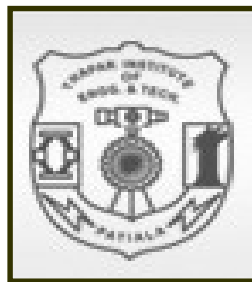


**CONSTRUCTION OF Ti-PLASMID BASED VECTORS USING TUBER-SPECIFIC  
GENE PROMOTERS FROM THE INDIAN POTATO CULTIVARS**

**A  
THESIS REPORT**

Submitted in partial fulfillment of the requirement for the award of  
the degree of Master of Science in Biotechnology



**Under the guidance of:**

**Dr. N. Das**

Asstt. Professor

**Submitted by:**

**Preeti Sharma**

Roll No. 3040022

---

**DEPARTMENT OF BIOTECHNOLOGY AND ENVIRONMENTAL SCIENCES  
THAPAR INSTITUTE OF ENGINEERING AND TECHNOLOGY  
(DEEMED UNIVERSITY)**

**PATIALA – 147 004**

**2006**

## **CANDIDATE'S DECLARATION**

I, hereby declare that the work presented in the thesis entitled, "**CONSTRUCTION OF Ti-PLASMID BASED VECTORS USING TUBER-SPECIFIC GENE PROMOTERS FROM THE INDIAN POTATO CULTIVARS**" in partial fulfillment of the requirement for the award of the degree of Master in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala, is an authentic record of my own work during the period of six months from January 2006 to June 2006, under the guidance of **Dr. N. Das, Assistant Professor, Thapar Institute of Engineering and Technology, Patiala**. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

Date:

Place: PATIALA

PREETI SHARMA

## **CERTIFICATE**

This is to certify that the thesis entitled, “**CONSTRUCTION OF Ti- PLASMID BASED VECTORS USING TUBER-SPECIFIC GENE PROMOTERS FROM THE INDIAN POTATO CULTIVARS**” submitted by Preeti Sharma in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, to Thapar Institute of Engineering and Technology (Deemed University), Patiala is a record of student’s own work carried out by her under my supervision and guidance. The thesis has not been submitted for the award of any other degree or certificate in this or any other University or institute.

(Dr. N. Das)

Supervisor  
Deptt. of Biotech. & Env. Sciences  
Thapar Institute of Engg. & Technology,  
Patiala.

(Dr. N. Das)

Head  
Deptt. of Biotech. & Env. Sciences  
Thapar Institute of Engg. & Technology,  
Patiala.

(Dr. T.P.Singh)

Dean, Academic Affairs  
Thapar Institute of Engg. & Technology,  
Patiala.

## **ACKNOWLEDGEMENT**

I wish to record my sincere gratitude to my guide and supervisor, **Dr. N. Das**, Assistant Professor and Head, Department of Biotechnology and Environmental Sciences, for his valuable guidance and constant encouragement throughout the duration of this work. He taught me how to plan, execute, record and interpret experimental findings.

My special thanks goes to all other staff and members of Department of Biotechnology and Environmental Sciences.

I extend my thanks to **Anshu** and **Vijay** for their invaluable help & support that they extended in clearing my doubts and queries during the course of this thesis work.

At last I wish to thank my parents & the almighty that has always bestowed their blessings on me.

(PREETI SHARMA)

## **CONTENTS**

	Page No.
1. INTRODUCTION	1-7
2. REVIEW OF LITERATURE	8-14
3. MATERIALS AND METHODS	15-24
4. RESULTS AND DISCUSSION	25-35
5. SUMMARY	36
6. REFERENCES	37-40

# **1. INTRODUCTION**

Regulation of gene expression is a vital process in the cell and involves the action of a host of specific protein factors, which can act at different steps in the gene expression pathway. This includes studies on the structure and regulation of the molecular machinery responsible for replication and transcription of DNA, the processing of RNA and the functional organization of chromatin, as well as studies on how genes and the factors acting on them are organized within the nucleus. Recombinant DNA technology allows us to manipulate the very DNA of living organisms and to make conscious changes in that DNA.

