CLONING AND FUNCTIONAL CHARACTERIZATION OF HEAT RESPONSIVE GENES FROM POTATO CULTIVAR KUFRI SURYA (*SOLANUM TUBEROSUM* L.)

A Thesis

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BIOTECHNOLOGY

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DECLARATION

I hereby declare that the dissertation entitled "Cloning and Functional Characterization of Heat Responsive Genes from Potato Cultivar Kufri Surya (*Solanum tuberosum* L.)" is conducted under the supervision of Dr. Umesh Goutam (14691), Associate Professor, LPU, Phagwara and co-supervision of Dr. Vinay Bhardwaj, Principle Scientist, CPRI, Shimla. This dissertation submitted for the PhD Degree is entirely my original work and all ideas and references have been duly acknowledged. It does not contain any work for the award of any other degree or diploma at any University.

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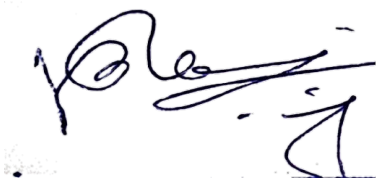
CERTIFICATE

This is to certify that Neha Salaria has completed PhD dissertation entitled "Cloning and Functional Characterization of Heat Responsive Genes from Potato Cultivar Kufri Surya (*Solanum tuberosum* L.)." under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study. No part of the dissertation has ever been submitted for any other degree or diploma at any University.

The dissertation is fit for the submission and the partial fulfilment of the conditions for the award of PhD Biotechnology.

Date: 26-09-2020



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ABSTRACT

Although tropicalization of potato is of utmost importance with respect to economic growth, it is still a major challenge to sustainable cultivation for major potato cultivars in India. Climate change has a negative impact on plant growth and yield. The expansion of the potato cultivation to the tropical parts of the country is constrained by a number of biotic and abiotic stresses. Thus, early maturing potato varieties could be a solution to tropicalization and yield loss due to climate change. Selection of elite germplasm lines is crucial in breeding programs for developing early maturing potato varieties. Germplasm selection requires a detailed understanding of molecular pathways for signal induction in potato tuberization. Tuberization in potato cultivar is a complex multigenic process and is induced under short days and low temperature while long days and high temperature inhibit it. Transcriptional factors are the prime determinants and regulators for the expression of genes involved in tuberization signals. Circadian clock regulated CDF (CYCLING DOF FACTORs) are a cluster of transcriptional repressors, that accentuates a major quantitative trait locus (QTL) for earliness in potato. Earliness in the plant is a desirable agronomic trait for higher productivity and also enables it to withstand various biotic and abiotic stresses. Since potato is a temperate crop, its tropicalization in the Indian subcontinent is a major agricultural challenge. StCDF1 induce a photoperiod-dependent tuberization pathway by interacting with other molecular regulators such as CONSTANS (StCO1/2) and StSP6A. The present study was conducted to elucidate the allelic variation, expression, role in flowering and tuberization of StCDF1 in contrasting tuberization behaviour of diverse Indian potato cultivars, Kufri Surya (KS) and Kufri Chandramukhi (KCM). Our results confirmed two allelic forms of StCDF1 viz. CDF1.2 and CDF1.3. Their expression profile suggests a higher upregulation of both CDF1.2 and CDF1.3 expression at dawn (6:00am) than noon (12:00pm). Virus-Induced Gene Silencing was used to validate the role of these alleles with respect to earliness and tuberization in KS. Real-time confirmed the upregulation of StCO and StSP5G and downregulation of StSP6A in VIGS plants. Phenotypic observations suggested delayed tuberization and a low tuber number in VIGS plants. . Based on this proof of concept, transgenic lines of late maturing cv. Kufri Girdhari

overexpressingStCDF1.2 gene were developed. Out of 95 putative shoots generated, 55 were found positive on primary screening by nptII PCR. These positive transformed lines were subjected to microtuberization for initial tuberization. In microtuberization, 27 transformed lines performed better and then these lines were cultivated in aeroponics facility. Out of twenty seven, five transgenic lines were selected for glass house and field trial. These lines were analyzed phenotypically (maturity and early tuberization) and molecular validation by qRT-PCR. The transformed lines were tuberizing 13 days early and senescence phenotype in comparison to control Kufri Girdhari.

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Neha Salaria

PREFACE

This thesis focuses on tropicalization of potato cultivars in India, as potato is temperate crop. To complete the demand of ever-increasing population, widening the potato cultivation need to be addressed and tropicalization of potato is need based. The expansion of the potato cultivation to the tropical parts of the country is constrained by the different environmental factors. Among these factors, photoperiod and temperature are the most significant one, which control major developmental stages like flowering and tuberization. These factors limit the distribution of potato in diverse geographical regions and hinder its spread in tropical and subtropical regions of the country. To increase the potato production, the research is being focused on understanding the molecular pathways involved in the tuberization initiation and induction. Transcriptional factors are reported to be very important and are involved in regulating the expression of the genes collaborating for tuberization signalling. Transcriptions factors belong to the CYCLING DOF (DNA-binding with one finger) FACTOR (StCDF) family are an important mediator of the tuberization process.

So, the present study demonstrates the expression of StCDF1 gene in different potato cultivars in different photoperiod. The early maturing allele StCDF1.2 expression in short and long adapted cultivars correlate the tuberization and maturity phenotype in region specific cultivars. Virus-Induced Gene Silencing was used to validate the role of these genes with respect to earliness and tuberization tetraploid potato. Overexpressing lines were developed to further autheicate the role of StCDF1 in tuberization.

The present research was carried out for the fulfilment of PhD thesis work at the Central Potato Research Institute, Shimla.

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Chapter I

INTRODUCTION

Introduction

Potato (*Solanum tuberosum L.)* is the most important non-cereal crop in the world, with the net production of 412 million tons in 2017. It ranks third as per the calorie consumption after major cereals like rice and wheat. In developing countries like India, potato is a very efficient crop on food security and farmers income perspective (Raymundo et al. 2018). With rapid climate change, temperature is becoming a major threat to the crop yield in agriculture sector (Zhao et al. 2018). In case of potato, the estimated yield loss due to the climate change is more than 15% worldwide. Various developmental processes are adversely affected by high temperature, but tuberization of the potato is very sensitive to the temperature change. Optimum night temperature of $18^{\circ}C$ is very crucial for the tuberization, as even increase of two degrees of temperature can reduce the tuber formation (Singh et al. 2015). Temperature also limits the distribution of potato in diverse geographical regions and hinders the efforts of yield enhancement (Dutt et al. 2017).

Recent biotechnological research focuses mainly on combating biotic constraints of potato crop but enhancing tuberization was not explored much. To combat the climate change and food scarcity, complete understanding of the tuberization process is necessary (Hannapel et al. 2017). The induction and initiation of the tuberization are controlled by different environmental cues like temperature and photoperiod. With respect to photoperiod, short-day conditions are reported to accelerate the overall tuberization process and suppress the flowering. Whereas long-day conditions are non-inductive for the tuberization and flowering process is upregulated (Abelenda et al. 2016). Reciprocation to the above-mentioned environmental cues depends upon the varietal variation, which is resulted from different origin and parental history of potato cultivars.

Andigenum group of the *S. tuberosum*, are the original Andean landraces adapted to the South American weather, where low night temperature and 12-hour day length is observed for the whole year. So, Andigena accession behaves as the obligate short day with respect to the tuberization, hence unable to tuberize in high temperature of the tropics and long days of temperate summers. After intercrossing and recurrent

selection of the Andigena group short day potatoes, long- day adapted Neo-tuberosum were developed by Cornell university under the potato breeding program in 1965. There's another potato accession from the plain region of the chile known as Chilotanum or Tuberosum, which tuberize only in long-day condition (Morris et al. 2014). Ghislan et al. (2009) reported the close relation of Chilotanum and Neo-tubersum based on DNA marker study. Many biomolecules positively or negatively involved in the regulation of the tuberization by perceiving and reciprocating to these factors.

Transcriptional factors are reported to be very important and are involved in regulating the expression of the genes collaborating for tuberization signalling. Transcriptions factors belong to the CYCLING DOF (DNA-binding with one finger) FACTOR (StCDF) family are an important mediator of the tuberization process. These factors regulate the tuberization by linking the circadian clock and the tuberization signals like StSP6A (Dutt et al. 2017). StSP6A is the Flowering Locus ortholog of *Arabidopsis thaliana*, which is a mobile signal required for tuberization in the potato and its regulation pathway is separate from flowering. But, tuberization and flowering both processes are dependent of photoperiod and light sensing in leaves and then transportation of signals to the respective organs (Kloosterman et al. 2013). StCDF also negatively control the expression of another transcription factor CONSTANS (StCO) by binding to a conserved site of CO promoter. Under unfavourable long day conditions, GIGANTEA (StGI) a clock gene interacts with the proteins like Flavin-binding Kelch repeat F-box protein 1 (FKF1), and lead degradation of StCDF by proteasome. In absence of StCDF, StCO stabilizes and further binds to the promoter of StSP5G and triggers the expression of this Flowering Locus homolog. StSP5G expression is directly under StCO and it further reduces the expression of the StSP6A and inhibits the tuberization process in potato (Abelenda et al. 2016).

StCDF 1, a plant maturity gene mapped to chromosome 5 and have three alleles designated as StCDF 1.1, StCDF1.2 and StCDF 1.3. Allele StCDF 1.1 is associated with the late maturity of some potato genotypes, whereas other two are present in early maturing genotypes. StCDF1.2 allele has an insertion of 7 bp in

StCDF 1.1, which resulted in early stop codon due to frameshift mutation. In case of StCDF 1.3 this insertion is of 865bp, hence the transcribed protein is 22 amino acid longer, than late maturing StCDF 1.1 (Morris et al. 2014, Hannapel et al. 2017). The DOF domain of this gene not only serve as the DNA binding site to interact with other downstream factors, but also as a potential target for Ubiquitination to reduce StCDF expression. Along with the DOF domain, other conserved domains are also present, which mediates the protein-protein interactions to regulate the signalling cascade (Kloosterman et al. 2013).

Singh et al. (2015) also reported the upregulation of the StCDF in the samples subjected to the high temperature stress. So, this gene is one of the potential candidates for conducting the study to increase tuberization process. To understand the functionality of this gene in tuberization VIGS (Virus Induced Gene Silencing) was performed using pTRV vector. It is the fast and efficient genomic technique used to explore the gene function in plants. VIGS is being frequently used in gene functionality studies because it is a simple, economic and swift functional genomics tool (Kant and Dasgupta, 2019). Both in case of abiotic and biotic stress, VIGS is reported to be successful in the identification of the candidate genes for facing theses constraints. VIGS aid the studies conducted to unravel the mechanism of the complex pathways involved in the regulation of the abiotic stress management. This technique is also helpful in the breeding programs in validation of the QTLs identified without any tedious plant transformation. pTRV vector system is the most versatile VIGS vector due to the wide host range and systemic infection. Due to advancement in the vector system the limitation like loss of efficacy with time and heritability can be surpassed. Modern vectors are more efficient, and transformation is stable for longer duration, hence resulted in stable transgenic plants (Ramegowda et al. 2014).

In the present study, to understand the role of StCDF1.2 in tuberization and plant maturity transient gene silencing was performed using pTRV-VIGS system. Validation of the VIGS was done by molecular analysis (qRT-PCR, sequencing) and phenotyping (tuberization study). Based on this proof of the concept, stable transgenic lines were developed using CDF1.2-pRI-101 gene construct. This is the first report with respect to the functionality study of CDF1.2 in Indian cultivars. This work can

from the foundation for the domestication of different potato cultivars in vast geographical region through tailored breeding programs.

Chapter II

REVIEW OF LITERATURE

Review of literature

Potato (*Solanum tuberosum* L.) is the perennial plant, which belongs to the Solanaceae family of the order Solanales. It is the most important and efficient non cereal food crop based on the food security. Potato is being cultivated in more than 140 countries, with the net production of 388 million tons worldwide (FAOSTAT, 2019). India is the second largest producer in the world after China with the net production of 51 million tons in year 2018. Potato is the primary source of carbohydrate consumed in different forms in many regions of the world (Dutt et al. 2017).As per the nutrition profile, other than starch it contains abundant quantity of vitamin C, vitamin B6, potassium, folate, and iron. If cooked with the skin on, potato can provide a good amount of the dietary fiber (Robertson et al. 2018). The colored potatoes are an important source of the antioxidants like vitamins, β-carotene, polyphenols, Anthocyanins, carotenoids, flavonoids, tocophenol and alpha linoleic acid (Zaheer and Akhtar, 2014).

2.1. Origin and history of the introduction

The cultivated tetraploid potato was earlier classified in two subspecies: Andigenum group and Chilotanum group. Andigenum landraces belongs to the Andes region (western Venezuela south to northern Argentina) of the South America. The potatoes of this landrace are obligate short-day with respect to tuberization, hence cannot tuberize in the long day conditions. Chilotanum landraces of the plain region of the Chile distributed from chiloe island to Chonos Archipelago. The modern potato is designated as the Neo-tuberosum subspecies and its origin is controversial (Morris et al. 2014). In 1969, Simmonds introduced this new subspecies by open pollination of the Andigenum landraces and selection for desired characteristics. This new accession was tuberizing early with reduced flowering, tubers were large but less in number. Resistance towards the major biotic constraints like late blight, viruses also gained attention for further breeding of these accessions (Ghislain et al. 2009).

The Neo-tuberosum is the modern tetraploid potato, adapted to the long day conditions for the tuberization. For more than forty years, the theory that Neo-

Tuberosum are derived from the Andigenum landraces was accepted. The vast diversity of the Andigenum landraces and availability of this germplasm for the breeding program has been supporting the above theory. The characteristic features of Neo-tuberosum accession are in-between of Andigenum and Chilotanum landraces. The high adaptability of the Andigenum landraces to Chilotanum conditions also supported the theory of recreation of Neo-tuberosum from the Andigenum landraces. But a study conducted by Ghislain et al. (2009) showed the close relation of the Neo-tuberosum group to the Chilotanum landraces through the molecular studies. The SSR (Simple sequence repeats) and plastid DNA makers were used to study the recreation theory for the origin of the Neo-tuberosum accession. As well as the tuber morphology of Neo-tuberosum and its adaptability to tuberize in long day conditions, challenges the theory of recreation.

2.2. Genetics of potato

The modern and common cultivated varieties of potato are tetraploid (2n=4x) with haploid set of 12 chromosome. Diploid (2x), triploid (3x) and pentaploid (5x) varieties are also available but not frequently used for the commercial cultivation (Hijmans and Spooner 2001). Diploid cultivars are significant in providing the gene pool for breeding programs targeting the tetraploid interploidy crosses (den Njis and Peloquin 1977). Triploid and pentaploid are not commonly cultivated but there are some regions in the Andes (South America). All the known potato cultivars known are important with respect to the genetic resources for breeding program targeting crop improvement and protection of potato (Watanabe 2015). Vegetative propagation of the potato can be achieved by using seed tuber and plant tissue culture plants. The seed in potato is referred as small tuber or tubercule which is used for cultivation. However botanical seed of potato is known as true potato seed (TPS) and it is collected from the berries (Harris 1978, Watanabe 2015). Grafting was also reported to be positive in case of potato for the propagation (Kasai et al. 2013).

2.3. Effect of heat stress on potato

Potato tuber production is reported to be induced and proceed well at optimum 18˚C-21˚C. High temperature is reported to reduce the total potato yield by affecting tuber number and size (Levy and Veilleux, 2007). Bulking of the potato is an important stage in potato tuberization and it determines the total yield. High temperature fastens the process of tuberization and reduces the bulking time required to attain proper size (Mihovilovich et al. 2014). Sink strength of the potato tubers is very important factor in the bulking of the tuber. Sink strength is the ability of the tubers to entice the photosynthesis products towards it, by deviating them from other paths. Sucrose is the most vital photoassimilate form which gets transported from leaves to the developing tubers (Zrenner et al. 1995). Sucrose is synthesized by photosynthesis in cytoplasm of the mesophyll cells of leaves. After synthesis sucrose is transported from one mesophyll cell to other using plasmodesmata (symplastic transport). Then from mesophyll cell it is transported to companion cells via active sucrose transporter. Then sieve element of phloem get sucrose through symplastic mode of transport. Final destination of the sucrose is sink (tuber in case of potato), hydrostatic pressure is generated between sieve elements and sink because of osmotic difference (Katoh et al. 2015). High temperature reduces the photosynthesis rate and sink strength of tuber is also altered. Hence the overall bulking of tuber is reduced by reducing the sink strength and increasing the stolon branching (Kim et al. 2017).

High temperature not only affects the tuber growth and development, but the overall plant growth is also altered. In case of tuber, not only it reduces the yield and size, quality of potato is also reduced. Especially in the tropical and subtropical regions, where day and night remain high. Efforts to increase potato cultivation are important for food security purposes in developing countries. But cultivation in tropical regions may results in reduced nutritional value of potato. Anthocyanins are the class of flavonoids responsible for the flesh color of the colored potatoes (Lewis et al. 1999). Their accumulations in the vacuoles are altered by various environmental factors and temperature is one of these factors. Anthocyanin accumulation is favoured by low temperature and hampered at elevated temperature (Dela et al. 2003, Fogelman et al. 2018). Another important class of nutrient carotenoids is present in significant amount in potato. In potato, majorly laxanthin, lutein, zeaxanthin, neoxanthin,

antheraxanthin, and β-cryptoxanthin comprise the carotenoids. Its biosynthesis is adversely affected by the change in the temperature during the development of the tubers. Steroidal glycoalkaloid are the secondary metabolites produced by plants during infection by pathogen for resistance. But this metabolite is highly lethal for the human and animals. It is resistance to the processing and cooking methods available for potatoes so far. Its production and accumulation increase in the abiotic (temperature, drought and salt) and biotic stress conditions.

2.4. Tuberization

Stolons are the specialized underground organs, which form the potato tuber on the induction. Stolon develops from the lateral bud at the bottom of the shoot and grows diagravitropically. On the induction of the tuberization, the longitudinal growth of stolon is inhibited, and radial growth is initiated. This initiation occurs by the cell growth in the pith and cortex region of the lower apical meristematic tissue (Xu et al. 1998). After initiation the cell of the perimedullary section starts dividing and expanding which results in enlargement of potato tuber. The whole growth pattern of stolon is changed on tuberization induction; this change includes changes in cell shape and type. The tuberization is favoured by short daylength, low temperature, nitrogen level and seed tuber physiological age (Morris et al. 2014). All the cells participating in the whole process connect to the phloem component of vascular system. The mobile signals originate from the leaf for the induction and initiations of the tuberization are transported through the phloem (Ewing and Struik, 1992, Hannapel et al. 2017).

2.4.1. Hormonal control of tuberization

The endogenous levels of plant hormones can be inhibitory or favourable to the tuberization process. The high endogenous levels of gibberellins are correlated to the delayed tuberization of the potato. Under inductive short-day conditions, concentration of gibberellins in leaves is less as compared to the long-day non-inductive conditions. Carrera et al. (1999) unravel the role $StGA_2ox1$ (GA_2- oxidase gene) in the regulation of the tuberization. The mRNA levels of this enzyme are increased on the induction of tuberization in leaves as this key enzyme in the synthetic

pathway of gibberellin. In another study, expression of $StGA_2ox1$ was reported to be upregulated on the onset of the tuberization in potato (Kloosterman et al. 2007).

On the contrary, cytokinin and auxin both complement the induction of the potato tuber formation (Banfalvi et al. 1997). Prior to the physiological changes in stolons the auxin transport genes (StPIN) were upregulated on induction of tuberization (Roumeliotis et al. 2013). Kolachevskaya et al. (2015) transformed potato using tms1-B33 promoter construct (tms1 is a gene involved in the biosynthetic pathway of auxin) and analysed different plant organs for the expression levels. The auxin (Indole acetic acid) content was higher in tubers in comparison to the stem and leaves. The complete regulatory mechanism of potato tuberization through ABA (abscisic acid) is not reported yet. Some of the studies suggest the upregulation of the tuberization induction on exogenous supply of ABA (Marschner et al. 1984).

2.4.2. Environmental regulation of tuberization

Among all the developmental processes, initiation and induction of tuberization are very sensitive to the external cues. Temperature and photoperiod are the most effective in the regulation of the overall process of the tuberization. High temperature is reported to be inhibitory for the initiation of the tuberization and tuber formation. Optimum 18˚C night temperature is very important for the tuber formation, as even increase of two degrees can reduce the process drastically. And at 25˚C or above, there will be no tuber initiation and formation. Heat stress affects the dry matter accumulation of the plant by altering the different metabolic pathways. Similarly, photo-period is also very significant environmental cue for the initiation and induction of the tuberization. The induction of the tuberization includes photoperiod sensing and then the signal transduction via tuberigen to the target organ (Martinez-Garcia et al. 2002). As per the recreation theory of the potato, modern cultivars are derived from short day Andigenum landraces. So, short day conditions (day-length should be less than 12hours) are inductive for the tuberization in potato crop. The short-day hastens the tuberization process, hence the vegetative growth is less, and flowering is reduced. Whereas, the long-day conditions (day-length more than 14 hours) are favourable for flowering and non-inductive for tuberization.

Photoperiod can only affect the initiation of tuberization and has no-effect on the growth and development of the potato tuber.

2.4.3. Proteins regulating tuberization

phyB is the photoreceptor involved in the photo-period sensing and regulation of induction of the tuberization. Being a photoreceptor, it is abundantly present in the leaf tissue and inhibits the tuberization. Under long day conditions, phyB get stabilized due to late afternoon and inhibit the induction of the tuberization in potato. Whereas, in short-day inductive conditions phyB levels are less, hence the inhibitory effect is circumvented (Batutis et al. 1982). Jackson et al. (1998) used antisense technique to understand the role phyB in tuberization. Andigena group was transformed using this anti-sense phyB construct, and tuberization was studied. Transformed lines were able to tuberize in both long and short-day conditions, whereas non-transformed lines were tuberizing only in inductive short-day conditions. This confirms the important role of phyB in the inhibition of the tuberization under non-inductive long day conditions.

Another protein found to have important role in flowering and tuberization of potato and other plants is Constans. This protein is characterized by B-box domain (N terminus) and CCT (Constans like TOC 1) region (C terminus). Initial this protein was reported to play crucial role in inducing flowering in Arabidopsis (Yano et al., 2000). Under favourable long day conditions, AtCO (Arabidoposis constans) induce flowering using photoperiodic cue. Other member of this family induces many physiological processes under different photoperiods. Hayama et al. (2017), reported Hd1 (heading date 1) member of CO family increase flowering under short day conditions. In potato, StCO delay tuberization and enhances flowering under long day condition (Talar et al. 2017). Under short day condition, AtCO overexpressing potato lines take longer time to tuberize than wild control potato (Martinez-Garcia et al., 2002).

2.4.4. Cyclic Dof factor

Zinc finger DOF family transcription factors are reported to be involved in various developmental processes of plant growth. The zinc finger domain present in

these transcription factors is the main distinguishing feature. This domain consists of a conserved C2-C2 finger structure of 50 amino acids and aids the DNA binding (Hir and Bellini, 2013). CDF is a member of this family, which is identified as the major QTL for initiation of the tuberization and plant maturity. It acts as mediator between circadian clock and the mobile tuberigens for regulating the tuberization and length of plant life-cycle (Kloosterman et al. 2013). Under short day conditions, StCDF 1 inhibits the flowering by downregulating the expression of StCO in leaf by binding to the receptors. And when conditions are not favourable for the tuberization StCDF 1 is degraded by the group of factors like StGI, FKF1, Kelch Repeat and Flavin-binding. All these factors interact in the presence of blue light, which is perceived by phyB and targets StCDF1 (Kim et al. 2017).

StCDF 1 not only interacts with DNA, but interaction with the proteins is also plays important role in overall regulation of the pathway. So, to interact with the proteins like FKF 1 and GI, StCDF have three conserved motifs at the C-terminal (Kloosterman et al. 2013). The interaction of StCDF1 with these proteins marks it for the proteasomal degradation by ubiquitination of the Dof factor. Under non-inductive long days, phyB perceive the signal and interact with clock protein StGI and targets the degradation of StCDF 1, which results in StCO upregulation and flowering. Pseudo-Response regulator (PRR) is another class of protein which regulates the expression of the StCDF 1. The PRR protein usually promotes the flowering by repressing the transcription of the StCDF 1 (Hayama et al. 2017). Ito et al. (2007) studied the effect of PRR mutation on the expression level of the CDF in *A. thaliana*. The CDF expression was downregulated in knock-down mutant lines of PRR proteins. The flowering was promoted in these PRR mutant lines because of upregulation of the expression of FT transcript. Kloosterman et al. (2013) mapped this maturity locus on the chromosome 5, using two diploid potato cultivars (CE3027 and CE3130). The suggested the CDF as the candidate gene the for-plant maturity and life cycle. The late maturing potato cultivars had showed homozygosity for the StCDF1.1 allele on sequence analysis. The other two alleles (StCDF1.2 and 1.3) were found to be have truncated C-terminal and corresponds to the early maturing phenotype. This truncation is due to the addition of 7 bp in allele 1.2 and 865bp in allele 1.3, in the StCDF 1.1

sequence. Overexpression of StCDF1.2 in the Andigena group enhanced the tuberization in the non-inductive long day conditions. Morris et al. (2014) studied the StCDF allelic variation in the Neo-tuberosum and Andigena landraces. Neo-tuberosum accessions were reported to comprise StCDF1.2 allele and StCDF1.1 was found to be absent.

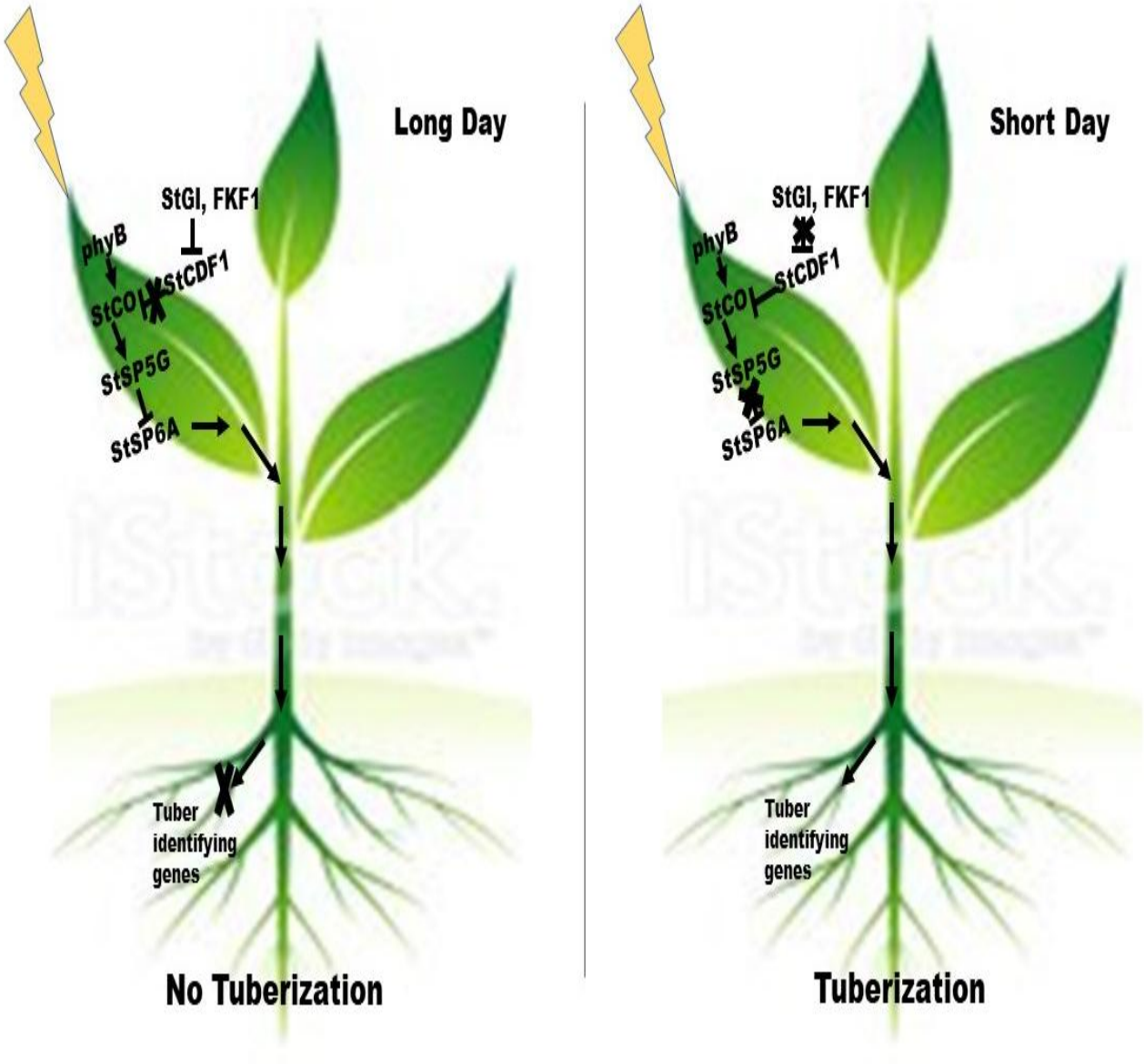

Figure 2.1. Model of transcriptional regulation of tuberization in S. *tuberosum*.

2.5. VIGS (Virus induced gene silencing)

Advances in the in-silico techniques and computational biology strategies had provided insight to the gene involved in the biotic and abiotic stress management. But this can only provide the initial cues, to validate the role of these candidate genes advancements in the functional characterization tools is necessary (Senthil-Kumar and Udayakumar, 2010). Earlier techniques like chemical mutagenesis, T-DNA (Transfer-DNA) tagging, targeting induced local lesions (TILLING), transposon tagging and RNAi (RNA interference) were used to understand the role of potential genes in stress management (Saleki et al. 1993, Koiwa et al. 2006, Zhu et al. 2007, Senthil-Kumar and Mysore 2014). All these tools are laborious and tedious, hence require a lot of time in the functional validation of the targeted genes. Genetic transformation of plant is required in RNAi technique, which restrict its use in genetically docile plants only and time consuming. So, there is a need of robust and efficient tools for the authentication of the candidates resulted from the in-silico analysis. VIGS is one of the high-throughput gene knock-down tool for functional analysis of the genes. It is not only a fast and efficient functional genomics tool and complete gene sequence is not required for analysis (Ramegowda et al. 2014).

2.5.1. Mechanism

It exploits the innate PTGS (post-transcriptional gene silencing) against virus pathogens and hinders the spreading of the infection. The host defense system initiating the production of the dsRNA (double stranded RNA) to facilitate the silencing. The targeted gene is ligated to the non-virulent virus-based vectors and plant is transformed using *Agrobacterium tumefaciens*. The selection of the fragment is very crucial, as the presence of off-targets must be avoided, and production of siRNA (short-interfering RNA) should be efficient (Xu et al. 2006). In the host plant, target gene is multiplied with viral genome and dsRNA production is initiated using RdRp (RNA-dependent RNA polymerase) either from virus or host. After production of dsRNA, Dicer (endoribonuclease) comes into action by recognizing and cleaving these dsRNA into 21-25nt siRNAs. These siRNA then binds to the RISC (RNA

induced silencing complex), RISC uses one strand of siRNA for homology-dependent cleaving of virus RNA (Deleris et al. 2006, Ramegowda et al. 2014).

2.5.2. VIGS vectors

Both DNA and RNA viruses have been altered to be used as the VIGS vectors. The potential of some viruses to infect a wide range of plants make the use of single VIGS vector for different host possible. TRV (*Tobacco rattle virus*) is one of the most frequently used VIGS vector with a wide host range (Senthil-Kumar and Mysore 2014). TRV is a positive ssRNA (single stranded) and its genome is bipartite (contain two segments). Two segments are: TRV1/RNA1 (include RdRp and protein for movement) and TRV2/RNA2 (genes for coat protein and non-essential proteins). TRV2 contain the genes which are not required in infection, hence can be replaced by the cloning sites. Further advancement in TRV vector makes it suitable for gateway cloning, cDNA library preparation and longer silencing duration. TRV1 is also altered for the insertion of the target genes and silencing without TRV2. TRV-VIGS system is preferred over other vector systems as it is highly efficient, wide host range, silencing can be maintained in grafted plants and silencing in detached plant parts is possible (Ramegowda et al. 2014).

