precisely in the following sections.

4.3.1 RNA isolation from different tissues of potato: Total RNA was isolated from potato cultivar namely Kufri Chipsona-1(CS-1) from leaf and tuber collected from field grown plant. The crude RNA samples were checked by agarose gel electrophoresis was done for checking quality of RNA. Spectrophotometer was also used to check the quality and quantity of RNA by measuring A_{260}/A_{280} . Ribosomal RNA bands were distinct indicating intactness of the total RNA preparations as shown in **Fig. 7**. Good quality of RNA was isolated from different tissues of the cultivar CS-1. In some of the RNA preparations, DNA impurities could be seen. Nano drop spectrophotometer was used to find the A_{260}/A_{280} ratio to assess the quality and quantity of RNA samples.

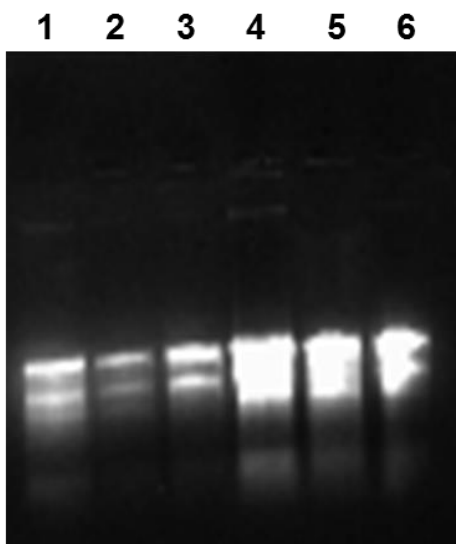


Fig .7: Total RNA isolation from potato tissues. Lane 1-3 specific to small tuber. Lanes 5& 6 specific to leaf.

4.3.2 Purification of RNA: Total RNA was purified by RNase- free DNase treatment followed by solvent extraction. Purified RNA samples were checked by Agarose gel electrophoresis from different potato tissues. The distinct RNA bands are shown in **Fig. 8**.

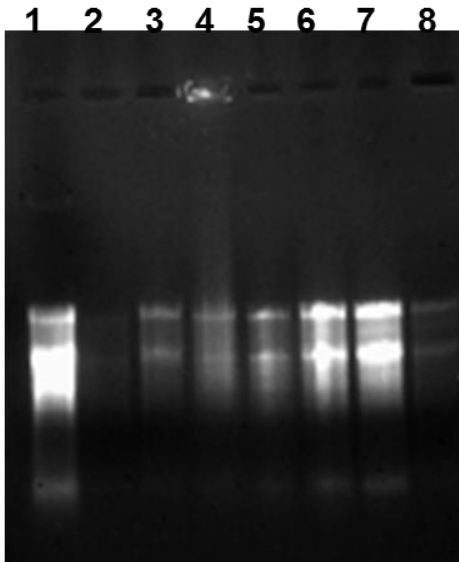


Fig. 8: Purified RNA bands. Lanes 2-4 specific to small tuber, Lanes 5-8 specific to leaf.

4.3.2 RT-PCR using specific primer set of *StBEL5* in different potato tissues: Reverse transcription was carried out using the purified total RNA. Total RNA from different tissues of potato was required for carrying out RT- PCR. *StBEL5* specific primer pair, SB5F2-0041, SB5R1-2229 was used. The amplicon size in each case was around 2.0 kb, as expected. The amplicons were used for cloning in the plasmid vector. The expression was shown in both the tissues. The RT-PCR data suggests that PCR amplification occurred in only two tissues namely small tuber and leaf.

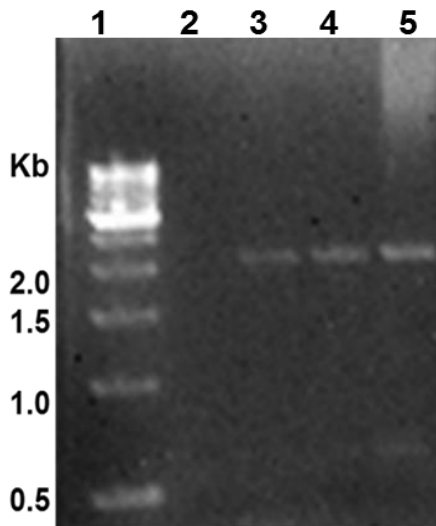


Fig .8: Agarose gel electrophoresis of RT- PCR products. Lane1- 500 bp ladder, Lanes 2& 3 PCR amplified bands specific to small tuber, Lane 4- PCR amplified band specific to leaf.