The ability of a gene to produce a biologically active protein is much more complex in eukaryotes than in prokaryotes. A major difference is the presence in eukaryotes of a nuclear membrane, which prevents the simultaneous transcription and translation that occurs in prokaryotes. Whereas, in prokaryotes, control of transcriptional initiation is the major point of regulation, in eukaryotes the regulation of gene expression is controlled nearly equivalently from many different points.

In eukaryotes, genomic DNA is packaged into chromosomes and kept in the cell nucleus, where genes are regulated and expressed. During each cell division cycle, the chromosomal DNA must be replicated exactly once, during S-phase, and then one copy of each replicated chromosome segregated into the two daughter cells during mitosis. When genes are expressed, the chromosomal DNA must be transcribed into RNA and the RNA then processed and transported to where it is needed.

If left to the molecular machinery of a cell without control, the transcription apparatus of the cell would express every gene in the genome at once: unwinding the DNA double helix, transcribing each gene into single-stranded mRNA and, finally, translating the mRNA into protein. But, no cell can function amid the resulting cacophony. So, there exists a mechanism by which cells muzzle many genes, so that, at a given point of time, only a fraction, about 15% of the genes are expressed. This phenomenon is known as "Regulation of Gene Expression".

There is another aspect to the control of gene expression in a cell, especially in eukaryotic cells and multicellular organisms. Each cell of a multicellular organism is "differentiated", i.e. it is specialized for a particular function. These cells are remarkably diverse, e.g. nerve cells, kidney cells, macrophages, myocytes, hair cells, etc. Some are

short and fat; some are long and thin; some have appendages; and others are roughly spherical. Yet all of these cells contain the same set of genes. So, how do we explain their highly diverse phenotypes? The answer lies in the regulation of gene expression, and it plays an important role in development and differentiation. Study of regulation of gene expression is one of the thrust areas of modern molecular biology and biochemistry.

**Role of promoters:** Promoter is the DNA region where the transcription initiation takes place. In prokaryotes, the sequence of a promoter is recognized by the Sigma ( $\sigma$ ) factor of the RNA polymerase. In eukaryotes, it is recognized by specific transcription factors. These promoters are regulated by many *cis*-acting elements, which are short DNA sequences present in promoters, which are activated or inhibited by proteins known as *trans*-acting factors. Some of these *cis*-acting elements are: Light-Responsive Elements (LREs), Glucocorticoid Response Elements (GREs), Sugar Responsive Elements (SUREs), Abscisic Acid Response Elements (ABREs), Enhancer and Silencer Sequences, etc.

**SOME ASPECTS OF TUBER DEVELOPMENT:** As we are interested in tuber-specific gene promoter in this study, it is relevant to discuss briefly about some aspects of tuber development. Potato tuber is a staple food in most countries of the temperate regions of the world. It is a modified stem, which grows underground and accumulates starch. Potato belongs to *Solanum* genus of the *Solanaceae* family. Potato is a rich source of starch. Freshly dug potatoes contain 75% water, 18% starch, 1½ % fiber, 2.2% protein, 1% ash (inorganic constituents), and 0.1% fat and a little sugar. Potatoes (tubers of *Solanum tuberosum*) are grown and eaten in more countries than any other crop, and in the global economy they are the fourth most important crops after the three cereals rice, wheat and maize. Therefore, research into potato tuber initiation and development, which enables our understanding and possible manipulation of these processes, is of great relevance. In addition to improving the yield and quality of potato harvests and increasing resistance to pathogen infection, research is also directed at improving the nutritional content of the tuber, and "pharming" which is removing the starch in the potato tuber and instead producing organic compounds such as proteins that are too expensive or cannot be produced in bacterial or yeast culture systems.

There are many factors that affect tuber formation the bacteria living in the root zone, but nitrogen levels, temperature and light have the greatest effect. High nitrogen

levels (supplied in the form of ammonium or nitrite ions) between the ranges of 1-3 mM have been found to completely inhibit or delay tuberization. If the plants are put into "excessive" nitrogen supply after they have started tuberizing, then tuber formation will cease and stolon growth may be resumed. High temperatures are inhibitory for tuberization in both short and long photoperiods, although the inhibitory effect is much greater in long photoperiods. High temperatures affect the partitioning of assimilates by decreasing the amount going to the tubers and increasing the amounts to other parts of the plant. There is also some evidence that the inhibitory effect of high temperatures is mediated through increased GA (Gibberellic Acid) levels. High light intensity is known to promote tuberization, and the promotive effects of high levels of irradiance can ameliorate the inhibitory effects of high temperatures.