Some new vector systems are based on two component systems: satellite virus and helper virus. Satellite virus works like TRV2 and aid the delivery of the fragment into the host plant, whereas helper virus provides the machinery for replication and movement. TYLCCNV (*Tomato yellow leaf curl china virus*) is an example of this vector system, where TYLCCNV itself works as helper virus and DNAβ is a satellite virus. Without helper virus silencing can be achieved, but the efficiency will be average, and silencing can be recovered easily (Guo et al. 2010). The monocotyledon plants have different vectors, which are only not effective in dicots. Among different vectors for monocots, BSMV (*Barley stripe mosaic*) is widely used for functional genomics studies. In wheat and Barley, BSMV was used for functional analysis of genes in management of drought (*TaEra1*, *TaBTF3*, *TdAtg8*, *HvDhn6* and *HvHVA1*) and light induced photo-inhibition (*TaPGR5*) stress (Manmathan et al. 2013). Atwood et al. (2014) used BPMV (*Bean pod mottle virus*) for the functional analysis of *GmRPA3* (Glycine max replication protein A) in iron procurement.

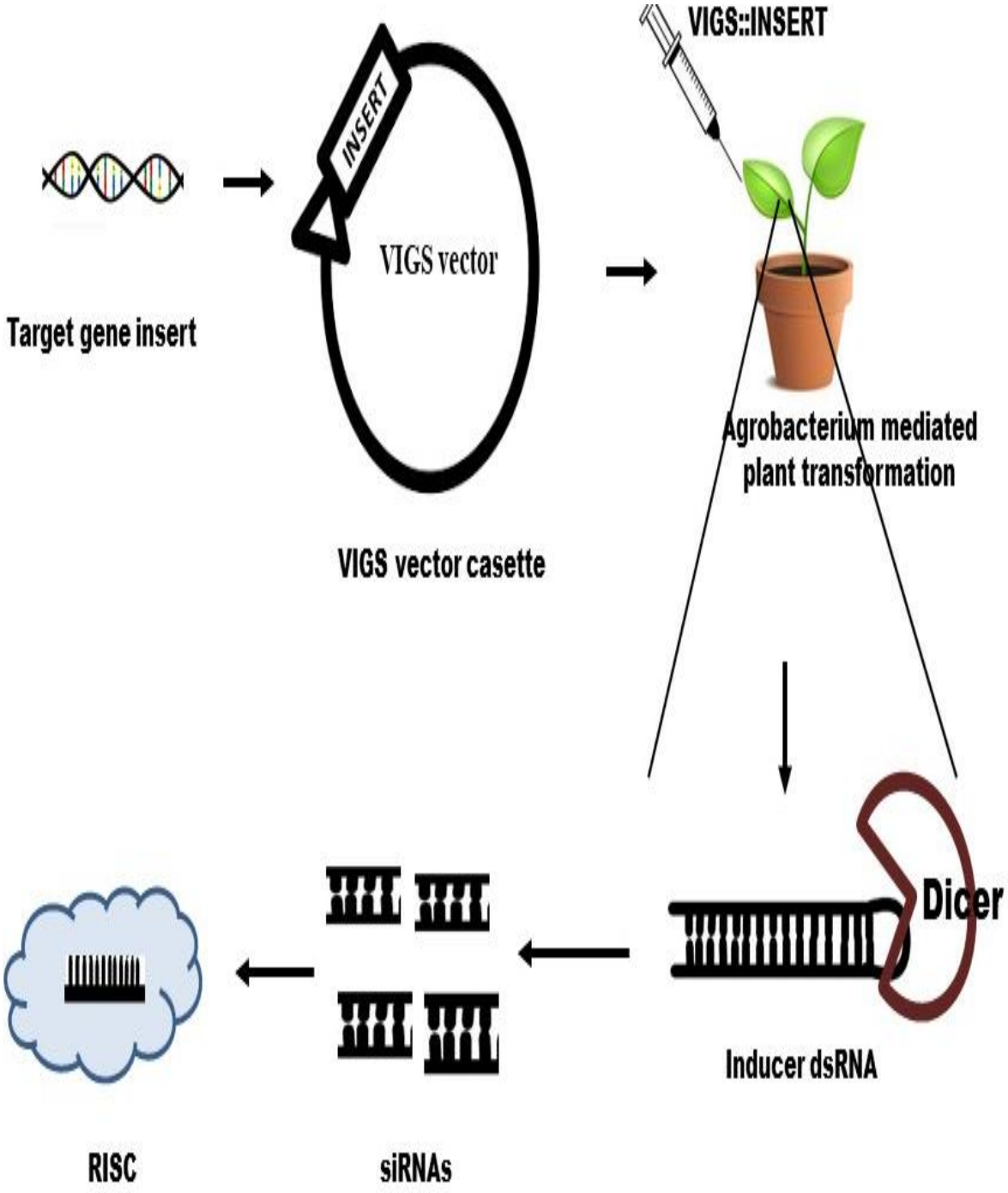

Figure 2.2 Diagrammatic representation of VIGS mechanism.

Table 2.1. List of the VIGS vectors and its application in abiotic stress related functional analysis.

S. No	Vector	Type	Abiotic stress	Reference
1	TRV (*Tomato rattle virus*)	ssRNA, Bipartite	Drought, oxidative, salt, osmotic, dehydration	Wang et al. 2019
2	PEBV	dsRNA	oxidative	Constantin et al. 2008
3	TYLCCNV	DNA	Oxidative, drought, salt	Yang et al. 2018
4	BSMV (*Barley stripe mosaic virus*)	RNA, tripartite	Drought	Wang et al. 2005

2.5.3. Advantages of VIGS in functional analysis of abiotic stress

VIGS is the high throughput reverse functional genomics tool and has many benefits over other available tools (Pfliger et al. 2013). It is faster and easier in comparison to the most known functional genomics tools. Silenced phenotype can be achieved in shorter duration which speeds up the whole characterization process. Plant transformation is not required for functional analysis; hence the host range can be widened to the recalcitrant species. In case of gene with lethal phenotype, VIGS is the

only option for the validation of the function of the candidate gene. For the traits which are under regulation of multigenic family, the entire gene can be silenced together via VIGS. Many protocols have been optimized for the functional analysis of genes involved in the abiotic stress management in recalcitrant plant species. VIGS has been reported to be successful in validating the QTLs (Quantitative trait loci) identified in the breeding programs (Ramegowda et al. 2014).

CHAPTER III

HYPOTHESIS

Hypothesis

Identification of upregulated genes during heat stress and their overexpression in the popular potato cultivar to make it thermotolerant.

Chapter IV

OBJECTIVES

Objectives

- Identification of heat responsive genes from Kufri Surya potato cultivar.
- Cloning and development of gene construct of identified genes.
- Functional validation of identified gene by transgenic approach in popular Potato cultivar.

Chapter V

MATERIALS AND METHOD

Materials and Methods

5.1. Plant material

Tubers of Kufri Surya (KS), Kufri Girdhari (KG), Kufri Chandramukhi (KCM), , Kufri Sindhuri (KSin), Kufri Aruna (KA) and in-vitro grown KG Plants were collected from Central potato research institute (CPRI), Bemloe, Shimla, Himachal Pradesh.

5.2. Bacterial strains and Vectors

The *Escherichia coli* strain DH5α for initial cloning and A*grobacterium tumefaciens* strain EHA105, was used for Plant transformation.pJET1.2/blunt cloning vector, pTRV VIGS vector and pRI-101 AN binary vector used in this study were procured from the crop improvement division of Central potato research institute, Shimla, Himachal Pradesh.

5.3. General protocol

5.3.1. Competent *E. coli* cells Preparation

DH5α strain was used for preparation of competent cells for cloning of target fragment. Bacteria was streaked on fresh LB(Luria Bertani) agar plate and from that single colony was used as inoculum for LB broth (5ml) and incubated overnight at 37°C with shaking at 200 rpm. From this culture 50ml of LB broth in conical flask was inoculated and incubated again with shaking (200rpm) at 37°C. After 30min, OD (Optical density) was measured at 600nm to check growth of culture. Bacterial culture was removed from incubation at OD 0.6 and incubated for 30min on ice. 12ml of bacterial culture was shifted in round bottom flask and centrifuged for 10min at 3000rpm (4°C). Prechilled 10mM$CaCl_2$was used to resuspend pellet and centrifuged again (3000g, 4°C and 10 min). The abovementioned step was repeated two more times and then pellet was resuspended in $CaCl_2$ and glycerol (20%) mixture (2ml glycerol + ml $CaCl_2$). Pre chilled microcentrifuge tubes (1.5ml) was used to make aliquots of prepared cells. competent cells were then freezed in liquid nitrogen and stored at –80°C.

5.3.2. Preparation of competent *A. tumefaciens* cells

The *A. tumefaciens*EHA105 was streaked on fresh LB(Luria Bertani) agar plate and from that single colony was used as inoculum for LB broth (5ml) and incubated overnight at 37°C with shaking at 200 rpm. From this culture 50ml of LB broth in conical flask was inoculated and incubated again with shaking (200rpm) at 37°C. After 30min, OD (Optical density) was measured at 600nm to check growth of culture. Bacterial culture was removed from incubation at OD 0.6 and incubated for 30min on ice. 12ml of bacterial culture was shifted in round bottom flask and centrifuged for 10min at 3000rpm (4°C). Prechilled 10mM$CaCl_2$was used to resuspend pellet and centrifuged again (3000g, 4°C and 10 min). The abovementioned step was repeated two more times and then pellet was resuspended in $CaCl_2$ and glycerol (20%) mixture (2ml glycerol + ml $CaCl_2$). Pre chilled microcentrifuge tubes (1.5ml) was used to make aliquots of prepared cells. competent cells were then freezed in liquid nitrogen and stored at –80°C.

5.3.3 Plasmid DNA isolation

The bacteria colony was inoculated in 5ml of LB broth and incubated overnight. Bacterial culture was centrifuged for 5min at 11000 rpm for 5 min and obtained pellet was re-suspended in solution I (200µl). After dissolving pellet, solution was cooled on ice for 10min and 200µl of solution II was added (always prepared fresh). Again solution was kept on ice for cooling for 10min and solution III (150µl). This mixture was mixed by inverting slowly (to avoid shearing of DNA) and incubated again on ice (10min). After incubation, mixture was centrifuged for 10-15min at 12000rpm. Supernatant was taken in fresh tube and RNase A (4µl) was added to it and incubation was done in water bath at 37°C for 45 min. Equal volume of chloroform (24): isoamyl alcohol (1) mixture was added to supernatant and centrifuged (12000rpm, 10min) again. For precipitation of plasmid DNA in solution 3M sodium acetate (0.1 volume of solution) and ethanol (2 volumes) was added and incubated for 60min in -80°C. The mixture was centrifuged at 12000rpm for 10min at 4°C. The obtained DNA pellet was washed two times with ethanol (70% V/V) and kept for drying at room temperature. Pellet was re-suspended in nuclease free water

(30 µl) and kept at -20°C. For quantity and quality estimation of Plasmid DNA spectrophotometrically nanodrop was used at 260/280.

5.3.4. Ligation

The final volume of 15µl was used to carry-out ligation reaction for cloning of target fragment. All the components (Table 5.1) of the reaction were added and reaction mixture was kept at 16°C for overnight incubation on heat block.

Table 5.1 Reaction mixture of Ligation reaction.

Components	Quantity
10X T4 DNA ligase buffer	1.5µl
DNA vector	1µl
T4-DNA ligase	1µl
DNA insert	3µl
RNase free water	8.5µl
Total	15µl

5.3.5. Transformation in *E. coli* cells

The 10µl ligation mixture was added to the 100µlof competent cells (DH5α) in laminar air flow. The mixture of ligated product and competent cells was incubated on ice for 30min. After 30mins, heat shock treatment was given in heat block at 42°C for 1min. Then immediately the tubes were placed on ice for 5min. 950µl of LB media was added to the tubes and incubated at 37°C for 60min at 120rpm. After incubation tubes were centrifuged at 3000rpm for 4mins. 900 µl of supernatant was discarded and pellet was then resuspended in the remaining 100µl media. The resuspended cells were plated onto the LB plates containing antibiotic as the selection agent. The LB agar plates were incubated at 37°C overnight and transformed colonies were observed.

5.3.6 Transformation in *A. tumefaciens* cells

Centrifuge tube containing 100µl competent cells was thawed on ice. After complete thawing 1µl of isolated plasmid was added in sterile condition in laminar hood. Cells and plasmid was mixed by tapping slowly and then cells were dipped in liquid nitrogen for freezing. After this mixture containing tubes were thawed on heat block at 37^{o}C about 5 min. In mixture containing tubes 1ml of Broth was added and incubated for growth for 4hr at 28^{o}C with shaking (140rpm). The tubes were centrifuged (4000rpm, 3min) and pellet was suspended in 100µl of supernatant. The resuspended cells were plated onto the LB plates containing antibiotic as the selection agent. The plates were incubated overnight at 28°C and transformed colonies were observed.

5.3.7. Restriction endonuclease treatments

Digestion of Plasmid DNA was performed by using restriction endonuclease specific to vector or gene fragment. Each reaction contain1µg of plasmid DNA cut smart buffer (1X) and restriction enzymes (5U). In double digestion reactions, buffer and temperature compatible to both enzymes was used. Restriction digestion products were resolved in 1.3% agarose gel and visualized with UV transilluminator.

5.3.8. RNA isolation

Total RNA was extracted was extracted using Trizol method from the leaf tissue. All the usable were treated with 0.1% DEPC (Diethyl pyrocarbonate) water and then autoclaved. 200mg of plant tissue crushed powder was added 1 ml Trizol and incubated for 10 min at 37ºC. Mixture was centrifuged at 12000g for 10 min and 200 µl chloroform was added to supernatant and incubated for 3-5min at 37ºC. Again centrifuged at 15000g for 10 min at 4ºC and equal volume of Phenol (25):chloroform(24):isoamyl alcohol(1) was added to the supernatant. Mixed by inverting and centrifuged at 10000g for 5min at 4ºC. Precipitation of RNA from the aqueous phase was done by using isopropyl alcohol and incubated at 37ºC for 5minutes. And again incubated for 10 minutes at -20ºC and centrifuged at 15000g for 10min at 4ºC. RNA pellet were washed using ethanol and then air dried pellet was dissolved in 30 µL RNase free water and stored at -80ºC.

5.3.9. DNA isolation

Genomic DNA was extracted from leaf samples using CTAB method with some modifications (Doyle and Doyle, 1990). 100 mg of leaf tissues were ground in liquid nitrogen to a powder form using a mortar and pestle. The powder was transferred to eppendorf tubes and suspended in 500µl DNA extraction buffer (CTAB buffer). The tubes were incubated at 65°C for 30 min in water bath. The mixture was centrifuged at 13000 rpm for 10 min and supernatant was transferred to a fresh eppendorf tube. To each tube equal volume of Phenol: chloroform (24:1) was added and mixed by inversion. After mixing, again centrifuged at 13000 rpm for 2 min and upper aqueous phase was transferred to a fresh eppendorf tube. To each tube 50µl of 7.5 M ammonium acetate and 500µl of chilled ethanol was added. Inverted the tubes slowly to precipitate DNA and centrifuged further at 10,000 rpm for 10 min to obtain DNA pellets. Washing was done 3-4 times with 70% ethanol and pellets were dissolved in 30µl TE buffer.

5.3.10 Colony PCR

8µl of sterile nuclease free water was taken in PCR tubes in sterile laminar conditions. Each bacterial colony was touched with pipette tip and then dipped in water. Master mix was prepared containing either gene specific or vector specific primers. 12µl of prepared master mix was then added to the PCR tubes and PCR was performed in a 20-µL volume (Table 5.2) using high fidelity polymerase. The thermocycling program consisted of initial denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 1min, 64°C for 1 min and 72°C for 1 min with a final extension of 72°C for 10 min.PCR products were resolved in 1.3% agarose gel and visualized with UV transilluminator.

5.3.11 Sequencing

Sequencing using primers internal to the vector was performed to determine the orientation and site of fragment insertion. Sequence information was obtained by the Sanger-Coulson method using the “Thermo Sequence fluorescent labelled primer cycle” sequencing-kit with 7-deazadGTP” (Amersam Pharmacia Biotech, Freiburg,

Germany). Software for the sequencing was Base Imagir 4.0 (L1-COR, MWG-Biotech GmbH, Ebersberg, Germany).

Table 5.2 Reaction mixture of colony PCR.

Components	Quantity
20X Emerald	10µl
Gene/Vector specific primer	2µl
RNase free water	8µl
Total	10µl

5.4. Expression studies

5.4.1 Plant Growth conditions

Well sprouted tubers of KS and KCM were planted in 15 cm pots containing the potting mixture in three replications including control. Plants were subjected to tuberization at 18ºC and 24ºC temperature with 8h light (600 $\mu Es^{-1}m^{-2}$) /16h dark photoperiod in a controlled environment chamber (Conviron, Model E-15, Canada).

5.4.2.cDNA Synthesis

For synthesis cDNA (Complementary DNA), RT-PCR (Reverse-transcriptase Polymerase Chain Reaction) reaction was performed in 10µl Volume (Table 5.3).Components (Table) of reaction was added to10µl of isolated RNA (1µg). Negative control (10µl of water +10µl of master mixture) was used to check contamination. The thermocycling program consisted of 25°C for 10min, 37°C for 60min, 37°C for 60min and 85°C for 5min.The cDNA was stored at -20°C for further use.

5.4.3. Quantitative RT-PCR

Reverse transcription quantitative real time PCR (RT-qPCR) was performed for expression analysis. An elongation factor (elf) gene primer from the potato

transcript was used to normalise the data. Expression analysis was performed using SYBR Green master mix (Applied Biosystems) on 7900HT Sequence Detection System (Applied Biosystems). The following reaction conditions were used: 95°C for 30s, 40 cycles of 95°C for 5s and 60°C for 31s, followed by 95°C for 15s, 60°C for 1min and 95°C for 15s to obtain melting curve. The expression of each gene relative to average C_t value of the housekeeping gene was calculated. Quantification of the relative change in the expression of the genes was calculated using $2^{-\Delta\Delta Ct}$method. The mean relative expression of the control sample was assigned 1 and then the relative expression of other lines was calculated. The mean value of three replicates was calculated and represented as results with standard deviation.

Table 5.3 Reaction mixture of RT-PCR for cDNA synthesis

Components	**Quantity**
100mM dNTPs	0.8µl
10X Buffer	2µl
10X Random primer	2µl
Reverse transcriptase enzyme	1µl
RNase free water	4.2µl
Total	10µl

5.5. Cloning of StCDF1.2 in pJET1.2/blunt cloning vector

5.5.1. Amplification of StCDF1.2

StCDF1 gene fragment was amplified using cDNA prepared from of K. Surya early morning. Gene specific primers were designed from Sequence obtained from PGSC, stCDF1 F and stCDF1 R. PCR was performed in a 25-µL volume (Table 5.4) using high fidelity polymerase. The thermocycling program consisted of initial denaturation at 94ºC for 4 min, followed by 30 cycles of 94ºC for 1min, 64ºC for 1 min and 72ºC for 1 min with a final extension of 72ºC for 10 min. PCR products were resolved in 1.3% agarose gel and visualized with UV transilluminator.

Table 5.4 Reaction mixture for amplification of StCDF1

Components	Quantity
cDNA	1µl
dNTPs	0.8µl
Buffer	2µl
Polymerase	0.1µl
Water	16.1µl
Total	20µl

5.5.2. Cloning of StCDF1

The identified and amplified gene products were then ligated in pJET1.2/blunt cloning vector. The vector containing gene fragment was transformed in competent *E. coli* cells (DH5α). Heat shock method explained in above section was used for transformation. Bacterial cells containing vector and gene fragment were inoculated (spreading) on LB agar plates containing ampicillin and kept at 37ºC for overnight incubation.

5.5.3. Analysis of recombinant clones

5.5.3.1. Colony PCR

As described earlier using gene specific primers.

5.3.2. Restriction Analysis

Plasmid from positive bacterial colonies was isolated and restriction digestion was performed using ***XbaI*** and ***Xhol I*** enzymes.

5.6. VIGS

5.6.1 Development of VIGS construct

5.6.1.1. Prediction of effective siRNA region and eliminating off-targets

The target gene sequences were obtained from published gene sequences in PGSC database (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml). The target gene CDS regions that could effectively yield the siRNA were identified with the help of bioinformatics (http://sidirect2.rnai.jp, http://bioinfo2.noble.org/RNAiScan.htm). Fragments were selected based on the number of effectively silenced siRNAs. The smaller fragments have a high silencing efficiency and the longer fragments could enhance the off-target effects and cause instability in some viral vectors. Thus the sequences were selected which fulfil requirement of having a length and untranslated region which are most effective with least possible silencing of off-target (Senthil-Kumar and Mysore, 2014).

5.6.1.2. Development of pTRV2 VIGS construct

The PCR products used for the development of VIGS constructs were amplified from KS cDNA using gene-specific primers. The primers were designed with the help of fast PCR software and were synthesized by IDT. The fragment of 530 bp on 1.3% agarose gel confirmed the successful amplification of StCDF1.2. These genes were first cloned in pTZ57R/T vector and then subcloned in pTRV2 vector in *E. coli* (strain DH5-α). The cloning was confirmed by colony PCR and restriction digestion using *EcoRI* and *BamHI* enzyme. The pTRV2::CDF1.2 vector construct was transformed into Agrobacterium tumefaciens strain GV3101 by a freeze-thaw method. All plasmid DNAs used for VIGS analysis were prepared using a Macherey-Nagel Nucleospin Plasmid isolation kit (Germany).

5.6.2. Agro-inoculation of Potato cultivar through pTRV2::VIGS constructs

The developed VIGS gene constructs (pTRV2::gene of interest) was mobilized in Agrobacterium. To carry out VIGS experiment, equal amount of developed construct and empty agro immobilised pTRV1 were used. K. Surya potato cultivar was used for inoculation and screening studies. Agro-immobilised construct was grown in LB Broth with Kanamycin (50µg/ml) and kept at 28ºC for overnight incubation with shaking. The well sprouted K. Surya tubers were used and the entire

meristematic region was pricked using 1ml insulin syringe. The overnight grown culture was centrifuged at 4000g and the pellet was re-suspended in VIGS induction medium with pH 5.5. After dissolving pellet 1ml of tobacco leaf extract was added and kept at 28ºC for 4hr with shaking (120rpm). The mixture was centrifuged at 4000g and pellet obtained was resuspended in the infiltration medium (pH 5.5). The tubers were injured at the meristematic region and agro inoculation was performed using 1ml insulin syringe. The treated tubers along with control (untreated) of KS were planted in pots. Three doses of Agro-inoculation were imposed, one just after sprouting, second at 30 days after planting and the third at 45 days after planting. The meristematic regions of plants were inoculated with agrobacterium culture having pTRV1 and pTRV2:: gene constructs using the agro-infiltration of leaf, stem and root through a 1ml insulin syringe injection (Senthil-Kumar and Mysore, 2014). Similarly, KS control plants were inoculated with agrobacterium culture having pTRV2 and pTRV1.

5.6.3. Validation of silencing

5.6.3.1. RNA isolation and quantification using qRT-PCR

Leaf samples were harvested at different time points (dawn, afternoon and dusk) from VIGS and KS control plants after the third inoculation. Total RNA was extracted from leaf tissues and was reverse transcribed into double-stranded cDNA. The expression of tuberization genes (StCDF1.2 and StSP6A) and complementary genes (StCO and StSP5G) relative to average Ct values of the housekeeping genes were determined and analyzed using ABI 7300 System Sequence Detection Software Version 1.4 (Applied Biosystems, USA). The protocol followed for quantification of gene expression was the same as mentioned earlier.

5.6.3.2 Sequencing

Sequencing of the PCR product amplified from StCDF1.2 silenced VIGS and control plants were performed to reconfirm the silencing. The obtained sequence was analysed using BLAST and pairwise alignment, with original sequence using Clustal omega.

5.6.4. Phenotypic observations

Flowering, senescence and tuberization stages were selected for the phenotypic study after silencing. Tubers were harvested from silenced and control plants after complete plant senescence.

5.7. Subcloning in binary vector

5.6.1. Cloning in pRI101 AN

Positive clone with StCDF1.2-pJET/Blunt gene cassette were grown overnight and plasmid was isolated from them using abovementioned method. After isolation restriction digestion was performed using ***NdeI*** and ***SalI*** enzymes. The fragments after Restriction digestion were electrophoresed on 1.3% agarose gel and extracted from gel and eluted using Qiagen gel extraction kit. The digested products were then cloned in pRI101 AN binary cloning vector. The vector containing gene fragment was transformed in competent *E. coli* cells (DH5α). Heat shock method explained in above section was used for transformation. Bacterial cells containing vector and gene fragment were inoculated (spreading) on LB agar plates containing ampicillin and kept at 37ºC for overnight incubation.

5.6.2. Agrobacterium Transformation of pRI101-StCDF1.2 gene

Positive clone with StCDF1.2-pRI101 AN gene cassette were grown overnight and plasmid was isolated from them using abovementioned method. The vector containing gene fragment was transformed in competent *A. tumefaciens* cells (EHA105). Freeze thaw method explained in above section was used for transformation. Bacterial cells containing vector and gene fragment were inoculated (spreading) on LB agar plates containing kanamycin (100µg/ml)/ rifampicin (50µg/ml)/ and kept at 28ºC for overnight incubation.

5.8. Plant transformation and Regeneration

5.8.1. Agrobacterium culture for co-cultivation

To prepare culture for infection, positive clones of *A. Tumefaciens* containing StCDF1.2-pRI101 AN gene cassette was freshly streaked on LB plate containing kanamycin (100µg/ml)/ rifampicin (50µg/ml)/. The plates were incubated at 28°C for 48 hr. After 48hr, 1ml of grown culture was used to inoculate 50 ml of YEM broth

and kept at 28°C for overnight with shaking (150rpm). The bacterial culture was centrifuged at 9000g (10min) and pellet was dissolved in liquid basal MS media. Acetosyringone (0.37mM) was added in mixture to increase efficacy of co-cultivation.

5.8.2. Plant transformation

On the day of infection, internodal stem sections were taken from four to six weeks plantlets and transferred to MS plates. Explants were incubated with 10 ml Agrobacterium inoculum for 20 min with 10µl of Acetosyringone occasionally swirling the plates. Then stem pieces after drying were transferred from the bacterial culture to new MS media plates (without PGRs and antibiotic) and incubated at 24°C in the dark. After three days, stem pieces were transferred to MS plates (BAP+NAA+GA_3) containing kanamycin (50µg/ml) and Cefotaxime (100mg/ml). These were incubated under fluorescent light at 100 µmol m-2s-1 with 16 hours light and 8 hours dark till putative shoots generated. Putative shoots generated were then transferred to tubes containing MS media (IAA+Kan) for root development.

5.8.3 Primary screening of putative transformants

DNA was isolated from leaves of transformed plants by method described above in section. nptII F and R primers were used to amplify fragment of nptII encoding gene. PCR was performed in a 25-µL volume using high fidelity polymerase. The thermocycling program consisted of initial denaturation at 94◦C for 4 min, followed by 30 cycles of 94ºC for 40 sec, 54ºC for 50 sec and 72ºC for 1 min with a final extension of 72ºC for 6 min.PCR products were resolved in 1.3% agarose gel and visualized with UV transilluminator.

5.9 Screening of transformants

5.9.1 Microtuberization

The transgenic lines and control K. Girdhari were grown in MS media (without PGRs and antibiotic) up to 8 node stage. Eight to ten nodal cutting having two nodes were subcultured in 100ml of MS basal (without PGRs and antibiotic) liquid media in tissue culture bottle. After 20 days of subculturing, the media was decanted off from bottle and 50ml of induction media (containing 10mg/L BAP) was

added for microtuber production. The bottles containing culture were then incubated in dark at 20ºC in tissue culture room. After incubation tubers from each line and control K. Girdhari were harvested (Hossain et al., 2017).

5.9.2 Minituberization

The transgenic lines and control K. Girdhari was planted in randomized block design on Styrofoam sheets. All in-vitro grown plants were cultivated in triplicates at spacing of 14cm x 16cm. Nutrient solution (macro and micronutrients) were sprayed on roots at regular intervals to keep them drenched. The pH of nutrient solution was maintained and solution was changed every week. Tubers were harvested every week and treated with bavistin (2%w/v) for 5min.

5.9.3 Pot experiment in glass house

Well sprouted minitubers of transgenic lines and control K. Girdhari from aeroponics facilities were planted in 15cm pots containing potting mixture in triplicates.

5.9.3.1 RNA isolation and quantification using qRT-PCR

Leaf samples were harvested at different time points from transformants and K. Girdhari control plants. The collected leaf samples were first dipped in liquid nitrogen and then were stored at -80ºC. Total RNA was extracted from leaf tissues and was reverse transcribed into double-stranded cDNA. The expression of tuberization genes (StCDF1.2 and StSP6A) and complementary genes (StCO and StSP5G) relative to average Ct values of the housekeeping genes were determined and analyzed using ABI 7300 System Sequence Detection Software Version 1.4 (Applied Biosystems, USA). The protocol followed for quantification of gene expression was the same as mentioned earlier.

5.9.3.2 Phenotypic studies

Flowering, senescence and tuberization stages were selected for the phenotypic study after silencing. Tubers were harvested from silenced and control plants after complete plant senescence.

5.9.4 Field trial

Tubers of five transgenic lines and control K. Girdhari were planted in contained field conditions in triplicates. Random block design was used to lay out the field and spacing of 0.5m X 1m between rows. Tubers were harvested from the field after complete senescence of plants.

5.9.4.1 Nutritional profiling

The tubers harvested from field of transformants and K Girdhari control were used to analyse different nutritional parameters (starch, protein and amylose).

5.9.4.1.1. Starch analysis

Tubers were crushed into powder after washing, cutting and drying them completely. 5ml of Ethanol (80%) was added to 100mg tuber powder and mixture was centrifuged after brief vortexing. After removing the supernatant, pellet was resuspended in 6.5ml perchloric acid (52%) and 5ml dH_2O. Again the mixture was centrifuged and supernatant was collected in falcon tube. Pellet was resuspended in perchloric acid and centrifuged. The supernatant was collected and pooled with earlier collected supernatant and dH_2O was added to make final volume of 50ml. For final sample preparation, 50µl sample + 950µl distilled water and 2ml chilled Anthrone reagent was added. Mixture was incubated at 85ºC in water bath for 8 min and then cooled at room temperature. The optical density was measured spectrophotometrically at 620nm.

5.9.4.1.2. Amylose estimation

Amylose content was estimated using the method given by Fajardo et al. (2013) with little modification.500µl of Perchloric acid (45%) was added to freezed powder and incubated for 4 min at room temperature. 16 ml of dH_2O was added to mixture and incubated again for 10 min after vortexing. 4ml aliquot from above mixture was mixed with potassium iodide and Iodine mixture. The optical density was measured spectrophotometrically at 550 and 620nm.

5.9.4.1.2. Total protein

Protein content was determined using the AOAC (2005) No. 2001:11 method with little modification (Rahman et al. 2016). 15ml of Sulphuric acid was used for hydrolyse 1 gm tuber and incubated in water bath for 2h at 42ºC. dH2O was added to hydrolysed material for titration. After titration total nitrogen content estimated was multiplied by conversion factor.

5.10. Statistical analysis

The statistical significance of obtained data was determined using an unpaired two-tailed Student's t-test (***, P/0.001: **, P/0.002:*P/ 0.005). All values reported mean ± Standard Error of three replicates.

Chapter VI

RESULTS AND DISCUSSION

Results and discussion

6.1. Expression analysis in potato cultivars grown under contrasting temperature

To quantify the relative expression difference of StCDF1 in heat tolerant (KS) and heat susceptible (KCM). qRT-PCR was performed using real time primers specific to StCDF1. The highest expression (1.92 fold) was observed in the morning sample (6am) of the KS and followed by 12 noon sample (1.53 fold) of KS. The morning transcript of KS was 0.92 fold higher than morning KCM and 0.39 fold higher than KS afternoon (**Fig 6.1a**). In case of KCM, endogenous transcript of morning sample was 0.747 fold higher than noon sample and 1 fold higher than evening sample (6pm) (**Fig 6.1b**). Upregulation of the StCDF1 was also observed in the heat tolerant KS in comparison to the KCM (heat susceptible). Similar increase of the expression of StCDF1 was reported by Singh et al. (2015) on increasing the temperature. Koolerstman et al. (2013) also observed the highest expression of StCDF1in sample collected just before dawn. This expression results can be explained by the phyB dependent regulation of StCO1. In potato, after perceiving red light phyB triggers the degradation of StCDF1.2 by interacting with other proteins like GI and FKF1 (Abelenda et al. 2016).

