Cloning, sequence characterization and heterologous expression of cold tolerance inducing transcription factor(s) (CBF) from tomato (*Solanum lycopersicum* Mill.)

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(2016-H-111-M)

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Thesis

Submitted to

The Faculty of Horticulture

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in partial fulfillment of requirements for the award of the degree of

Master of Science in Biotechnology

2019

DEDICATE THIS THESIS

TO MY

BELOVED PARENTS

&

MY ADVISOR

DR. KHALID ZAFFAR MASOODI

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Certificate – I

This is to certify that the thesis entitled, **"Cloning, sequence characterization and heterologous expression of cold tolerance inducing transcription factor(s) (CBF) from tomato (*Solanum lycopersicum* Mill.)"** submitted in partial fulfilment of the requirements for the award of the degree of **Master of Science in Biotechnology,** to the **Faculty of Horticulture**, **Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** is a record of bonafide research work carried out by **Ms. Anisa Sajad (Regd. No. 2016-H-111-M)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that any help or information received during the course of investigation has duly been acknowledged.

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We, the members of the Advisory Committee of **Ms. Anisa Sajad (Regd. No. 2016-H-111-M)** a candidate for the degree of **Master of Science in Biotechnology** have gone through the manuscript of the thesis entitled, **"Cloning, sequence characterization and heterologous expression of cold tolerance inducing transcription factor(s) (CBF) from tomato (*Solanum lycopersicum* Mill.)"** and recommend that it may be submitted by the student in partial fulfilment of the requirements for the award of the degree.

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Certificate – III

This is to certify that the thesis entitled, **"Cloning, sequence characterization and heterologous expression of cold tolerance inducing transcription factor(s) (CBF) from tomato (*Solanum lycopersicum* Mill.)"** submitted by **Ms. Anisa Sajad (Regd. No. 2016-H-111-M)** to the **Faculty of Horticulture, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir** in partial fulfilment of the requirements for the award of the degree of **Master of Science in Biotechnology** was examined and approved by the Advisory Committee and External Examiner on …….

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ABSTRACT

The research study entitled "Cloning, sequence characterization and heterologous expression of cold tolerance inducing transcription factor(s) (CBF) from tomato (*Solanum lycopersicum* Mill.)" was conducted to clone and characterize the member of CBF gene family, CBF1 from *Solanum lycopersicum* Mill. The CBF1 gene cloned in this study showed high similarity to the previously characterized CBF genes from *Solanum lycopersicum* Mill. Bioinformatic analysis showed that the CBF1 sequence showed high degree of similarity with that of the *Solanum lycopersicum* Mill. as expected and that CBF1 gene cloned has no sequence variation with the already submitted sequence in GenBank by our research group. BLAST of CBF1 cloned from Pusa Sheetal resulted in hits with CBF1. CLUSTALW alignment of the CBF1 of Pusa Sheetal with that of GenBank CBF1 did not show any sequence variation. The CBF1 sequence analysis indicated nuclear localization signal sequence and other conserved motifs typical to CBF type of transcription factors of *Solanum lycopersicum* Mill. In summary, our results indicate cloning of a gene, CBF1 that established high homology with *Solanum lycopersicum* Mill. CBF transcription factors. The CBF1

was cloned as fusion protein with GFP. This study can be further exploited for increasing tomato production in Kashmir in future by initiating transgenic research to develop cold stress tolerant tomato varieties. The outcomes of this research can be applied to different crops that are severely affected by cold and freezing temperatures

Keywords: Cold stress, *Solanum lycopersicum*, CBF1, Cloning

Signature of Student

Dated: _____

Signature of Major Advisor

Dated: _____

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Anisa Sajad

Place: Shalimar, Srinagar

Dated: _____

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Chapter 1

INTRODUCTION

Plants respond and adapt to the stresses (low temperature, drought, high salinity etc.) at the molecular and cellular levels as well as at the physiological and biochemical levels. Expression of a variety of genes has been demonstrated and can be induced by these stresses in a variety of plants. The products of these genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction in stress response. Cold is a major environmental limitation to plant distribution and crop productivity. Sudden frosts can cause large scale crop damage with concomitant loss of millions of dollars. This has encouraged physiological, structural and biochemical investigations of cold-induced injuries in plants as well as plant responses to cold stress (Kirkham, 2016). It has been found that plants vary greatly in their ability to tolerate cold temperatures (Sakai and Larcher, 1987). Many species from tropical regions such as tomato, maize and rice are unable to tolerate freezing and as a consequence suffer chilling injury when exposed to temperatures ranging from 0-10°C. In contrast, plants from temperate regions such as wheat, canola and *Arabidopsis* are capable of tolerating both chilling and freezing temperatures. The ability of these plants to withstand freezing, however is not a constant property, but increases dramatically upon exposure to low, non-freezing temperatures, a phenomenon known as cold acclimation. It has been established that an important component of cold acclimation in *Arabidopsis* is the CBF cold response pathway and within 15 minutes of exposing plants to low temperature, genes are induced that encode a small family of transcriptional factors known as CBF1, CBF2 and CBF3 or DREB1b, DREB1c and DREB1a respectively. By this property some plants develop increased cold tolerance upon pre-exposure to low, non-freezing temperature (Phoon *et al*., 2016). Researchers have identified multiple signal pathways involved in cold acclimation. In one of the pathways, the *CBF/DREB1* genes play a key role in regulating the expression of other cold-related genes and

the degree of cold acclimation response (Lv *et al*., 2017). The *CBF/DREB1* genes were first detected in *Arabidopsis*, then in a number of plant taxa including barley, canola, tomato, rice, sour cherry and strawberry (Shi *et al*., 2017).

The tomato plant (*Solanum lycopersicum*) is highly cold-sensitive and low field temperatures can result in shortened growth periods and decreased crop yield. At temperatures of 10, 8 and 5°C, its growth is respectively affected, incrementally decreased and then stops completely. In order to adapt to low temperatures plants usually promote mechanism to mitigate chilling stress. (Yuan *et al*., 2017). In recent years, studies addressing cold tolerance have found that cold-induced genes play important role in the tomato plants resistance to low temperatures and cold acclimation. Studies of genes stimulated by low temperatures have revealed that many genes, including cold regulated (COR) genes are expressed together, suggesting that a cis-acting factor might be involved in regulating their expression. Further studies showed that related c-repeat (CRT) and dehydration responsive element (DRE) motifs are in the COR gene promoter sequences. The transcription factor, CRT/DRE-binding factor (*CBF*) binds to the promoter of a low temperature-induced gene containing the CRT/DRE regulatory elements to regulate the gene's expression level when induced by a series of low temperatures and to improve the plant's cold resistance (Pareek *et al*., 2017). *CBF* has been widely used in genetic engineering to improve cold resistance in crops. *CBF* can also regulate the expression levels of multiple functional genes in plants under drought, high salt and low-temperature stresses (Ahanger *et al*., 2017).

The regulation of transcription factors and effector genes, collectively called cold-regulated (*COR*) genes, is necessary for cold tolerance. Inducer of CBF EXPRESSION1 (*ICE1*) is an upstream transcription factor that controls the transcription of *CBF*, which activates many downstream genes to confer cold tolerance on plants. *ICE1* encodes a bHLH transcription factor similar to the transcriptional activation gene, *MYC*, which is inactive at normal temperatures but specifically binds with *CBF3* promoter sequences at low temperatures, inducing

CBF3 expression(Copley *et al*., 2017). When *CBF3* binds to the DRE sequence in the promoter of a downstream gene, it induces the expression of a series of downstream *COR* genes and other genes that play a role in the plant's adaptation to cold. Up-regulating this pathway, using an *ICE1* transgene, has been shown to improve cold resistance and growth, without producing abnormalities (Ghneim-Herrera *et al*., 2017). Presently, transgenic *ICE1* plants, including *Arabidopsis,* citrus, *Populus suaveolens*, apple, *Cymbidium*, rice, tobacco, cucumber and chrysanthemum, have improved cold resistance when compared with non-transgenic plants (Ribeiro *et al*., 2017).

In Kashmir valley, the growing season of different crops is limited due to lack of cold acclimation, making these crops susceptible to chilling, which drastically influences the quality and quantity of the produce. Chilling stress is a major production constraint of tomato which is a cold-sensitive horticultural crop. The development of chilling tolerant tomato varieties thus has significant potential to impact tomato production.

The current work is focused on sequence analysis of cloned CBF gene(s) for cold tolerance in *Solanum lycopersicum* and to study the heterologous expression of CBF in *E.coli* (DH5□) for expression of cold stress proteins. This work will pave the way to tackle the food production insufficiency due to climate change and may contribute to food security by stabilizing the yield of major crops that nurtures a large human population on this planet.

The present investigation "Cloning, sequence characterization and heterologous expression of cold tolerance inducing transcription factor(s) (CBF) from tomato (*Solanum lycopersicum*)" will therefore, be undertaken with the following objectives;

- To clone cold tolerance gene(s) (CBF) in *Solanum lycopersicum* with subsequent gene sequence analysis;
- To evaluate heterologous expression of CBF gene(s) in *E.coli* (DH5α) using pEGFP-C1 as vector and translational confirmation of cold stress expressed proteins.

Chapter 2

REVIEW OF LITERATURE

Cold is a major environmental limitation to plant distribution and crop productivity throughout the state of J&K. Sudden frosts during winter months can cause crop damage in large area. Many studies have been carried out to understand the physiological, structural and biochemical basis of cold-induced injuries in plants as well as plant responses to cold stress. In most plants and tissues, freezing-induced injuries result largely from the severe cellular dehydration that occurs upon ice formation and the cellular membrane systems are the primary site of freeze-induced injury and multiple forms of membrane damage can occur as consequence of freeze-induced cellular dehydration (Thomashow, 1999). Thus the mechanisms plants use to respond to cold-stress overlap with responses to dehydration and other abiotic or biotic stress(Shinozaki and Yamaguchi-Shinozaki, 2000). Dehydrins, which stabilizes macromolecules and act as cytoprotectants, are synthesized by cells in response to low temperature, ABA (abscisic acid), or any environmental influence that has a dehydration component, such as drought, salinity, or extracellular freezing(Xu *et al*., 2010; Yordanov *et al*.). Cold acclimation is a process whereby some plants increase in cold tolerance upon pre-exposure to low, non-freezing temperature (Thomashow, 1999). Changes due to freezing include reduction or cessation of growth, reduction of tissue water content, transient increase in ABA levels, changes in membrane lipid composition, accumulation of compatible osmolytes such as proline, betaine and soluble sugars, as well as increased levels of antioxidants(Xin and Browse, 2000). Over a thousand genes are involved in these processes in Arabidopsis (Bechtold *et al*., 2016; Kreps *et al*., 2002).However, there are many other cold-up-regulated genes whose sub-cellular functions have not been well defined and none of these cold-related genes can induce full cold acclimation alone, although each of them has some cell-protective functions under cold stress. The small contributions of these genes to cold tolerance encouraged scientists to

identify the cold-responsive signal transduction pathway (cold STP) and possible regulator genes. Manipulation of the STP could be a powerful method of modifying cold tolerance in plants.

Researchers have identified multiple signal pathways involved in cold acclimation (Xin and Browse, 2000; Yang *et al*., 2005; Zhang *et al*., 2004b). In one of the pathways, the CBF/DREB1 genes play a key role in regulating the expression of other cold-related genes and the degree of cold acclimation response. The CBF/DREB1 genes were first detected in Arabidopsis (Gilmour *et al*., 1998; Liu *et al*., 1998) and were subsequently discovered in a number of plant taxa including barley(Skinner *et al*.), Canola (Jaglo *et al*., 2001), tomato (Zhang *et al*., 2004b), rice (Dubouzet *et al*., 2003), grapevine (Karimi *et al*., 2015), papaya (Zhu *et al*., 2013) and many more. CBF/DREB1 stands for C-repeat (CRT)-binding factor/dehydration responsive element (DRE) binding protein, while CRT/DRE (core sequence: CCGAC) is a cis-acting element present in promoters of multiple cold-regulated (COR) genes (Baker *et al*., 1994; Jiang *et al*., 2013). The CBF proteins contain a conserved AP2 (APETALA2)/ERF (ethylene responsive element-binding factor) domain which recognizes and binds to the CRT/DRE element, thus regulating the expression of downstream genes (Jiang *et al*., 2013; Van-Buskirk and Thomashow, 2006; Yamaguchi-Shinozaki and Shinozaki, 2005).

In Arabidopsis, the CBF gene transcripts appear within 15 min after exposure to low temperature, followed by the increase of transcripts of CRT/DRE-containing target genes at about 2hours(Van Buskirk and Thomashow, 2006). Constitutive overexpression of AtCBF1, AtCBF2 and AtCBF3 in transgenic Arabidopsis plants leads to significant increases in freezing, drought and salt tolerance (Gilmour *et al*.; Gilmour *et al*., 2000). These transgenic plants show enhanced expression of multiple COR genes and elevated levels of various cryoprotectants such as proline and soluble sugars under warm-temperature growth conditions (Gilmour *et al*., 2000; Gilmour *et al*., 1998). The target genes

that respond to both CBF overexpression and low temperature stimulus have been identified by microarray experiments and defined as a transcription unit "CBF regulon" (Nakashima and Yamaguchi-Shinozaki, 2006). At present, more than 100 genes have been assigned to the CBF regulon, encoding proteins with a wide range of functions such as transcription factors, signal transduction pathway components, biosynthetic proteins, cryo-protectant proteins and other stress-related proteins (Van Buskirk and Thomashow, 2006). Thus, the CBF transcription factors and CBF regulon are involved in multiple components of the cold acclimation process. There have been several reports of transformation of Arabidopsis CBFs into economically important crops, such as wheat (Pellegrineschi *et al*., 2004) and rice (Oh *et al*., 2005) to increase resistance to low temperature and other abiotic stresses.