4.3.3 Purification of *StBEL5* RT-PCR products: The PCR amplified products of ~2.0 kb was treated with Klenow enzyme and then purified. These products were resolved in agarose gel and eluted using Silica bead DNA extraction kit as shown in **Fig. 9**.

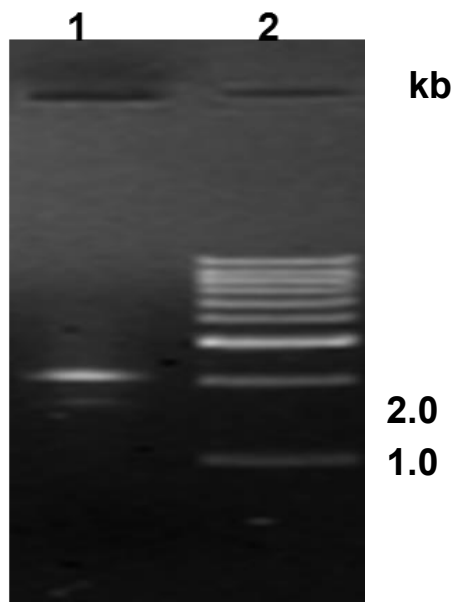
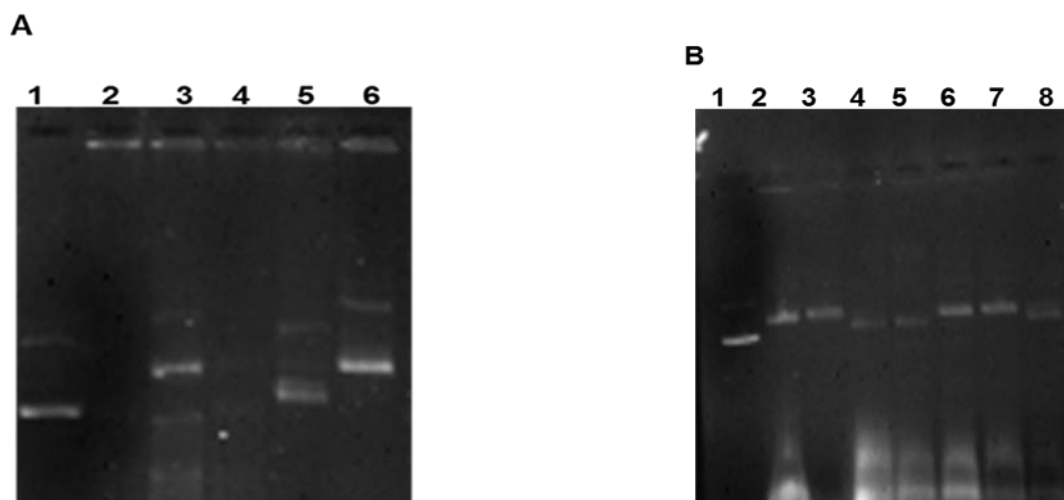


Fig.9: Elution of DNA bands. Lane 1- eluted DNA band (~2.0 kb), Lane 2- 1.0 kb ladder

4.3.4 Cloning of *StBEL5* specific cDNA in the *pUC19* and *pMD20-T* vector: First, the *StBEL5* specific eluted RT-PCR products corresponding to leaf and tuber were ligated to *SmaI* treated *pUC19* and *pMD20-T* vectors. Transformation of *E.coli* DH5 α was carried out with the above ligation mixtures for further blue-white screening of the recombinant plasmid clones. The procedure in detail is provided in materials and methods. A number of white colonies were clonally purified by streaking, and the individual white colonies were picked up for isolation of plasmid DNA in mini scale by alkali lysis method. The crude plasmid DNA samples were analysed by agarose gel electrophoresis. Mobility clearly suggests in 3 out of 5 plasmid DNA specific to leaf (**Fig. 10A**) and 6 out of 6 (**Fig. 10B**) appeared to be recombinant plasmid clones in *pUC19* vector; whereas 5 out of 6 specific to leaf (**Fig. 10C**) were putative clones based on the *pMD20-T* vector.