6.2. StCDF1 expression in short-day and long-day adapted Indian cultivars

In case of short day (KA) and long day (KG and KSin) adapted Indian varieties,StCDF1 expression was studied using qRT-PCR. The highest expression (3.34 fold) was studied in KA morning under short day conditions and lowest (0.331 fold) was observed in KG evening under long conditions. In this study also level of transcript of StCDF1 in all the early morning samples was higher than their respective noon and evening samples. In all Indian varieties used for the study, short day conditions led to the higher expression of StCDF1 in comparison to the plants grown under long day conditions (**Fig 6.2**). The KA short day 6am expression was 0.64 fold higher than KA long day 6am. The short-day conditions are inductive for tuberization

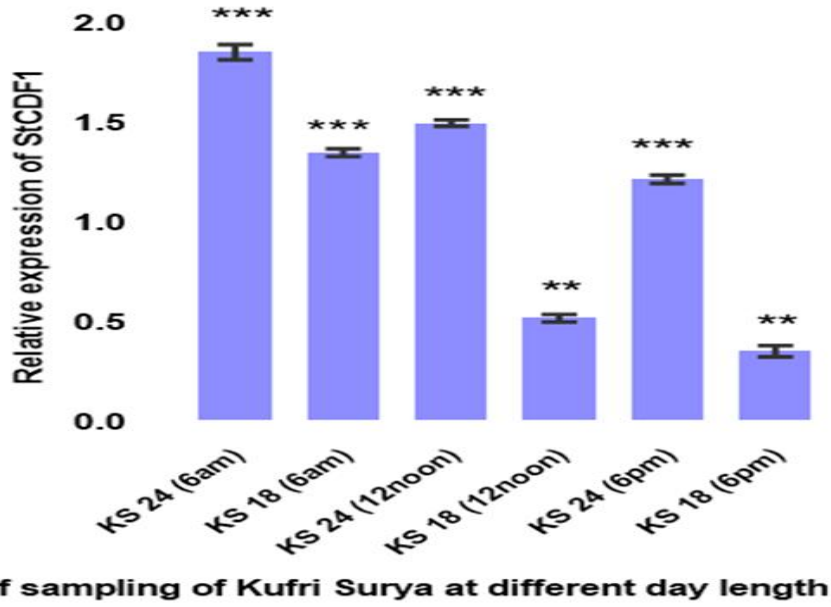

Figure 6.1aGene expression analysis of StCDF1 in heat-tolerant (KS) Indian potato cultivar by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates.

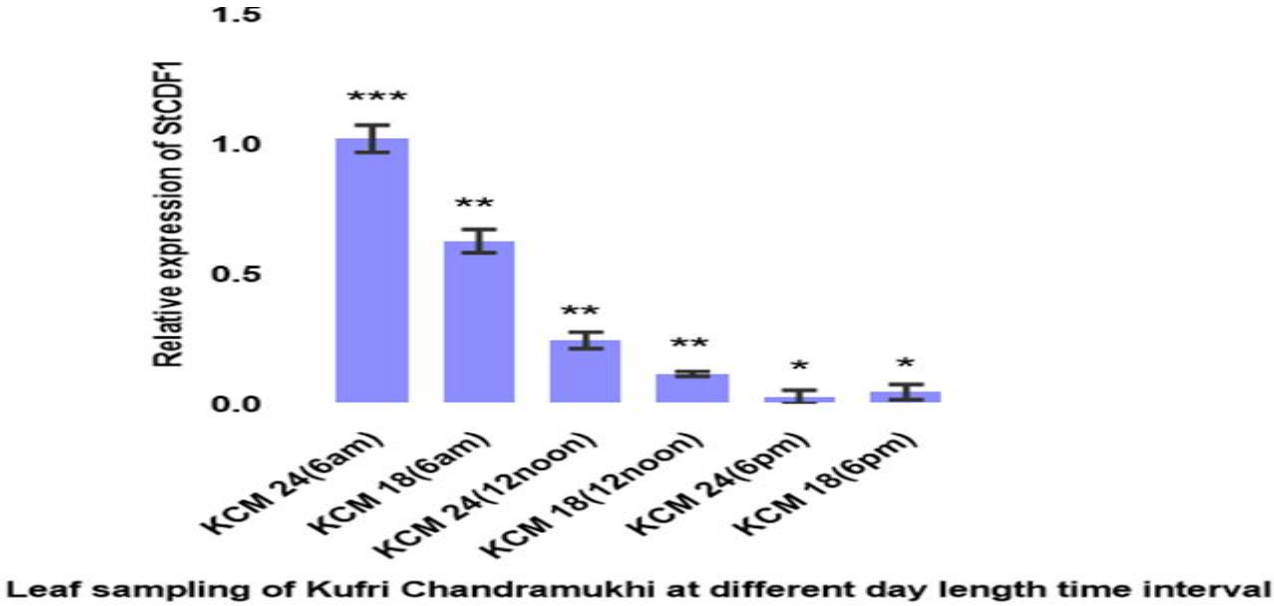

Figure 6.1bGene expression analysis of StCDF1 in heat-susceptible (KCM) Indian potato cultivar by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates.

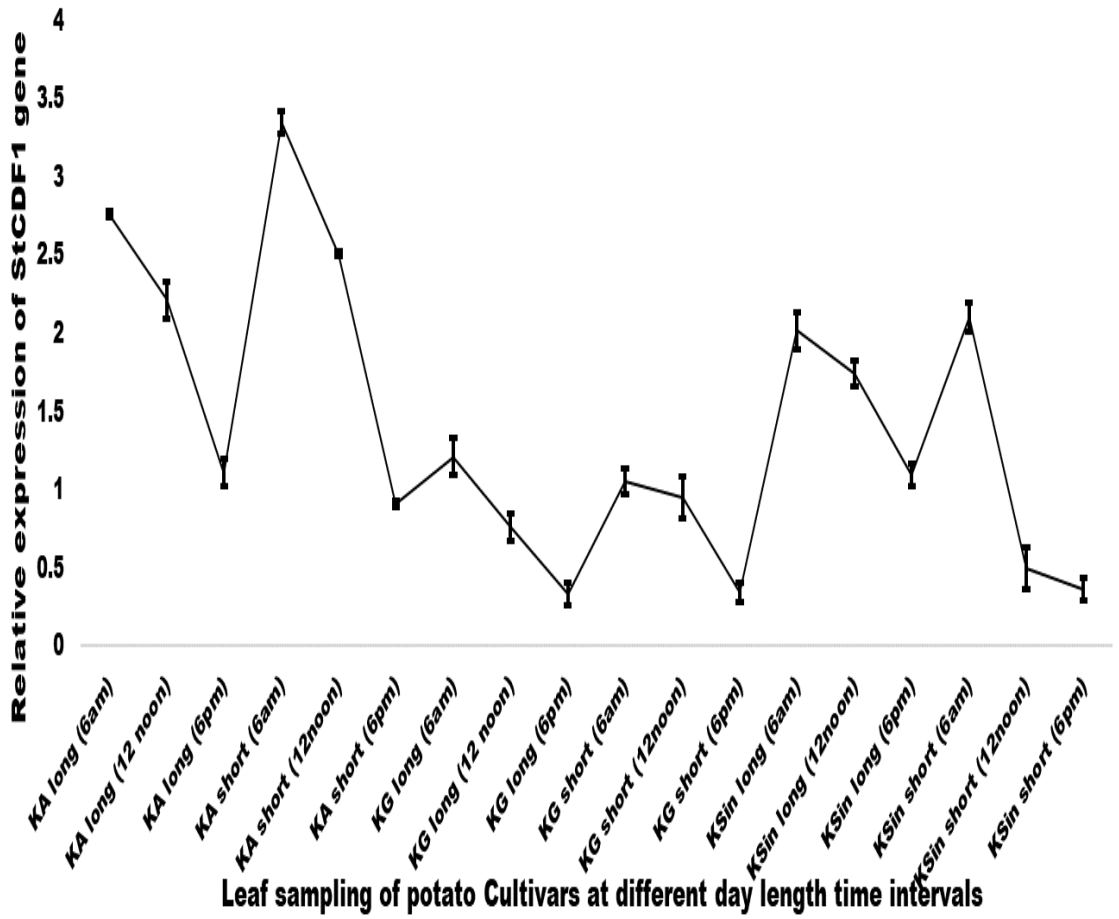

Figure 6.2 Gene expression analysis of StCDF1 in long day and short day adapted Indian potato cultivars by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates.

So, expression of the StCDF 1 is upregulated by these conditions. Whereas the long day conditions are favourable for the flowering, which in result delays tuberization (Hannapel et al. 2017). In long day conditions, the long afternoon aids degradation of StCDF1 and StCO gets stabilized. Whereas in short day conditions StCDF1 binds to the conserved domain of the promoter of the StCO and downregulate StCO expression (Dutt et al. 2017). Kloosterman et al. (2013) observed the downregulation of the StCO1 in the StCDF1.2 overexpressed lines, confirming the abovementioned StCDF1 role in regulation of StCO1. StCO1 doesn't directly affect the tuberization in non-inductive conditions, it regulate the expression of StSP6A via StSP5G

6.3. Allelic variation of StCDF1 in Indian potato cultivars

To study the allelic variation in StCDF1 gene, the selected long day and short-day Indian varieties were grown in constraint conditions. On the gel electrophoresis of the PCR products generated by using the high-fidelity polymerase, two fragment were observed corresponding to CDF allele 1.2 and 1.3 (**Fig 6.3**). Both alleles CDF1.2 and CDF 1.3 correspond to the earliness and plant maturity. There are three conserved domains present in the CDF translated protein, which aid the protein-protein interaction. These domains facilitate the binding of StGI and FKF1 with StCDF1, and lead to StCDF1 degradation. In case of CDF 1.2 and 1.3 allele, the C-terminal is lost due to the insertion at C-terminal. The binding and degradation of StCDF1 is impaired and affect the onset of tuberization (Kloosterman et al. 2013). As also reported in the *Arabidopsis thaliana*, CO promoter is the binding site for CDF gene and this binding alter the expression of CO gene. Similarly, in potato CO homologue (StCO) is present and its role in inhibition of the tuberization is well documented (Martinez-Gracia et al. 2002).

6.4. Cloning of StCDF1.2 in cloning vector pJET1.2/blunt

Putative full-length StCDF1.2 was amplified by using gene specific primers form KS early morning leaf sample. KS was selected because of highest expression in expression profile studies. The primers were designed based on sequence obtained from Potato Genome Sequencing Consortium (PGSC). PCR amplified products were electrophoresed on 1.3% agarose gel and two fragments of 1329 and 2300bp were observed (**Fig 6.4**). The 1329bp fragment corresponds to allele StCDF1.2 was extracted from gel and eluted using Qiagen gel extraction kit. The allele StCDF1.2 is linked with the early maturity, other counterparts of the allele StCDF1.1 and StCDF1.3 are not as effective (Kloosterman et al. 2013). The eluted fragment was ligated in the linearized cloning vector pJET1.2/blunt.

Vector pJET1.2/blunt is high efficiency cloning vector used to clone blunt end PCR products. Mostly high-fidelity polymerase mostly results blunt end PCR products, can be ligated in this vector. This vector is a positive selection vector, which This vector contains for a gene, which encodes for a lethal restriction enzyme. The

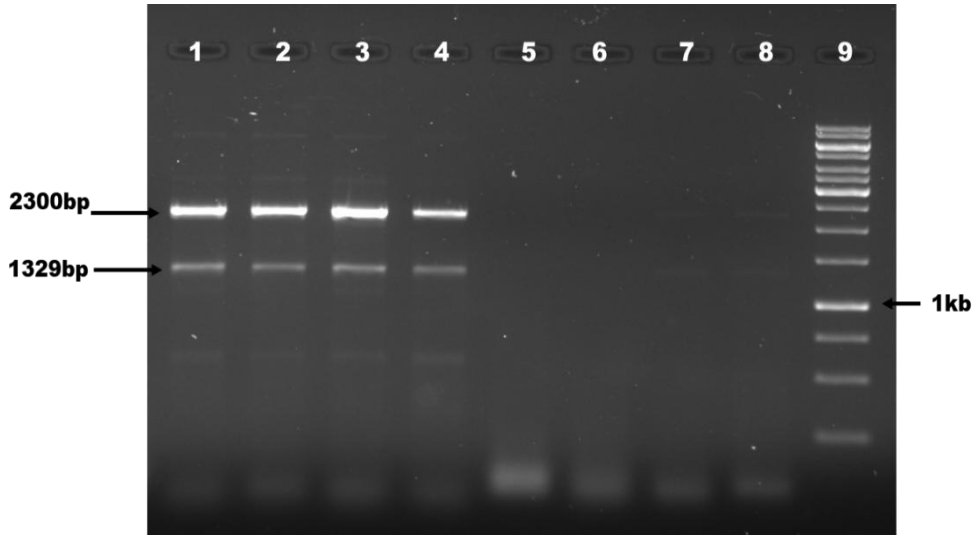

Figure 6.3 Amplification of StCDF1 gene by using gene specific primers in Indian potato cultivars. 1) KA(Short-day) **2)** KA(Long-day **3)** KSin (Short-day) **4)** KSin (Long-day) **5)** Blank **6)** Negative control **7)** KG (Short-day) **8)** KG (Long-Day) **9)** Ladder (1kb)

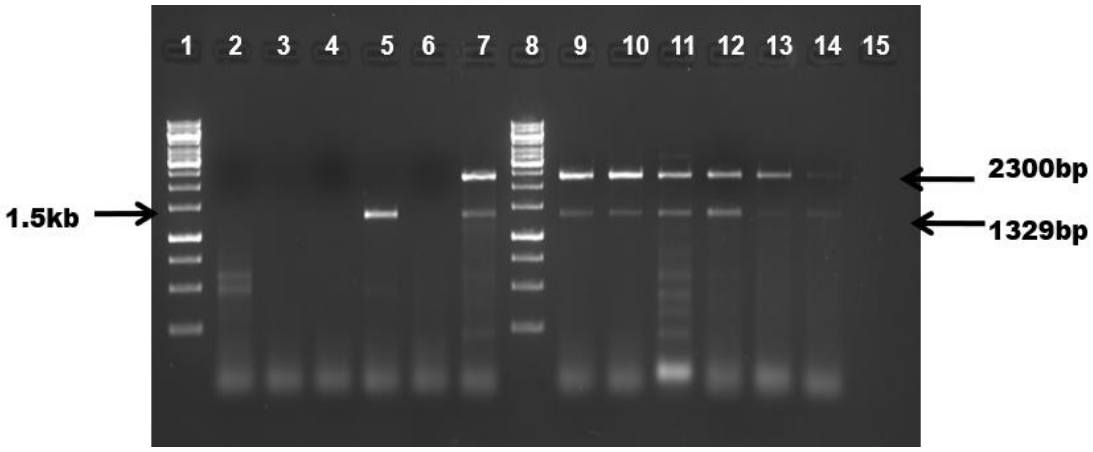

Figure 6.4 Agarose gel electrophoresis of PCR amplification of StCDF1 at different temperature- 1) Ladder (1kb) **2)** KS 18ºC(early morning) **3)** KS 18ºC(afternoon) **4)** KS 18ºC(evening) **5)** KCM 18ºC(early morning) **6)** KCM 18ºC(afternoon) **7)** KCM 18ºC(evening) **8)** Ladder (1kb) **9)** KS 24ºC(early morning) **10)** KS 24ºC(afternoon)**11)** KS 24ºC(evening) **12)** KCM 24ºC(early morning) **13)** KCM 24ºC(afternoon)**14)** KCM 24ºC(evening)**15)** Blank

Ligation of DNA in the vector disrupts the gene in the vector. On transformation, only transformed cells will be able to survive and form colonies. In non-transformed cells the vector gets recircularized and gene will able to eliminate the need of selection of transformants using high-throughput techniques. express the lethal gene. This positive selection vector increases the efficiency and pace of the transformation process. It uses the lac promoter with UP mutation to enhance the expression of the inserted DNA in bacterial cell. To maintain its high copy number in bacteria, it contains ColE1/pMB1/pBR1/pBR322/pUC origin of replication (**Fig 6.5**). The ligated product was then transformed with vector into high efficiency competent *E. coli* cells (DH5α) by heat shock method (www.snapgene.com).

To provide another selectable agent, vector harbour AmpR gene which encode for β-lactamase. This gene confers resistance against antibiotic like ampicillin and related class. The cells that have transformed completely become resistance to the antibiotic and form colonies on the plates (Manna et al. 2013). Whereas, cells with non-ligated vector or no vector cannot survive on the plate. So, after transformation, bacterial cells were plated on LB media containing ampicillin and incubated at 37ºC. Ampicillin hinders the cell wall synthesis process by inhibiting the enzyme action involved in the synthesis. In case of transformed cells, the β-lactamase hydrolyse the β-lactam ring of the ampicillin (Law et al. 2002). After overnight incubation, the transformed colonies (**Fig 6.6**) were observed on the LB plate containing ampicillin. More than hundred transformed colonies were observed, which confer the high efficiency of pJET1.2/blunt. The conformation of the transformed colonies was done

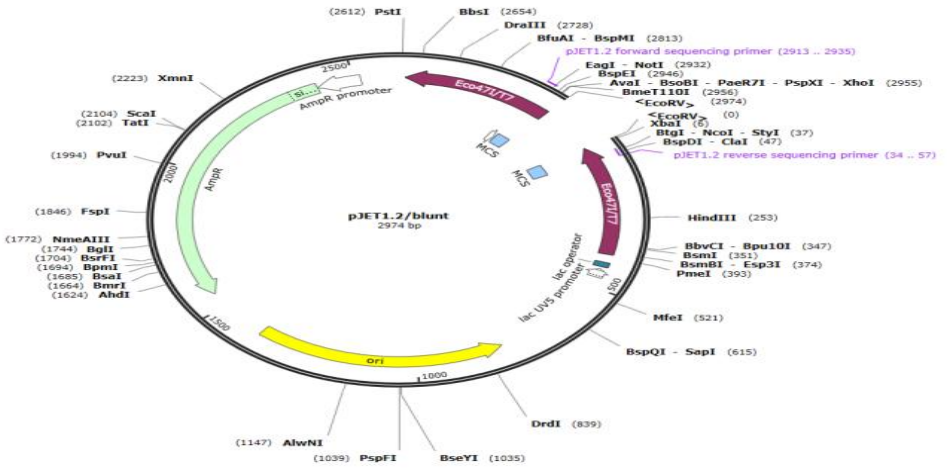

Figure 6.5 Vector map of pJET1.2/Blunt (www.snapgene.com)

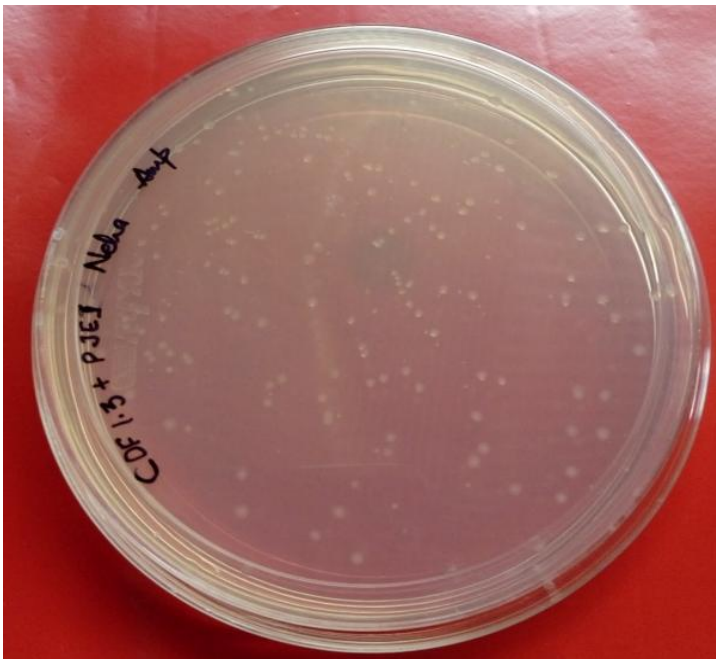

Figure 6.6 Transformed colonies of St-CDF1.2-pJET1.2/blunt on the LB plate containing ampicillin

Table 6.1 Quantitative and Qualitative estimation of *E. coli* plasmid isolated from the positive clones harbouring StCDF1.2-pJET1.2/blunt

S.No.	Sample	Conc. (ng/µl)	A_{260}	A_{280}	A_{260}/A_{280}
1	CDF 1	159.8	3.196	1.703	1.88
2	CDF 3	284.8	5.696	3.047	1.87

by colony PCR and restriction digestion. The reconfirmation of the cloning was done by sequencing of the plasmid. The confirmed colonies can be used for the downstream process.

6.4.1. Analysis of recombinant clones

6.4.1.1. Colony PCR

Sometimes colonies on the plates may be due to some other reason, then stable transformation. Retention of the selectable marker is also possible without the DNA insert. So, colonies on the plate is not always conferring the stable transformation of the gene cassette in competent *E. coli*. Initial screening method is very crucial to distinguish colonies with target gene from false positives. Colony PCR is technique for the primary screening of the bacterial colonies directly. The bacterial colonies are subjected to PCR directly by using target DNA sequence specific primers. It is the fast and efficient tool for screening of large number of colonies (Bergkessel and Guthrie, 2013). Transformed bacterial colonies were directly subjected to PCR using stCDF1 F and stCDF1 R primers. The amplified products were resolved on 1.3% agarose gel. The fragment of 1329bp was observed on the gel (**Fig 6.7**), which confirmed the cloning of StCDF1.2 in pJET1.2/blunt. Two positive colonies were selected for the further analysis.

6.4.1.2. Restriction Analysis

After confirmation from colony PCR, positive colonies were selected and inoculated in the LB broth containing ampicillin. After overnight incubation, plasmid was isolated by alkaline lysis method using Qiagen kit and concentration was determined spectrophotometrically using nanodrop (Table 6.1). Isolated plasmids were subjected to restriction digestion using ***Xba*I** and ***Xho*I** enzymes. The enzymes were selected by analysing the gene and vector sequence for the restriction enzyme site. The selected restriction enzymes should cut at the flanking regions, not within the DNA insert and vector (www.addgene.org). Analysis of restriction digestion products on 1.3% agarose gel electrophoresis confirmed the presence of stCDF1.2 inpJET1.2/blunt cloning vector. The vector backbone of 2.9kb and fallout of 1329bp was observed on the gel (**Fig 6.8).**

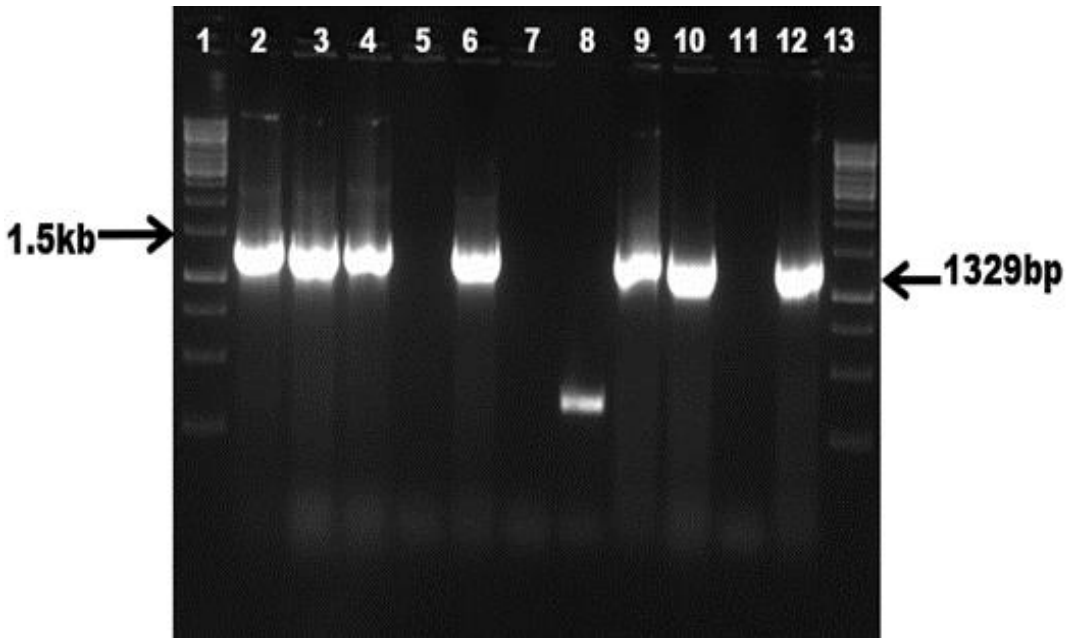

Figure 6.7 Agarose gel electrophoresis of Colony PCR of *E. coli* StCDF1.2-pJET1.2/blunt. 1) Ladder (1kb) **2)** Colony 1 **3)** Colony 2 **4)** Colony 3 **5)** Colony 4 **6)** Colony 5 **7)** Blank**8)** Colony 6**9)** Colony 7**10)** Colony 8 1**1)** Colony 9 **12)** Colony 10**13)** Ladder (1kb)

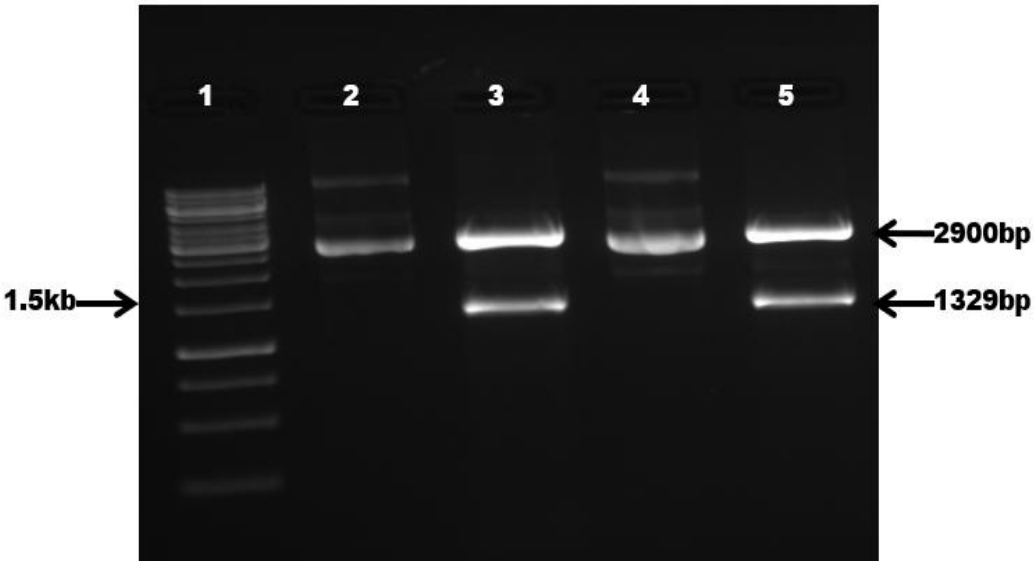

Figure 6.8 Agarose gel electrophoresis of restriction digestion of *E. coli* StCDF1.2-pJET1.2/blunt 1) Ladder (1kb) **2)** uncut plasmid colony 1 **3)** a) cut (T/A) vector pJET1.2/blunt. b) StCDF1.2 (gene) fragment (1329bp) **4)** uncut plasmid colony 3 **5)** a) cut (T/A) vector pJET1.2/blunt. b) StCDF1.2 (gene) fragment (1329bp)

6.4.2. Sequencing

Sequencing using primers internal to the vector pJET1.2/blunt was performed to determine the orientation and site of fragment insertion. Primers internal to vector are helpful in providing the complete coverage of the target sequence for consistent characterization. Sequencing provides the complete characterization of the cloned plasmid DNA. Identity of nucleotide, order of nucleotide and junction between plasmid and DNA insert can be determined by the sequencing (Alberts et al. 2002). Sequence information was obtained by the Sanger-Coulson method using Big Dye terminator kit by applied biosystems (**Fig 6.9**). This kit provides the all four ddNTPs labelled with different fluorophores. The addition one of these ddNTPs cease elongation of the product and product are separated according to the size (www.neb.com).

6.4.2.1. Analysis of the sequence

6.4.2.1.1. BLAST (Basic Local Alignment Tool)

The sequence obtained by sequencing was analysed using BLAST and Clustal omega. BLAST is one of the most frequently used bioinformatic tool for searching sequence similarity with database sequences. It uses heuristic method for the data-mining of the raw sequence (Neumann et al. 2014). The overview of the aligned query sequence was obtained in the graphical form (**Fig 6.10**). The alignment was represented in colour coded form ranging from black (<40 hits) to red (>200 hits). E-value in the BLAST represents the chance alignments of the query sequence (Altschul et al. 1990). Lower the E-value means chance alignments are less in number, hence better match with published sequences (McGinnis and Madden, 2004). In this study, E-value of 0 was obtained with 99% query cover for StCDF1 sequence (**Fig 6.11**). For detailed analysis alignment was studied with different available sequences in database. Orientation and strand for the obtained sequence was also obtained from this alignment studies (**Fig 6.12**).

GATGTCTGAAGTTAGAGATCTGCTATTAAGCTGTTTGGTAAAACAATTGGTATGACACAACAAGAAC
AATTGTGTTTATCTTCATGATGATCATACAACCTCATCCCCTCTTTCAATTGATGATGATAAGATCCTT
TAAGGAGAATTTACACAAAGCAAACAAGATGATGAACTTGTCGATCCAACTGCAGACTCGTCGATTG
AACCAGAAACATCATCTGGTATAAGCGATGACATCAAGATGCAGGATGCAGAAAAGAAACATTATC

ATCAAAATCTATCGAGGAGGAGGATTCGAGTGAGGAAAAGGCACTCAAGAAGCCTGATAAATTGAT
TCCATGTCCGCGATGTAATAGCATGGAAACAAAGTTCTGTTATTACAACAATTACAACGTCAACCAG
CCTCGTTACTTCTGCAAGAACTGCCAGAGATATTGGACTGCTGGAGGGACAATGAGAAATGTGCCTG
TGGGATCTGGTCGCCGAAAGAACAAGAGTTCTTCCATTTCAAATTATCCTCTTCAAGCAGGTCGGGTC
GAAGCAGCAGCTCACGGAATGCATCTTCTGCTTTAAGGACAAATGGAACTGTCCTTACATTTGGATCA
GATAAACCCCTTTGTGATTCAATGGTTTCTGCATTGAACTTAGCTGAGAATTCACATAATATGAATCG
AAATGAATTCCATGGATCCGAACGAAGAATGCCTGCAATCGGGAATGATCAATCAAATGGAACTTGT
AGTACAGCCTCAAGTGTAACTGACAAAGAAAGCAGTGCTGGTACTCATGATTTAGCAAATTGGAATA
ATTTCCAGCCATTTCCTCCTCAAGTACCGTACTTTCAGGGCGCTCCGTGGCCTTATTCTGGCTTTCCAG
TATCATTCTATCCAGCAACACCGTACTGGGGCTGCACCGTAGCAAACCCTTGGAACGTACCTTGGCTT
TCTTCTGATCAATCGTCAGTCCAGAACAACAGTCCTACTTCACCAACATTAGGAAAACATTCTCGGGA
TGAAAGCAAGATTGATCCATCACAATCACGGAGAAGAGATGCTAATTTGCAGAATAGAGAAGGCGA
GAGATGTGTACTGATTCCGAAGACATTAAGGATTCATGATCCAAATGAAGCGGCTAAAAGCTCTATA
TGGTCAACACTAGGTATCAGGAATGAGAAGATTGATTCAGCTCGTGGTACAATGCTCTTCAGTGCCTT
CAATCCAAAAGCTGATCATAGAAATCGCGAACATGACACTTCTTTTGCCCTGCAAGCTAATCCAGCA
GCCTTGTCTAGATCACTTCATTTCCGCGAGAGCACACAATGA

Figure 6.9 Sequence obtained from the sequencing of pJET1.2/blunt:StCDF1.2 positive clones.