Tomato, like Arabidopsis, encodes three CBF homologs, LeCBF1, LeCBF2 and LeCBF3 that are present in tandem array in the genome(Zhang *et al*., 2004a).Constitutive over-expression of LeCBF1 in transgenic Arabidopsis plants induced expression of CBF-targeted genes and increased freezing tolerance indicating that LeCBF1 encodes a functional homolog of the Arabidopsis CBF1–3 proteins.It has also been established that an important component of cold acclimation in Arabidopsis is the CBF cold response pathway (Thomashow, 2001). Within 15 min of exposing plants to low temperature, genes are induced that encode a small family of transcriptional activators known as either CBF1, CBF2 and CBF3 (Gilmour *et al*., 1998) or DREB1b, DREB1c and DREB1a, respectively (Liu *et al*., 1998). This is quickly followed by activation of the 'CBF regulon,' the set of genes that are induced in response to the CBF/ DREB1 transcriptional activators. The immediate target genes of the CBF/DREB1 proteins have CRT (C-repeat)/DRE (dehydration responsive element) elements in their promoters, the DNA regulatory sequence to which the CBF/DREB1 transcriptional activators bind (Baker *et al*., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994).

Attempts to improve stress tolerance of tomato (*Lycopersicon esculentum*) plants have been made by expressing an Arabidopsis C-repeat/dehydration responsive element binding factor 1 (CBF1) cDNA driven by a cauliflower mosaic virus 35S promoter and the degree of chilling tolerance of transgenic plants was found to be significantly greater than that of wild-type tomato plants as measured by survival rate, chlorophyll fluorescence value and radical elongation (Hsieh *et al*., 2002).The expression pattern of CpCBF2 in papaya under low (7ºC) and high temperature (35ºC) stresses was examined and showed that high temperature stress had little effect on the expression of CpCBF2 but low temperature repressed CpCBF2 expression (Zhu *et al*., 2013). *Brassica napus*CBFs reportedly function as trans-acting factors in low-temperature responses in Brassica, controlling the expression of cold-induced genes through an ABA-independent pathway. Like CBF1, both CBF2 and CBF3 activated expression of reporter genes in yeast that contained the CRT/DRE as an upstream activator sequence (Gilmour *et al*., 1998). CBF transcripts also accumulated rapidly in response to mechanical agitation. The promoter regions of the CBF genes do not contain the CRT sequence, CCGAC and overexpression of CBF1 did not have a detectable effect on CBF3 transcript levels, suggesting that the CBF gene family is not subject to auto-regulation. It was proposed that cold-induced expression of CRT/DRE- containing COR genes involved a low temperature-stimulated signaling cascade in which CBF gene induction was an early event. Recently it has been reported that the tomato plant is sensitive to cold and low field temperatures can result in shortened growth periods and decreased crop yield (Hu *et al*., 2015).

Transcriptome-profiling analysis in Arabidopsis indicates the existence of multiple low-temperature regulatory pathways in addition to the CBF cold response pathway and also indicating the extensive down-regulation of gene expression during cold acclimation (Fowler and Thomashow, 2002). The expression of CBF genes in different tissues of Arabidopsis, during development

and in response to low temperature was analyzed and further characterization of RNA interference (RNAi) and antisense lines failed to accumulate CBF1 or/and CBF3 mRNAs under cold conditions(Novillo *et al*., 2007). It was also found that CBF1 and CBF3 are regulated in a different way than CBF2. Moreover, in contrast to CBF2, CBF1 and CBF3 are not involved in regulating other CBF genes and positively regulate cold acclimation by activating the same subset of CBF-target genes(Novillo *et al*., 2007). It has also been demonstrated that CBF1 and CBF3 have different functions than CBF2 and CBF regulon is composed of at least two different kinds of genes, one of them requiring the simultaneous expression of both CBF1 and CBF3 to be properly induced. This indicates that CBF1 and CBF3 have a concerted additive effect to induce the whole CBF regulon and the complete development of cold acclimation (Novillo *et al*., 2007). Isolation of one CBF/DREB1- like gene, CBF4, from both freezing-tolerant wild grape (Vitis riparia) and freezing-sensitive cultivated grape (*Vitis vinifera*) has been reported. Expression of the endogenous Vitis CBF4 genes was low at ambient temperature, but enhanced upon exposure to low temperature (4 °C)(Xiao *et al*., 2008).

Low temperature causes a negative impact on plant growth and development, but plants evolve a series of mechanisms to respond to chilling stress and one of them is CBF [C-repeat (CRT)/dehydration-responsive element (DRE) binding factor] gene family which has been well studied in different crops. A new gene SpCBF1, was isolated from frost-tolerant *Solanum pinnatisectum* by PCR and analyzed for its function in cold-tolerance by over-expression of CBF1which increased the level of COR (cold- regulated) gene transcripts in OE (over expression) lines and the physiological indexes related to cold tolerance like the contents of SOD, soluble protein, MDA, proline and soluble sugar were higher in OE lines than in WT (untransformed control) except Relative Water content (RWC) which was lower. All these results indicated that SpCBF1 gene plays a promoting role in potato responding to cold stress.

Tomato inducer of CBF expression has been reported to be involved in cold and salt stress signaling. The investigate functions of tomato in cold and salt tolerance, c index CS1, immunochemical assay of endogenous ICE1 protein and RT-PCR of cold inducible genes were conducted with tomato plants. An ICE1 related protein with molecular mass of 55 Dalton was induced under chilling and salt stresses. The expression of tomato ICE1 gene under chilling stress was maintained at constant level in contrast to the protein level. Chilling stress sequentially up-regulated CBF homologue, SICBF1 and trehahalose-6-phosphate synthase (SITPS1). It was concluded that tomato ICE1 specific cis-elements, leading to induction of cold tolerance by trehalose synthesis.

Studies related to cloning and transformation of inducer of CBF expression in tomato indicated that transcription of CRT/DRT binding factor (CBF) was regulated by inducer of CBF expression (Yuan *et al*., 2017).

Chapter 3

MATERIALS AND METHODS

The present investigation entitled "Cloning, sequence characterization and heterologous expression of cold tolerance inducing transcription factor (s) (CBF) from tomato (*Solanum lycopersicum* Mill.)" was carried out at Transcriptomics Laboratory, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir, Shalimar during years 2016 and 2017. Flowchart of work protocol is depicted in Fig. 1.

3.1 Plant material

Tomato seed material of Pusa Sheetal variety was collected from seed repositories of Indian Agricultural Research Institute (IARI), New Delhi. One varieties Pusa Sheetal of tomato was selected.

3.2 Microbial cultures and plasmids

E. coli strain viz. BL-21 (DE3) (Novagen) was obtained from IISc Bangalore India. *E. coli* (DH5α) was obtained from National Institute of Immunology, New Delhi, India. The pEGFPC1 was kindly supplied by Prof Zhou Wang, University of Pittsburgh, Pennsylvania, USA.

3.3 Sterilization

The seed material was soaked in water for 10 minutes, washed twice with sterile water, and then sterilized seeds were germinated in petriplates at 24°C in the incubator. Germinated seedlings were transferred to petri plates and grown under controlled conditions in growth chamber at 24°C/15°C (Day/Night).

3.4 Growth condition and cold shock of tomato

Seedlings were given cold stress at 4°C for 24 hrs at germination stage (10-12 days after sowing). About 100 mg (fresh weight) of seedlings were

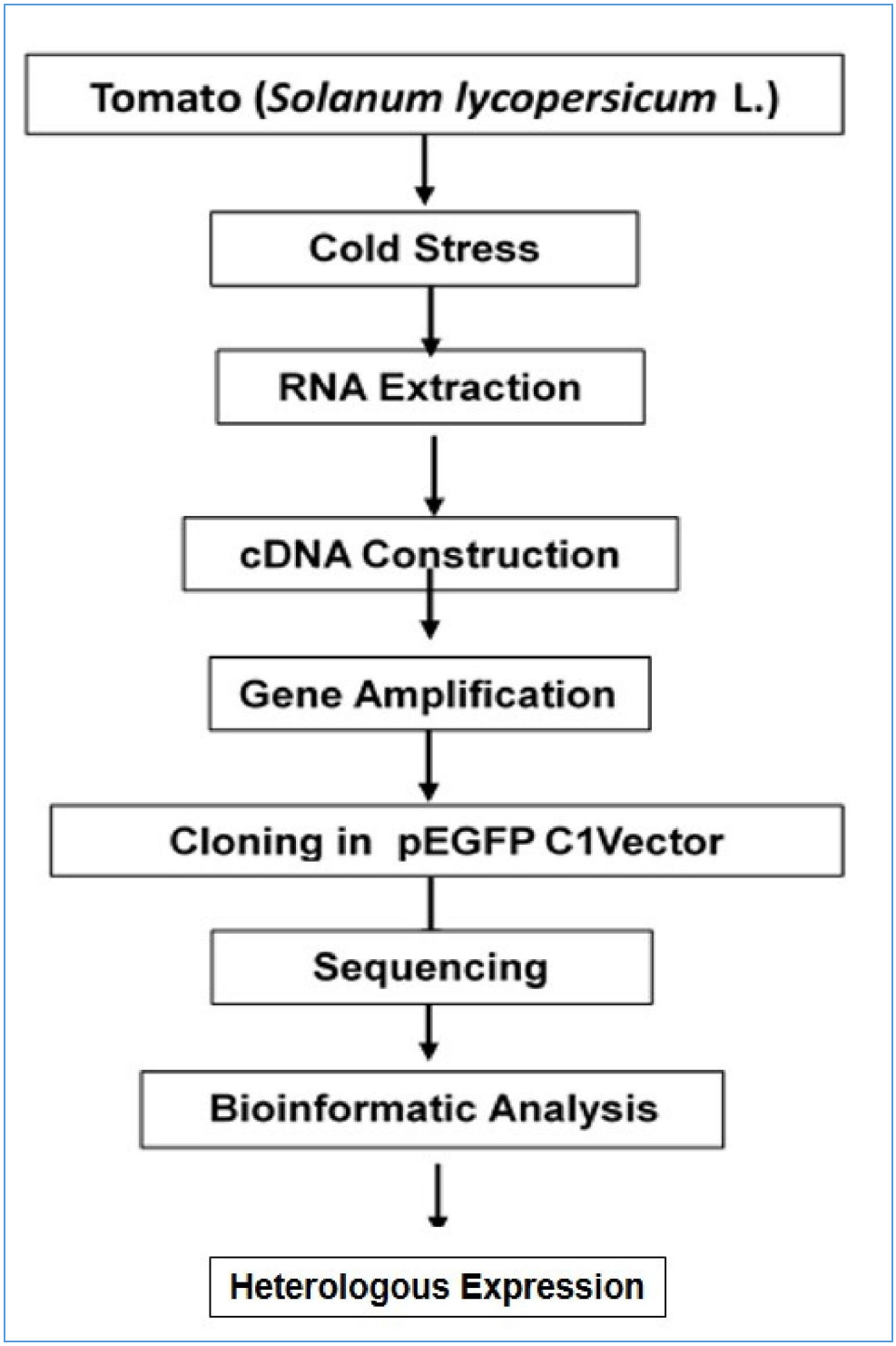


Fig. 1: Flow chart showing work protocol

collected after 24 hours of stress alongwith control seedlings and were processed for RNA extraction.

3.5 DEPC treatment

Microfuge tubes, tips, tip boxes, mortar pestle, spatula, were DEPC treated overnight and then autoclaved followed by drying in hot air oven.

3.6 RNA extraction and qRT-PCR

Total RNA was extracted at germination stage after cold stress from the seedlings of control as well as cold stressed samples of Pusa Sheetal variety of tomato using Trizol (Invitrogen) (Chomczynski and Sacchi, 1987) method as follows.

Tissue samples (100mg) was grinded in a mortar and pestle in liquid nitrogen and transferred to a fresh microfuge tube.1ml of Trizol was added to the homogenized sample and mixed thoroughly and incubated at room temperature for 5 minutes 0.2 ml of chloroform was added and the tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature for 2-3 minutes. Centrifugation of the samples at 12,000 x g for 15 minutes at 4º C was done. Following centrifugation, the mixture separated into lower red, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase was carefully transferred into a fresh 1.5ml tube without disturbing the interphase. The aqueous phase was processed for RNA precipitation.0.5 ml of 100% isopropanol was added to the aqueous phase. Incubation at room temperature for 10 minutes was done. Centrifugation was done at 12,000 × g for 10 minutes. The supernatant was removed from the tube leaving only the RNA pellet. The pellet was washed with 1 ml of 75% ethanol. The samples were vortexed briefly and then the tubes were centrifuged at 7500×g for 5 minutes at 4º C. The wash was discarded. The RNA pellet was air dried for 5-10 minutes. The RNA pellet was resuspended in RNase free water

(DEPC water). After dissolving, samples were run in 1% agarose gel to check quality of RNA and presence of any DNA contamination.

3.7 DNase treatment of RNA samples

Thermo Scientific DNase kit was used for removing traces of DNA in the extracted RNA. The DNase treatment was given following the manufacturers protocol.

3.8 Quantitative and qualitative analysis of RNA

The quality of RNA was checked by using Nanodrop (*Thermo Fisher SCIENTIFIC*) at optical density (OD) of 260 nm and 280 nm. The samples showing $OD_{260/280}$ ratio between 1.9 and 2 were used for further experimental studies. The quantity of RNA was checked by using an OD of 260 nm.

3.9 cDNA synthesis

cDNA synthesis was done using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit using oligodTprimers.The first strand cDNA was synthesized by using manufacturers protocol and stored at -20˚ C. The protocol for synthesis of first strand is given below.

The following reagents were added in an RNase free tube on ice as per the indicated order.

Template	Total RNA	1.5 µg
Primers	Oligo $(dT)_{18}$ primer	1 µl
Nuclease free water		Final VolTo 12 µl
Total volume		**12 µl**

The components were mixed gently and a brief spin was given. Incubation at 65º C for 5 minutes was given. The tube was chilled on ice and brief spin was

given. The tube was again placed on ice and the following components were added as per the indicated order.