c

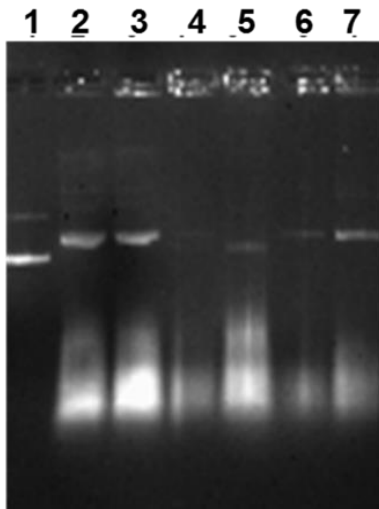


Fig. 10: Gel electrophoresis of *StBEL5* specific putative clones. (A) Specific to leaf, Lane 1- pUC19 vector , Lanes 2, 5 & 6 -putative clones.

(B) Specific to small tuber, Lane 1 – pUC19 vector, Lanes 2, 5& 6- putative clones

(C) Specific to leaf, Lane1- pMD20-T vector, Lanes 2-7 -putative clones.

4.3.5 Partial characterization of the *StBEL5* putative clones: Plasmid DNA was isolated from white colonies by alkalysis method and further analysed to check whether the plasmid contains desired inserts or not. Putative clones were analysed in agarose gel on the basis of mobility of bands.

4.3.5.1 RNase treatment: Two pMD20-T vector-based putative clones corresponding to leaf were treated with RNase as shown in **Fig. 11**.

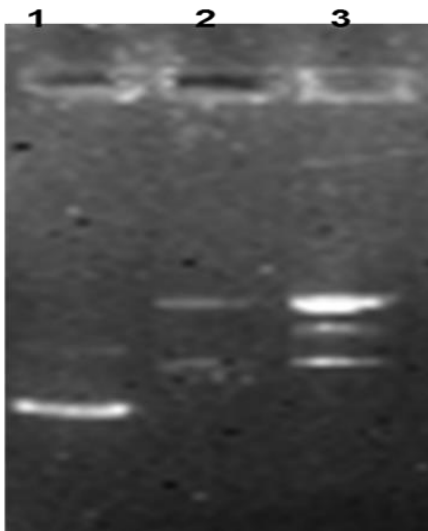


Fig. 11: RNase treatment. Lane 1- pMD20-T vector, Lanes 2 & 3- RNase treated putative clones

4.3.5.2 Restriction digestion of recombinant plasmids: Restriction enzymes namely *EcoRI* was used for performing restriction digestion of recombinant plasmids carrying desired inserts.

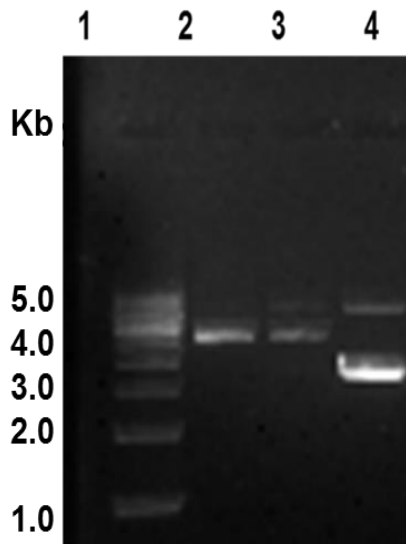


Fig. 12: Restriction analysis of the putative clone of *StBEL5*. Lane1- 1.0 kb ladder, Lanes 2 & 3 putative clones, Lane 4-Uncut recombinant clone

4.3.5.3 *Polymerase chain reaction (PCR)*: PCR approach was adopted for initial characterization of *StBEL5* specific putative clones as shown in **Fig. 13** using same set of primers as employed earlier in the RT-PCR approach. The size of the amplicon was more than ~2.0 kb as expected.