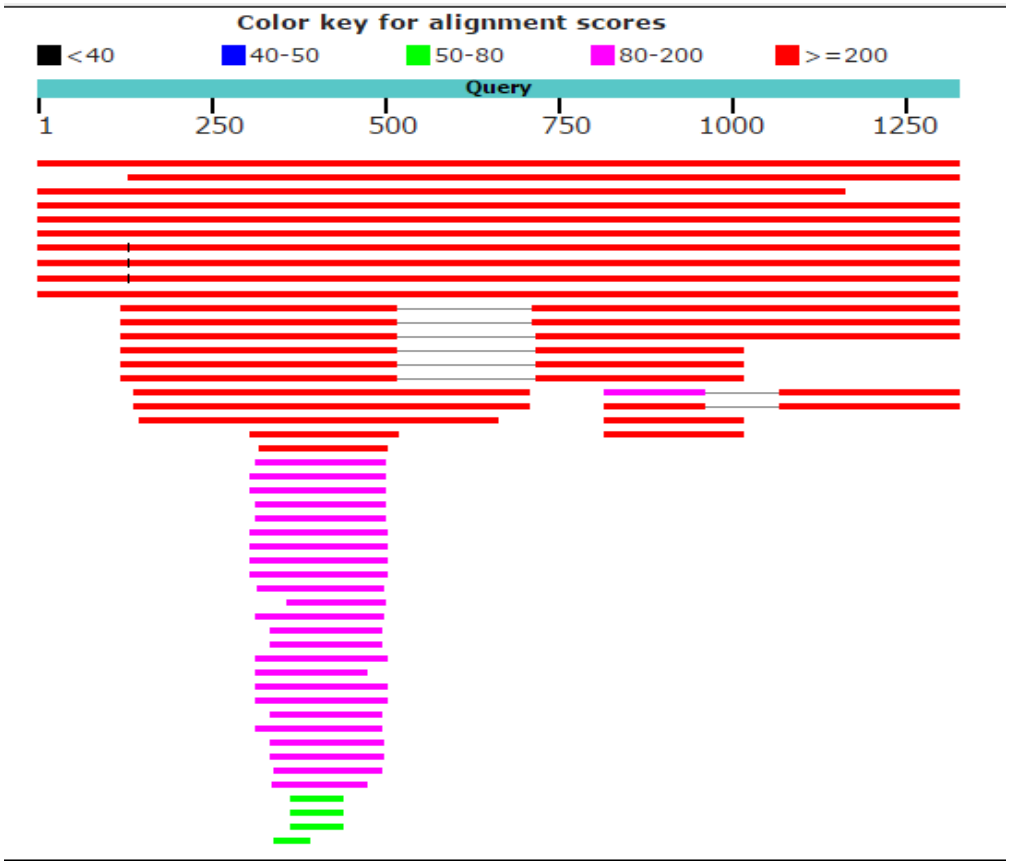

Figure 6.10 Graphical representation of BLAST hits on the query sequence.

Sequences producing significant alignments:

Select: All None Selected:3

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☑	PREDICTED: Solanum tuberosum cyclic dof factor 1 (LOC102603307), transcript variant X1, mRNA	2386	2386	99%	0.0	99%	XM_006355049.2
☑	PREDICTED: Solanum tuberosum cyclic dof factor 1 (LOC102603307), transcript variant X2, mRNA	2163	2163	90%	0.0	99%	XM_015312183.1
☑	Solanum tuberosum cyclic dof factor 1 (LOC102603307), mRNA	2089	2089	87%	0.0	99%	NM_001318682.1
☐	PREDICTED: Solanum pennellii cyclic dof factor 1 (LOC107018556), mRNA	2084	2084	99%	0.0	95%	XM_015219065.1
☐	Solanum lycopersicum cDNA, clone: LEFL2017H08, HTC in fruit	2041	2041	99%	0.0	95%	AK326937.1
☐	PREDICTED: Solanum lycopersicum cyclic dof factor 1 (LOC101248009), mRNA	2039	2039	99%	0.0	95%	XM_004238773.2
☐	Solanum pennellii chromosome ch05, complete genome	1879	2092	99%	0.0	95%	HG975444.1
☐	Solanum lycopersicum chromosome ch05, complete genome	1834	2048	99%	0.0	94%	HG975517.1
☐	Solanum lycopersicum cDNA, clone: LEFL1062CB10, HTC in leaf	1829	2042	99%	0.0	94%	AK247208.1
☐	PREDICTED: Capsicum annuum cyclic dof factor 1-like (LOC107868098), mRNA	1637	1637	99%	0.0	89%	XM_016714674.1

Figure 6.11 List of sequence producing significant alignment with the query sequence.

PREDICTED: Solanum tuberosum cyclic dof factor 1 (LOC102603307), transcript variant X1, mRNA

Sequence ID: XM_006355049.2 **Length:** 1980 **Number of Matches:** 1

Range 1: 342 to 1670 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
2386 bits(1292)	0.0	1318/1329(99%)	8/1329(0%)	Plus/Plus

```
Query  2    ATGTCTGAAGTTAGAGAT-CTGCTATTAAGCTGTTTGGTAAAACAATTGGTATGACACAA  60
            ||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  342  ATGTCTGAAGTTAGAGATCCTGCTATTAAGCTGTTTGGTAAAACAATTGGTATGACACAA  401

Query  61   CAAGAA--CAATTGTGTTTATCTTCATGATGATCATACAACCTCATCCCCTCTTTCAATT  118
            ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  402  CAAGAAACCAATTGTGTTTATCTTCATGATGATCATACAACCTCATCCCCTCTTTCAATT  461

Query  119  GATGATGATAAGATC-CTTT--AAGGAGAATTTACACAAAGCAAACAAGATGATGAACTT  175
            ||||||||||||||| ||||  ||||||||||||||||||||||||||||||||||||||
Sbjct  462  GATGATGATAAGATCACTTTAGAAGGAGAATTTACACAAAGCAAACAAGATGATGAACTT  521

Query  176  GTCGATCCAACTGCAGACTCGTCGATTGAACCAGAAACATCATCTGGTATAAGCGATGAC  235
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  522  GTCGATCCAACTGCAGACTCGTCGATTGAACCAGAAACATCATCTGGTATAAGCGATGAC  581

Query  236  ATCAAGATGCAGGATGCAGA-AAAGAAACATTATCATCAAAATCTATCGAGGAGGAGGAT  294
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  582  ATCAAGATGCAGGATGCAGATAAAGAAACATTATCATCAAAATCTATCGAGGAGGAGGAT  641

Query  295  TCGAGTGAGGAAAAGGCACTCAAGAAGCCTGATAAATTGATTCCATGTCCGCGATGTAAT  354
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  642  TCGAGTGAGGAAAAGGCACTCAAGAAGCCTGATAAATTGATTCCATGTCCGCGATGTAAT  701
```

Figure 6.12 Detailed alignment of query sequence with the homologous sequence using BLAST.

6.4.2.1.2. Clustal omega

It is the most widely used bioinformatics tool for multiple sequence alignment of the query sequence with published sequences. Alignments show the significant relationship between two sequences. It is also important for the protein structure and function analysis for future work. Clustal omega also uses heuristic method for the alignment results. Heuristic or best guess method is one of most accurate and efficient algorithms for alignment of the sequences (Daugelaite et al. 2013). The obtained sequence was aligned with the sequence obtained from PGCS (PGSC0003DMC400032069). Pairwise alignment tool was used for the alignment analysis. The query sequence showed 99% similarity or homology with the published sequence (**Fig 6.13**).

```
CLUSTAL O(1.2.2) multiple sequence alignment


st       -ATGTCTGAAGTTAGAGATCCTGCTATTAAGCTGTTTGGTAAAACAATTGGTATGACACA
KS       GATGTCTGAAGTTAGAGAT-CTGCTATTAAGCTGTTTGGTAAAACAATTGGTATGACACA
          ****************** ****************************************

st       ACAAGAAACCAATTGTGTTTATCTTCATGATGATCATACAACCTCATCCCCTCTTTCAAT
KS       ACAAGAAACCAATTGTGTTTATCTTCATGATGATCATACAACCTCATCCCCTCTTTCAAT
         ************************************************************

st       TGATGATGATAAGATCACTTTAGAAGGAGAATTTACACAAAGCAAACAAGATGATGAACT
KS       TGATGATGATAAGATCACTTT--AAGGAGAATTTACACAAAGCAAACAAGATGATGAACT
         ********************  ***********************************

st       TGTCGATCCAACTGCAGACTCGTCGATTGAACCAGAAACATCATCTGGTATAAGCGATGA
KS       TGTCGATCCAACTGCAGACTCGTCGATTGAACCAGAAACATCATCTGGTATAAGCGATGA
         ************************************************************

st       CATCAAGATGCAGGATGCAGATAAAGAAACATTATCATCAAAATCTATCGAGGAGGAGGA
KS       CATCAAGATGCAGGATGCAGAAA-AGAAACATTATCATCAAAATCTATCGAGGAGGAGGA
         **********************:* ***********************************

st       TTCGAGTGAGGAAAAGGCACTCAAGAAGCCTGATAAATTGATTCCATGTCCGCGATGTAA
KS       TTCGAGTGAGGAAAAGGCACTCAAGAAGCCTGATAAATTGATTCCATGTCCGCGATGTAA
         ************************************************************

st       TAGCATGGAAACAAAGTTCTGTTATTACAACAATTACAACGTCAACCAGCCTCGTTACTT
KS       TAGCATGGAAACAAAGTTCTGTTATTACAACAATTACAACGTCAACCAGCCTCGTTACTT
         ************************************************************

st       CTGCAAGAACTGCCAGAGATATTGGACTGCTGGAGGGACAATGAGAAATGTGCCTGTGGG
KS       CTGCAAGAACTGCCAGAGATATTGGACTGCTGGAGGGACAATGAGAAATGTGCCTGTGGG
         ************************************************************

st       ATCTGGTCGCCGAAAGAACAAGAGTTCTTCCATTTCAAATTATCCTCTTCAAGCAGGTCG
KS       ATCTGGTCGCCGAAAGAACAAGAGTTCTTCCATTTCAAATTATCCTCTTCAAGCAGGTCG
         ************************************************************
```

```
st      GGTCGAAGCAGCAGCTCACGGAATGCATCTTCCTGCTTTAAGGACAAATGGAACTGTCCT
KS      GGTCGAAGCAGCAGCTCACGGAATGCATCTT-CTGCTTTAAGGACAAATGGAACTGTCCT
        ****************************** ***************************

st      TACATTTGGATCAGATAAACCCCTTTGTGATTCAATGGTTTCTGCATTGAACTTAGCTGA
KS      TACATTTGGATCAGATAAACCCCTTTGTGATTCAATGGTTTCTGCATTGAACTTAGCTGA
        ************************************************************

st      GAATTCACATAATATGAATCGAAATGAATTCCATGGATCCGAACGAAGAATGCCTGCAAT
KS      GAATTCACATAATATGAATCGAAATGAATTCCATGGATCCGAACGAAGAATGCCTGCAAT
        ************************************************************

st      CGGGAATGATCAATCAAATGGAACTTGTAGTACAGCCTCAAGTGTAACTGACAAAGAAAG
KS      CGGGAATGATCAATCAAATGGAACTTGTAGTACAGCCTCAAGTGTAACTGACAAAGAAAG
        ************************************************************

st      CAGTGCTGGTACTCATGATTTAGCAAATTGGAATAATTTCCAGCCATTTCCTCCTCAAGT
KS      CAGTGCTGGTACTCATGATTTAGCAAATTGGAATAATTTCCAGCCATTTCCTCCTCAAGT
        ************************************************************

st      ACCGTACTTTCAGGGCGCTCCGTGGCCTTATTCTGGCTTTCCAGTATCATTCTATCCAGC
KS      ACCGTACTTTCAGGGCGCTCCGTGGCCTTATTCTGGCTTTCCAGTATCATTCTATCCAGC
        ************************************************************

st      AACACCGTACTGGGGCTGCACCGTAGCAAACCCTTGGAACGTACCTTGGCTTTCTTCTGA
KS      AACACCGTACTGGGGCTGCACCGTAGCAAACCCTTGGAACGTACCTTGGCTTTCTTCTGA
        ************************************************************

st      TCAATCATCAGTCCAGAACAACAGTCCTACTTCACCAACATTAGGAAAACATTCTCGGGA
KS      TCAATCGTCAGTCCAGAACAACAGTCCTACTTCACCAACATTAGGAAAACATTCTCGGGA
        ******.*****************************************************

st      TGAAAGCAAGATTGATCCATCACAATCACGGAGAAGAGATGCTAATTTGCAGAATAGAGA
KS      TGAAAGCAAGATTGATCCATCACAATCACGGAGAAGAGATGCTAATTTGCAGAATAGAGA
        ************************************************************

st      AGGCGAGAGATGTGTACTGATTCCAAAGACATTAAGGATTCATGATCCAAATGAAGCGGC
KS      AGGCGAGAGATGTGTACTGATTCCGAAGACATTAAGGATTCATGATCCAAATGAAGCGGC
        ************************.***********************************

st      TAAAAGCTCTATATGGTCAACACTAGGTATCAGGAATGAGAAGATTGATTCGGCTCGTGG
KS      TAAAAGCTCTATATGGTCAACACTAGGTATCAGGAATGAGAAGATTGATTCAGCTCGTGG
        ************************************************.********

st      TACAATGCTCTTCAGTGCCTTCAATCCAAAAGCTGATCATAGAAATCGCGAACATGACAC
KS      TACAATGCTCTTCAGTGCCTTCAATCCAAAAGCTGATCATAGAAATCGCGAACATGACAC
        ************************************************************

st      TTCTTTTGCCCTGCAAGCTAATCCAGCAGCCTTGTCTAGATCACTTCATTTCCGCGAGAG
KS      TTCTTTTGCCCTGCAAGCTAATCCAGCAGCCTTGTCTAGATCACTTCATTTCCGCGAGAG
        ************************************************************

st      CACACAATGA
KS      CACACAATGA
        **********
```

Figure 6.13 Pairwise alignment of the query sequence with the published sequence obtained from PGSC.

6.5. Virus Induced Gene Silencing

VIGS has been frequently used to validate the role of the candidate gene in the abiotic stress (Ramegowda et al. 2014). Before proceeding to the downstream process for the overexpression of StCDF1.2, VIGS was performed for the functional validation of this gene in tuberization. TRV-VIGS mediated silencing of StCDF1.2 was performed and validated by q-RTPCR.

6.5s.1. Identification of the target sequence

Selection of the target sequence in VIGS protocol for the construct development is very crucial. Target sequence should produce maximum number of siRNA (**Fig 6.14**) with no off targets for gene silencing. To increase the efficiency of the silencing, length of the sequence should be less than 1kb and more than 100bp (Senthil-Kumar and Mysore, 2014).The CDS regions (**Fig 6.15**) that can produce the siRNA were selected from gene sequences by using bioinformatics tools siDirect (http://sidirect2.rnai.jp/) and siRNA-scan (http://bioinfo2.noble.org/RNAiScan.htm).

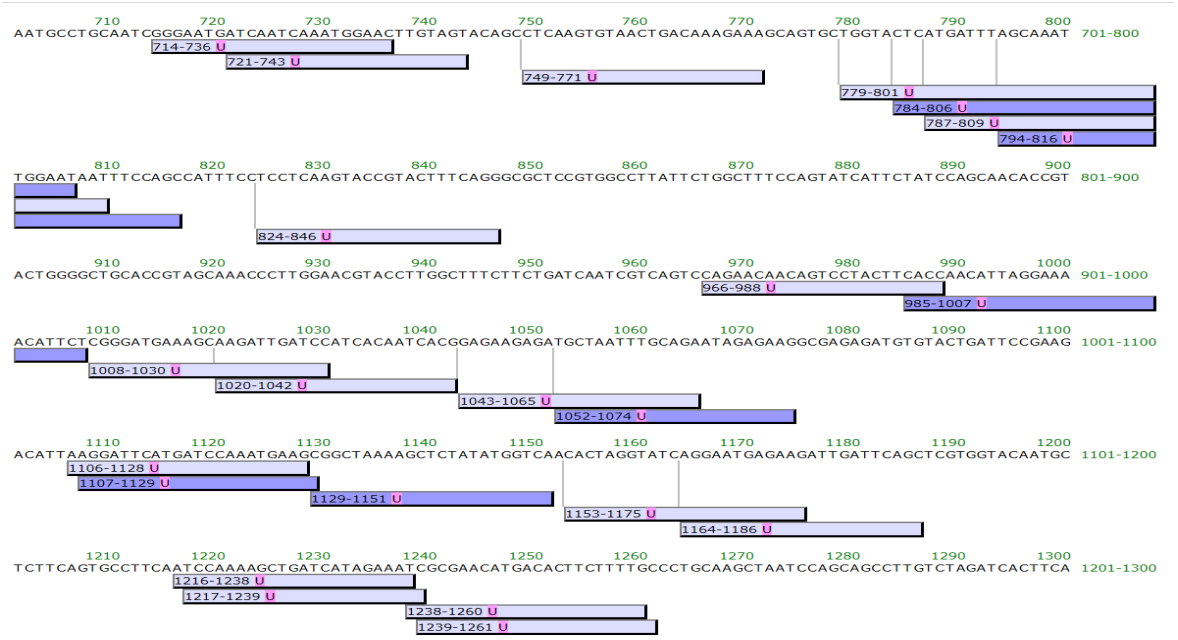

Figure 6.14 Graphical representation of the siRNAs produced using bioinformatics tool.

ATGTCTGAAGTTAGAGATCCTGCTATTAAGCTGTTTGGTAAAACAATTGGTATGACACAACAAGAAA
CCAATTGTGTTTATCTTCATGATGATCATACAACCTCATCCCCTCTTTCAATTGATGATGATAAGATCA
CTTTAGAAGGAGAATTTACACAAAGCAAACAAGATGATGAACTTGTCGATCCAACTGCAGACTCGTC
GATTGAACCAGAAACATCATCTGGTATAAGCGATGACATCAAGATGCAGGATGCAGATAAAGAAAC
ATTATCATCAAAATCTATCGAGGAGGAGGATTCGAGTGAGGAAAAGGCACTCAAGAAGCCTGATAA
ATTGATTCCATGTCCGCGATGTAATAGCATGGAAACAAAGTTCTGTTATTACAACAATTACAACGTCA
ACCAGCCTCGTTACTTCTGCAAGAACTGCCAGAGATATTGGACTGCTGGAGGGACAATGAGAAATGT
GCCTGTGGGATCTGGTCGCCGAAAGAACAAGAGTTCTTCCATTTCAAATTATCCTCTTCAAGCAGGTC
GGGTCGAAGCAGCAGCTCACGGAATGCATCTTCCTGCTTTAAGGACAAATGGAACTGTCCTTACATTT
GGATCAGATAAACCCCTTTGTGATTCAATGGTTTCTGCATTGAACTTAGCTGAGAATTCACATAATAT
GAATCGAAATGAATTCCATGGATCCGAACGAAGAATGCCTGCAATCGGGAATGATCAATCAAATGGA
ACTTGTAGTACAGCCTCAAGTGTAACTGACAAAGAAAGCAGTGCTGGTACTCATGATTTAGCAAATT
GGAATAATTTCCAGCCATTTCCTCCTCAAGTACCGTACTTTCAGGGCGCTCCGTGGCCTTATTCTGGCT
TTCCAGTATCATTCTATCCAGCAACACCGTACTGGGGCTGCACCGTAGCAAACCCTTGGAACGTACCT
TGGCTTTCTTCTGATCAATCATCAGTCCAGAACAACAGTCCTACTTCACCAACATTAGGAAAACATTC
TCGGGATGAAAGCAAGATTGATCCATCACAATCACGGAGAAGAGATGCTAATTTGCAGAATAGAGA
AGGCGAGAGATGTGTACTGATTCCAAAGACATTAAGGATTCATGATCCAAATGAAGCGGCTAAAAGC
TCTATATGGTCAACACTAGGTATCAGGAATGAGAAGATTGATTCGGCTCGTGGTACAATGCTCTTCAG
TGCCTTCAATCCAAAAGCTGATCATAGAAATCGCGAACATGACACTTCTTTTGCCCTGCAAGCTAATC
CAGCAGCCTTGTCTAGATCACTTCATTTCCGCGAGAGCACACAATGA

Figure 6.15 Targeted gene fragment from the complete StCDF1 gene sequence for VIGS (Highlighted in yellow).

6.5.2. Cloning of target StCDF1.2-VIGS in pTRV2

Putative full-length StCDF1.2-VIGS was PCR amplified from the plasmid of pJET1.2/blunt::StCDF1.2 by using gene specific primers (StCDF1-VIGS F and StCDF1-VIGS R) flanking enzyme sites for ***EcoRI*** and ***BamHI***. PCR amplified fragment of 530bp was observed on 1.3% agarose gel (**Fig 6.16**). After confirmation on gel electrophoresis, the amplified PCR fragment was extracted from gel and eluted using Qiagen gel extraction kit. The eluted gene fragment was then cloned in pTRV2-VIGS vector. pTRV2 contains the non-essential structural genes which are replaced by cloning site for the DNA insert. It contains MCS (multiple cloning site) between CaMV35S (cauliflower mosaic virus) enhancer and NOS (nopaline synthase terminator).The cloned gene fragment was then transformed with vector into high efficiency competent *E. coli* cells (DH5α) by heat shock method. For the screening of the transformed colonies KanR gene is present in pTRV2 vector, which encode for

After transformation, bacterial cells were plated on LB media containing kanamycin and incubated at 37ºC overnight.

6.5.3. Analysis of recombinant clones

6.5.3.1. Restriction Analysis

Plasmid from overnight grown culture of positive clones harbouringVIGS-pTRV2:: StCDF1.2 gene cassette was isolated by alkaline lysis method using Qiagen kit and concentration was determined spectrophotometrically (Table 6.2). Isolated plasmids were subjected to restriction digestion using ***EcoRI*** and ***BamHI*** enzymes. In pTRV2 vector restriction site for ***EcoRI*** is present at 1647bp and ***BamHI*** site is at 1671 bp. Analysis of restriction digestion products on 1.3% agarose gel electrophoresis confirmed the presence of stCDF1.2-VIGS in pTRV2 vector (**Fig 6.17**).

Table 6.2 Quantitative and Qualitative estimation of plasmid isolated from *E. Coli* StCDF1.2-VIGS-pTRV2.

S.no.	Sample	Conc. (ng/µl)	A_{260}	A_{280}	A_{260}/A_{280}
1	CDF-VIGS 1	159.8	3.196	1.703	1.88
2	CDF-VIGS	284.8	5.696	3.047	1.87

6.5.4. Agrobacterium transformation of stCDF1.2-VIGS-pTRV2 gene

Plasmid from overnight grown culture of *E. coli* positive clones harbouring pTRV2::StCDF1.2-VIGSgene cassette was isolated by alkaline lysis method using Qiagen kit. The plasmid from positive clones was then transformed into high efficiency competent *A. tumefaciens* cells (EHA105) by freeze thaw method. EHA105 is the frequently used *A. tumefaciens* strain in the plant biotechnology. It is derived from *A. tumefaciens*EHA101 by deleting nptII (for kanamycin resistance) gene from Ti plasmid by site-directed mutagenesis (Chetty et al. 2013). LB media containing kanamycin/rifampicin and incubated at 28ºC.

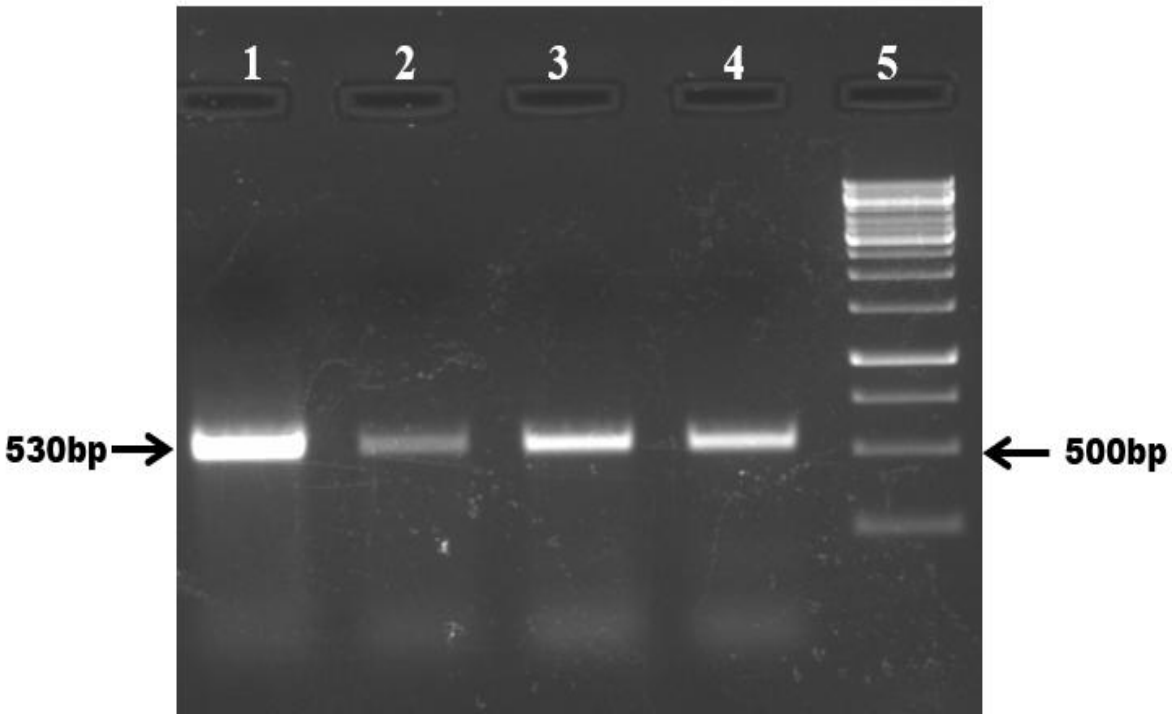

Figure 6.16 Agarose gel electrophoresis of PCR amplification of StCDF1.2-VIGS.1) Sample 1 **2)** Sample 2 **3)** sample 3 **4)** Sample 4 **5)** Ladder 1kb

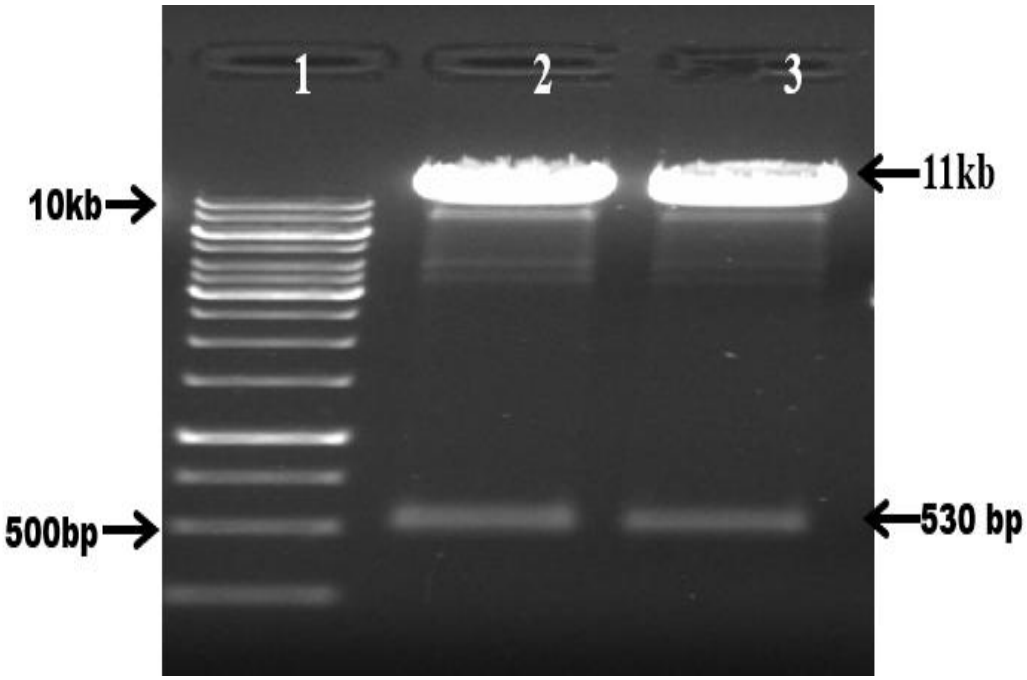

Figure 6.17 Restriction digestion of *E. coli* harbouring pTRV2::StCDF1.2-VIGS gene cassette.1) Ladder (1kb) **2)a)** cut vector pTRV2**b)** stCDF-VIGS (gene) fragment (530bp) **3)a)** cut vector pTRV2**b)** stCDF-VIGS (gene) fragment (530bp)

As kanamycin was used as selectable marker for the screening of the transformants, EHA105 was the most suitable strain for transformation. After transformation, bacterial cells were plated on

6.5.4.1. Analysis of recombinant clones

6.5.4.1.1 Colony PCR

Bacterial colonies were directly subjected to PCR for initial screening by using gene specific primer. The PCR amplified products were analysed on 1.3% agarose gel and fragment of size 530bp was observed (**Fig 6.18**). Out of five colonies screened by colony PCR, two were selected for further confirmation.

6.5.4.1.2. Plasmid PCR

After confirmation from colony PCR, plasmid from overnight grown culture of two positive clones harbouring pTRV2::StCDF1.2-VIGSgene cassette was isolated by alkaline lysis method and concentration was determined spectrophotometrically (Table 6.3). Isolated plasmids were subjected to PCR using gene specific primer. The PCR amplified products were analyzed on 1.3% agarose gel and fragment of size 530bp was observed (**Fig 6.19**). This reconfirms the transformation of pTRV2::StCDF1.2-VIGSgene cassette in the *A. tumefaciens* EHA105.

Table 6.3 Quantitative and Qualitative estimation of plasmid isolated from *A. tumefaciens* harbouring pTRV2::StCDF1.2-VIGS.

S.no.	Sample	Conc. (ng/µl)	A_{260}	A_{280}	A_{260}/A_{280}
1	Colony 1	17	0.34	0.18	1.9
2	Colony 2	14.5	0.28	0.15	1.9

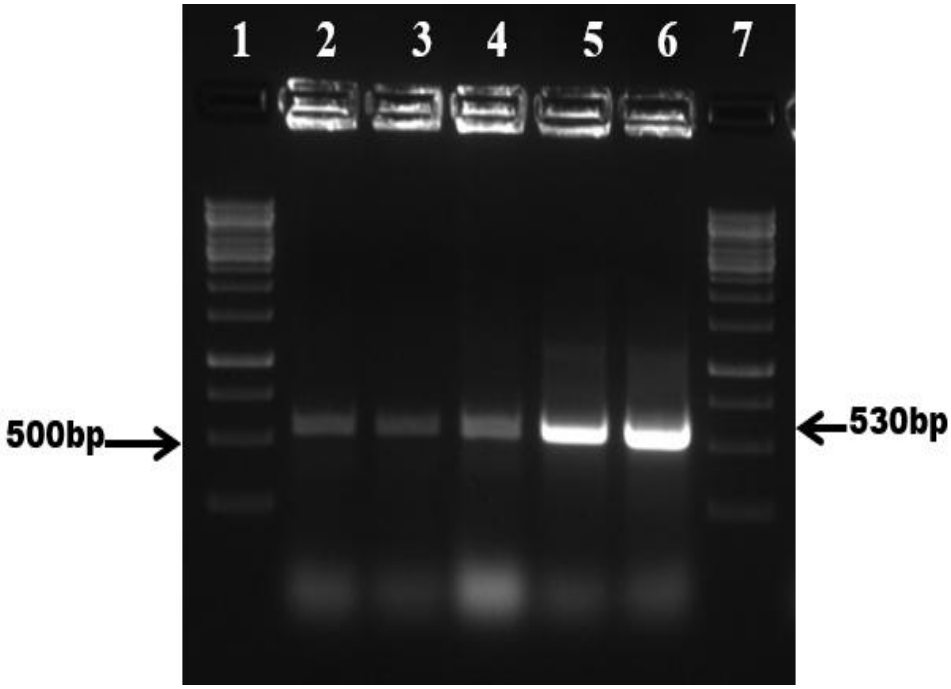

Figure 6.18Agarose gel electrophoresis of *A. tumefaciens* colony PCR for screeningpTRV2::StCDF1.2-VIGS positive clones.1) Ladder 1kb **2)** Colony 1 **3)** Colony 2 **4)** Colony 3 **5)** Colony 4 **6)** Colony5**7)** Ladder 1kb

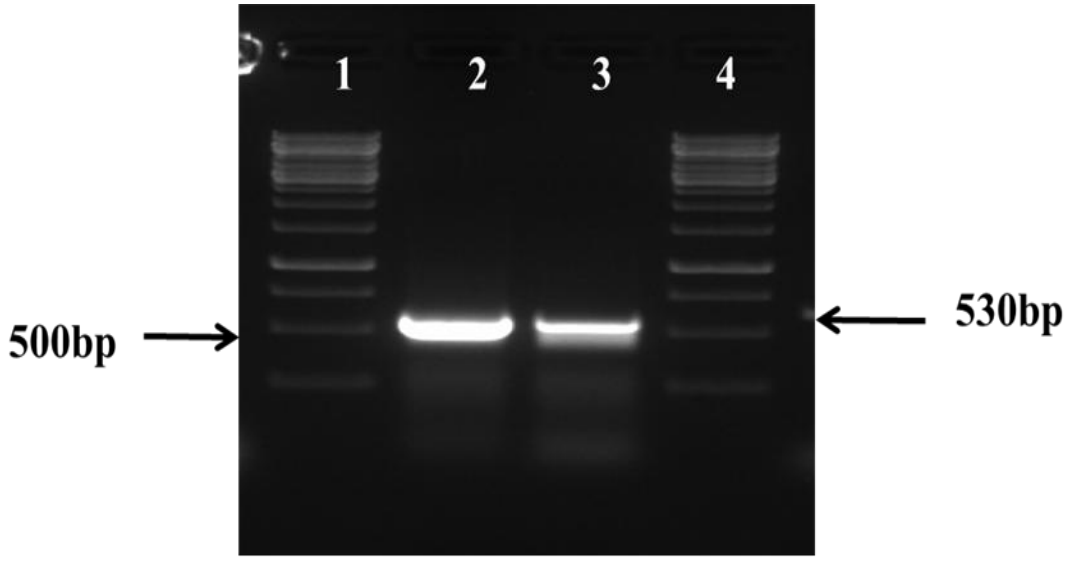

Figure 6.19Agarose gel electrophoresis of *A. tumefaciens* plasmid PCR for pTRV2::StCDF1.2-VIGS.1) Ladder (1kb) **2)** Colony 1 **3)** Colony 2**4)** Ladder (1kb)

6.5.5. Plant transformation of stCDF-VIGS-pTRV2 cassette

For transformation of the KS plants, LB broth was inoculated using *A. tumefaciens* culture containing StCDF1.2-VIGS-pTRV2 cassette and pTRV1. pTRV is a bipartite vector hence pTRV1 is required for the movement of the vector after plant transformation. There are three methods available for plant inoculation: Syringe inoculation, agrodrench and prick inoculation. For the large-scale experiments, prick inoculation is efficient for inoculating large sample size. Syringe inoculation and agrodrench are for smaller sample size, so we used these methods for inoculation. For the inoculating a fixed amount of the inoculum to the plants syringe inoculation method is very useful. Both methods are very efficient, but agrodrench enhances the efficacy by involving complete plants. Agrodrench method is also easy to perform, as culture just has to drench on plants.