5X Reaction Buffer	4µl
RiboLock RNase Inhibitor (20U/µl)	1µl
10 mM dNTP Mix	2µl
RevertAid M-MuLV RT (200U/µl)	1µl

The components were mixed gently and a brief spin was given. The components were incubated at 42º C for 60 minutes. The reaction was terminated by heating at 70º C for 5 minutes.

3.10 Validation of cDNA and primers using PCR

To validate cDNA synthesis and primers, polymerase chain reaction (PCR) was carried out using primers of GAPDH gene. The detail of the primers used is given in Table 1.

Table 1: Primers for amplification of CBF-1 and GAPDH genes. The bold underlined sequence represents the site for restriction enzyme

Nature of primer	Primer sequence		Size of Amplicon	Tm °C	Restriction Site
GAPDH	Forward primer	5′ CCTTAGTTGATTCTGTACC3′	169bp	59.7	Nil
	Reverse primer	5′ GCCAGTTAGTAGTAATTGCCA 3′		58.3	Nil
CBF1	K-Lab-CBF1-LS-F	CTCAGT**AGATCT**ATGAATATCTTTGAAACCTA	618bp	55	BglII
	K-Lab-CBF1-LS-R	CGTACT**CTGCAG**TTAGATAGAATAATTCCATA		55	PstI

3.11 PCR Reaction mixture

PCR amplification for CBF-1 and GAPDH gene was carried out in a reaction volume of 20 µl in 0.2 ml PCR tubes. The reaction mixture contents are given in Table 2.

Table 2: PCR reaction mixture for amplification of CBF-1 and GAPDH genes

Constituents	**Volume**
Autoclaved water	13.6 µl
PCR buffer (1 X)	2 µl
$MgCl_2$ (25 mM)	1.2 µl
dNTPs (25 mM)	0.4 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
TaqPolymerase (5 U/µl)	0.3 µl
cDNA sample (70 ng/µl)	0.5 µl
Total volume	**20 µl**

3.12 PCR cyclic conditions

The amplification reaction was carried out in thermocycler (*Applied Biosystems*). The cycling conditions are shown in Table 3.

Table 3: Thermal cycling conditions for amplification of CBF-1 and GAPDH genes

Steps		Temperature ^{o}C	Time	Number of cycles
1. Hot-Start		94	2 min	1
2. Denaturation		98	10 sec	35
3.Annealing	GAPDH	61	30 sec	
4. Extension		68	2 min	
5. Final extension		68	10 min	1

3.13 Gel Electrophoresis

The amplified products of GAPDH and CBF1 genes were electrophoresed on 2% agarose gel and compared with 100 bp DNA ladder respectively.

3.14 cDNA Quantification

cDNA quantification was done using Qubit$^{@}$ 2.0 Fluorometer (Invitrogen).

3.15 Confirmation of cDNA synthesis

The internal gene primers for GAPDH used for confirmation of cDNA synthesis were designed (Table 1.0) using protocol outlined in the primer3 online software (http://frodo.wi.mit.edu/primer3/) (Krawetz, 2000). The primers were checked for folds and self-complementarity by oligocalc software (http://www.basic.northwestern.edu/biotools/oligocalc.html) (Kibbe, 2007). These GAPDH primers were used for confirmation of cDNA synthesis. The predicted size of amplicon was 169bp.

3.16 Gene cloning

CBF1 were cloned from cDNA of *Solanum lycopersicon* Mill. Var Pusa Sheetal. Strategy from cloning of CBF-1 in pEGFPC-1 is depicted in Fig. 2.

3.17 Primer designing

For designing primers for amplification of genes coding for CBF1 from

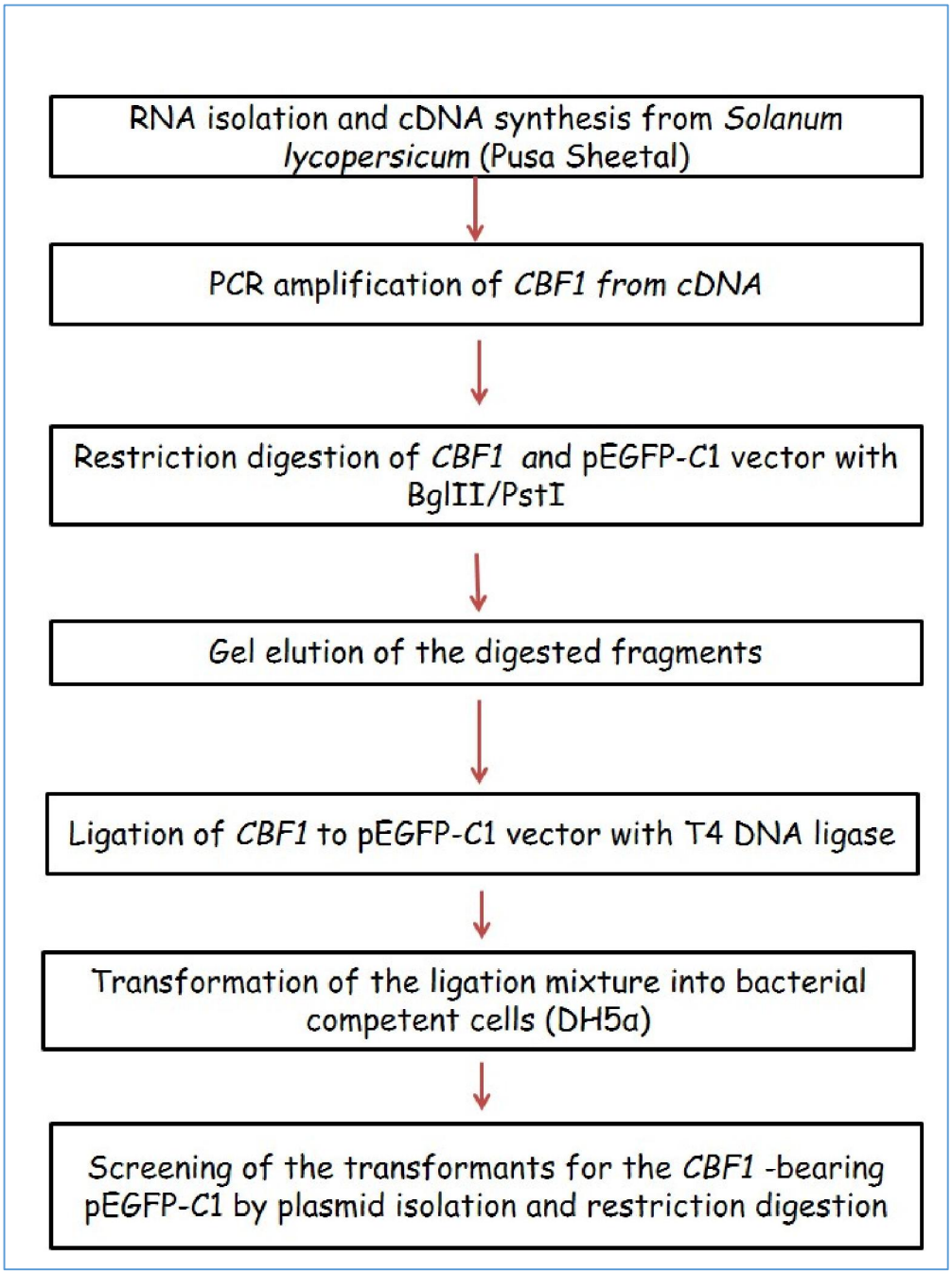


Fig. 2: Strategy for heterologous Protein Expression CBF1-GFP

Solanum lycopersicon Mill. Var Pusa Sheetal, amino acid sequences for previously submitted Genbank sequence were retrieved from GenBank (Farheena, 2018). The mRNA sequence was aligned using Clustal W (www.ebi.ac.uk/clustalw) to check for conserved motifs (Chenna *et al.*, 2003; Larkin *et al.*, 2007). Two oligonucleotide primers for CBF1 gene were designed (Table 3) using the primer 3 online software. Restriction site was added in the primers at the 5′ and the 3′ ends.

3.18 PCR amplification of full length genes (CBF-1)

Total RNA was extracted from 1-2 week old seedlings of *Solanum lycopersicum* Mill. Var Pusa Sheetal variety. First strand cDNA was prepared with 2µg of total RNA. The putative full-length *CBF1*cDNAs was amplified with K-Lab-CBF1-LS-F and K-Lab-CBF1-LS-R primers. After an initial denaturation at 94°C for 2 min, 35 cycles were run each with 30s of denaturation at 94°C, followed by 40s annealing at 55°C, and 1min extension at 72°C.

Following constituents were gently mixed and the tubes were spin briefly:

Constituents	***CBF1***
Buffer (10X)	2.5µl
dNTP's (2.5mM each)	2.5 µl
$MgCl_2$ (25mM)	1.0 µl
cDNA (PB)	1.0 µl
Vent DNA Pol.	1U
Gene specific primers F+R (10mM)	2+2µl

The PCR reaction was run in an Eppendorf thermocycler as per the following protocol.

The protocol followed for the PCR is as follows:

S. No.	Gene	*CBF1*	
	Step	**Temperature °C**	**Time (min)**
1.	Initial denaturation	95	3:00
2.	Denaturation	95	0:30
3.	Tm	50	0:30
4.	Elongation	72	1:30
5.	Step 2 repeat 30 cycles		
6.	Final extension	72	10:00
7.	Hold	4	∞

The products were run on a 1% agarose TAE gel and visualized on an UV-transilluminator and photographed using a gel documentation system. 1kb/100bp ladder (Genetix) was used as a molecular weight marker. The DNA bands were excised from the gel with a sharp sterilized blade, weighed and put in an autoclaved 2 ml microfuge tube. DNA purification from the excised band was carried out by using MinElute gel purification kit (QIAGEN) according to the manufacturers' instruction

3.19 Restriction digestion of the PCR fragments and Vector

The eluted fragments of CBF1 and pEGFPC1 Vector were double digested simultaneously with the specific restriction enzymes using the following protocol.

Constituents	*CBF1*	*pEGFPC1*
DNA	5 µl	5 µl
Buffer (5X)	2 µl	2 µl
Restriction Enzyme (Thermo) (1U)	BglII and PstI	BglII and PstI
H_2O	1 µl	1 µl

The above constituents were gently mixed and the tubes were spun briefly and incubated at 37°C for 1hr. The products were run on a 1% agarose TAE gel and visualized on a UV-transilluminator and photographed using a gel documentation system. 1Kb ladder was used as a molecular weight marker. The restricted fragments were gel purified using MinElute gel purification kit (QIAGEN) according to the manufacturers' instruction.

3.20 Expression vector

The expression vectors used was pEGFPC1 obtained from Zhou Wang, University of Pittsburgh, PA, USA) (Fig. 1). Vector preparation was carried out by double digestion of pEGFPC1 expression vectors using restriction enzymes as given in Table above. Schematic diagram for cloning of CBF-1 gene in pEGFPC-1 vector is depicted in Fig. 3.

3.21 Ligation

The ligation reaction was performed using 0.2µg of digested vector, 0.4µg insert, 1µl ligation buffer and 1U T4 DNA ligase in a final volume of 10µl. The samples were incubated at 4°C for 24 hrs and the recombined plasmid was transformed into *E.coli* DH5α and BL21 (DE3).

3.22 Transformation

A single colony of *E. coli*DH5α/BL-21 was inoculated into 10ml LB broth in a 50 ml falcon tube. It was grown at 37°C for 16 hrs. 1ml of the above grown

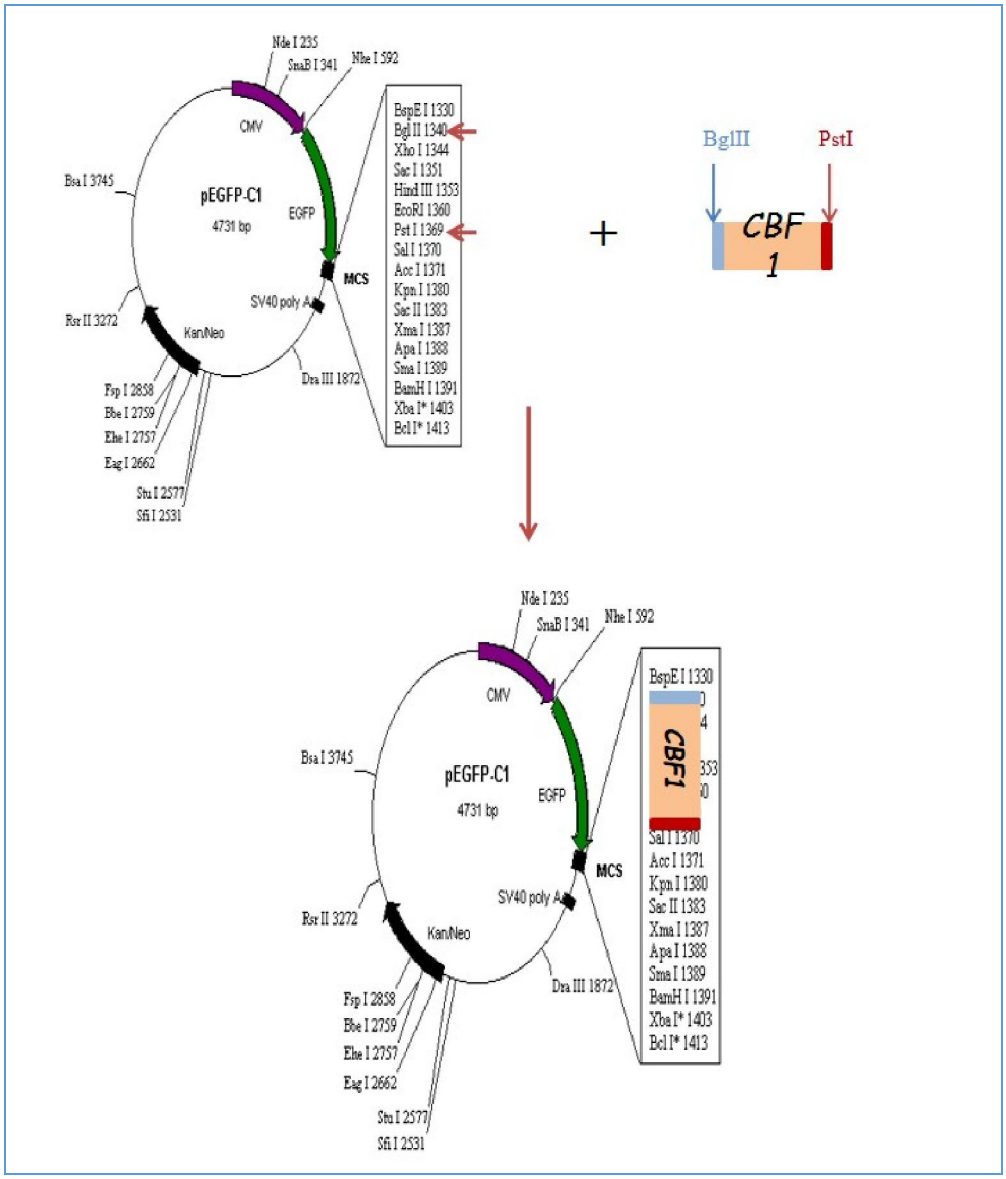


Fig. 3: Schematic diagram for cloning CBF1 gene from *Solanum lycopersicum* Mill. Variety Pusa Sheetal in pEGFP-C1 vector