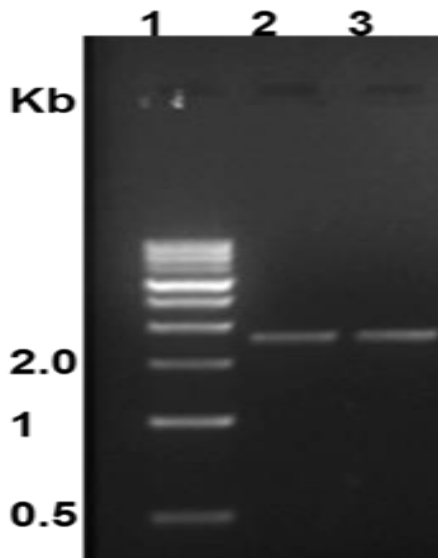


Fig .13: Characterization of the putative *StBEL5* cDNA clones through PCR. Lane 1- 1.0 kb ladder, Lanes 2&3- PCR amplified product using leaf specific cDNA clone as template

The putative *StBEL5* cDNA clones as obtained in the study appeared to be quite promising which need to be sequenced for further analysis and molecular studies.

Concluding remarks

StBEL5 is a long distance transcript in potato which belongs to TALE superfamily. It is one of the important transcription factors and involved in the regulation of various genes associated with the complex tuberization process in potato. Overall functionality of *StBEL5* depends on its salient sequence features. Earlier studies revealed that this transcription factor was expressed differentially in the potato tissues. Currently, molecular and biochemical studies on *StBEL5* gained considerable importance because of its crucial role in gene expression. Several modern bioinformatics tools have been employed in the study to generate data comprising of search protein motifs, phylogenetic tree, analysis of amino acid composition and some other biochemical attributes. Some of these features were not reported earlier as revealed from the literature survey. All the above information as generated in the study would be quite useful to carry out various biochemical and molecular studies in order to gain in-depth understanding of this transcription factor. Apart from *in silico* approaches, total RNA was isolated from different tissues of a commercially important Indian potato cultivar namely Kufri Chipsona-1 (CS-1). RT-PCR approach was adopted to get a number of putative *StBEL5* cDNA clones specific to both leaf and tuber.. The cDNA as obtained in this study were partially characterized by restriction analysis and PCR, and appeared to be promising clones. However, the putative clones need to be sequenced to carry out further genetic manipulations.

References

- Bachem CWB, van der Hoeven, Lucker J, Oomen R, Casarini E, Jacobsen E, Visser RGF (2000) Functional genomic analysis of potato tuber life-cycle. *Potato Res* 43:297–312
- Banerjee AK, Chatterjee M, Yu Y, Suh S-G, Miller WA, Hannapel DJ (2006) Dynamics of a mobile RNA of potato involved in a long distance signaling pathway. *Plant Cell* 18:3443–3457
- Bellaoui M, Pidkowich MS, Samach A, Kushalappa K, Kohalmi SE, Modrusan Z, Crosby WL, Haughn GW (2001) The Arabidopsis BELL1 and KNOX TALE homeodomain proteins interact through a domain conserved between plants and animals. *Plant Cell* 13: 2455–2470
- Burglin TR (1997) Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. *Nucleic Acids Res* 25: 4173–4180
- Chan RL, Gago GM, Palena CM, Gonzalez DH (1998) Homeoboxes in plant development. *Biochim Biophys Acta* 1442: 1–19
- Chen H, Banerjee AK, Hannapel DJ (2004) The tandem complex of BEL and KNOX partners is required for transcriptional repression of *ga20ox1*. *Plant J* 38:276–284
- Chen H, Rosin FM, Prat S, Hannapel DJ (2003) Interacting transcription factors from the TALE superclass regulate tuber formation. *Plant Physiol* 132:1391–1404
- Chen H, Rosin FM, Prat S, & Hannapel DJ (2003) Interacting transcription factors from the three amino acid loop extension superclass regulate tuber formation. *Plant Physiology*, 132(3), 1391-1404.
- Clark SE (1997) Organ formation at the vegetative shoot meristem. *Plant Cell* 9: 1067–1076
- Dinant S, & Lemoine R. (2010) The phloem pathway: new issues and old debates. *Comptes rendus biologies*, 333(4), 307-319
- Ding B, Itaya A, Qi Y (2003) Symplasmic protein and RNA traffic: regulatory points and regulatory factors. *Curr. Opin. Plant Biol.* 6:596–602
- Dong YH, Yao JL, Atkinson RG, Putterill JJ, Morris BA, Gardner RC (2000) MDH1: an apple homeobox gene belonging to the BEL1 family. *Plant Mol Biol* 42: 623–633
- Ferrandon D, Koch I, Westhof E, & Nüsslein-Volhard, C (1997) RNA–RNA interaction is required for the formation of specific bicoid mRNA 3' UTR–STAUFEN ribonucleoprotein particles. *The EMBO journal*, 16(7), 1751-1758
- Jackson SD (1999) Multiple signaling pathways control tuber induction in potato. *Plant Physiol* 119:1–8 23.
- Kerstetter R, Vollbrecht E, Lowe B, Veit B, Yamaguchi J, Hake S (1994) Sequence analysis and expression patterns divide maize knotted1-like homeobox genes into two classes. *Plant Cell* 6: 1877–1887
- Kerstetter RA, Hake S (1997) Shoot meristem formation in vegetative development *Plant Cell* 9:1001–1010
- Reiser L, Sanchez-Baracaldo P, Hake S (2000) Knots in the family tree: evolutionary relationships and functions of knox homeobox genes. *Plant Mol Biol* 42: 151–166
- Lough TJ, & Lucas WJ. (2006). Integrative plant biology: role of phloem long-distance macromolecular trafficking. *Annu. Rev. Plant Biol.* 57, 203-232
- Lucas WJ (1999) Plasmodesmata: intercellular channels for macromolecular transport in plants. *Curr. Opin. Cell Biol.* 7:673–80