6.5.6.Validation of silencing

6.5.6.1. Quantification of gene expression in VIGS treated Plants

The expression of target gene StCDF1.2 was reduced in VIGS plants, compared to control (6:00 am). Reduced expression of StCDF1.2 in VIGS plant was observed at all-time points. The highest expression of 0.286 fold was observed at 6:00 am in VIGS plants. During dawn (6:00 am) the expression of StCDF1.2 was 0.741 fold higher than that of VIGS treated plants. Reduction in the expression by 0.263 fold was recorded in VIGS plant at noon (12:00 pm) when compared to the VIGS plants at dawn (6:00 am) (**Fig 6.20a**). The expression of genes acting downstream to StCDF1.2 (StSP6A, StSP5G and StCO) was also checked during the same time points. The expression of potato FT homologue StSP6A was down-regulated in correspondence to StCDF1.2 silencing. The expression of StSP6A was reduced by 0.5, 0.784 and 1.39 fold at 6:00 am, 12:00 pm and 6:00 pm respectively. While contrastingly the expression of StSP5G, another FT homologue was upregulated by (2 fold) in VIGS treated plants during noon (12:00 pm) (**Fig 6.20b**). The silencing of StCDF1.2 resulted in the 0.6 fold upregulation of StCO, expression, which causes the upregulation of StSP5G expression. Expression analysis studies revealed a downregulation in the expression of StSP6A and upregulation in StSP5G and StCO

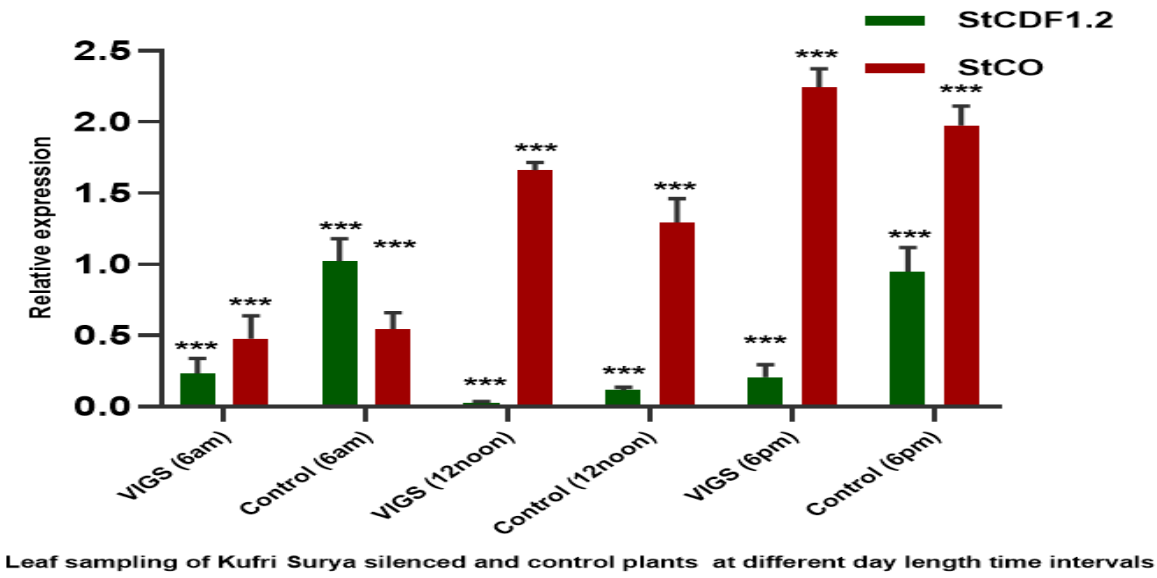

Figure 6.20a Gene expression analysis of StCDF1 and StCO in silenced and control potato plants by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates. Three time points for leaf harvest (time is shown on the x axis) from the plants.

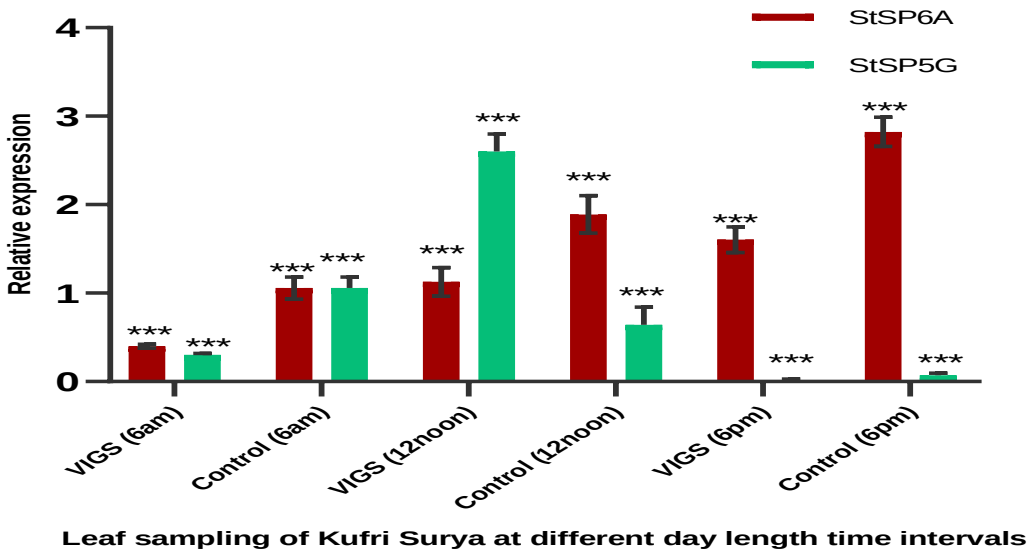

Figure 6.20b Gene expression analysis of StCDF1 and StCO in silenced and control potato plants by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates. Three time points for leaf harvest (time is shown on the x axis) from the plants.

transcripts in VIGS plants. This could be due to the fact that StCDF1.2 has a central role in tuberization by indirectly controlling the expression of StSP6A, StSP5G and StCO. StCDF1.2 downregulates the expression of StCO, which then causes a suppression of StSP5G transcripts. This suppression of StSP5G induces the expression of a mobile tuberization signal, StSP6A which induces the tuber development in stolon tips (Koolerstman et al. 2013). Since there was a downregulation of StCDF1.2 in VIGS plants, StCO was relieved from its inhibition casing its upregulation. This indicates a conserved repressive function of potato StCDF1.2 on StCO. StCO now transcriptionally activates StSP5G, functions as a transcriptional repressor for StSP6A. This substantiates the notion that StCO does not directly affect the tuberization in non-inductive photoperiods, it indirectly regulates the expression of StSP6A via StSP5G (Navarro et al. 2011; Dutt et al. 2017).

6.5.6.2. Reconfirming the silencing of StCDF1.2 gene by Sanger Sequencing

The VIGS treated and non-treated samples were amplified by PCR using VIGS specific primers. The fragments were then purified by PCR and subjected to Sanger sequencing. The quality values of pure base of chromatograph showed Q value 5 and medium bases and exhibited the low quality amplification of the target gene due to interruption of mRNA sequence StCDF1.2 in comparison to the control sample sequences (**Fig. 6.21**) showed pure and high quality base value of 20 and above. Sanger sequencing is one way to reconfirm the silencing in VIGS plants.

6.5.6.3 Effect of StCDF1.2 on flowering

Regardless the upregulation of StCO and StSP5G transcripts in VIGS plants, there was no change in flowering, flower initiation, flower number and flowering pattern between VIGS and control plants. Since flowering is a multigenic trait and upregulation of StCO and StSP5G via gene silencing through VIGS is incomplete and transient. This silencing might have not complete effect on other key regulators of flowering, as its flowering signal transduction pathway is different from that of tuberization. We also observed no change in flowering pattern and flowering initiation in both control and VIGS plants. Similar results were observed by Koolerstman et al. (2013), where there was no difference in flowering between StCDF1.2 overexpressing

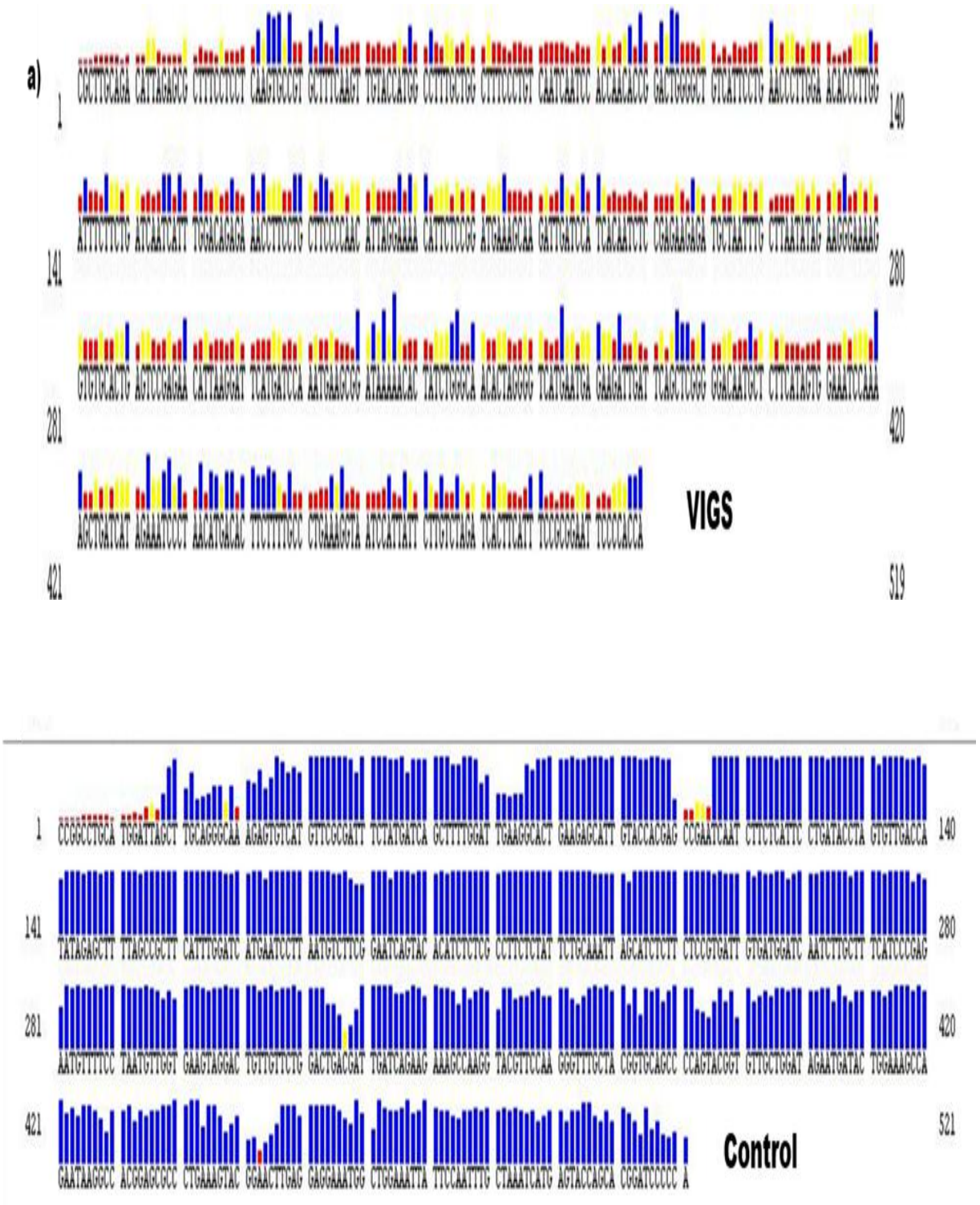

Figure 6.21 Representative Sanger sequencing electropherogram of sequences obtained after silencing of StCDF1.2 in K. Surya.

transgenic lines and non-transgenic control plants. These findings clearly establish a divergence in signalling pathways for both flowering and tuberization

6.5.6.4. Effect of StCDF1.2 on tuberization

Tubers were harvested from VIGS treated and control plants (95 days matured plants). Since there was a downregulation of StCDF1.2 and StSP6A expression in VIGS treated plants, they exhibited senescence while the control KS plants had more tubers (Table 6.4) and were phenotypically mature and healthy (**Fig. 6.21**).VIGS plants also showed delayed tuberization, indicating the important role of StCDF1.2 in tuberization initiation. Since other genes from the tuberization signalling cascade may also play an important role in tuber formation, a complete suppression in tuberization was not observed. Our present findings indicate the role of StCDF 1.2 gene in governing the initiation of tuberization. This conceptualizes the earliness and delayed tuberization pathway in potato cultivars. The same has been well demonstrated in earlier cDNA microarray studies showing the StSP5G and StSP6A gene expression in KS and KCM cultivars (Singh et al. 2015).

6.5.6.5. Effect of StCDF1.2 on senescence

Change in tuberization process can affect the overall plant physiology and growth, specifically plant senescence. The early growth stages (vegetative growth and flowering) of VIGS and control plants were similar. The difference was observed after induction of tuberization in both sets of plants. Silenced plants showed senescence, which could lead to a reduced number of tubers when compared to the control plants.

Table 6.4 Number of tubers harvested from silenced and control K. surya.

S.no	Potato cultivar	No. of tubers
1	Silenced	7
2	Control	20

Figure 6.22Tubers of Control and silenced K. Surya harvested after maturation.

Table 6.5 Quantitative and Qualitative estimation of *E. coli* plasmid isolated from the positive clones harbouring StCDF1.2-pRI101 AN.

S.no.	Sample	Conc. (ng/µl)	A_{260}	A_{280}	A_{260}/A_{280}
1	Colony 1	582.17	11.641	6.261	1.86
2	Colony 2	334.0	6.681	3.560	1.87

6.6. Subcloning of StCDF1 in pRI101-AN Binary vector

Plasmid isolated from positive clones harbouring pJET/Blunt1.2::StCDF1.2 gene cassette was digested using ***NdeI*** and ***SalI*** enzymes. The binary vector was also digested with same enzymes. While designing primers NdeI restriction site was added to the forward primer and SalI site was added to reverse primer. pRI101-AN is plasmid DNA series of the binary vector used for transgenic production in dicotyledons. The digested products were then cloned in pRI101AN binary cloning vector. It contains CaMV 35s promoter and 5'UTR (untranslated region) of ADH (alcohol dehydrogenase) gene from *A. thaliana*. The cloned product was then transformed with vector into high efficiency competent *E. coli* cells (DH5α) by heat shock method. After transformation, bacterial cells were plated on LB media containing kanamycin 100μg/ml and incubated at 37ºC.

6.6.1. Analysis of recombinant clones

6.6.1.1. Colony PCR

Transformed bacterial colonies (**Fig 6.23**) were subjected to PCR using CaMV 35S promoter forward and StCDF1 R primers. The PCR amplified products were analyzed on 1.3% agarose gel and fragment of size 2000bp was observed (**Fig6.24**). Ten colonies were PCR amplified and two positive colonies were selected for were confirmation.

6.6.1.2. Restriction Analysis

After confirmation from colony PCR, plasmid from overnight grown culture of positive clones harbouring pRI101::StCDF1.2 gene cassette was isolated by alkaline lysis method using Qiagen kit and concentration was determined spectrophotometrically (Table 6.5). Isolated plasmids were subjected to restriction digestion using ***Nde*I** and ***Sal* I** enzymes. Analysis of restriction digestion products on 1.3% agarose gel electrophoresis confirmed the presence of StCDF1.2 in pRI101 AN binary vector (**Fig 6.25**).

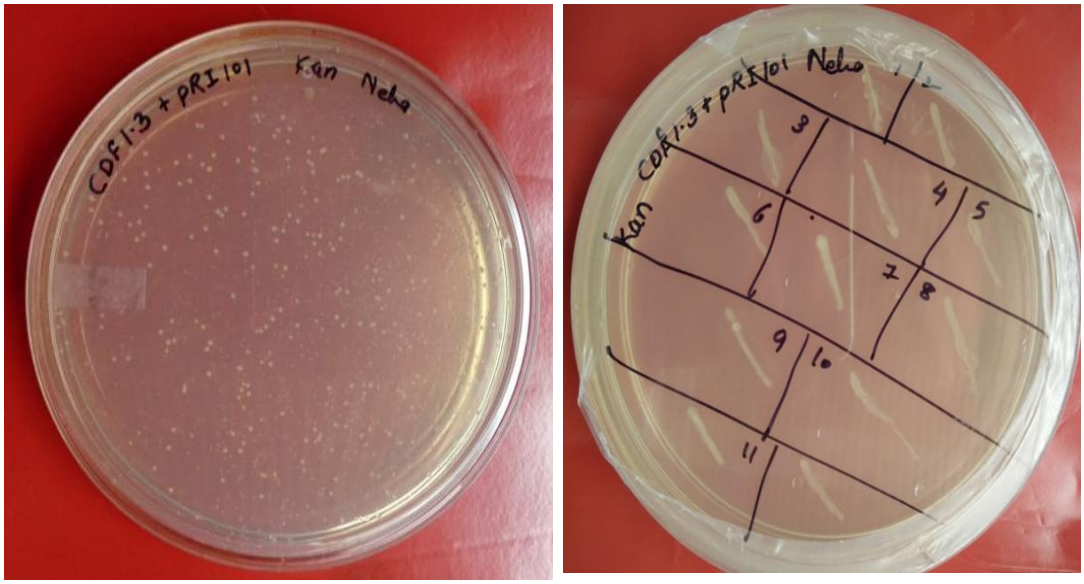

Figure 6.23 Colonies of *E. coli* St-CDF1.2-pRI101 AN transformants b) Colonies streaked on LB plate containing kanamycin100µg/ml

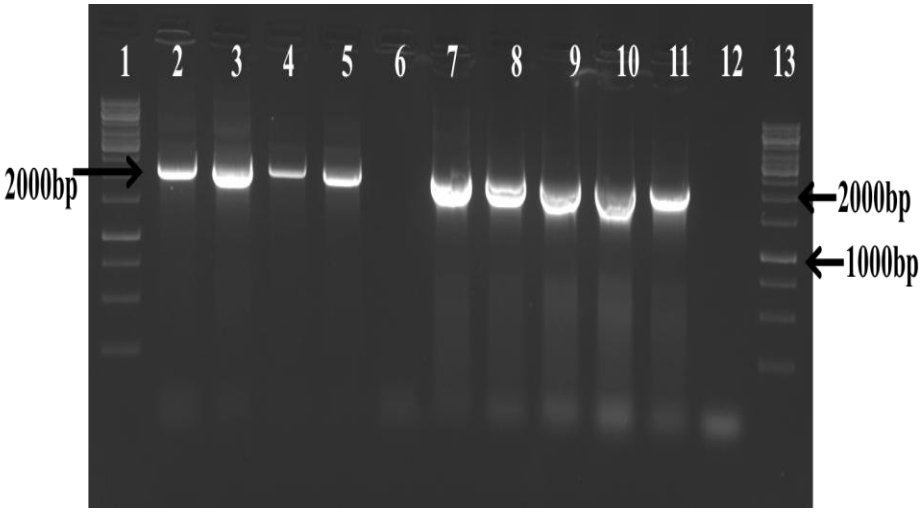

Figure 6.24 Agarose gel electrophoresis of *E.coli* StCDF-pRI101 AN colony PCR

1) Ladder (1kb) 2) Colony 1 3) Colony 2 4) Colony 3 5) Colony 4 6) Colony 5 7) Colony 6 8) Colony 7 9) Colony 8 10) Colony 9 11) Colony 10 12) Colony 11 13) Ladder (1kb)

6.6.2. Agrobacterium Transformation of pRI101-StCDFgene

Plasmid from overnight grown culture of positive clones harbouring stCDF-pRI101 AN gene cassette was isolated by alkaline lysis method using Qiagen kit. The cloned product was then transformed with vector into high efficiency competent *A. tumefaciens* cells (EHA105) by freeze thaw method. After transformation, bacterial cells were plated on LB media containing kanamycin (100µg/ml)/ rifampicin (50µg/ml) and incubated at 28ºC.

6.6.2.1. Analysis of recombinant clones

6.6.2.1.1. Colony PCR

Transformed colonies (**Fig 6.26**) were subjected to PCR using CAMV 35S promoter and NOS terminator. The amplified products were analyzed on 1.3% agarose gel and amplified product of size 2300bp was observed (**Fig 6.27**).

6.6.2.1.2. Plasmid PCR

After confirmation from colony PCR, plasmid from overnight grown culture of positive clones harbouring StCDF-pRI101 AN gene cassette was isolated by alkaline lysis method and concentration was determined spectrophotometrically (Table 6.6). Isolated plasmids were subjected to PCR using CAMV 35S promoter and NOS terminator. The amplified products were analyzed on 1.3% agarose gel and amplified product of size 2300bp was observed (**Fig 6.28**).

Table 6.6 Quantitative and Qualitative estimation of *A. tumefaciens* plasmid isolated from the positive clones harbouring StCDF1.2-pRI101 AN

S.no.	Sample	Conc. (ng/µl)	A_{260}	A_{280}	A_{260}/A_{280}
1	Colony 1	17	0.34	0.18	1.9
2	Colony 2	14.5	0.28	0.15	1.9

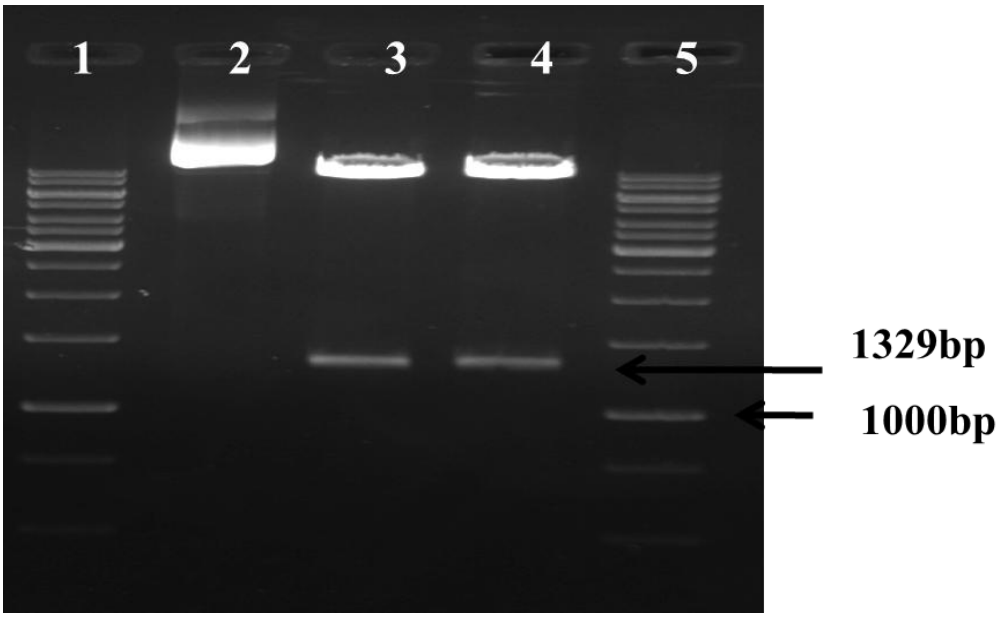

Figure 6.25Agarose gel electrophoresis of restriction digestion of *E. coli* StCDF1.2-pRI101 AN 1) Ladder (1kb) **2)** uncut plasmid colony 1 **3)** a)cut binary vector pRI 101 AN. b) StCDF(gene) fragment(1329bp) **4)** a)cut binary vector pRI 101 AN. b) StCDF(gene) fragment(1329bp)**5)** Ladder (1kb)

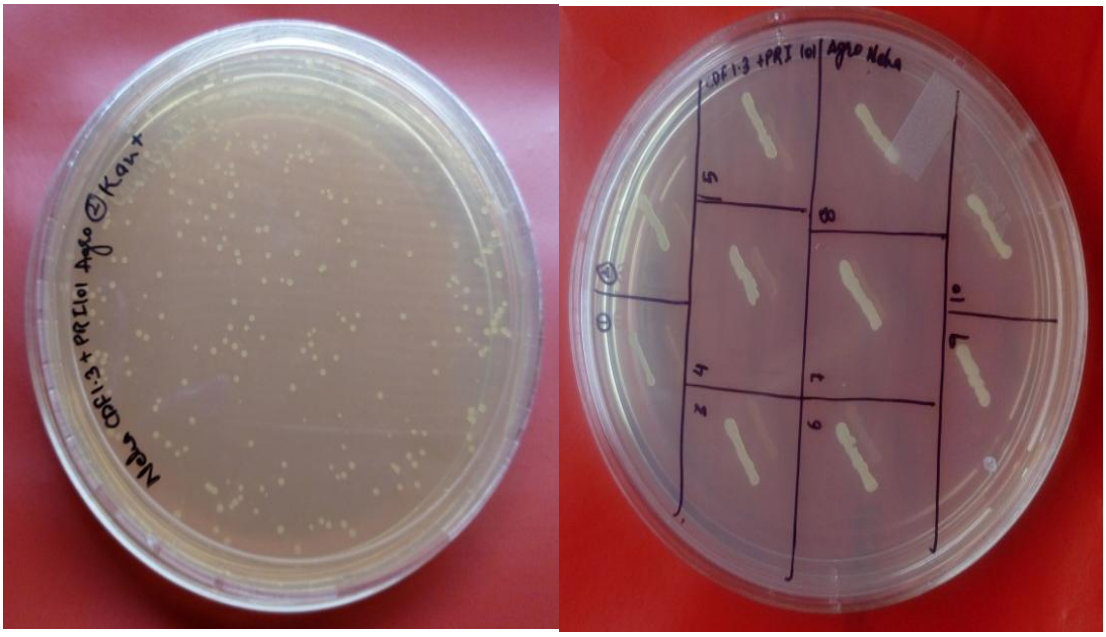

Figure 6.26 a) Colonies of*A. tumefaciens* St-CDF1.2-pRI101 AN transformants. b) Colonies streaked on LB plate containing kanamycin (50mg/ml) and rifampicin (50mg/ml)

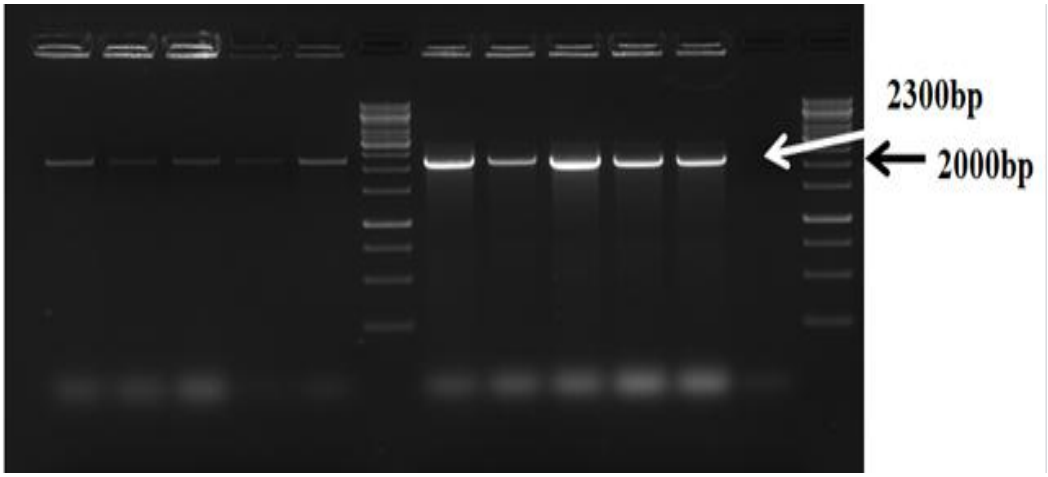

Figure 6.27Agarose gel electrophoresis of colony PCR of *A. tumefaciens*StCDF1.2-pRI101 AN 1) Ladder (1kb) 2) Colony 1 3) Colony 2 4) Colony 3 5) Colony 4 6) Colony 5 7) Colony 6 8) Colony 7 9) Colony 8 10) Colony 9 11) Colony 10 12) Colony 11 13) Ladder (1kb)

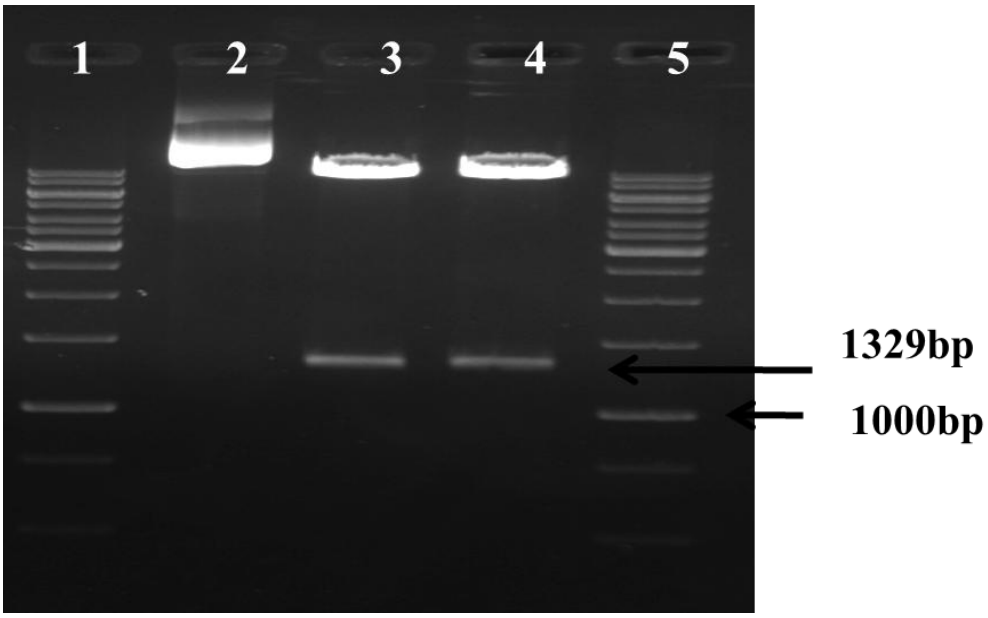

Figure 6.28Agarose gel electrophoresis of Plasmid PCR *A. tumefaciens*StCDF1.2-pRI101 AN 1) Ladder (1kb) 2) Colony 1 3) Colony 2 4) Ladder (1kb)

6.7 Plant transformation and regeneration

As potato is a dicot system, binary vector should contain suitable selectable marker and dicot promoter. Binary vector pRI-101 AN contain CaMV35s promoter, which is reported efficient in expressing target gene in potato cultivars (Bruce and Rupp, 2019). During transformation T-DNA from RB (Right border) to LB (Left border) was transferred via *A. tumefaciens* (Han et al. 2015). The Agrobacterium mediated plant transformation is reported to be highly efficient method for generation of stable transgenic plants. Traditional transformation method (Polyethylene glycol-mediated, pollen pathway) are not frequently used in plant transformation as these are laborious and efficiency is low (Hwang et al. 2015). Low copy number is important in transgenic production, physical transformation method (particle bombardment, electroporation) introduce large number of insert in host. Therefore Agrobacterium mediated transformation is the preferable method of plant transformation (Keshavareddy et al. 2018, Craze et al. 2018).