culture was used to inoculate 100ml of LB in 250ml flask and kept in an incubator shaker at 37°C for 4h with constant shaking (250 rpm). The culture was kept on ice for 10 min and transferred to 50ml falcon tube and centrifuged at 5000 rpm for 5 min at 4°C. After centrifugation the supernatant was decanted and the cells were resuspended in 1ml cold 0.1M $CaCl_2$. The cells were vortexed and again centrifuged at 5000 rpm for 5 min at 4°C. After centrifugation the supernatant was decanted and the cells were resuspended in1 ml cold 0.1M $CaCl_2$. The cells were vortexed and incubated on ice for 20 min to make them competent. The competent cells were dispensed in 2ml microfuge tubes (200 µl/tube) and stored at -80°C for further use. 5µl of ligation mixture was pipetted gently into 200µl of competent cells. The tubes were incubated for 30 min on ice, transferred for 2 min to 42°C (heat shock), and immediately put back on ice. 900 µl of LB was added and the tubes were incubated at 37°C for 2 h. 100 µl of the cells were plated on LB Ampicillin (50µg/ml)/plates. 100 µl of the control DNA was also plated on LB Ampicillin (50µg/ml) plates. The plates were grown overnight at 37°C in an incubator.

3.23 Confirmation of cloning by restriction digestion

The clones were separately inoculated in 5ml LB broth and incubated for 16 hrs with 250 rpm constant shaking at 37°C. Plasmid DNA was isolated from clones using plasmid purification kit (Sigma) following manufacturers' instructions. The plasmids were stored at -20°C for further use. Restriction digestion of the vectors was carried out by the following protocol:

Gene	***CBF1***	***Empty Vector***
Plasmid clone (0.5µg)	pKZ-CBF1	pEGFPC1
Buffer (1.5 µl)	Tango	Tango
R. E – 1	BglII	BglII
R. E – 2	PstI	PstI

The samples were incubated at 37°C for 4 h for complete digestion. Enzymes were inactivated at 65°C for 10 min. The products were run on 0.7% agarose TAE gel at 8 volts/cm for 30-45 min. The products were visualized and photographed.

3.24 Heterologous expression

The expression vector pEGFP-C1 (Clontech) was used to generate fusion protein constructs with GFP at the N terminus of CBF1 for convenient visualization using fluorescent microscopy as described earlier (Dar, Masoodi *et al.*, 2014).

3.25 Cell culture experiments

Human C4-2 prostate cancer cells were obtained from Dr. Zhou Wang, University of Pittsburgh, PA, USA and maintained in RPMI1640 medium supplemented with 10% FBS, 1% glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) at 37ºC in thepresence of 5% CO2 in a humidified incubator. GFP-CBF1 and GFP expression vectors was transiently transfected into C4-2cells using Lipofectamine Reagent according to the manufacturer's protocol (Thermo). Cells were transfected at >60% confluence in phenol red-free OptiMEM. The Expression of GFP-CBF1 fusion proteins was imaged 16hours after transfection using LMI Fluorescent Microscope, ABE, London, UK.

Chapter- 4

EXPERIMENTAL FINDINGS

The results of the present investigation entitled "Cloning, sequence characterization and heterologous expression of cold tolerance inducing transcription factor (s) (CBF) from tomato (*Solanum lycopersicum*) Mil" are described in the present chapter under the following headings.

4.1 RNA Isolation

Single variety of tomato (*Solanum lycopersicum* Mil) viz. Pusa Sheetal was selected for the present investigation (Fig 4). The seeds were incubated at 25 degree Celsius on petriplates for 7 days and further cold stress was given at 4 degrees Celsius for 24 hrs. Total RNA was isolated from the germinated seedlings of stressed and control treatments and integrity and purity of the isolated RNA was confirmed by subjecting the samples to laboratory analysis. RNA samples showing $OD_{260/280}$ ratio >1.9 was an indication of good quality RNA. The quantity of RNA was checked at OD of 260 nm and based on the observations, it could be classified as of good quality. Gel profile of RNA also demonstrated good quality of RNA bands with clear distinction between 28SRNA and 18SRNA (Fig 5 a) in stressed and non-stressed samples.

4.2 cDNA synthesis

PCR amplification reaction for the confirmation of cDNA and also an appropriate annealing temperature for gene of interest (CBF1) and housekeeping gene (GAPDH) was identified as 53°C and 61°C, respectively. The amplified products were run on 1.5% agarose gel and Fig 5(b) shows the amplified product of GAPDH gene (162bp) with ladder (1kb) and fig 6 shows the amplified product of CBF1 gene (618 bp) with ladder (1kb).

4.3 Gene cloning

RNA isolated from 7 days old germinated seedlings of *Solanum*

lycopersicum (Pusa Sheetal) (Figure 5a). cDNA synthesis done using GAPDH primers (Fig. 5b), and the PCR amplification resulted in a 618 bp band as expected. The quality and brightness of the band reflected the quality of the cDNA formed (Fig. 5b). PCR resulted in the amplification of full length genes (Fig. 6), where the length of gene CBF1 was 618bp (Fig 7). The full length genes were treated with restriction enzymes Bgl11 and Pst1 whose sites were embedded in the primers used for gene amplification. The fragments gel purified and ligated with the vector PEGFP-C1 followed by transformation in *E. coli*, DH5α strain Fig. 8 (1&2). Plasmid DNA was successfully extracted and confirmed {Fig. 8 (3)}. There was upshift of bands in positive clones (Fig. 9). Further cloning was confirmed restriction digestion with the suitable restriction enzyme that which released the insert (Fig. 7). Restriction digestion of vector PEGFP-C1 with *BglII*and *Pst*I released 618bp insert and 4.7kb vector band.

4.4 Sequencing and Bioinformatic analysis of CBF gene(s)

The PCR products of CBF1 gene were sequenced and results are presented in Fig. 11. The deduced amino acid sequences of CBF1 were compared to those of other organisms from GenBank, and showed high degree of similarity with that of the *Solanum lycopersicum* Mill. as expected. The CBF1 comprised of 618 bp coding region which coded for a protein of 205 amino acids. BLAST of CBF1 cloned from PS resulted in hits with CBF1.

4.5 Vector construction

Expression vector was designed and constructed for transformation of *E. coli* (Fig. 12), and BLAST used to find regions of similarity between the query sequence and the sequence present in the NCBI (National Center For Biotechnology Information) database. Top hits with 100% sequence similarity are shown in Fig. 13 and Fig. 14. Pairwise and Multiple sequence alignment carried out by Clustal Omega revealed that comparing the query sequence of CBF1 with the database sequence, there was no difference between the query sequence of

Pusa Sheeetal with the database sequence. The nucleotide sequence at various positions with respect to the database sequence are presented in Fig. 15. Subsequently, putative translated sequence obtained from the nucleotide sequence showed no differences in Pusa Sheetal as shown in Fig. 16 and Fig. 17 using EXPASY translate tool.

Table 4: The expression vectors constructed for expression studies

S. No.	Expression/Cloning Vector	Genes 1	Vector Constructed
1	PEGFP-C1	*CBF1*	pKZ100

4.6 Protein expression

C4-2 cells were transfected with plasmid PEGFP-C1 and protein was isolated from those cells. SDS-PAGE was run for the isolated proteins and visualized using Coomassie stain. Microscopic examination of CBF1 bearing cells transfected using fluorescent microscope showed that localization of protein in the cells and under different resolutions as shown in Fig. 19 and Fig. 20.

Fig. 4: Germinated seedlings after 7 days of incubation at cold stress

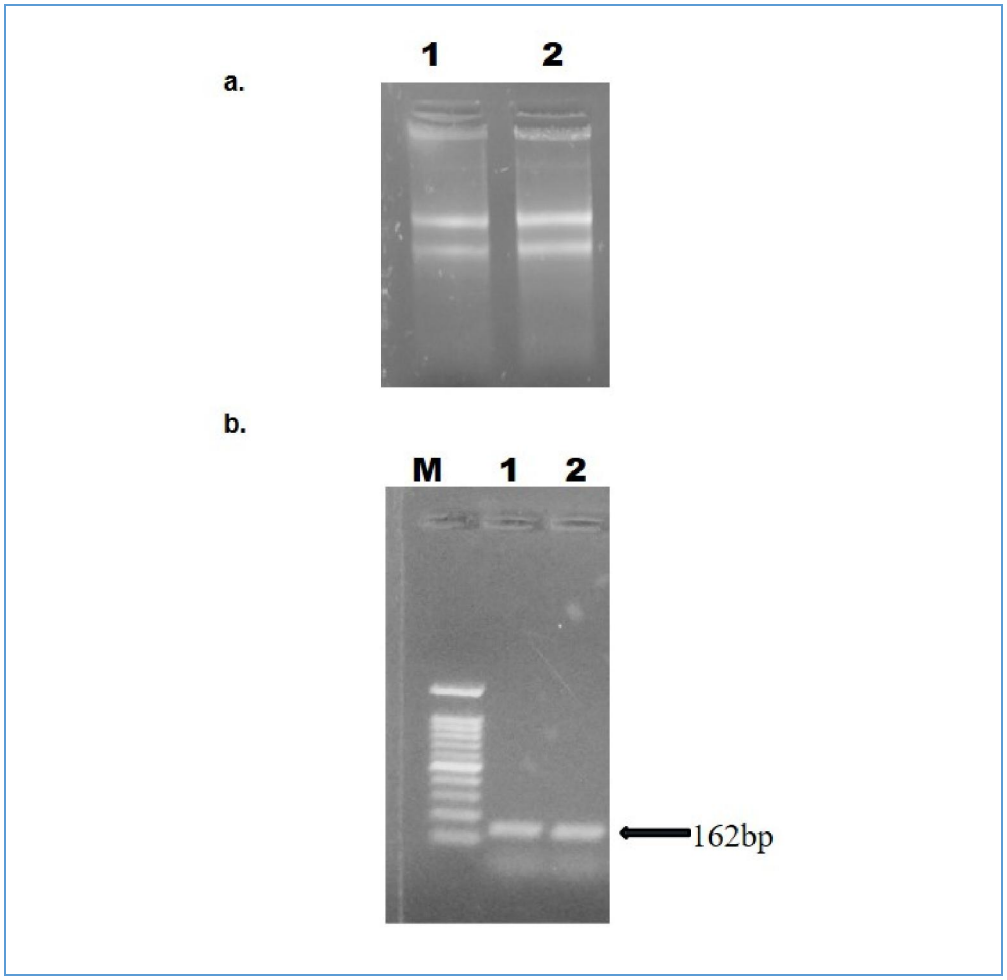


Fig. 5:

a. **RNA isolation from *Solanum lycopersicum* Mill. Variety Pusa Sheetal**
Lane1: Cold stress, Lane 2: Control.

b. **Confirmation of cDNA synthesis using GAPDH primers which resulted in a 162pb fragment**
Lane M: 100bp Marker, Lane 1: Cold Stress, Lane 2: Control