- Muller J, Wang Y, Franzen R, Santi L, Salamini F, Rohde W (2001) In vitro interactions between barley TALE homeodomain proteins suggest a role for protein-protein associations in the regulation of Knox gene function. *Plant J* 27: 13–23
- Oparka KJ, Santa Cruz S (2000) The great escape: phloem transport and unloading of macromolecules. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51:323–47
- Reiser L, Modrusan Z, Margossian L, Samach A, Ohad N, Haughn GW, Fischer RL (1995) The BELL1 gene encodes a homeodomain protein involved in pattern formation in the Arabidopsis ovule primordium. *Cell* 83: 735–742
- Rosin FM, Hart JK, Horner HT, Davies PJ, Hannapel DJ (2003a) Overexpression of a knox gene of potato alters vegetative development by decreasing gibberellin accumulation. *Plant Physiol* 132: 106–117
- Sasaki T, Chino M, Hayashi H, Fujiwara T (1998) Detection of several mRNA species in rice phloem sap. *Plant Cell Physiol.* 39:895–97
- Smith HM, Boschke I, Hake S (2002) Selective interaction of plant homeodomain proteins mediates high DNA-binding affinity. *Proc Natl Acad Sci USA* 99: 9579–9584
- Smith HMS, Hake S (2003) The interaction of two homeobox genes BREVIPEDICELLUS and PENNYWISE, regulates internode patterning in the Arabidopsis inflorescence. *Plant Cell* 15:1717–1727
- Tamaoki M, Kusaba S, Kano-Murakami Y, Matsuoka M (1997) Ectopic expression of a tobacco homeobox gene, NTH15, dramatically alters leaf morphology and hormone levels in transgenic tobacco. *Plant Cell Physiol* 38: 917–927.
- Tauberger E, Fernie AR, Emmermann M, Renz A, Kossmann J, Willmitzer L, Trethewey RN (2000) Antisense inhibition of plastidial phosphoglucomutase provides compelling evidence that potato tuber amyloplasts import carbon from the cytosol in the form of glucose-6-phosphate. *Plant J* 23:43–53 47.
- Taylor MA, Mad Arif SA, Kumar A, Davies HV, Scobie LA, Pearce SR, Flawell AJ (1992) Expression and sequence analysis of cDNAs induced during the early stages of tuberization
- Van den Berg JH, Ewing EE, Plaisted RL, McMurry S, Bonierable MW (1996) QTL analysis of potato tuberization. *Theor Appl Genet* 93:307–316
- Vreugdenhil D, Xu X, Jung CS, van Lammeren AAM, Ewing EE (1999) Initial anatomical changes associated with tuber formation on single-node potato (*Solanum tuberosum* L.) cuttings: re-evaluation. *Ann Bot* 84:675–680
- Xu X, Vreugdenhil D, van Lammeren AAM (1998b) Cell division and cell enlargement during potato tuber formation. *J Exp Bot* 49:573–582