As reported in different studies on callus induction and shoot regeneration using different explants (Internode, leaf etc.), internodes are most efficient and frequently used explant in potato transgenic production (Mall et al. 2011). For the induction of the callus and shoot regeneration from internodes of K. Girdhari, different PGRs (Plant growth regulators) were used. Different concentration of these PGRs was added to MS media and their efficacy was studied. K. Girdhari is a late blight resistance and high yielding variety suitable for cultivation in the hills of India. Its long shelf life for the storage and high dry matter content makes it desirable variety (Joseph et al. 2011). It is reported to be recalcitrant for the callus induction and shoot regeneration in in-vitro conditions (Kumari et al. 2015). In this study, PGRs like 2,4D (2, 4-Dichlorophenoxyacetic acid), IAA (3-Indole acetic acid), NAA (1-Naphthalene acetic acid), Zeatin, Kinetin, TDZ (Thidiazuron), GA_3 (Gibberellin A3) and BAP (6-Benzylaminopurine) were used in different combinations. PGRs are the plant hormones (Auxin, cytokinin and gibberellins), which aid callus formation and shoot regeneration. Auxins (2,4D, IAA and NAA) added in plant tissue culture media for callus induction and initiate organ formation. Cytokines (BAP, TDZ, Zeatin and kinetin) stimulate and complete the organ formations specially shoot formation.

Table 6.7 Effect of PGRs (BAP+NAA) on callus formation and shoot regeneration.

S.NO.	PGRs (mg/L)		Callus	Shoot regeneration
	BAP	NAA		
1	4	6	No	No
2	6	8	No	No
3	8	10	Yes	No
4	10	12	Yes	Yes
5	12	14	No	No

Table 6.8 Effect of PGRs (BAP+NAA+GA_3) on callus formation and shoot regeneration.

S.NO.	PGRs (mg/L)			Callus	Shoot regeneration
	BAP	NAA	GA_3		
1	4	6	0.1	No	No
2	6	8	0.2	No	No
3	8	10	0.3	Yes	Yes
4	10	12	0.4	No	Yes
5	12	14	0.5	No	No

Table 6.9 Effect of PGRs (BAP+IAA+GA_3) on callus formation and shoot regeneration.

S.NO.	PGRs (mg/L)			Callus	Shoot regeneration
	BAP	IAA	GA3		
1	4	6	0.1	No	No
2	6	8	0.2	Yes	No
3	8	10	0.3	No	Yes
4	10	12	0.4	No	Yes
5	12	14	0.5	No	No

Gibberellins (GA_3) are found to be effective in potato transformation for the stem elongation (Bruce and Rupp, 2019).

In media containing 2,4D alone (0.5 mg/L, 1mg/L, 1.5mg/L and 2mg/L), no shoots were observed after callus induction. 2,4D was found efficient in inducing callus in short period of 20 days. But, when these calluses were transferred to shoot regeneration media, organ formation was hampered and callus turned brown. Other combination such as BAP+ NAA, BAP+NAA+GA_3, Zeatin+GA_3 with the antibiotic as selectable marker were also found ineffective in K. Girdhari transformation (Han et al.2015, Davidson et al. 2004).When BAP was used with NAA and antibiotic (Kanamycin) callus and shoot regeneration was observed, when concentration of both were more than 9mg/L (Table 6.7). But shoots were fragile and browning started after transferring in tubes containing MS media with antibiotic. In another experiment, GA_3 was added to above combination (BAP+NAA), GA_3 was found to be effective at 0.3mg/L and 0.4 mg/L (Table 6.8). Shoot regenerated at similar concentration, but regenerated shoots were sturdy and responded further in rooting media also. Combination of BAP+IAA+ GA_3 was found efficient in comparison to the other PGRs combination used. Shoot regeneration (**Fig 6.29**) was initiated after 45 days of subculturing in MS media plates (containing BAP + IAA + GA3 + Kanamycin + cefotaxime + Carbellicin) (Table 6.9). Whereas, in other combinations shoot regeneration initiated after 50 days. Direct shoot regeneration is preferred over callus induction for stable and efficient transformation (Dandekar and Fisk, 2005). Kumlay and Ercili (2015) also observed the use of GA_3 and IAA reduces the duration of shoot regeneration in comparison to other PGRs combination in MS media.

After shoot regeneration (**Fig 6.30**), selection of transgenic with target gene and parental elite genes are desirable. Somaclonal variations which occur during tissue culture limit this effort (Han et al. 2015). The 'off-types' formed due to somaclonal variation could be genetically or phenotypically modified from the desired transgenic. Duration of cultivation in plant tissue culture facility and culture room conditions are reported to alter somaclonal variation frequency. In case of potato, genetic makeup of cultivar also modifies frequency of variation (Keshavareddy et al. 2018). Barrel and Conner (2011) reported initial shoots regenerated from callus have more chances to be

somaclonal variant. Later shoots from same callus are phenotypically normal or less mutated. So, regeneration of multiple shoots from same callus is advised to get phenotypically desired plants.

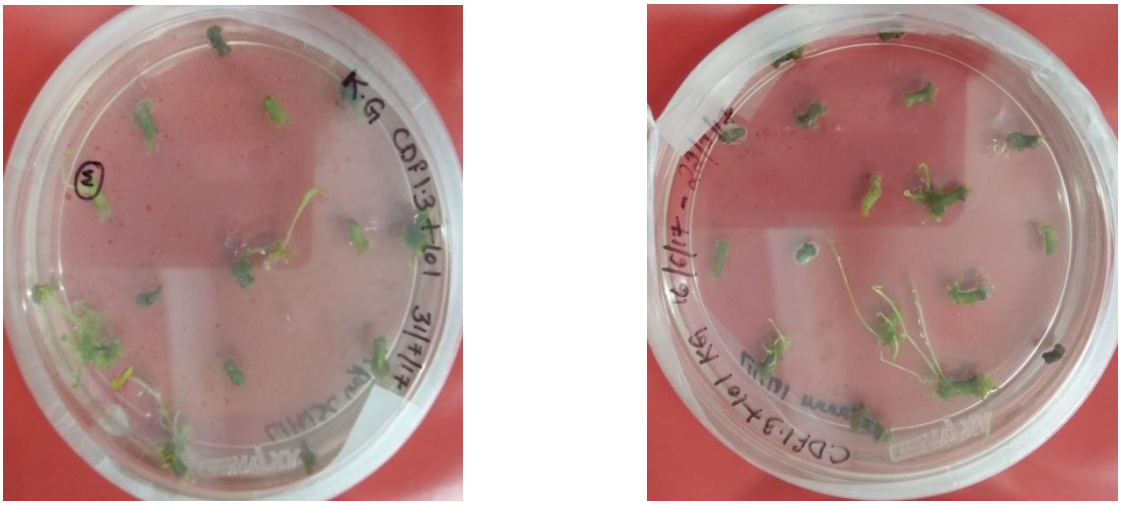

Figure 6.29 Shoot regeneration in MS media containing PGRs of potato cultivar K. Girdhari.

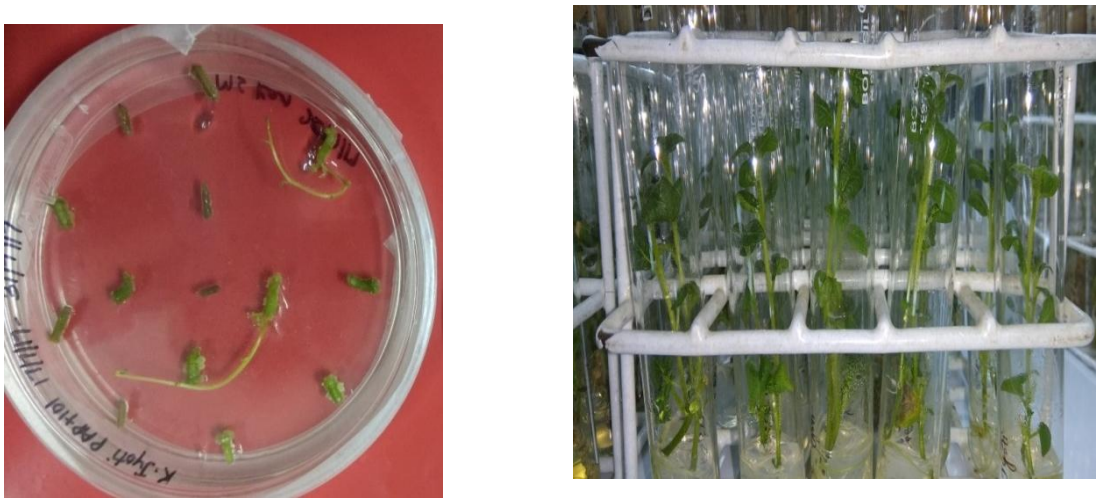

Figure 6.30 a) Shoot regeneration in MS media containing PGRs of potato cultivar K. Girdhari. b) *in vitro* rooting

6.7.1 Primary screening

For primary screening of regenerated StCDF1.2-pRI 101 AN lines presence of nptII was analysed. pRI10-AN contain NeoR gene, which confers resistance against kanamycin and neomycin (www.snspgene.com). Kanamycin was used in all early selection medias for bacteria's. In regenerated plants primer specific to this gene was used for screening (Meiyalaghan et al. 2011). DNA from all lines was subjected to PCR and product was electrophoresed on gel (**Fig 6.31**). Amplified product of 700 bp was observed on electrophoresis confirms the transformation (**Fig 6.32**). Out of 90 rengenerated lines, 55 were found positive in primary screening. These 55 transformed lines were used in further screening. Transformation effcicency was calculated;

$$\text{Transformation efficiency} = \frac{\text{No. transformed plantlets X 100}}{\text{Total no. of regenerated plantlets}}$$

Transformation efficiency of 61% was calculated by using abovementioned formula. Donmez et al. (2019) reported that internodal explant shows higher transformation efficiency in comparison to leaf explant. Callus induction of 33% was observed, using AGL1 strain of Agrobacterium in cv. Desiree. In another study conducted by Chetty et al. (2013), EHA105 was found to be most efficient Agrobacterium strain. Transformation rate of 65% was reported using this Agrobacterium strain. Bakhsh et al. (2014), observed highest transformation using CaMV35s promoter for potato transformation, as this is a ubiquitous expression vector for dicot transformation. Han et al. (2015) used cv. Altantic and Jowon to study transformation efficiency and effect of *in-vitro* cultivation duration on transformation. Altantic transformation efficiency depends on duration of in-vitro cultivation, whereas Jowon efficiency was no affected. In another study, sixteen varieties of potato were transformed using two different protocols. On calculating both regeneration capacity and transformation efficiency, no correlation can be interpreted. On molecular screening of transformants using nptII gene highest transformation of 90% was observed (Heeres et al. 2002).

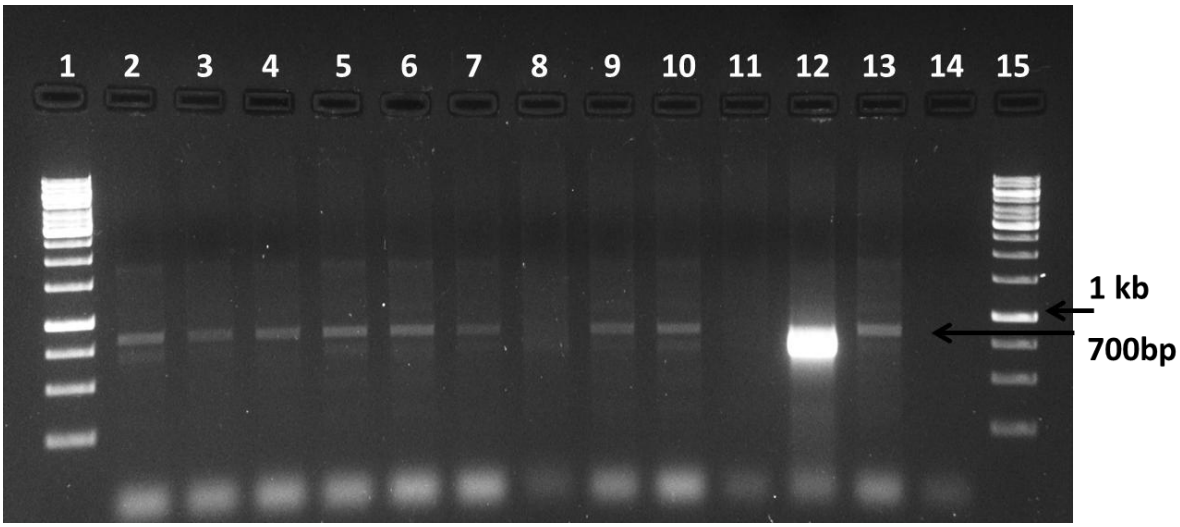

Figure 6.31Agarose gel electrophoresis of nptII PCR for primary screening of StCDF1.2-pRI101 AN transgenic lines 1) Ladder (1kb) 2) A1 3) C1 4) E1 5) F1 6) G1 7) H1 8) I1 9) J1 10)L1 11) K1 10) M1 11) S1 12) T1 13)T2 14) Control 15) Ladder (1kb)

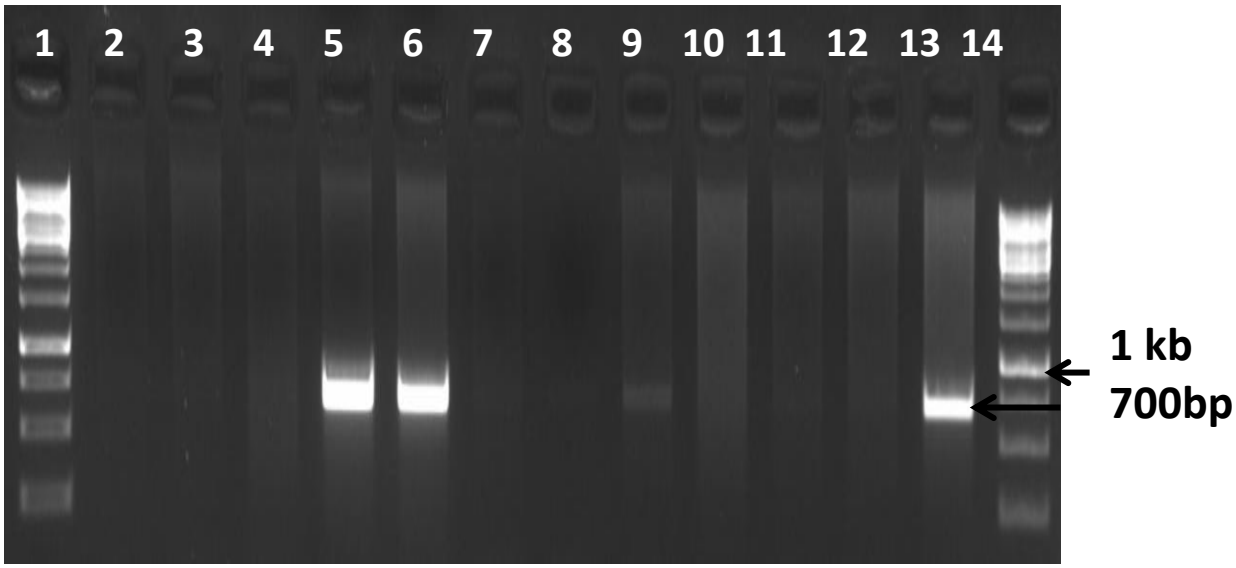

Figure 6.32Agarose gel electrophoresis of nptII PCR for primary screening of StCDF1.2-pRI101 AN transgenic lines 1)Ladder (1kb) 2) A3 3) B1 4) C3 5) F2 6) K2 7) H4 8) I3 9) M3 10) Y2 11) G4 10) M2 11) V1 12) Control 13) T2 14) Ladder (1kb)

6.8 Microtuber production

Microtuber production was chosen because this technique offers more advantageous than plantlet regeneration. Microtuber are sturdy, hence chances of damage are less in handling than *in-vitro* plant regeneration (Rahman et al. 2015). Microtubers are produced in in-vitro environment, so pathogen free tubers are produced. Handling, storage and other downstream processes are easy to conduct and observe. Higher survival and direct filed cultivation without acclimatization make it better technique for potato transgenic and seed production (Imani et al. 2010). For microtuberization initiation and shoot growth, number of explants is very important. Optimum number of explants enhances the shoot growth and later microtuber production (Rahman et al. 2015). Gopal et al. (2004) studied effect of light and sucrose on tuberization induction and dormancy on microtubers produced from Indian potato cultivars. When induction and development was performed under complete darkness, yields were high and dormancy was low. Whereas under diffused light condition dormancy was longer, which is useful in germplasm conservation. Higher sucrose concentration in MS media in both multiplication and tuberization induction media promote tuberization and biomass accumulation. So, in present study microtubers of transformed lines were produced in dark conditions with high concentration of sucrose.

After one week of induction buffer addition microtubers were observed in transformed lines (**Fig 6.33**). In control K. Girdhari tuberization was observed after five days in comparison to transformed lines. The microtubers were collected once leaves started yellowing and senescence (**Fig 6.34**) (Table 6.10). Hossain et al. (2017), studied efficiency of microtuber of three Bangladeshi potato varieties (Diamant, Asterix and Granola) in field conditions. Microtubers of all three varieties performed better in survival, vegetative and tuberization analysis in field conditions. Yield of microtuber derived plants were double in comparison to the plants form in-vitro plantlets. On studying different media supplements in MS basal media, BAP+IAA+GA3 combination was reported to be most efficient in induction of microtuber formation from in-vitro potato stem explants. 4mg/L of BAP was most efficient in establishing good shoot system in initial growth stage (Borna et al. 2019).

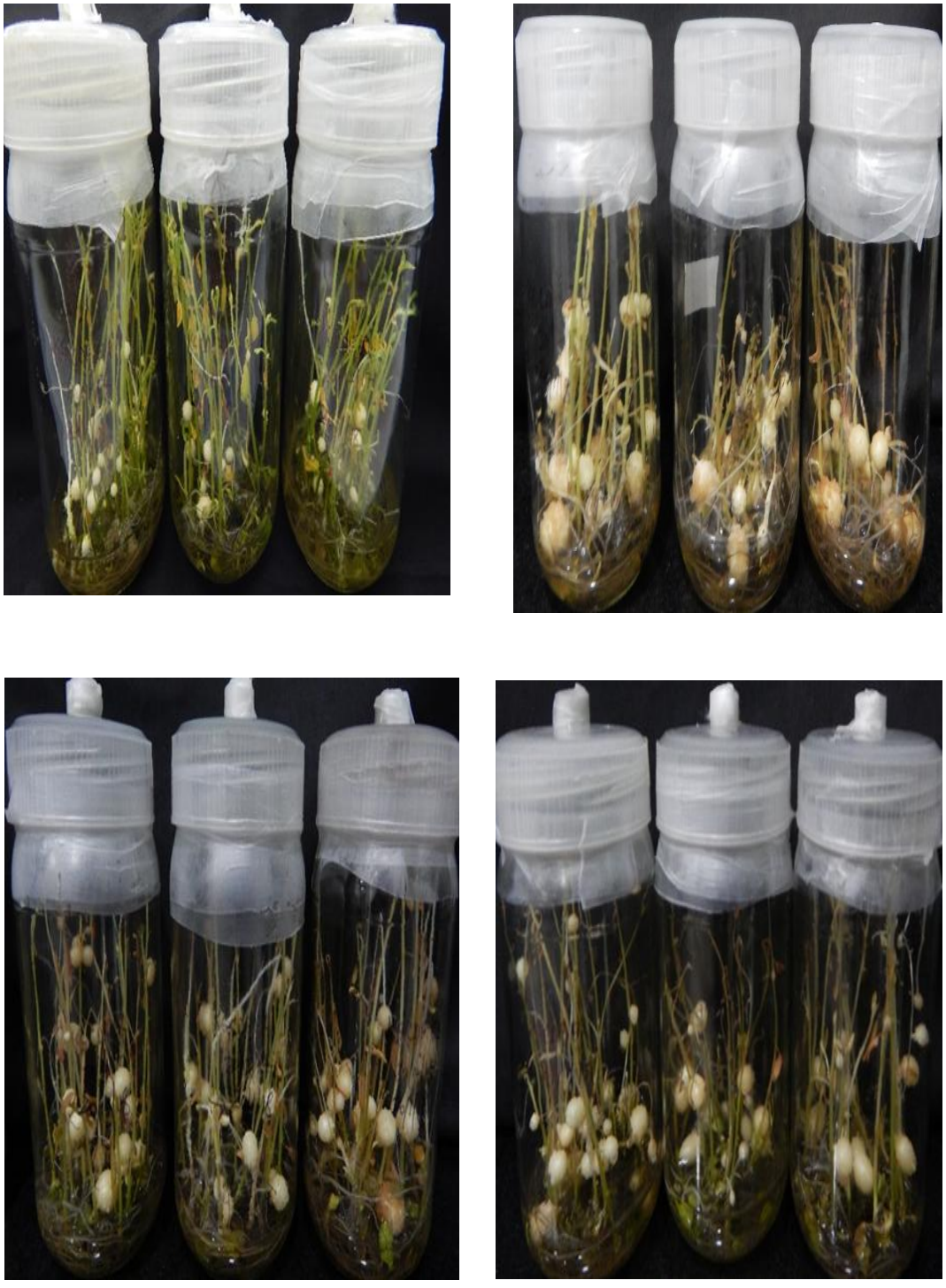

Figure 6.33 Microtuber production in glass jar of transgenic lines and K. Girdhari control.

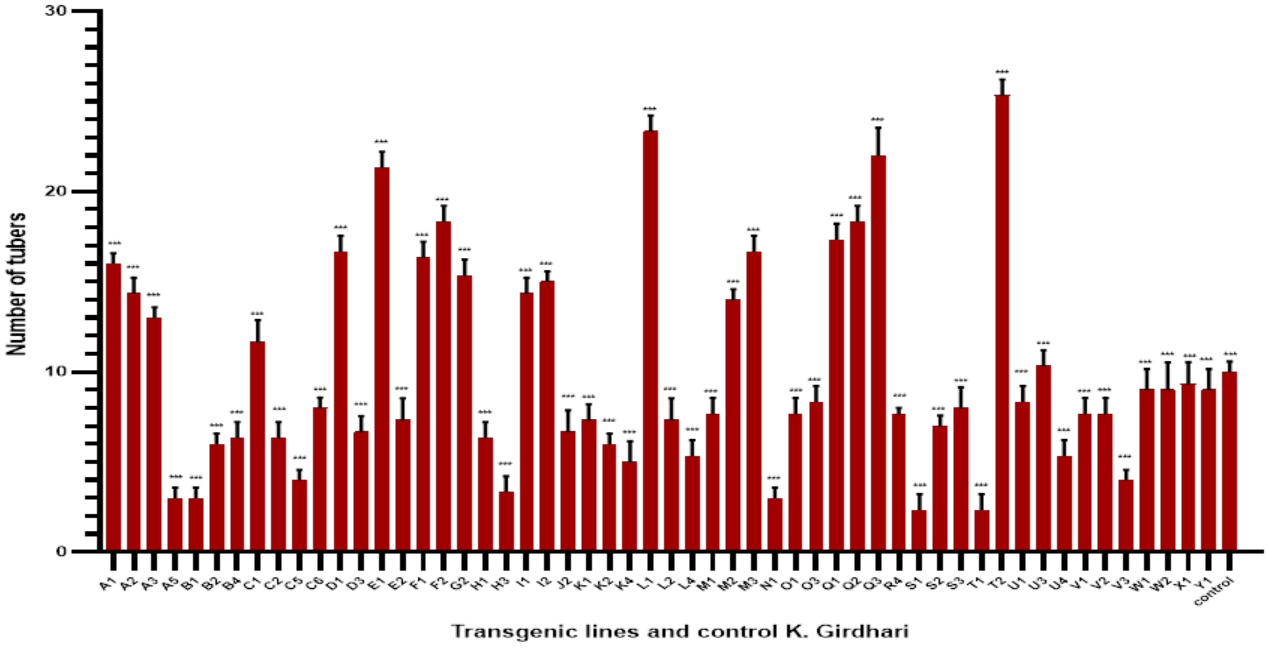

Figure 6.34 Number of tubers harvested from transgenic lines and control K. Girdhari

Table 6.10 Number of tubers harvested from transgenic lines and control in microtuberization

S.No.	Transgenic lines	No. of tubers
1	A5, B1, B2, B4, C2, C5, C6, D3, E2, H1, H3, J1, J2, J4, K2, K4, L2, L4, M1, N1, O1, O3, R4, S1, S2, S3, T1, U1, U3, U4, V1, V2, V3, W1, W2, X1, Y1, control	0-10
2	A1, A2, A3, C1, D1, F1, F2, G2, I1, I2, K1, M2, M3, Q1, Q2, T2	10-20
3	E1, L1, Q3	20-30

6.9 Minituber production

Minituber production from putative shoots of transgenic plants results in faster multiplication of plants for field trials. Aeroponics has been frequently used technique for minituber production. It is a soilless technique where growth and tuberization occur in controlled environment (Farren and Mingo-Castel, 2006). In case of potato, aeroponics was reported to be more efficient in increasing yield by optimum aeration of root system (Buchseth et al. 2016). In some countries quality of soil and water is poor, which make it unsuitable to growth potato in field conditions. Aeroponics system reported to be very beneficial in certified pathogen-free potato seed production (Mbiyu et al. 2012). This soilless cultivation technique also offers non-invasive method to study root system and tuberization of transgenic or stressed plants (Tunio et al. 2019). So, in this study twenty six positive and negative lines from nptII and microtuber analysis were selected and cultivated in aeroponics facility with control K.Girdhari.

The overall vegetative growth (plant and stem height) of the plant was increased in comparison to field grown plants (**Fig 6.35**). Similar higher plant growth was also observed by Farren and Mingo-Castel (2006), on cultivating extra-early potato cv Zorba in aeroponics facility. Initiation of tuberization in transgenic was observed after 22 days of cultivating. At initiation average number of 2-3 tubers was observed in control, whereas in positive lines average was 5-6 tubers. Tubers were harvested every week and kept at room temperature for drying after bavistin treatment. Maximum number of tubers (73) were collected from transgenic line E1, followed by A1 (52 tubers) and I2 (44 tubers) (Table 6.11).Leaf primordias were also present in control and in transgenic lines no leaf primordias were absent. This indicates the early onset of the tuberization as all the biomass is being used in tuber formation. In a study conducted to analyse productivity of minitubers of two potato cv Ametyst and tofu in field conditions. Ametyst was reported to be a better performer in field conditions than Tajfun (Rykaczewska, 2016). Tiwari et al. (2020) used plants grown under aeroponics facility to study genes responsible for nitrogen metabolism in potato. Variable concentration of nitrogen was used and transcriptome of different tissues (shoot, root and stolon) of potato was analysed.

Table 6.11 Number of mini-tubers produced by K.G. control and transgenic lines in the aeroponics.

S.no	Transgenic lines	No. of tubers
1	K.G. Control	44
2	K.G. E1	73
3	K.G.T2	27
4	K.G. M2	11
5	K.G. A1	52
6	K.G. C1	21
7	K.G. F1	24
8	K.G. L1	46
9	K.G. K1	14
10	K.G. Q3	16
11	K.G. D1	20
12	K.G. F2	35
13	K.G. I2	44
14	K.G. G2	9
15	K.G. A2	19

16	K.G. I1	4
17	K.G. Q1	10
18	K.G. Q3	31
19	K.G.M3	3
20	K.G.A3	3
21	K.G.O2	16
22	K.G. K4	12
23	K.G. B1	11
24	K.G. L4	1
25	K.G. G1	6
26	K.G. N1	3
27	K.G. T3	6

6.10 Glass house

Sprouted minitubers from aeroponics facility of five StCDF1.2 overexpressing transgenic lines and control were planted in pots in glass house conditions. Selected lines (F2, A1, T2, C1 and E1) were nptII PCR positive and performed well in tuberization studies (microtuberization and aeroponics). The overexpression in transgenic lines was confirmed by gene expression and phenotypic studies.

Figure 6.35 Microtuber productions of transgenic lines and K. Girdhari control in Aeroponics facility

6.10.1Quantification of gene expression in transgenic lines

In transgenic plants target gene (StCDF1.2) was strongly upregulated compared to control K. Girdhari. Maximum upregulation of 56.77 fold was observed in transgenic line T2 (**Fig 6.36**). Koolsertman et al. (2013) also reported upregulation of more than 30 fold in transgenic lines overexpressing StCDF1.2/1.3 in *S. andigenum*. Another transgenic line StCDF1.2-F2 showed upregulation of 14.803 fold, followed by E1 (13.01 fold), C1 (3.306 fold) and A1 (2.567 fold). The expression of other signalling molecule (StSP6A, StSP5G and StCO) was also followed. StSP6A, a mobile signal for onset of tuberization was also upregulated in transgenic lines. Here also highest expression (65.43 fold) was observed in KG-StCDF1.2-T2 transgenic line (**Fig 6.37**) by normalizing control. Another clade member of FT is StSP5G, its expression was followed and downregulation was observed (**Fig 6.38**). Downregulation of 0.964 fold was showed by T2 transgenic line in comparison to control K. Girdhari. Expression of StCO was also decreased in transgenic lines overexpressing StCDF1.2 (**Fig 6.39**).

Similar upregulation of StSP6A and downregulation of StSP5G and StCO was reported by Kloosterman et al. (2013) in StCDF1.2 overexpressed lines. Abelenda et al. (2016) reported the suppression of StSP6A expression by a member of its FT clade StSP5G. StSP5G expression was reported to be positively regulated by StCO1/2 and these are stabilized by phytochromes under favourable photoperiod. On silencing of StPHYF (Phytochrome F) a light receptor, accumulation of StCO1 protein was less than control plants. This decreased accumulation represses StSP5G expression and StSP6A levels were elevated. These phytochromes plays crucial role in the photoperiod-dependent tuberization. StPHYF regulates StCO pathway and stabilize it in light (long afternoon) and aid StCDF1.2 degradation (Zhou et al. 2019). Plantega et al. (2018) indicated additional regulation of both StSP5G and StSP6A, as overexpression of StCO does not affected StSP6A expression in short day conditions. In short day conditions, the tuber-inducing role of StCDF1 by repressing StCO may be compensated by the overexpression of StCO, but StCDF1 may also directly activate StSP6A.

6.11 Phenotypic studies

6.11.1 Effect of *StCDF1.2* on flowering

Regardless of the down regulation of StCO and StSP5G transcripts in transgenic line overexpressing StCDF1.2, there was no change in flowering, flower initiation, flower number and flowering pattern between transgenic and control plants. Since flowering is a multigenic trait and down regulation of StCO and StSP5G via overexpression of StCDF1.2 is incomplete and transient. This overexpression might have not complete effect on other key regulators of flowering, as its flowering signal transduction pathway is different from that of tuberization. AtCDF1 (Arabidopsis ortholog of CDF1) was reported to suppress flowering under non- inductive conditions. When conditions are favourable for flowering AtCDF1 was found to be degraded by FKF1-GI complex (Sawa et al. 2007). Imaizumi et al. (2005) developed transgenic lines of Arabidopsis overexpressing AtCDF1 and reported delayed flowering in comparison to wild type. In case of Arabidopsis, CO have positive effect on flower development and under long conditions accumulation of CO flowering was induced and promoted. Whereas in potato overexpression of Arabidopsis CO delay tuber induction and formation, but has no effect on flowering (Martinez-Garcia et al., 2002, Hannapel et al. 2017).

6.11.2 Effect of *StCDF1.2* on tuberization

Overexpression of StCDF1.2 in K. Girdhari enhanced the tuberization and number of tubers was also increased. Overexpression of StCDF1.2 suppresses StCO expression, which is reported to be correlated with StSP6A higher accumulation and enhanced tuberization (Kloosterman et al. 2013).