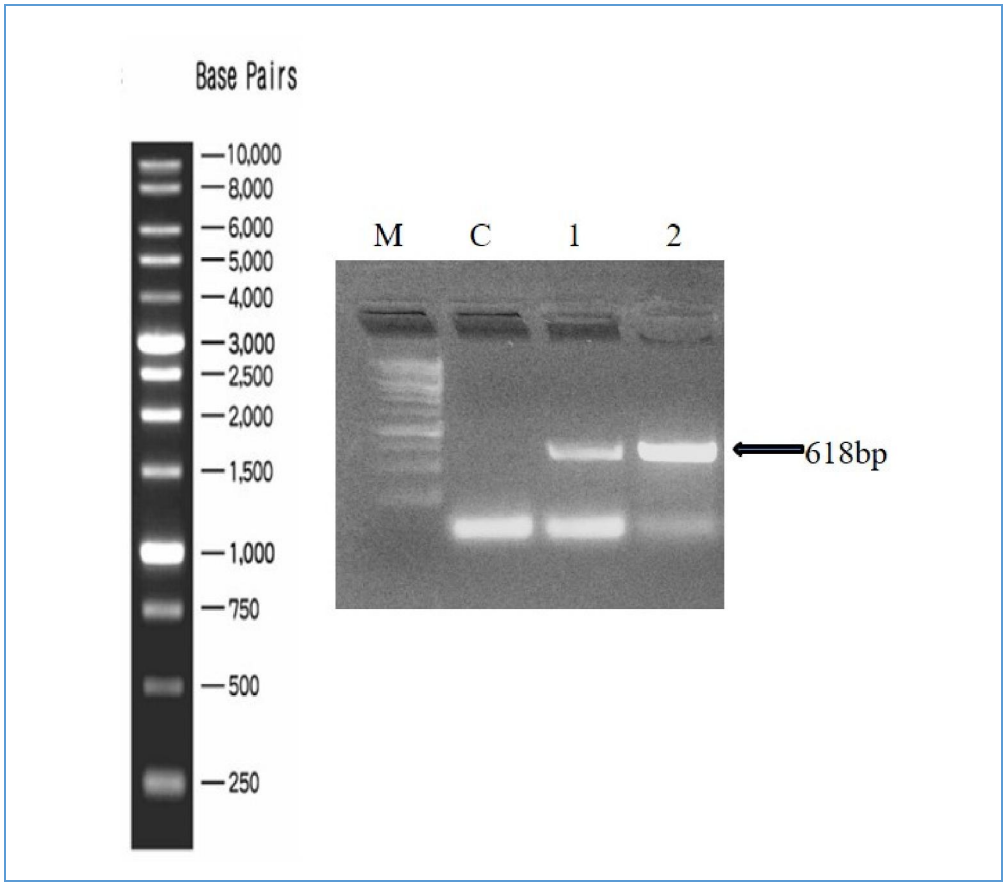


Fig. 6: PCR amplification of *CBF1 gene from Solanum lycopersicum* Mill. Variety Pusa Sheetal

Lane M: Gene ruler 1Kb
Lane C: Negative control
Lane 1 and 2: PCR amplification of *CBF1* (Expected size 618bp)

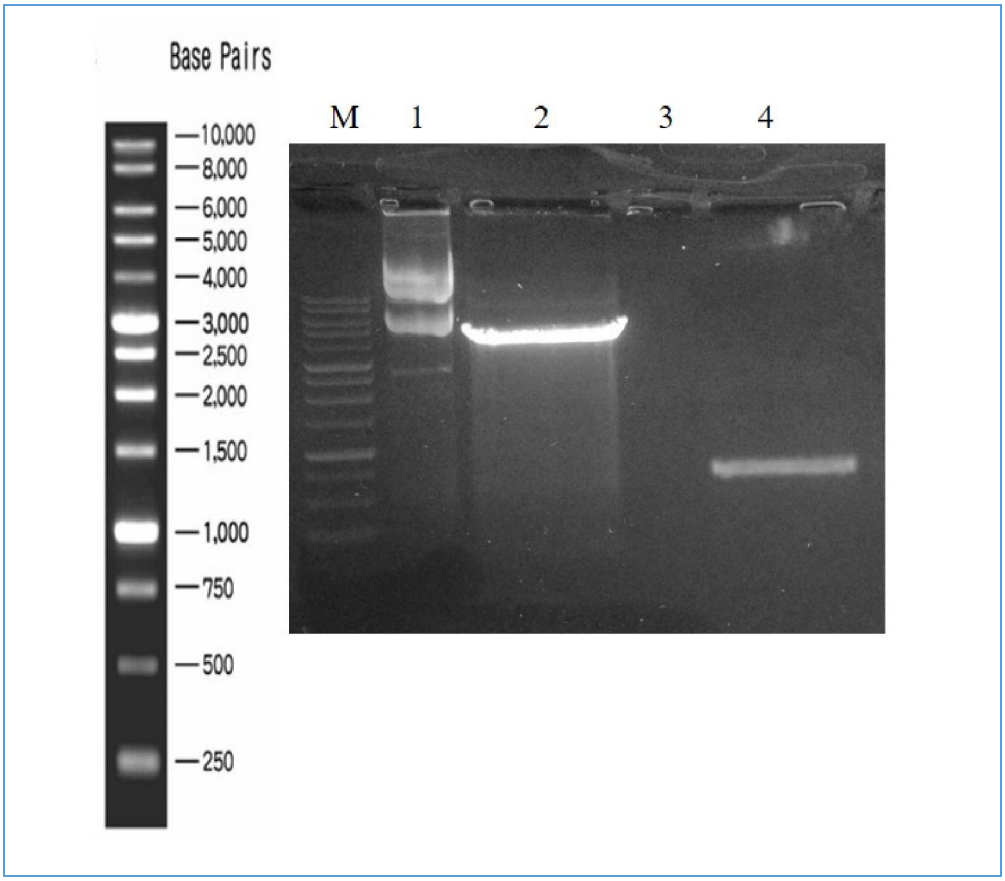


Fig. 7: Restriction digestion of Plasmid pEGFPC1 and 618bp Insert with BglII and PstI restriction enzymes

Lane M: Gene ruler 1Kb
Lane 1: uncut Plasmid
Lane 2 : Restriction digested Plasmid with BglII and PstI
Lane 3: empty
Lane 4: Restriction digested CBF1 with BglII and PstI

Fig. 8: Transformation of Ligated plasmid and insert in *E. coli* DH5α

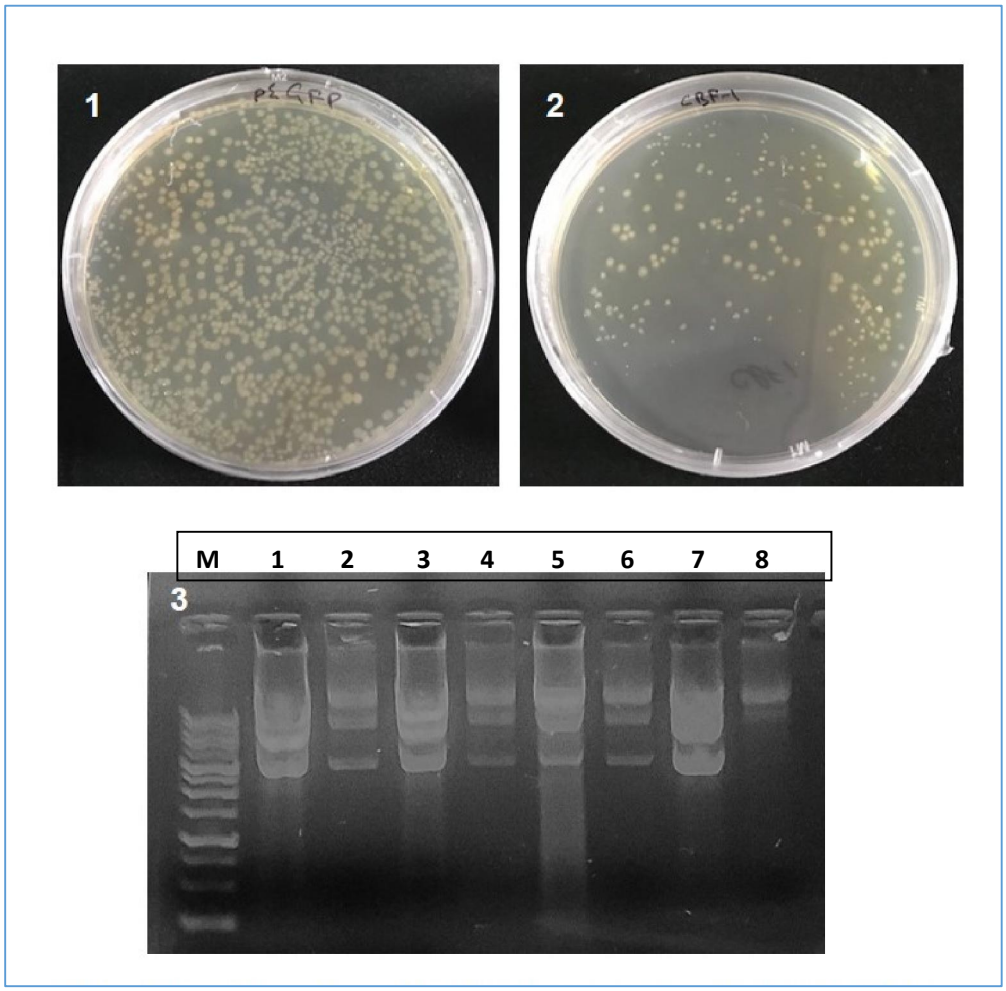


Plate 1: Control pEGFPC1 Transformed in *E.coli* DH5α cells on Kanamycin LB plates

Plate 2: CBF1 product ligated with pEGFPC1 vector and transformed in *E. coli* DH5α cells and grown on Kanamycin LB plates.

Plate 3: Gel Pic of Isolated plasmids run on 0.7% agarose gel. Lanes 1-8 contains plasmids isolated from colonies obtained after transformation.

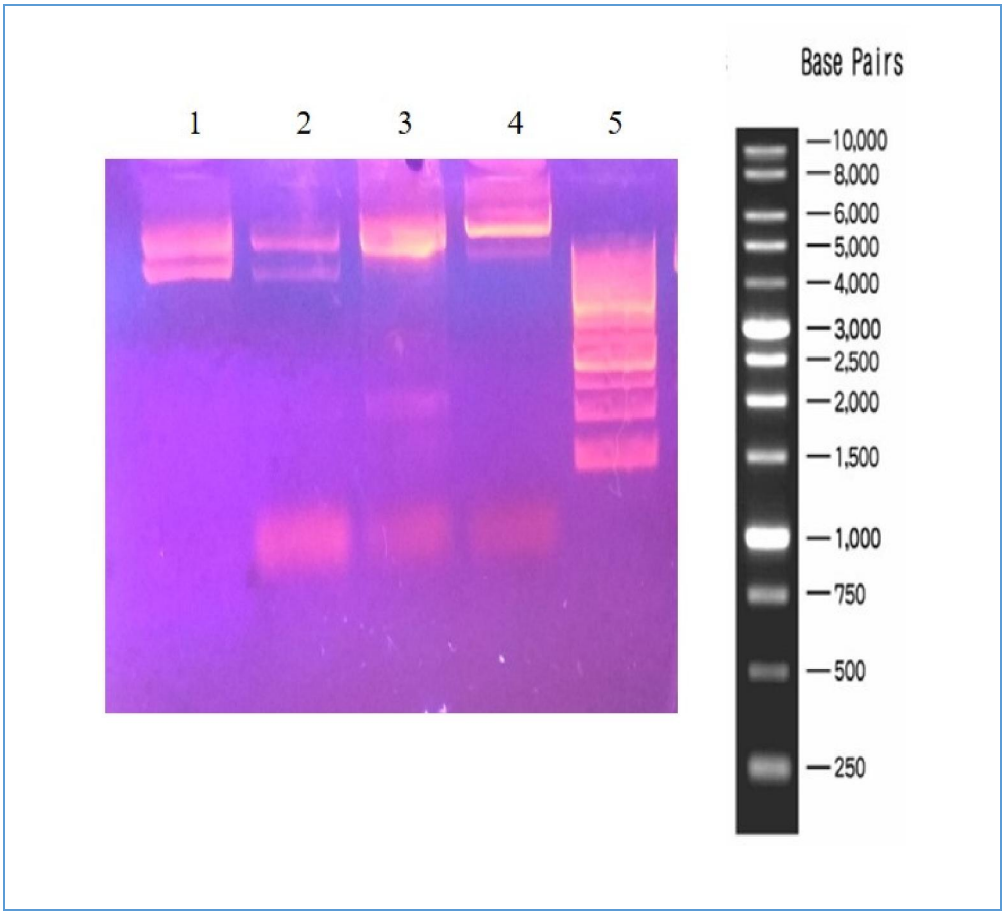


Fig. 9: Screening of isolated plasmids for CBF1 insertion in pEGFP-C1

Lanes1-4: isolated plasmids
Lane5: Gene ruler 1kb

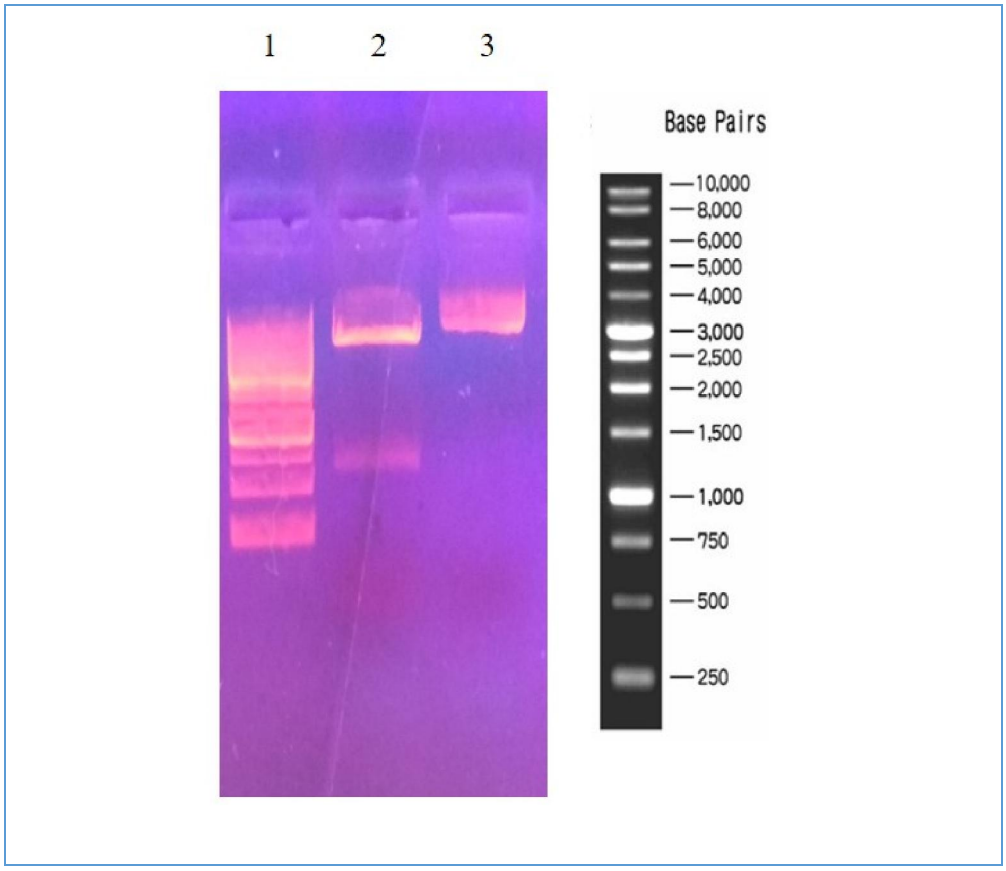


Fig. 10: Verification of cloning of plasmids by restriction digestion

Lane1: Generuler1kb
Lane2: BglII/PstI digestion of plasmid harboring CBF1 gene (Expected sizes: 4.7kb, ~600bp)
Lane3: BglII digestion of plasmid harboring CBF1 gene (Expected size: ~5.3kb)

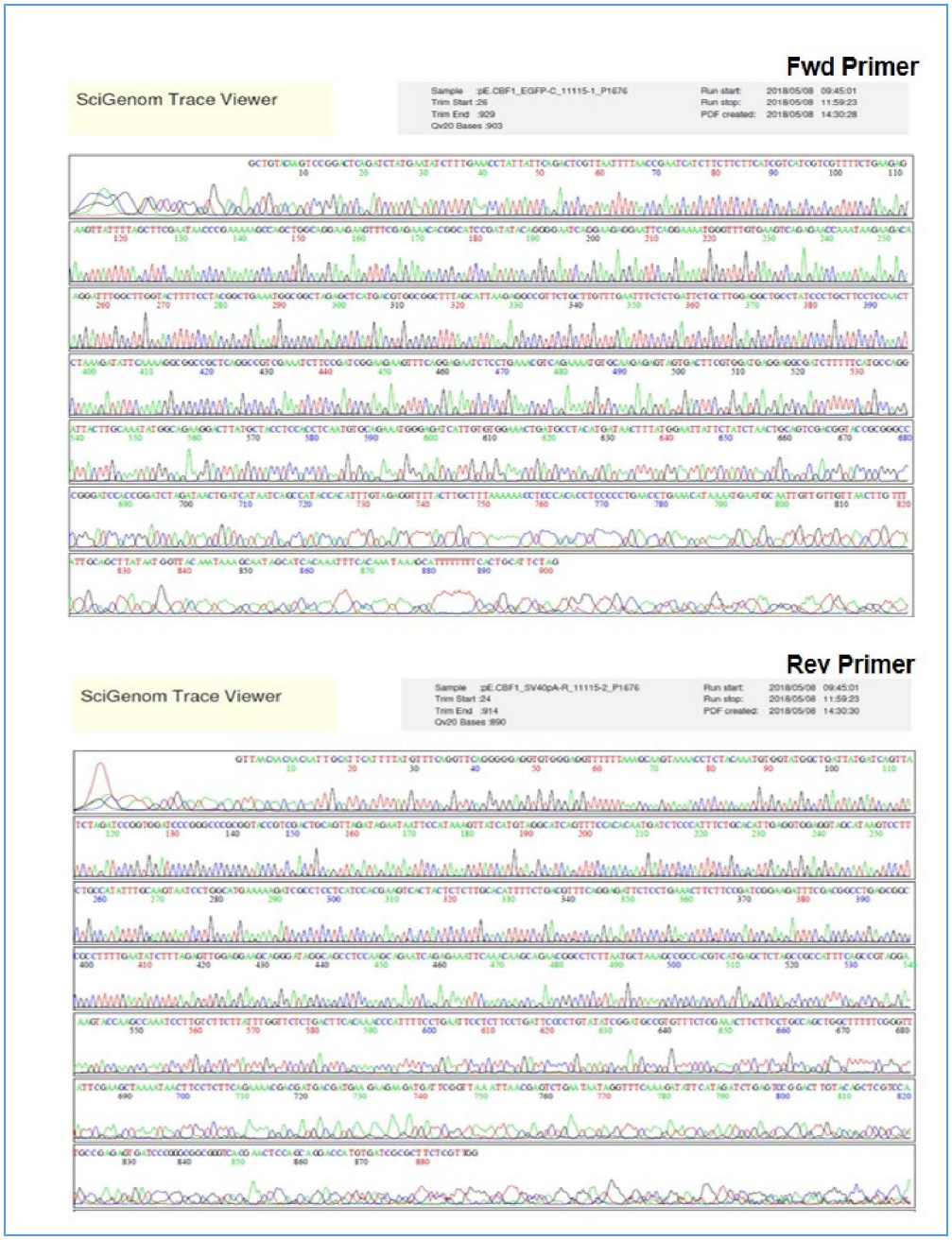


Fig. 11: Sequence chromatogram showing sequencing results with Forward and reverse Primer

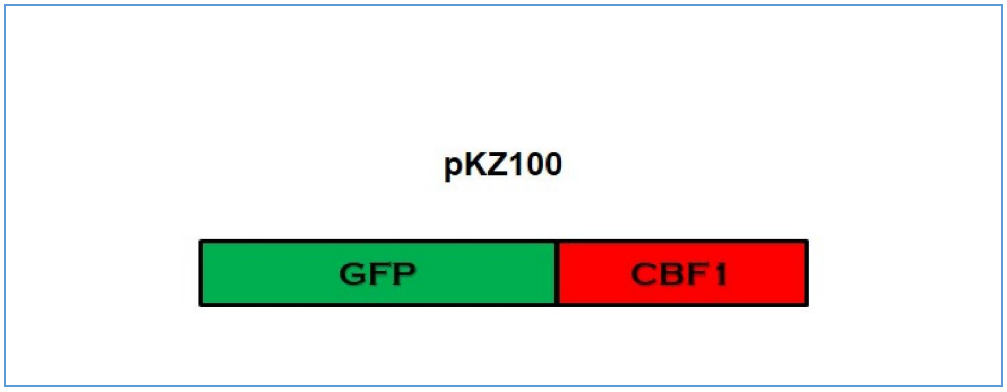
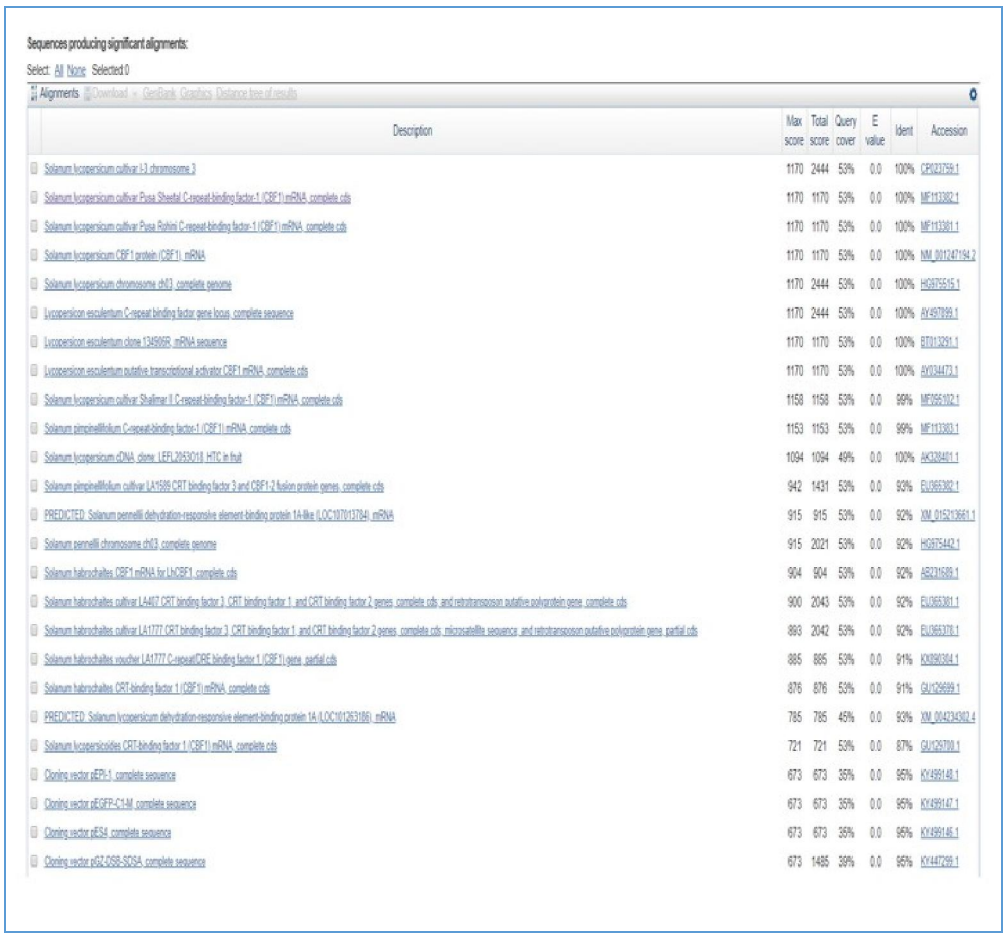


Fig. 12: Construction Map of plasmid pKZ100 harbouring GFP-CBF1 fusion protein



Sequences producing significant alignments:

Select: All None Selected 0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Solanum lycopersicum cultivar I-3 chromosome 3	1170	2444	53%	0.0	100%	CP032756.1
Solanum lycopersicum cultivar Pusa Sheetal C-repeat-binding factor-1 (CBF1) mRNA, complete cds	1170	1170	53%	0.0	100%	MF113382.1
Solanum lycopersicum cultivar Pusa Rohini C-repeat-binding factor-1 (CBF1) mRNA, complete cds	1170	1170	53%	0.0	100%	MF113381.1
Solanum lycopersicum CBF1 protein (CBF1), mRNA	1170	1170	53%	0.0	100%	NM_001247184.2
Solanum lycopersicum chromosome ch03, complete genome	1170	2444	53%	0.0	100%	HG975515.1
Lycopersicon esculentum C-repeat binding factor gene locus, complete sequence	1170	2444	53%	0.0	100%	AY497899.1
Lycopersicon esculentum clone 134566R, mRNA sequence	1170	1170	53%	0.0	100%	BT013291.1
Lycopersicon esculentum putative transcriptional activator CBF1 mRNA, complete cds	1170	1170	53%	0.0	100%	AY034473.1
Solanum lycopersicum cultivar Shalimar II C-repeat-binding factor-1 (CBF1) mRNA, complete cds	1158	1158	53%	0.0	99%	MF995102.1
Solanum pimpinellifolium C-repeat-binding factor-1 (CBF1) mRNA, complete cds	1153	1153	53%	0.0	99%	MF113383.1
Solanum lycopersicum cDNA, clone: LEFL20430I18, HTC in fruit	1094	1094	49%	0.0	100%	AK328401.1
Solanum pimpinellifolium cultivar LA1589 CRT binding factor 3 and CBF1-2 fusion protein genes, complete cds	942	1431	53%	0.0	93%	EU565362.1
PREDICTED: Solanum pennellii dehydration-responsive element-binding protein 1A-like (LOC107013784), mRNA	915	915	53%	0.0	92%	XM_015213661.1
Solanum pennellii chromosome ch03, complete genome	915	2021	53%	0.0	92%	HG975442.1
Solanum habrochaites CBF1 mRNA for LhCBF1, complete cds	904	904	53%	0.0	92%	AB231685.1
Solanum habrochaites cultivar LA407 CRT binding factor 3, CRT binding factor 1, and CRT binding factor 2 genes, complete cds, and retrotransposon putative polyprotein gene, complete cds	900	2043	53%	0.0	92%	EU565361.1
Solanum habrochaites cultivar LA1777 CRT binding factor 3, CRT binding factor 1, and CRT binding factor 2 genes, complete cds, microsatellite sequence, and retrotransposon putative polyprotein gene, partial cds	893	2042	53%	0.0	92%	EU565370.1
Solanum habrochaites voucher LA1777 C-repeat/DRE binding factor 1 (CBF1) gene, partial cds	885	885	53%	0.0	91%	KX090364.1
Solanum habrochaites CRT-binding factor 1 (CBF1) mRNA, complete cds	876	876	53%	0.0	91%	GU126699.1
PREDICTED: Solanum lycopersicum dehydration-responsive element-binding protein 1A (LOC101265186), mRNA	785	785	45%	0.0	93%	XM_004234392.4
Solanum lycopersicoides CRT-binding factor 1 (CBF1) mRNA, complete cds	721	721	53%	0.0	87%	GU126700.1
Cloning vector pEPI-1, complete sequence	673	673	35%	0.0	95%	KY499148.1
Cloning vector pEGFP-C1-M, complete sequence	673	673	35%	0.0	95%	KY499147.1
Cloning vector pES4, complete sequence	673	673	35%	0.0	95%	KY499146.1
Cloning vector pGZ-DSB-SDSA, complete sequence	673	1485	39%	0.0	95%	KY447299.1

Fig. 13: BLAST result of cloned sequence from www.ncbi.nlm.nih.gov

Fig. 14: Blast hit confirms CBF-1 cloned from *Solanum lycopersicum* cultivar Pusa Sheetal