6.11.3 Effect of *StCDF1.2* on senescence

Change in tuberization process can affect the overall plant physiology and growth, specifically plant senescence. The early growth stages (vegetative growth and flowering) of transgenic and control plants were similar. The difference was observed after induction of tuberization in both sets of plants. Transgenic plants showed early

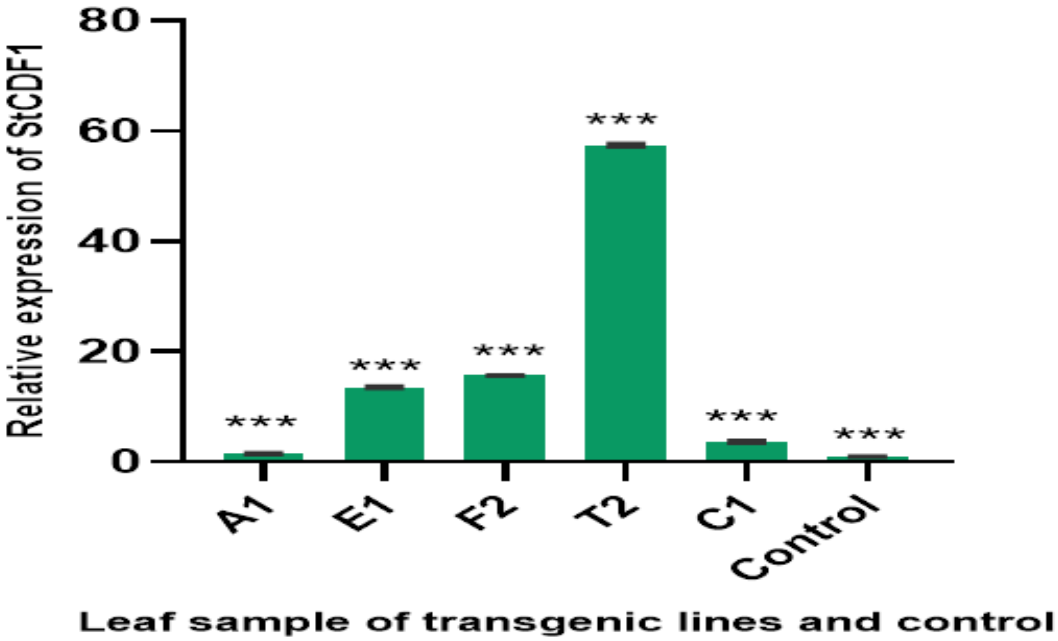

Figure 6.36Gene expression analysis of StCDF1.2 in transgenic and control potato plants by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates.

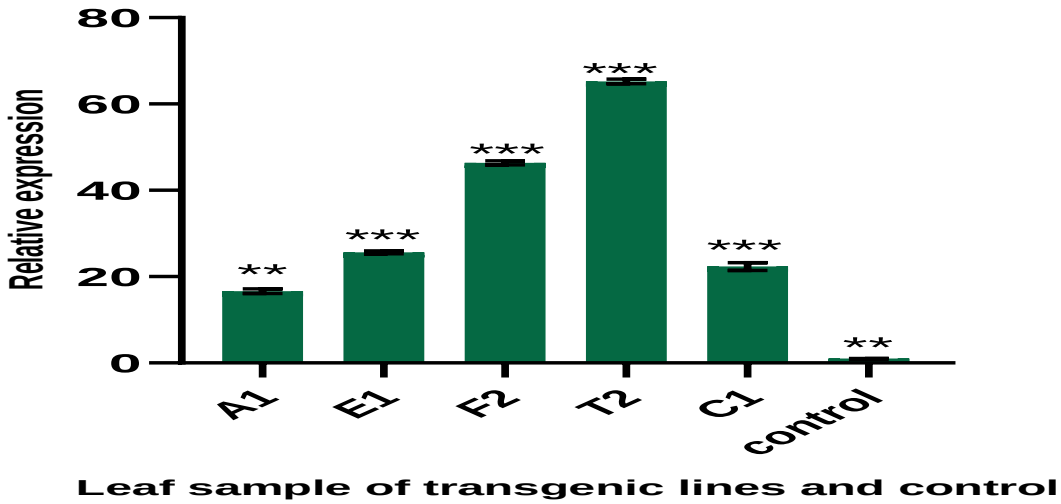

Figure 6.37 Gene expression analysis of StSP6A in transgenic and control potato plants by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates.

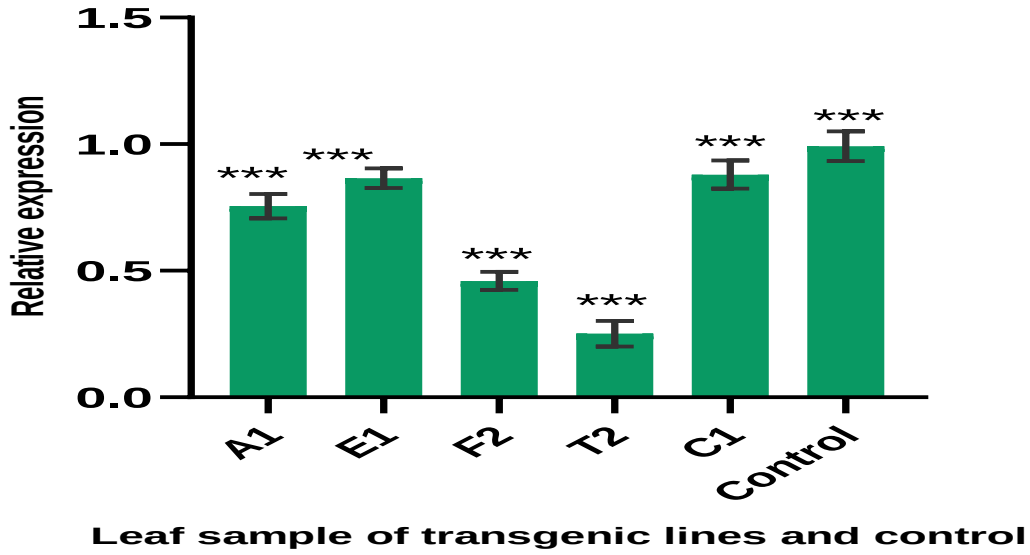

Figure 6.38 Gene expression analysis of StSP6A in transgenic and control potato plants by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates.

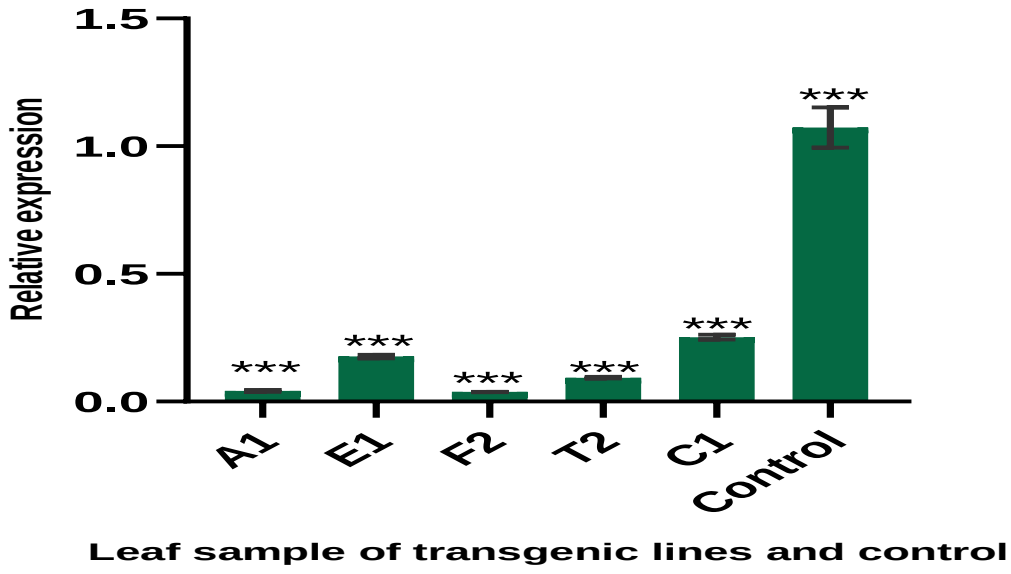

Figure 6.39 Gene expression analysis of StSP6A in transgenic and control potato plants by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates.

Figure 6.40 StCDF1.2 overexpressing transgenic lines and control K. Girdhari after germination showing yellowing of leaves.

signs of senescence (**Fig 6.40**) and completed their life cycle earlier than control K. Girdhari. Similar five week early termination of life cycle was reported by Kloosterman et al. (2013) in S. andigenum transgenic lines overexpressing stCDF1.2. Gene StCDF1 is associated with plant maturity and tuberization induction. Senescence after completion of tuberization is normal physiological process in potato life cycle. So, earliness (fast senescence) is linked with early induction and completion of tuberization.

6.12 Nutritional profiling

6.12.1. Starch content

In plants starch is a major reservoir of carbohydrate and provides basic nutrition to animals. Potato starch not only provides nutrition but also serves as raw material for starch based industries (Schall et al. 2000). It consists of amylose [linear, α (1 → 4) linkage] and amylopectin [branched, β (1 → 6) linkage] molecules. Starch synthesis occurs in plastids and then gets assembled as granules (Brammell et al. 2015). Many studies have been conducted on potato starch as its in great demand for industries. In Indian potato varieties starch was reported to vary between 11% to 18% using anthrone reagent (Kaur and Aggarwal, 2014). No change in starch content of transgenic lines was observed and it varies between normal ranges (**Fig 6.43**).

6.12.2 Amylose content

About 70-80% of starch molecule is made up of amylopectin and rest is amylose. This ratio of amylose to amylopectin determines and alters many physiochemical properties of starch. Amylose content in potato starch is important for industrial as well as medical perspective. Earlier studies have focused on genetic manipulation of potato starch to make it more suitable for industries (Xu et al. 2014). But now paradigm has shifted to alter potato starch to reduce its side effect for obese and diabetic patients. In both industries and medical field, high amylose content is desirable (Tuncel et al. 2019). Resistant starch (high amylose containing) usually donot get digested in upper gut and get attacked by gut micoflora, which have

beneficial effect on health. So, many studies have been published on altering starch composition (Raigond et al. 2013).

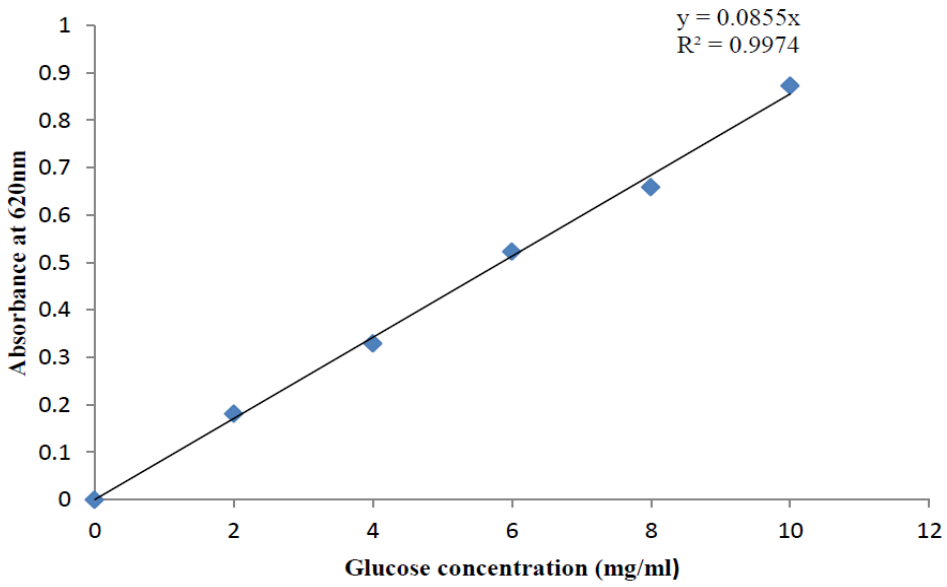

Figure 6.41 Standard curve of starch

In Indian potato varieties amylose content reported to vary from 10-35% of the total starch content (Raigond et al. 2017). In present study amylose content was found to vary in normal range (19-21%) (**Fig 6.44**). Raigond et al. (2017) reported the amylose content of K. Girdhari control to be 20.1 % of the total starch.

6.12.3. Total protein

In previous studies, total protein of different potato varieties was reported to range between 0.70-4.60 percent (Abbas et al. 2011). Potato is the second largest grown protein providing crop after wheat. Protein from potato provides some essential amino acids, which are lacking in common crops and vegetables. These include essential amino acids like lysine, threonine, tryptophan, and methionine etc. (Kärenlampi and White, 2009). They are associated with several health benefits such as regulation of blood pressure, allergy and cholesterol. Proteins like patatin, protease inhibitors, and HMW (High molecular weight) proteins are reported to have

antimicrobial and anticarcinogenic properties (Waglay and Karboune, 2016). The protein content (**Fig 6.45**) in transgenic plants observed to vary in normal range (2.14-3.21).

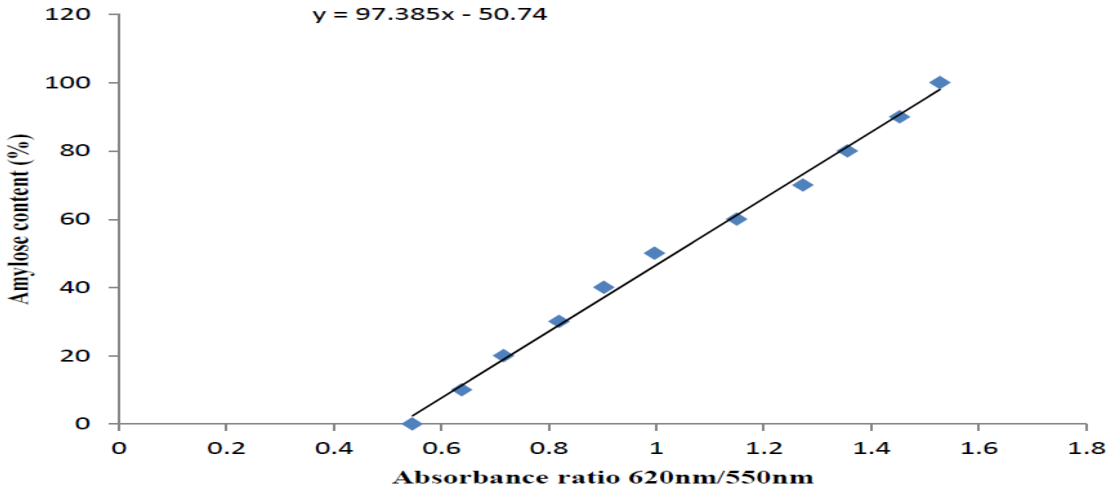

Figure 6.42 Standard curve of amylose

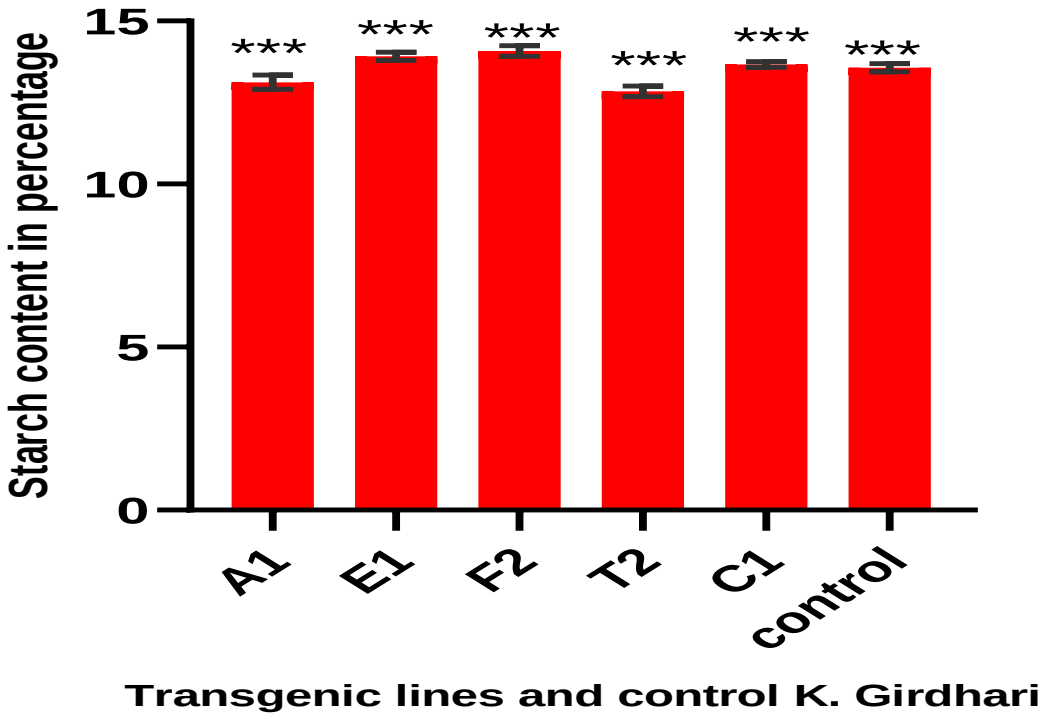

Figure 6.43 Starch content in transgenic lines and control K. Girdhari

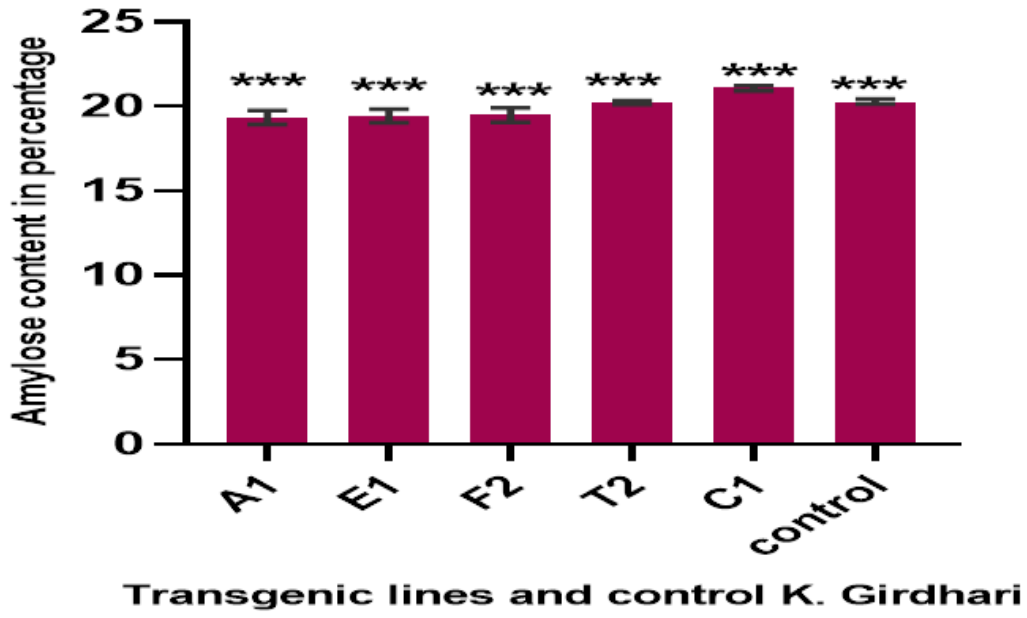

Figure 6.44 Amylose content in transgenic lines and control K. Girdhari.

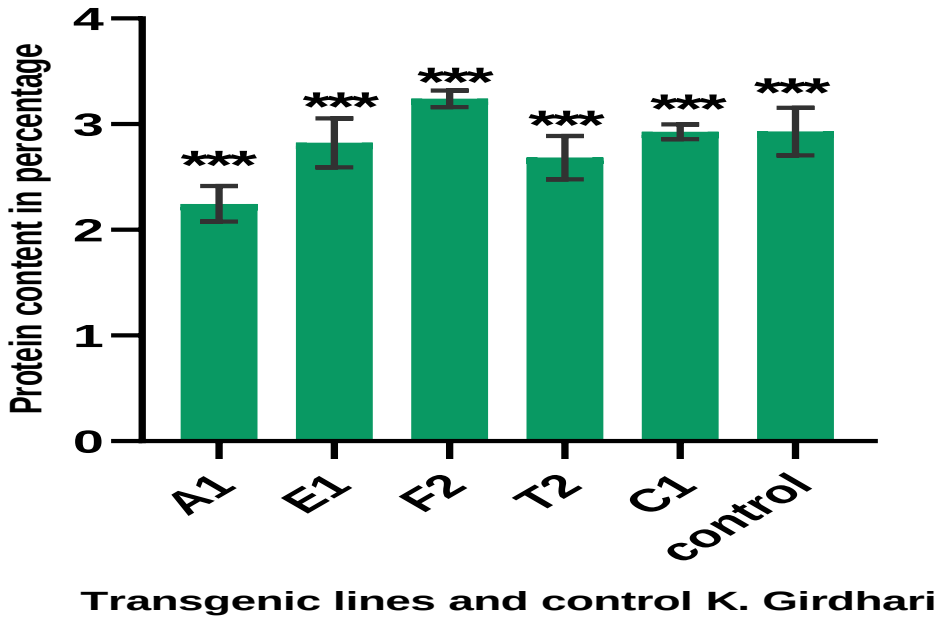

Figure 6.45Protein content in transgenic lines and control K. Girdhari

6.13 Field trial

Field assay was performed to compare performance of minitubers in glass house and field conditions. These same five transgenic lines (F2, A1, C1, T2 and E1) and control K. Girdhari was grown in field condition under contained environment. Tubers were harvested after complete senescence of plants (Table 6.12) and stored in cooling chamber for further use. The number of tubers were highest in C1 line (67 tubers), followed by F2 (65 tubers) and others lines also showed higher tuberization in comparison to control K. Girdhari (**Fig 6.46**). Overexpression of StCDF1.2 in K. Girdhari enhanced the tuberization and number of tubers was also increased. Overexpression of StCDF1.2 suppresses StCO expression, which is reported to be correlated with StSP6A higher accumulation and enhanced tuberization (Kloosterman et al. 2013).

Table 6.12 Number of tubers harvested from transgenic lines and control K.Girdhari from field.

S.no.	Lines	No. of tubers
1	F2	65
2	E1	59
3	A1	55
4	T2	41
5	C1	67
6	Control	25

Figure 6.46 Tubers of transgenic lines and K. Girdhari control in field.

SUMMARY AND CONCLUSION

Summary and conclusion

Our present findings indicate the role of StCDF 1.2 gene in governing the initiation of tuberization. Early maturing potato varieties could be a solution to problems caused by climate change. Being a temperate crop, efforts of potato tropicalization in India were hindered. The present study was conducted to elucidate the allelic variation, expression, role in flowering and tuberization of StCDF1. The importance findings of the study are as follows:

- In expression study of StCDF1 in KS (Heat tolerant) and KCM at different tuberization temperature, highest expression (1.92 fold) was observed in the morning sample (6am) of the KS. High temperature upregulates the expression of StCDF1.
- To studyStCDF1 expression in different daylength adapted Indian varieties, qRT-PCR was performed. The highest expression (3.34 fold) was studied in KA morning under short day conditions and lowest (0.331 fold) was observed in KG evening under long conditions. In all Indian varieties used for the study, short day conditions led to the higher expression of StCDF1 in comparison to the plants grown under long day conditions.
- To study the allelic variation in StCDF1 gene, PCR was performed using the high-fidelity polymerase, two fragments were observed corresponding to CDF allele 1.2 and 1.3.
- To validate role of StCDF1.2 in tuberization and earliness, Virus-Induced Gene Silencing was performed on KS. Real-time confirmed the upregulation of StCO and StSP5G and downregulation of StSP6A in VIGS plants. Phenotypic observations suggested delayed tuberization and a low tuber number in VIGS plants.
- Based on this proof of concept, transgenic lines of late maturing cv. Kufri Girdhari overexpressing StCDF1.2 gene were developed.

- Out of 95 putative shoots generated, 55 were found positive on primary screening by nptII PCR. These positive transformed lines were subjected to microtuberization for initial tuberization. In microtuberization, 27 transformed lines performed better than other lines and control K.Girdhari.
- Then these better performing lines were cultivated in soilless aeroponics facility. Out of twenty seven, five transgenic lines were selected on tuberization study basis for glass house and field trial.
- In transgenic plants, StCDF1.2 was strongly upregulated and maximum upregulation of 56.77 fold was observed in transgenic line T2. StSP6A was also upregulated with highest expression of 65.43 fold in KG-StCDF1.2-T2. Expression of StCO and StSP5G was decreased in transgenic lines overexpressing StCDF1.2.
- On phenotypic study, flowering process was not affected whereas tuberization and senescence was enhanced.
- Field cultivation of transgenic lines conferred that overexpression of StCDF1.2 enhances the tuberization and number of tubers was also increased.

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Appendices

Appendix I

Bacterial growth medium

Luria Bertani

Components	Concentration(g/L)
Bacto-yeast extract	5
Bacto-Tryptone	10
NaCl	10

Yeast extract Mannitol

Components	Concentration(g/L)
Mannitol	10
Yeast extract	0.4
NaCl	0.1
$MgSO_4.7H_2O$	0.02
K_2HPO_4	0.8

Appendix II

Plasmid isolation solution

Solution I (pH-8.0)

Components	Concentration
Tris	25 mM
EDTA	10 mM
Glucose	50 mM

Solution II

Components	Concentration
NaOH	0.2 M
SDS	1 % (w/v)

Solution III

Components	Volume (ml)
5 M Potassium acetate	60
Glacial acetic acid	11.5
Water	28.5

Appendix III

List of Indian potato cultivars

Variety	Year of release	Parentage
Kufri Sindhuri	1967	Kufri Red x Kufri Kundan
Kufri Chandramukhi	1968	Seedling 4485 x Kufri Kuber
Kufri Jyoti	1968	3069d(4) x 2814a(1)
Kufri Arun	2005	Kufri Lalima x MS/82-797
Kufri Surya	2006	Kufri Lauvkar x LT-1
Kufri Girdhari	2008	Kufri Megha x Bulk pollen (10 genotype)

Appendices IV

Murashige Skoog (MS) media

Stock-1 50X

Components	
Potassium Nitrate (KNO_3)	47.5g
Ammonium Nitrate (NH_4NO_3)	41.25g
KH2PO4	4.25g
Magnesium Sulphate ($MgSO_4.7H_2O$)	9.25g

Stock-2 50X (500ml)

Components	
Calcium Chloride ($CaCl_2.2H_2O$)	11g

Stock-3 100X (500ml)

Components	
Boric acid (H_3BO_3)	310mg
$MnSO_4.H_2O$	845mg
$ZnSO_4.7H_2O$	430mg
KI	41.5mg
$Na_2MoO_4.2H_2O$	12.5mg
$CuSO_4.5H_2O$	1.25mg
$CoCl_2.6H_2O$	1.25mg

Stock-4 100X (500ml)

Components	
$FeSO_4.7H_2O$	1390mg
$Na_2EDTA.2H_2O$	1865mg

Stock-5 50X (500ml)

Components	
Myoinositol	2-5g
Glycine	50mg
Thiamine HCl	1.25ml
Nicotinic acid	6.25ml
Pyridoxin HCl	6.25ml

MS media

Components	**Concentration**
Stock I,II,III,IV,V	1X
Sucrose	30g/litre
Gelrite	2.1 g/litre
Calcium pantothenate	2ml/litre (100mg/ml stock)

Appendix V

Antibiotics stocks

Dissolved in distilled water and filter sterilized. Stored at -20ºC for further use.

Antibiotic	**Concentration (mg/ml)**
Kanamycin	50
Ampicillin	100
Rifampicin	20
Cefotaxime	1
Carbenicillin	1

Appendix VI

Growth regulators stocks

Filter sterilized and stored at -20ºC for further use.

Growth regulator	Solvent	Concentration (mg/ml)
IAA	DMSO	1
GA3	EtOH	3
BAP	NaOH	1

Appendix VII

Buffers used in VIGS

Induction buffer	10mM of MES (2-(N-morpholino)ethanesulfonic acid) solution was prepared in d-H_2O and 200µl of Acetosyringone or Tobacco leaf extract was added to MES solution. The pH of the buffer was adjusted at 5.5 by adding NaOH.
Infiltration buffer	10mM of MES (2-(N-morpholino)ethanesulfonic acid) solution was prepared in d-H_2O and pH of the buffer was adjusted at 5.5 by adding NaOH.

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Solanum tuberosum (CYCLING DOF FACTOR) CDF1.2 allele: A candidate gene for developing earliness in potato

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ABSTRACT

Circadian clock regulated CDF (CYCLING DOF FACTORs) are a cluster of transcriptional repressors, that accentuates a major quantitative trait locus (QTL) for earliness in potato. Earliness in the plant is a desirable agronomic trait for higher productivity and also enable it to withstand various biotic and abiotic stresses. Since potato is a temperate crop, its tropicalization in the Indian subcontinent is a major agricultural challenge. *StCDF1* induce a photoperiod-dependent tuberization pathway by interacting with other molecular regulators such as CONSTANS (*StCO1/2*) and *StSP6A*. The present study was conducted to elucidate the allelic variation, expression, role in flowering and tuberization of *StCDF1* in contrasting tuberization behaviour of diverse Indian potato cultivars, Kufri Surya (KS) and Kufri Chandramukhi (KCM). Our results confirmed two allelic forms of *StCDF1* viz. *CDF1.2* and *CDF1.3*. Their expression profile suggests a higher upregulation of both *CDF1.2* and *CDF1.3* expression at dawn (6:00 am) than noon (12:00 pm). Virus-Induced Gene Silencing was used to validate the role of these alleles with respect to earliness and tuberization in KS. Real-time confirmed the upregulation of *StCO* and *StSP5G* and downregulation of *StSP6A* in VIGS plants. Phenotypic observations suggested delayed tuberization and a low tuber number in VIGS plants. This present study confirms the allelic variation, the importance and functionality of *StCDF1.2* in conferring earliness in tetraploid potato cultivars.

1. Introduction

Although tropicalization of potato is of utmost importance with respect to economic growth, it is still a major challenge to sustainable cultivation for major potato cultivars in India. Climate change has a negative impact on plant growth and yield (Dahal et al. 2019). The expansion of the potato cultivation to the tropical parts of the country is constrained by a number of biotic and abiotic stresses. Thus, early maturing potato varieties could be a solution to tropicalization and yield loss due to climate change (Kawar et al. 2018).

Selection of elite germplasm lines is crucial in breeding programs for developing early maturing potato varieties. Germplasm selection requires a detailed understanding of molecular pathways for signal induction in potato tuberization. Tuberization in potato cultivar is a complex multigenic process and is induced under short days and low temperature while long days and high temperature inhibit it. Transcriptional factors are the prime determinants and regulators for the expression of genes involved in tuberization signals (Hannapel et al. 2017).

StCO negatively regulates tuberization by activating the transcription of *StSP5G* repressor. *StSP5G* suppresses the expression of a mobile signal for tuberization, StSP6A, thus inhibiting the tuberization under non-inductive long day photoperiods. *StCDF1* gene of potato belongs to DOF (DNA-binding with one finger) family and are important mediators of tuberization. Previously conducted cDNA microarray studies on Indian potato cultivars also depicted the importance of *StCDF1* gene in early tuberization (Singh et al. 2015). *StCDF1* was mapped as a plant maturity gene on chromosome 5 and had three alleles, designated as *StCDF 1.1, StCDF1.2* and *StCDF1.3*. Allele *StCDF1.1* is related to late maturity of potato genotypes, whereas *StCDF1.2* and *StCDF1.3* are associated with early maturing genotypes (Kloosterman et al. 2013; Kondhare et al. 2019). *StCDF1.2* indirectly induce tuberization by upregulating the expression of *StSP6A*. *StCDF1.2* allele was reported to be most effective in early initiation and induction of tuberization, making it a potential candidate for study to induce earliness in Indian potato cultivars.

Abbreviations: KCM, Kufri Chandramukhi; KS, Kufri Surya; pTRV, Tobacco Rattle virus vector (VIGS vector); qRT-PCR, quantitative real-time Polymerase chain reaction; StCDF, Cycling DNA with one finger protein; StCO, Constans; StSP5G, Self pruning 5G; StSP6A, Self pruning 6A; VIGS, Virus Induced Gene Silencing

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Even though the role of *StCDF1.2* in inducing earliness is well demonstrated in Andigena population. No study has been reported in tetraploid potato regarding its role and efficacy in inducing earliness. *StCDF1.2* gene was targeted through VIGS (Virus-Induced Gene Silencing) to fill the knowledge gap in understanding the diversity of Indian potato cultivars for earliness and maturity. In comparison to any other Post-Transcriptional Gene Silencing (PTGS) technique, genes through VIGS can be functionally characterized in a high-throughput and remarkably short time duration, without changing the target plant genome (Senthil-Kumar and Mysore, 2014; Rojas et al. 2012). There are no reports until now on the functional assessment of CDF genes with respect to earliness and maturity in Indian potato cultivars. The present study was designed to validate the functionality of *StCDF1.2* gene in an early maturing and heat-tolerant potato cultivar KS using VIGS technology. The Indian potato cultivars, KS was initially reported to be heat tolerant and early maturing (Minhas et al. 2006), which was later validated by cDNA microarray studies (Singh et al. 2015). The present study confirms and authenticates the molecular function of *StCDF 1.2* allele with reference to earliness and maturity in Indian potato cultivar.

2. Materials and methods

2.1. Plant material and growth conditions

Kufri Surya (KS) and Kufri Chandramukhi (KCM) are tetraploid Indian potato cultivars. KS have parental lineage from the andigenum group, is early maturing and heat tolerant, whereas KCM has neo-tuberosum parental line, is late maturing and heat susceptible. Well sprouted tubers of KS and KCM were planted in 15 cm pots containing the potting mixture in three replications including control. Plants were subjected to tuberization at 18 °C and 24 °C temperature with 8h light (600 $\mu Es^{-1}m^{-2}$) /16h dark photoperiod in a controlled environment chamber (Conviron, Model E-15, Canada).