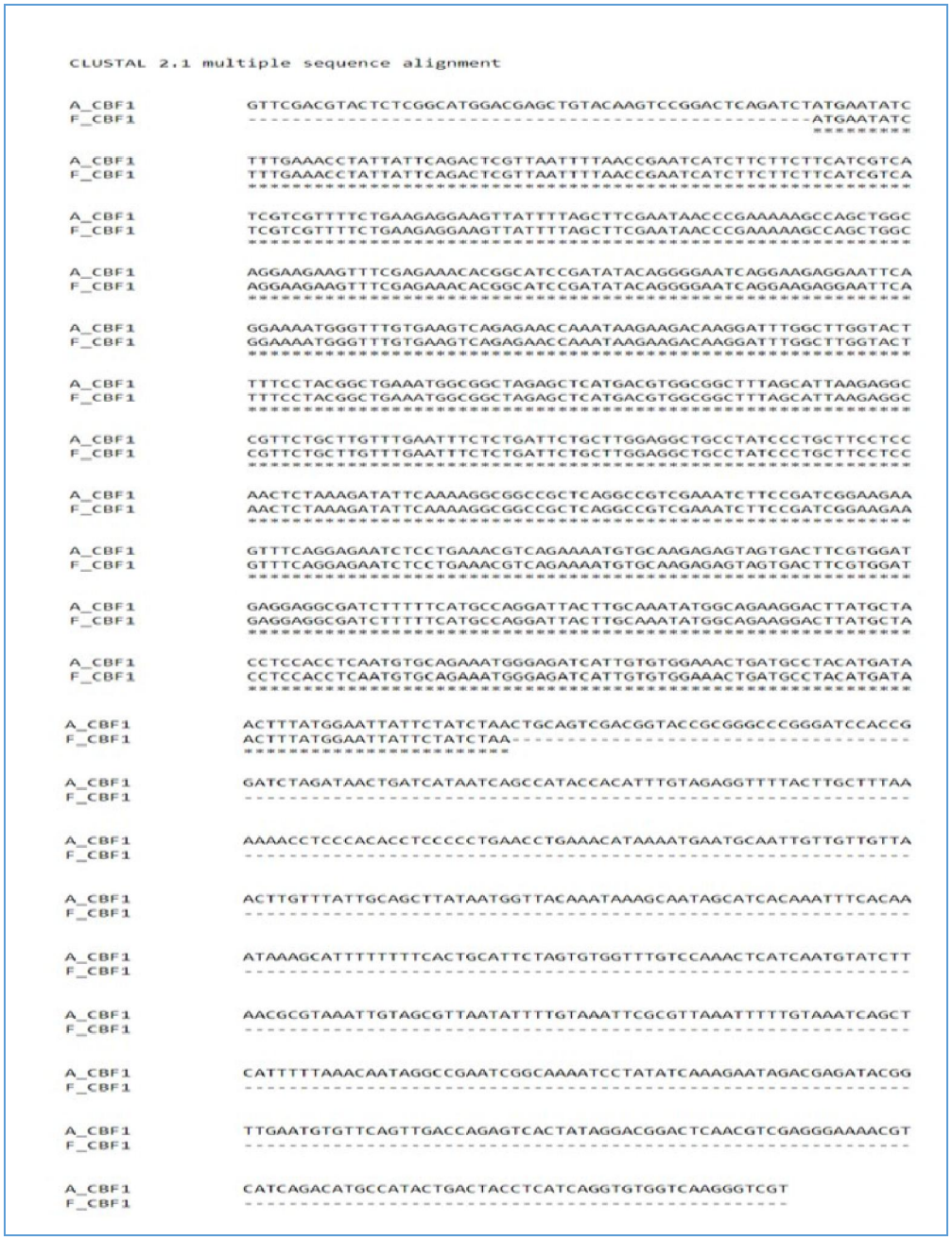


Fig. 15: ClustalW Alignment of query sequence with Genbank Sequence showing multiple alignment

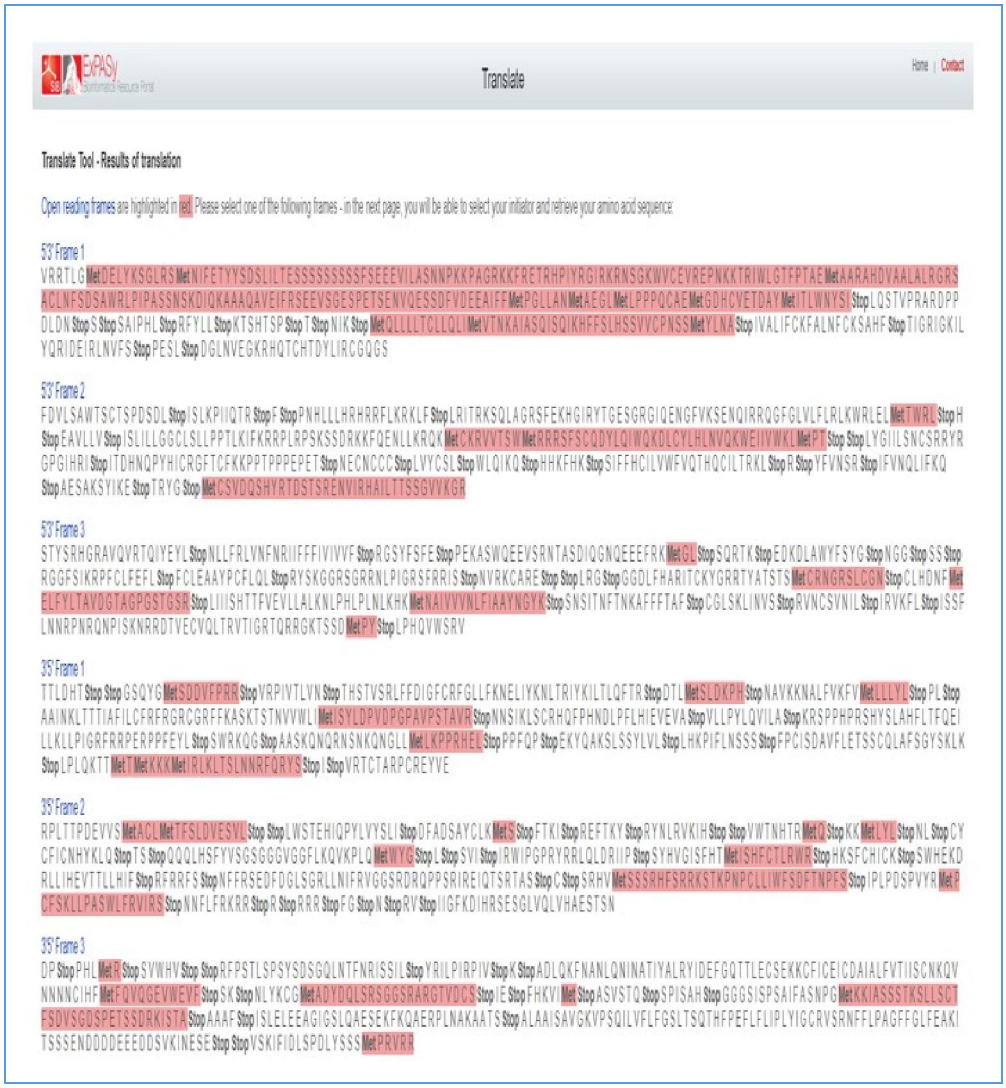


Fig. 16: Translated Protein sequence using EXPASY translate tool

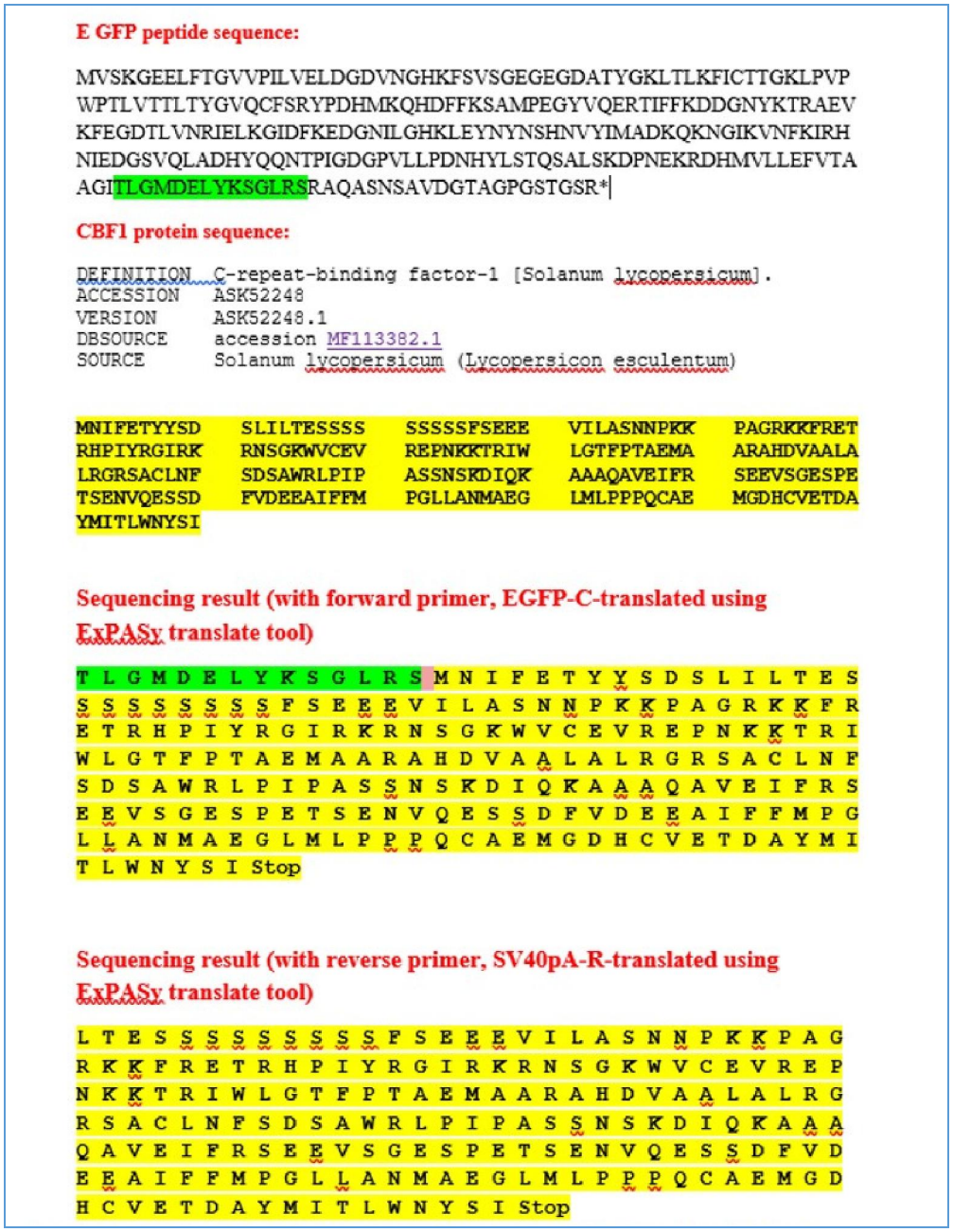


Fig. 17: Translated Protein sequence using EXPASY translate tool confirming in frame cloning of GFP-CBF-1 fusion protein obtained from sequencing

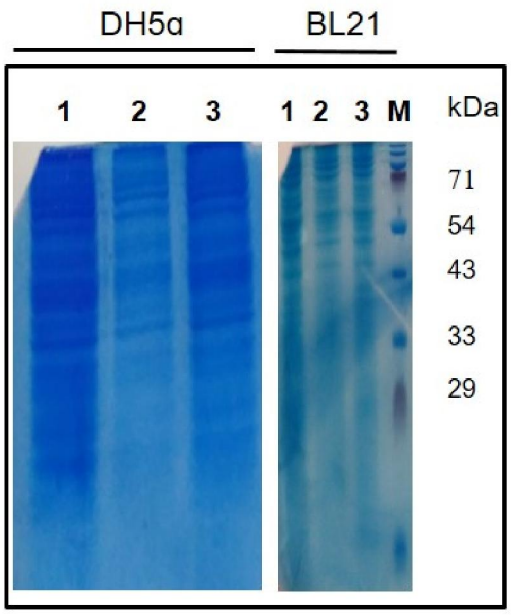


Fig. 18: Coomassie staining of protein extracts from *E. coli* cells transfected with the indicated plasmids

Lane 1: Untransformed plasmid (-ve control)
Lane 2: GFP tagged CBF1 harboring cells (Expected size: 49kDa)
Lane 3: GFP (+ve control) (Expected size: 26.9kDa)
Lane M: Pre-stained Protein marker

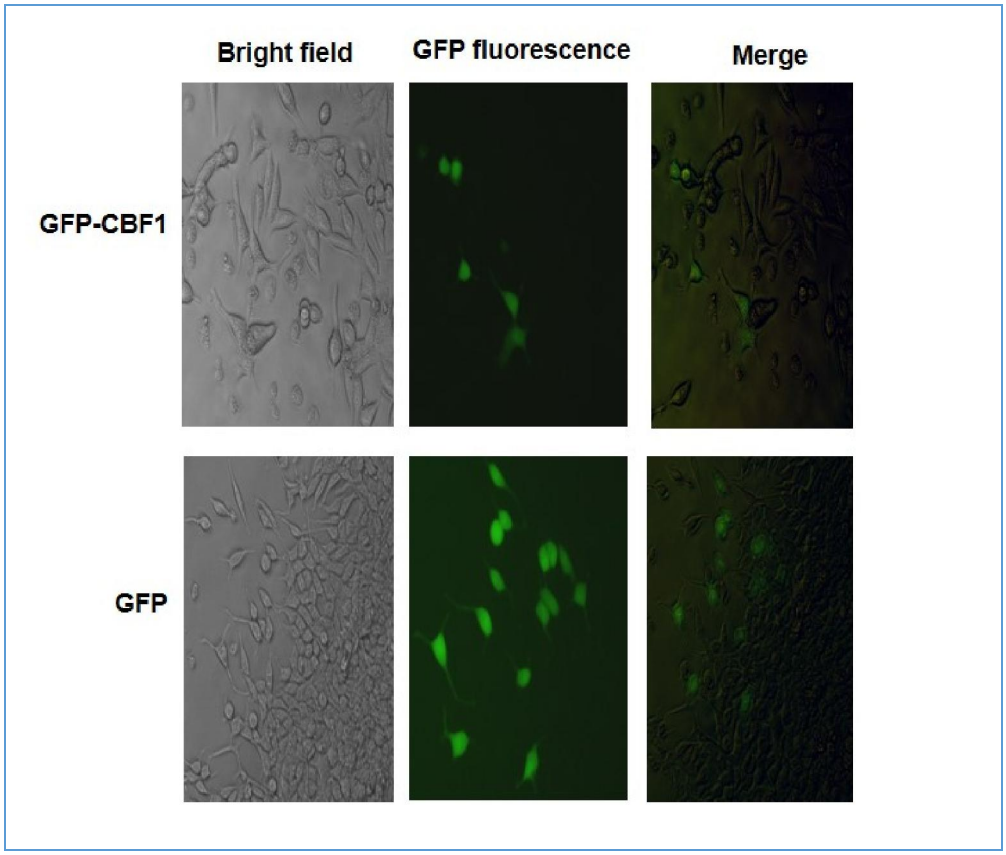


Fig. 19: Confirmation of functional fusion protein through Fluorescence microscopy after transfection of GFP-CBF1 in human C4-2 cells with the indicated plasmids

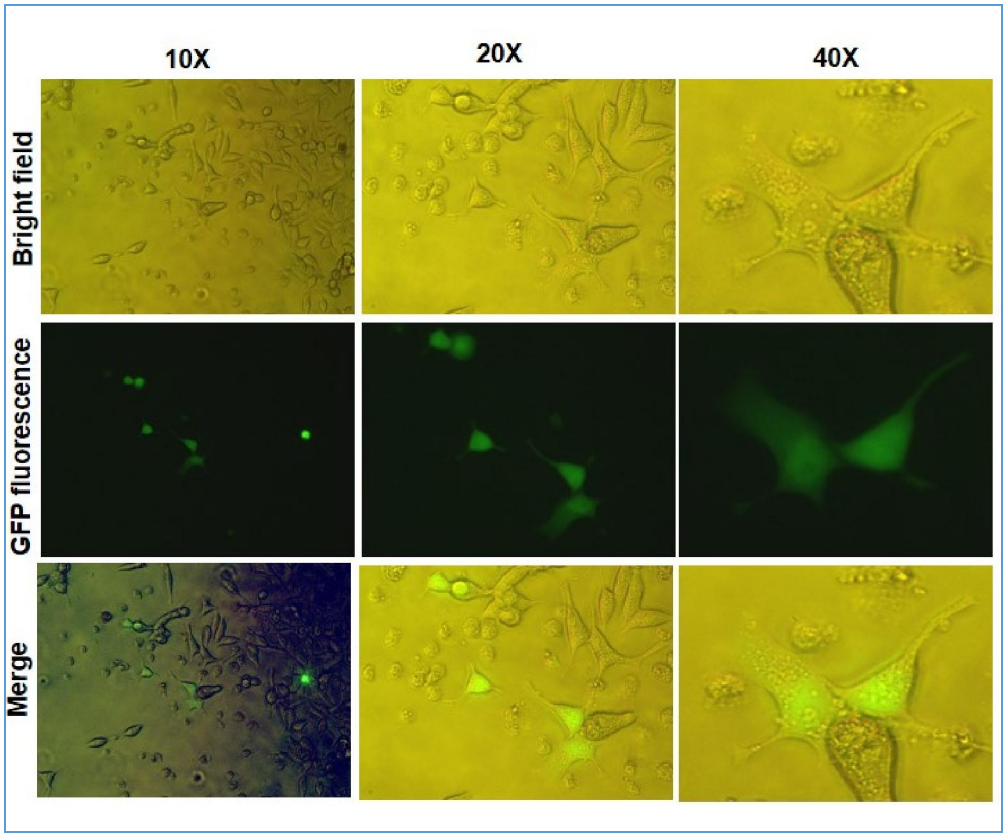


Fig. 20: Fluorescence microscopy after transfection of GFP-CBF1 in human C4-2 cells at different resolutions

Chapter 5

DISCUSSION

Abiotic stress which include cold, drought, and salinity restrict the growth of plants and yield of the crops. Hence crop plants are therefore required to acclimatize to different kinds of abiotic stress for their survival and produce. Cold stress is considered to be one of the major factors which influences the development and growth of plants and confines the geographical distribution of plant species which are sensitive to cold. Most of the plants have developed a mechanism known as cold acclimation which provides resistance to withstand freezing and/or cold stress (Mahajan and Tuteja, 2005).

During cold acclimation gene expression of plant gets changed through various mechanisms such as transcription, post transcription and post translation. The ability of the plants to withstand cold is due to some specific signal transduction pathways which result in the activation of many cold regulated (COR) gene (Wani *et al.*, 2018).

The ICE1-CBF/DREB1-dependent cold signaling cascade is the most prominent pathway induced under cold stress and controls cold-regulated genes (COR genes) which inturn induces cold tolerance in plants. The positive cold signaling regulator that binds to the MYC-recognition cis-element in the CBF3/DREB1A promoter is ICE1, a MYC-type helix-loop (bHLH) transcription factor (Tester and Bacic, 2005).

Upon few minutes of exposure of plants to cold stress the CBF genes are induced and encode transcription factors known as CBF1, CBF2, and CBF3 (also known as DREB1B, DREB1C and DREB1A respectively. CBFs are one of the types of ethylene-responsive element binding factor/APETALA2 (ERF/AP2)-type transcription factor family and have a significant role in cold acclimation in plant species which are evolutionary diverse (Mittler, 2006).

Since the valley of Kashmir has very cold winters and remains covered under snow for many months altogether, we only grow one crop/season. In Kashmir valley, the growing season of different crops is limited due to lack of cold acclimation, making these crops susceptible to chilling, which drastically influencing the quality and quantity of the produce. Chilling stress is a major production constraint of tomato which is a chilling-sensitive horticultural crop. The development of chilling tolerant tomato thus has significant potential to impact tomato production (FAO, 2015). So, if somehow we are able to manipulate the genes in such a way so that the plant acclimatizes to cold stress we will be able to grow two crops/season. In view of this hypothesis, the present investigation was carried out to clone and check the heterologous expression of CBF1 transcription factor and sequence verification of this cold responsive transcription factors (CBF1) in tomato (*Solanum lycopersicum* L.). In light of the available literature and data generated in the present study the results are in close conformity with them (Rabbani *et al.*, 2003).

Cold tolerant variety of *Solanum lycopersicum* L. variety was selected for the present investigation depending upon its resilience to acclimatize to colder temperatures. Tomato (*Solanum lycopersicum* L.) is considered to be the most important horticulture crop. Like other crops of temperate climates, tomato is very much sensitive to chilling (0-12 ºC). Due to cold stress growth and development is severely negatively affected in tomato which results in losses in yield. Cold temperatures are also known to inhibit germination and under water deficit conditions vegetative growth is negatively affected and photosynthesis is impaired. Reproductive development of plants in all the phases gets affected and hence cold stress causes homeotic floral transformation. (Akula and Ravishankar, 2011). Fruit set also tends to decrease because of poor pollen germination andchilling injury in fruits occur at temperatures below 12^{o}C (Zandalinas *et al.*, 2018).

In Arabidopsis, CBF/DREB family of transcription factors are involved in controlling a part of cold response (Gilmour etal.1998), so their loss of the function causes lack of acclimation and their gain of function induces tolerance to freezing. Arabidopsis CBF2 is one major QTLs for inducing cold tolerance and the interaction between cold acclimation and photoperiod. The variations in cold response are due to the differences in the promoter region of AtCBF2.

However, in our previous investigation, it was observed that CBF2 and CBF3 were not expressed in any of the four tomato varieties under study under normal and under cold stress, suggesting that these tomato varieties does not have a functional CBF2 and CBF3. This suggests thatCBF2 and CBF3 are not expressed in these four tomato varieties and henceforth do not play any role in inducing cold tolerance in the four tomato varieties under study. The finding is substantiated by the previous studies which also show that CBF2 and CBF3 does not impart cold tolerance in tomato. However, CBF2 is known to enhance cold tolerance in Arabidopsis, rice, wheat, peanut and barley. Since only CBF1 is expressed in pusasheetal so we cloned the gene in pFGFPC1 vector. The study can further be extrapolated to check nucleo-cytoplasmic translocation of the GFP tagged CBF1 gene in plants (Ashraf *et al.*, 2018).

Previous study from our lab has shown increased expression of CBF-1 upon cold stress at germination stage in all four varieties. We cloned CBF-1 gene and successfully expressed it in heterologous host. In tomato the heterologous ectopic expression of Arabidopsis CBF1 caused vegetative resistance to cold and to methyl viologen which is an oxidative stress causing compound. This suggests that CBF1 can induce cold acclimation in Shalimar-II and *Lycopersicum pimpinilli folium* varieties as these are grown in template region and over expression of this genes can further impart increased tolerance against cold stress (Vishwakarma *et al.*, 2017).

Similar results were found in a study carried out by Zhang *et al.*, (2004), that among the three CBF homologs, LeCBF1-3 (*Lycopersicon esculentum* CBF1-

3), that are present in tandem array in the genome. LeCBF1 gene was the only gene found to be cold inducible further supporting our findings. In transgenic Arabidopsis plants, overexpression of LeCBF1 induced expression of CBF-targeted genes enhanced freezing tolerance which reveals that LeCBF1 encodes a functional homolog of the Arabidopsis CBF1-3 proteins (Shi *et al.*, 2018). This GFP-CB-1 fusion protein can further help in studying interacting partners of CBF1.

Comparative sequence analysis of fusion protein

Comparative transcriptome sequence studies are gaining the momentum in the current era, as they decipher key information which helps to link genes across and within the species. The comparative transcriptomics holds its scope to compare sequence information of species, transfer this information (as model candidate genes) into related organisms and then compare their expression levels. The crop improvement programs can use the information of comparative sequence analysis to transfer superior genes into species of their choice (Ohta *et al.*, 2018).

The current investigation lead to confirmation of successful cloning of CBF1 gene in pEGFPc1 vector.

Since, the protein structural analysis was carried out using predicted structures as templates, further isolation of CBF-1 protein can further be structurally elucidated upon intervention of X-ray crystallography and NMR techniques. Therefore, it is anticipated that future intricate studies needs to be carried out to check the nucleo-cytoplasmic translocation dynamics of CBF1 in response to cold stress.

Understanding the mechanisms of tolerance to environmental stresses in plants has the potential to provide novel strategies for genetic improvement of stress tolerance in plants. The transcription factors play a paramount role in controlling various component traits of stress tolerance by regulating an array of genes. Earlier studies demonstrated that the expression of the CBF genes in

Arabidopsis resulted in an increased tolerance to cold, salt and drought stresses. The cDNAs encoding CBF transcription factors have been identified and cloned from various plant species including Arabidopsis, rice, wheat, canola, soybean. In the present investigation, we have cloned and characterized the member of CBF gene family, CBF1 from *Solanum lycopersicum*. Cloning and characterization of CBF1 gene from such a plant is of prime importance as it will elucidate the mechanisms underlying the CBF-mediated abiotic stress tolerance and their role in adaptation of this plant to harsh environmental conditions (Khan *et al.*, 2018). The CBF1 gene cloned in this study showed high similarity to the previously characterized CBF genes from *Solanum lycopersicum*. Bioinformatic analysis showed that the CBF1 sequence showed high degree of similarity with that of the *Solanum lycopersicum* Mill. as expected and that CBF1 gene cloned has no sequence variation with the already submitted sequence in GenBank by our research group. BLAST of CBF1 cloned from PS resulted in hits with CBF1. CLUSTALW alignment of the CBF1 of Ps with that of GenBank CBF1 did not show any sequence variation. The CBF1 sequence analysis indicated nuclear localization signal sequence and other conserved motifs typical to CBF type of transcription factors of *Solanum lycopersicum*. In summary, our results indicate cloning of a gene, CBF1 that established high homology with *Solanum lycopersicum* CBF transcription factors. The CBF1 encodes a protein having a conserved domain and other typical features of CBF TFs like nuclear localization signal (NLS) of *Solanum lycopersicum*. The expression patterns indicate that CBF1 is early responsive gene to cold stresses.

Chapter – 6

SUMMARY AND CONCLUSIONS

The present investigation, "Cloning, sequence characterization and heterologous expression of cold tolerance inducing transcription factor (s) (CBF) from tomato (*Solanum lycopersicum)*" was carried out at Transcriptomics Laboratory,Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology, Kashmir, Shalimar, Srinagar during the year 2017-2018.

The results obtained and observations recorded during the period of study are summarized as under :

- CBF1 gene of *solanum lycopersicum*, crop under study was successfully cloned in pEGFPc1 vector. Higher constitutive expression of CBF1 will help in developing high yielding freezing tolerant tomato capable of growing at low temperatures.
- CBF1 was successfully cloned as fusion protein with GFP in the study.
- It was determined during the study that the gene cloned has no sequence variation with the already submitted sequence in GenBank by our research group.
- During the study it was determined that the fusion protein showed successful expression in heterologous host.
- This study can be further exploited for increasing tomato production in Kashmir in future by initiating transgenic research to develop cold stress tolerant tomato varieties.
- The outcomes of this research can be applied to different crops that are severely affected by cold and freezing temperatures.

CONCLUSION

From the study it is concluded that:

- This study will be of value for establishing a comprehensive biotechnological programme for transgenic study of this crop for increasing its production in Kashmir and thus, will pave the way to tackle the food production insufficiency due to climate changes and may contribute to food security by stabilizing the yield of major crop that nurtures a large human population on this planet.

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CERTIFICATE

Certified that all the corrections/amendments as suggested by External Examiner Dr. Fouzia Rashid, Assistant Professor, Department of Clinical Biochemistry, University of Kashmir during Viva-Voce examination held on 15-01-2019 have been incorporated in the manuscript entitled **"Cloning, sequence characterization and heterologous expression of cold tolerance inducing transcription factor(s) (CBF) from tomato (*Solanum lycopersicum* Mill.)"** submitted by **Anisa Sajad (Regd. No. 2016-H-111-M)**.

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