2.2. RNA isolation

The leaf samples were collected at dawn (6:00 am), afternoon (12:00 noon) and dusk (6:00 pm) for *StCDF1* expression analysis. Total RNA was isolated from the harvested leaf sample by using a NucleoSpin® RNA Plant kit (MACHEREY-NAGEL) by following the manufacturer's protocol. The quantity and quality of isolated RNA were assessed using a NanoDrop Micro Photometer (Thermo Scientific, USA) and agarose gel electrophoresis. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for the synthesis of double-stranded cDNA(complementary DNA).

2.3. PCR amplification and sequencing for allelic variation studies

The *StCDF1* gene was amplified from the cDNA of KS and KCM using specific primers designed from the *StCDF1* sequence (PGSC003DMG400018408) obtained from Potato Genome Sequencing Consortium (PGSC). The primers were designed with the help of fast PCR software and synthesized by Integrated DNA Technologies (IDT). PCR products were sequenced by the Sanger-Coulson method using Applied Biosystems. The obtained sequence was analyzed using BLAST (Basic Local Alignment Sequence Tool) and pairwise alignment was done by Clustal omega.

2.4. Quantitative RT-PCR

Gene expression was analyzed by Reverse transcription-quantitative real-time PCR (RT-qPCR). Expressions of target genes were studied with reference to the elongation factor (*alpha ELF1a*) using SYBR Green master mix (Applied Biosystems) in a 7900HT Sequence 115 Detection System (Applied Biosystems). PCR reactions (20μL) included 20ng cDNA, 0.2mM of each primer and 10 μL SYBR Premix (Applied Biosystems, USA) under the following conditions: 95°C for 30 s, 40 cycles at 95°C for 5 s, and at 60°C for 31 s; followed by 95°C for 15 s, at 60°C for 1 min, and at 95°C for 15s to obtain the melt curves. The expression of each gene relative to average C_t values of the housekeeping genes was determined and analyzed using ABI 7300 System Sequence Detection Software Version 1.4 (Applied

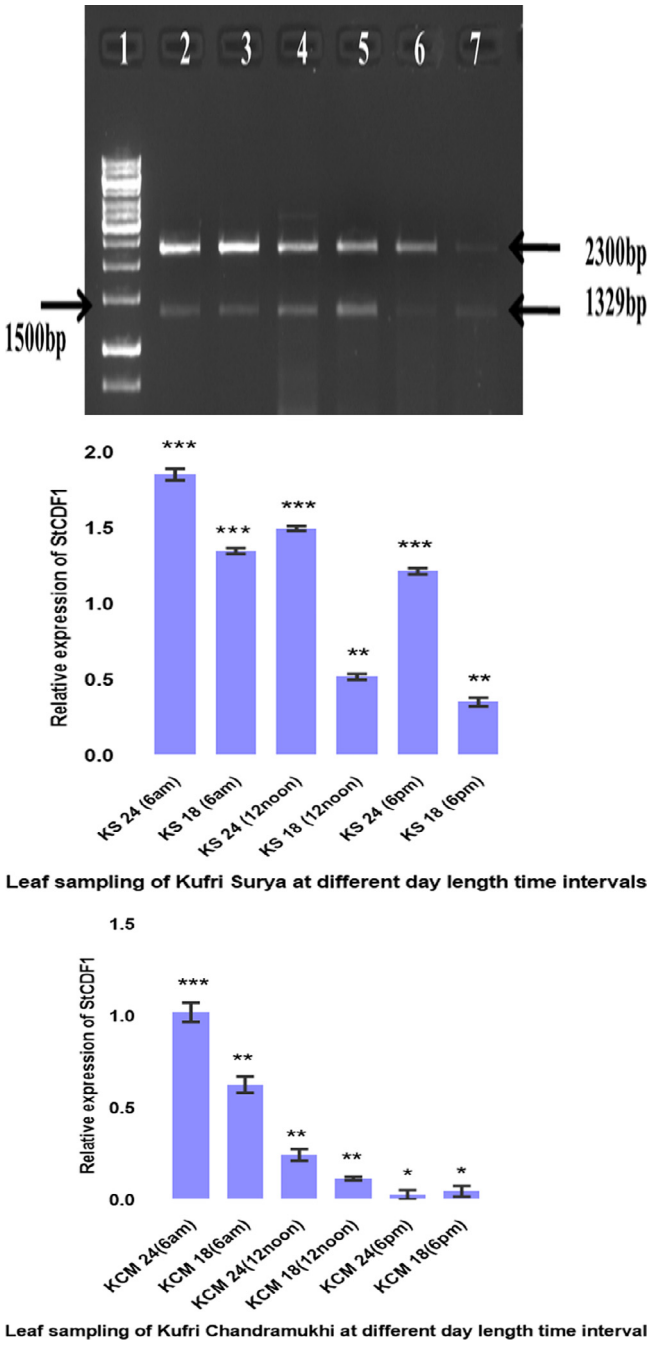

Fig. 1. (a). PCR amplification of *stCDF1* gene showing fragments corresponding StCDF1.2 and 1.3 allele. **b).** Gene expression analysis of *StCDF1* in heat-tolerant (KS) and heat susceptible (KCM) Indian potato cultivars by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates. Three time points for leaf sampling (time is shown on the x-axis) from plants grown at 18 °C and 24 °C temperature conditions with 8h light/16h dark photoperiod. **c)** Gene expression analysis of *StCDF1* heat susceptible (KCM) Indian potato cultivars by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates. Three time points for leaf sampling (time is shown on the x axis) from plants grown at 18 °C and 24 °C temperature conditions with 8h light/16h dark photoperiod.

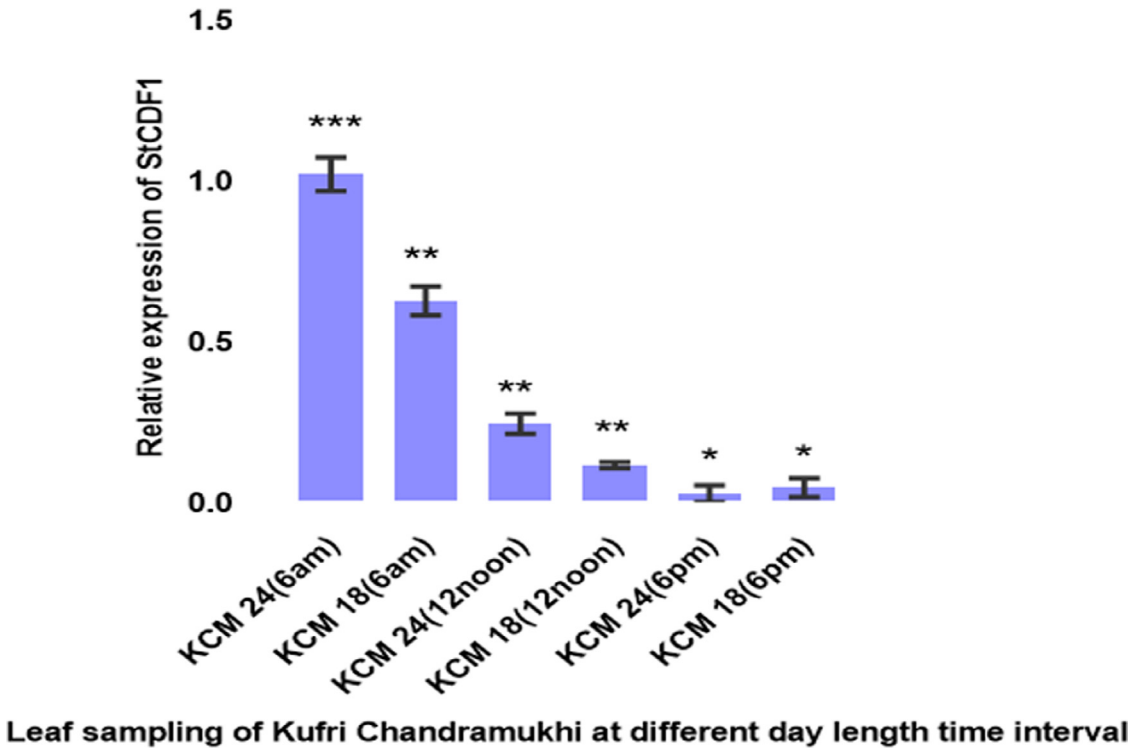

Fig. 1 Continued.

Biosystems, USA). Quantification of the relative changes in gene transcript level was performed in accordance with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Standard deviations were calculated for all lines tested using SDSRQ manager software (Applied Biosystems, USA).

2.5. Development of VIGS construct

2.5.1. Prediction of effective siRNA region and eliminating off-targets

The DNA sequences of the target gene were taken from the PGSC database (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml). The CDS regions that could effectively produce the siRNA were selected from the target gene sequences by using bioinformatics tools (http://sidirect2.rnai.jp, http://bioinfo2.noble.org/RNAiScan.htm). Fragments were selected based on the number of effectively silenced siRNAs. The smaller fragments have a high silencing efficiency and the longer fragments could enhance the off-target effects and cause instability in some viral vectors. Thus the sequences were selected to meet the criteria of having a length and untranslated region, which are most effective with minimum off-target silencing. Further, the BLAST tool was used to ensure that there is no off-target silencing (Senthil-Kumar and Mysore, 2014).

2.5.2. Development of pTRV2 VIGS construct

The PCR product used for the development of VIGS construct was amplified from KS cDNA using gene-specific primers. The primers were designed with the help of fast PCR software and were synthesized by IDT. The fragment of 530 bp on 1.3% agarose gel confirmed the successful amplification of *StCDF1.2*. These genes were first cloned in pTZ57R/T vector and then subcloned into pTRV2 vector in *E.coli* (strain DH5-α). The cloning was confirmed by colony PCR and restriction digestion using *EcoRI* and *BamHI* enzyme. The pTRV2:: CDF1.2 vector construct was transformed into *Agrobacterium tumefaciens* strain GV3101 by a freeze-thaw method. All plasmid DNAs used for VIGS analysis were prepared using a Macherey-Nagel Nucleospin Plasmid isolation kit (Germany).

2.6. Agro-inoculation of Potato cultivar through pTRV2:: VIGS constructs

The developed VIGS gene construct (pTRV2:: gene of interest) was mobilized in Agrobacterium. The developed agro construct was used for the VIGS experiment along with pTRV1 mobilized in Agrobacterium (1:1). The screening for gene knock-down using VIGS was carried out in the KS potato cultivar. pTRV2:: gene of interest was grown in LB broth with Kanamycin (50μg/ml) and incubated at 28 °C overnight. The sprouted tubers were pricked using a 1ml insulin syringe at the meristematic region. The culture was centrifuged, and the pellet was dissolved in the induction medium (pH 5.5). 1ml of tobacco leaf extract was added and incubated at 28 °C with shaking at 120 rpm. The cell suspension was centrifuged, and the pellet was dissolved in the infiltration medium (pH 5.5). The tubers were injured at the meristematic region and agroinoculation was performed using 1ml insulin syringe. The treated tubers along with control (untreated) of KS were planted in pots. Three doses of Agro-inoculation were imposed, one just after sprouting, second at 30 days after planting and the third at 45 days after planting. The meristematic regions of plants were inoculated with agrobacterium culture, having pTRV1 and pTRV2:: gene constructs using the agro-infiltration of leaf, stem and root through a 1ml insulin syringe injection (Senthil-Kumar and Mysore, 2014). Similarly, KS control plants were inoculated with agrobacterium culture, having pTRV2 and pTRV1.

2.7. Validation of silencing

2.7.1. RNA isolation and quantification using qRT-PCR

Leaf samples were harvested at different time points (dawn, afternoon and dusk) from VIGS and KS control plants after the third inoculation. The collected leaf samples were first dipped in liquid nitrogen and then were stored at -80 °C. Total RNA was extracted from leaf tissues and was reverse transcribed into double-stranded cDNA. The expression of tuberization genes (*StCDF1.2* and *StSP6A*) and complementary genes (*StCO* and *StSP5G*) relative to average C_t values of the housekeeping genes were determined and analyzed using ABI 7300

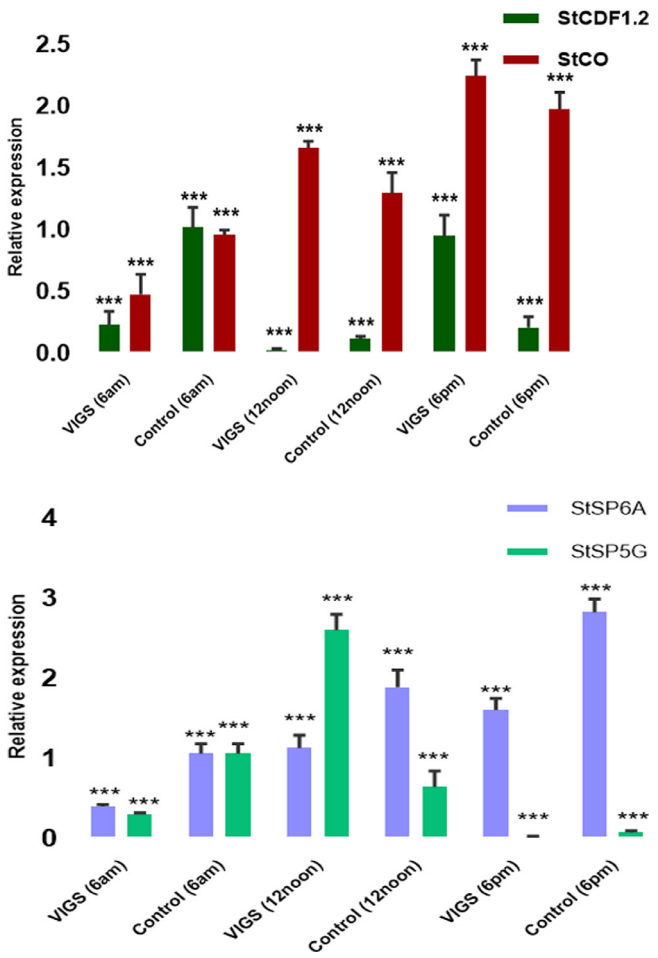

Fig. 2. (a). Gene expression analysis of *StCDF1.2* and *StCO* in VIGS and control potato plants by qRT-PCR.

qRT-PCR was performed using RNA extracted from KS and KCM plants using primers designed against the *StCDF1.2* gene.

Data are the average of three biological replicates. The statistical significance of differences between different time intervals were determined using an unpaired two-tailed Student's t-test (***, P\0.001). The relative expression value of the sample was normalised with endogenous reference gene with early morning time sample. Compared to early morning sample, there was a significantly decreased level of the transcript according to ABI real-time SD RQ manager. Error bars represent mean ± SEM. Transcript levels quantified by qRT-PCR analyses of *StCDF1.2* gene from the KS and KCM samples.

2b. Gene expression analysis of *StSP6A* and *StSP5G* in VIGS and control potato plants by qRT-PCR. Y-axis shows mean relative expression values ± standard error from three biological replicates. Three time points for leaf harvest (time is shown on the x axis) from the plants.

qRT-PCR was performed using RNA extracted from VIGS KS and control KS plants using primers designed against the *StCDF1.2, StCO, StSP5G* and *StSP6A* gene.

Data are the average of three biological replicates. The statistical significance of differences between control plants and VIGS plants were determined using an unpaired two-tailed Student's t-test (***, P\0.001). The relative expression value of the sample was normalised with control sample value. Compared to VIGS plants, there was a significant increase level of the transcript in control leaf samples according to ABI real-time SD RQ manager normalise the control sample RQ value as 1. Error bars represent mean ± SEM. Transcript levels quantified by qRT-PCR analyses of *StCDF1.2, StCO, StSP5G* and *StSP6A* genes from the VIGS plants.

System Sequence Detection Software Version 1.4 (Applied Biosystems, USA). The protocol followed for quantification of gene expression was the same as mentioned in section 2.4.

2.7.2. Sequencing

Sequencing of the PCR product amplified from *StCDF1.2* silenced VIGS and control plants was performed to reconfirm the silencing. The obtained sequence was analyzed using BLAST and pairwise alignment, with the original sequence using Clustal omega.

2.8. Phenotypic observations

Flowering, senescence and tuberization stages were selected for the phenotypic study after silencing. Tubers were harvested from silenced and control plants after complete plant senescence.

2.9. Statistical analysis

The statistical significance of obtained data was determined using an unpaired two-tailed Student's t-test (***, P/0.001: **, P/0.002:*P/0.005). All values reported mean ± Standard Error of three replicates.

3. Results

3.1. Allelic variation studies

PCR was performed to study the allelic variation of *StCDF1* using KS and KCM cDNA by using a high-fidelity polymerase, which resulted in two fragments, each about 1.3 kb and 2.3 kb (Fig. 1a). These fragments correspond to *StCDF1.2* and 1.3 alleles, which was later confirmed by sequencing. The same was reported by Kloosterman et al. (2013) in early maturing potato varieties of Andigena species. In this study, E-value of 0 was obtained with 99% query cover for *StCDF1* sequence. For detailed analysis, alignment was studied with different available sequences in the database. 99% sequence homology was obtained on analysis of the obtained alleles by BLAST. Clustal omega was used for pairwise alignment study which gave 99% similarity with the published sequence.

3.2. Expression profile of StCDF1 in KS and KCM

qRT-PCR was performed to understand the diurnal regulation of *StCDF1* expression in short-day (KS) and long-day (KCM). The highest expression (1.92 fold) was observed in the samples collected at dawn (6:00 am) followed by (1.53 fold) in samples collected at noon (12:00 pm) in KS. The expression of *StCDF1* transcript was 0.92 fold higher in KS when compared to KCM during dawn (6:00 am) and 0.39 fold higher in KCM when compared to KS at noon (12:00) (Fig. 1b). In KCM, the expression of endogenous transcripts was 0.747 fold higher during dawn than noon and 1 fold higher than during dusk (6:00 pm) (Fig. 1c). Upregulation of the *StCDF1* was also observed in the short day adapted KS in comparison to the long day adapted KCM (heat susceptible).

3.3. VIGS

3.3.1. Quantification of gene expression in VIGS treated Plants

The expression of target gene *StCDF1.2* was reduced in VIGS plants, compared to control (6:00 am). Reduced expression of *StCDF1.2* in VIGS plant was observed at all-time points (6:00am, 12:00noon and 6:00pm) (Fig. 2a). The highest expression of 0.286 fold was observed at 6:00 am in VIGS plants. During dawn (6:00 am) the expression of *StCDF1.2* was 0.741 fold higher than that of VIGS treated plants.

Reduction in the expression by 0.263 fold was recorded in VIGS plant at noon (12:00noon) when compared to the VIGS plants at dawn (6:00 am). The expression of genes acting downstream to *StCDF1.2* (*StSP6A, StSP5G* and *StCO*) was also checked during the same time points. The expression of potato FT homologue *StSP6A* was down-regulated in correspondence to *StCDF1.2* silencing. The expression of *StSP6A* was reduced by 0.5, 0.784 and 1.39 fold at 6:00 am, 12:00noon and 6:00 pm respectively. While contrastingly the expression of *StSP5G*, another FT homologue was upregulated by (2 fold) in VIGS treated plants during noon (12:00noon) (Fig. 2b). The silencing of *StCDF1.2* resulted in the 0.6 fold *StCO*, expression, which governs the upregulation of *StSP5G* expression.

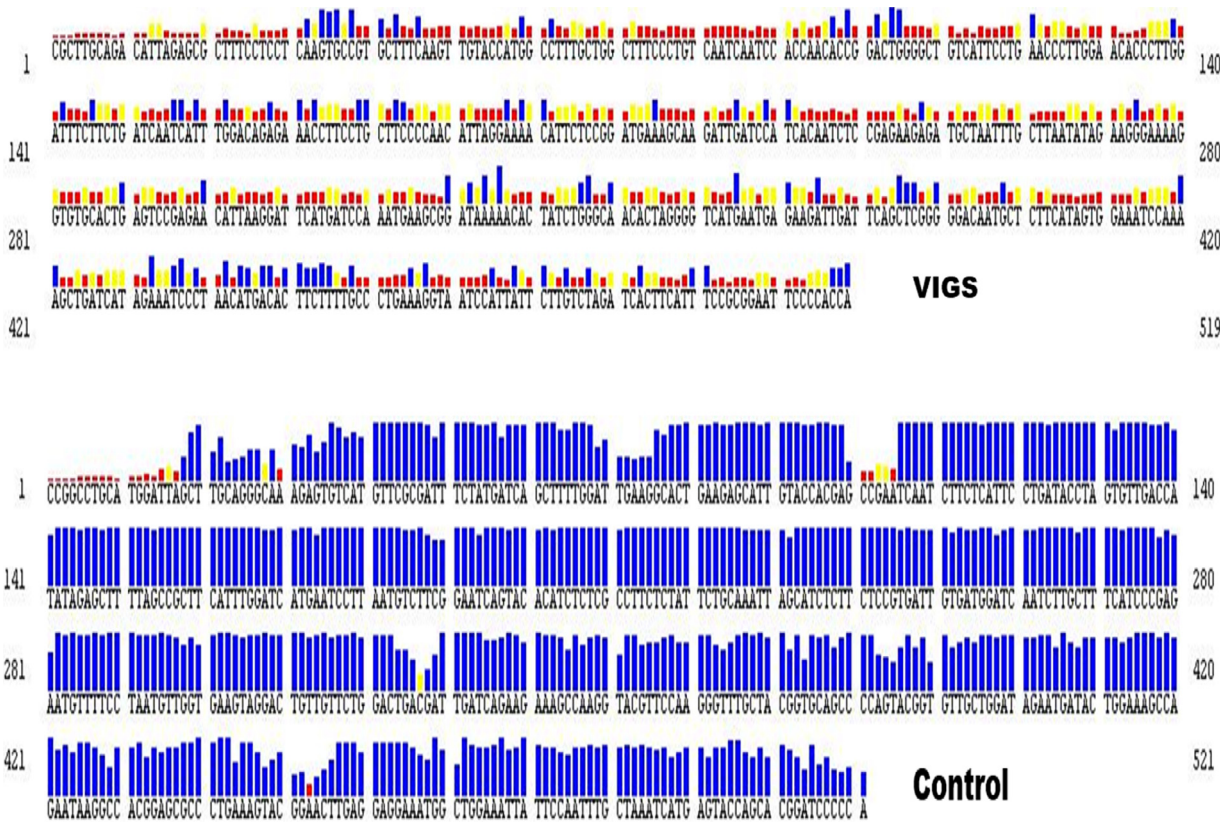

Fig. 3. (a). Representative Sanger sequencing electropherogram of sequences obtained after silencing of *StCDF1.2* in VIGS treated K. Surya.
3b. Electropherogram of sequences obtained from control K. Surya.

3.3.2. *Reconfirming the silencing of* StCDF1.2 *gene by Sanger Sequencing*

The VIGS treated and non-treated samples were amplified by PCR using VIGS specific primers. The fragments were then purified by PCR and subjected to Sanger sequencing. The quality values of pure base of chromatograph showed Q value 5 and medium bases and exhibited the low quality amplification of the target gene due to interruption of mRNA sequence *StCDF1.2* in comparison to the control sample sequences (Fig. 3a and 3b) showed a pure and high quality base value of 20 and above. Sanger sequencing is one way to reconfirm the silencing in VIGS plants.

3.3.3. *Effect of* StCDF1.2 *on flowering*

Regardless of the upregulation of *StCO* and *StSP5G* transcripts in VIGS plants, there was no change in flowering, flower initiation, flower number and flowering pattern between VIGS and control plants. Since flowering is a multigenic trait and upregulation of *StCO* and *StSP5G* via gene silencing through VIGS is incomplete and transient. This silencing might have not complete effect on other key regulators of flowering, as its flowering signal transduction pathway is different from that of tuberization.

3.3.4. *Effect of* StCDF1.2 *on tuberization*

Tubers were harvested from VIGS treated and control plants (95 days matured plants). Since there was a downregulation of *StCDF1.2* and *StSP6A* expression in VIGS treated plants, they exhibited senescence while the control KS plants had more tubers and were phenotypically mature and healthy (Fig. 4).VIGS plants also showed delayed tuberization, indicating the important role of *StCDF1.2* in tuberization initiation. Since other genes from the tuberization signalling cascade may also play an important role in tuber formation, a complete suppression in tuberization was not observed.

3.3.5. *Effect of* StCDF1.2 *on senescence*

Change in tuberization process can affect the overall plant physiology and growth, specifically plant senescence. The early growth stages (vegetative growth and flowering) of VIGS and control plants were similar. The difference was observed after induction of tuberization in both sets of plants. Silenced plants showed senescence, which could lead to a reduced number of tubers when compared to the control plants.

4. Discussion

Development of early maturing potato variety is essential to meet the demands of farmers and to increase productivity. With the advancement in molecular tools for functional studies, identification of candidate genes and their efficient functional validation is now

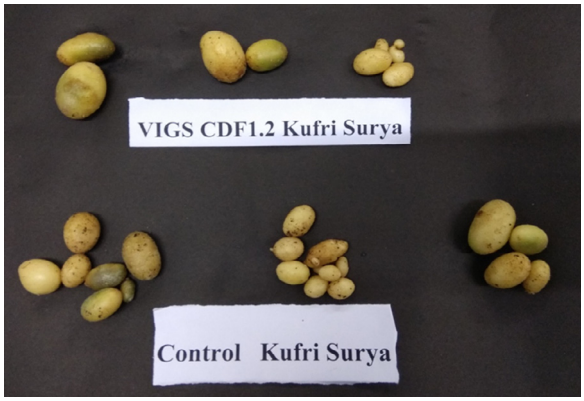

Fig. 4. Tubers yield from VIGS plants transformed with *StCDF1.2* and control KS plants.

less complex. *StCDF1.2* is a prime candidate gene that determines the extent of plant maturity and is linked with *StSP6A* and *StCO* for the regulation of tuberization signal. Variation in the expression pattern on *StCDF1* observed by us clearly corroborates with the findings of Koolerstman et al. (2013), who also reported a similar variation in the expression patterns of *StCDF1* in diploid potato cultivars at different time points during a 24-hour day cycle. Koolerstman et al. (2013) also observed the highest expression of *StCDF1* in the sample collected just before dawn (6:00 am). Expression of *StCDF1* decreased with the progression of the day and reached to the lowest point by dusk (6:00 pm). This variation in the expression could be due to the degradation of *StCDF1* by dusk. This could be mediated by a class of blue-light (long day) perceiving ubiquitin ligase, *StFKF1* (FLAVIN-BINDING KELCH REPEAT F-BOX PROTEIN 1) and a clock protein *StGI* (GIGANTEA) which interact with *StCDF1* and marks it for proteasomal mediated protein degradation (Abelenda et al. 2016). In short-day conditions, suppression of few clock genes does not allow the formation of StFKF1/StGI/StCDF1 complex and results in a high StCDF1 level (Kim et al. 2017). Talar et al. (2017), reported the same diurnal control of tuberization and flowering genes using qRT-PCR. The expression of BBX gene family revealed that some members show maximum expression in the light phase. Phylogenetic analysis of B-box proteins revealed that most of them were orthologs to tomato and Arabidopsis B-box proteins. These members of the BBX have a domain, termed CCT (CONSTANS like), composed of more than 40 amino acids located in the C-terminus. Till date, the best-characterized plant BBX protein is CONSTANS (CO, *AtBBX1*) in Arabidopsis.

Expression analysis studies revealed a downregulation in the expression of *StSP6A* and upregulation in *StSP5G* and *StCO* transcripts in VIGS plants. This could be due to the fact that *StCDF1.2* has a central role in tuberization by indirectly controlling the expression of *StSP6A, StSP5G* and *StCO. StCDF1.2* downregulates the expression of *StCO* which causes a suppression of *StSP5G* transcripts. This suppression of *StSP5G* induces the expression of a mobile tuberization signal, *StSP6A* which induces the tuber development in stolon tips (Koolerstman et al. 2013). Since there was a downregulation of *StCDF1.2* in VIGS plants, *StCO* was relieved from its inhibition casing its upregulation. This indicates a conserved repressive function of potato *StCDF1.2* on *StCO. StCO* now transcriptionally activates *StSP5G*, which functions as a transcriptional repressor for *StSP6A*. This substantiates the notion that *StCO* does not directly affect the tuberization in non-inductive photoperiods, it indirectly regulates the expression of *StSP6A* via *StSP5G* (Fig. 5) (Navarro et al. 2011; Dutt et al. 2017). Silencing of *StPHYF* (Phytochrome F) a light receptor, reduced the accumulation of StCO1 protein, compared to the control plants. This decreased accumulation represses *StSP5G* expression, elevating StSP6A levels. These phytochromes play a crucial role in the photoperiod-dependent tuberization. StPHYF regulates StCO pathway and stabilize it in light (long afternoon) and aid StCDF1.2 degradation (Zhou et al. 2019).

We also observed no change in flowering pattern and flowering initiation in both control and VIGS plants. Similar results were observed by Koolerstman et al. (2013), where there was no difference in flowering between *StCDF1.2* overexpressing transgenic lines and nontransgenic control plants. These findings clearly establish a divergence in signalling pathways for both flowering and tuberization. Plantega et al. (2018), indicated additional regulation of both *StSP5G* and *StSP6A*, as overexpression of *StCO* is not affected by *StSP6A* expression in short-day conditions. In short-days, the tuber inducing role of *StCDF1* by repressing *StCO* may be compensated by the overexpression of *StCO*, while *StCDF1* may also directly activate *StSP6A*.

Our present findings indicate the role of *StCDF 1.2* gene in governing the initiation of tuberization This conceptualizes the earliness and delayed tuberization pathway in potato cultivars. The same has been well demonstrated in earlier cDNA microarray studies showing

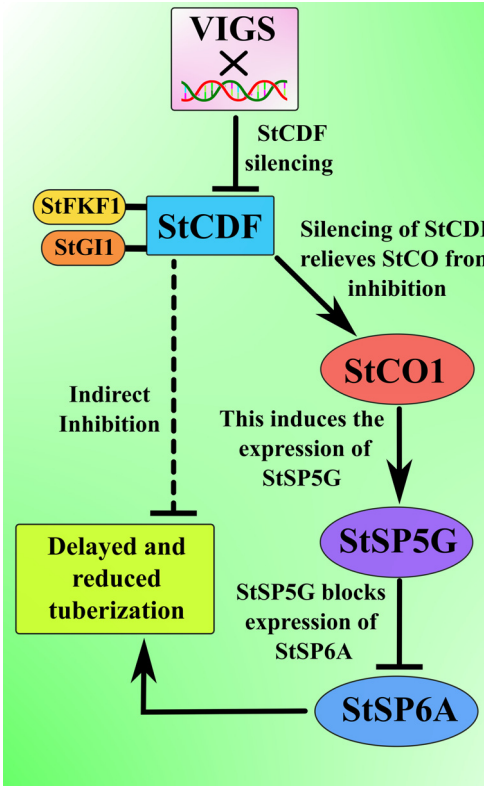

Fig. 5. Represents the molecular regulation of tuberization in VIGS plants. *StCDF1.2* down-regulates *StCO* expression. This represses *StSP5G* transcription, facilitating *StSP6A* expression and tuberization initiation. The silencing of *StCDF1.2* by VIGS relieves the *StCO* inhibition. This *StCO* inhibits tuberization by indirectly inhibiting *StSP6A* expression through *StSP5G* repressor. The dashed line represents the indirect inhibition of tuberization in VIGS plants with silenced *StCDF1.2*.

the *StSP5G* and *StSP6A* gene expression in KS and KCM cultivars (Singh et al. 2015). VIGS was used to authenticate the role of *StCDF1.2* as a candidate gene for earliness. qRT-PCR was performed to assess and validate the extent of gene silencing of *StCDF1.2*, using gene-specific primers. More than 80% of target gene expression can be suppressed by the TRV-VIGS system in the host plant (Senthil-Kumar and Mysore, 2014). As *StCDF1.2* is the indirect inducer of *StSP6A*, its downregulation confers its role as a StSP6A inducer for tuberization. To further authenticate the silencing, PCR was performed, to confirm the degradation of *StCDF1.2* gene sequence, which further reconfirms the silencing and functionality of *StCDF 1.2* in KS and KCM cultivars.

5. Conclusion

Development of early maturing potato cultivars is required in wide agro-climatic zones of the country. *StCDF1* gene is a key factor for earliness and initiation of tuberization signalling in potato. The expression profile of *StCDF1, StSP6A, StCO, StSP5G* and their functional validation by VIGS, is a basic strategy to understand the role of *StCDF1.2* allele in Indian potato cultivar. This could help to understand the mechanism of early tuberization in contrasting Indian potato cultivars. This could further help in designing breeding programs for the development of early maturing potato cultivars using routine conventional breeding and or genome editing approaches.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.sajb.2020.05.008.

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