A method for synchronized *in vitro* tuber induction in a Hungarian cultivar of *Solanum tuberosum* designated "Keszthelyi 855" has been developed. It was shown that in this system tuberization and stolon elongation primarily depend on the level of sucrose in the medium. The cytokinin, 6-benzylaminopurine (BAP), also enhances the efficiency of tuber formation, however, only at sucrose concentration above 4% (w/v). The synchronized plant culture provided starting material for isolation of genes specifically expressed in tuberizing *Solanum* species during the early stage of tuber development. In comparison with the non-tuberizing *Solanum brevidens*, three types of specific transcripts have been obtained by differential screening. Based on DNA sequence analysis the genes isolated code for the major tuber proteins, patatin and proteinase inhibitors.

**Tuber initiation and enlargement:** After induction of tuberization, next step is tuber initiation and enlargement. During enlargement, tubers become the storage sinks of the plant and store massive amounts of carbohydrates and also significant amounts of protein.

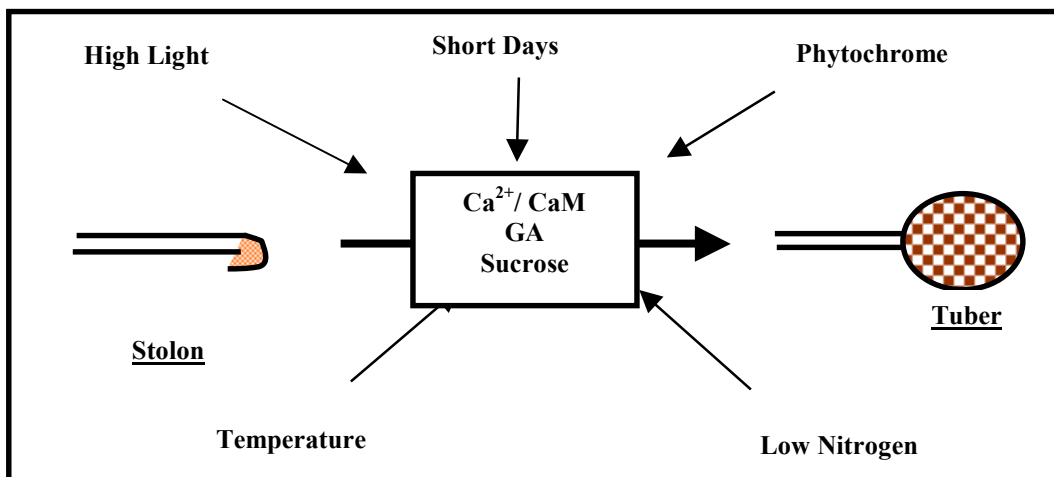
**Changes on the Protein Level:** About 2% of the fresh weight of a potato tuber is present as protein. The protein composition changes dramatically during stolon-tuber transition resulting in the formation of a much-simplified protein complement consisting of only a few highly abundant proteins such as patatin. Other proteins include type II proteinase inhibitor, a Bowman-Birk proteinase inhibitor and a Kunitz trypsin inhibitor. Experiments have indicated that these proteins have no role in tuber development.

**Carbohydrate Metabolism:** One of the most pronounced changes observed during tuber initiation and enlargement is the massive formation of starch, which in the mature

tuber typically represents 20% of the fresh weight. But, surprisingly, starch biosynthesis is not essential for tuber formation, as has been experimentally demonstrated in potato tubers in which AGPase was reduced by antisense repression, thus showing a significantly reduced starch level. These plants display normal tuber formation (Fernie and Willmitzer, 2001).

Soluble carbohydrates, most notably sucrose, have convincingly been described to be strong inducers of tuberization. However, the path of Sucrose unloading into the growing tuber has been recently found, consisting of both apoplastic and symplastic pathways. With the first visible sign of tuber initiation, there is a switch from predominantly apoplastic unloading into stolons that are undergoing extension growth into towards predominantly symplastic unloading into tubers.

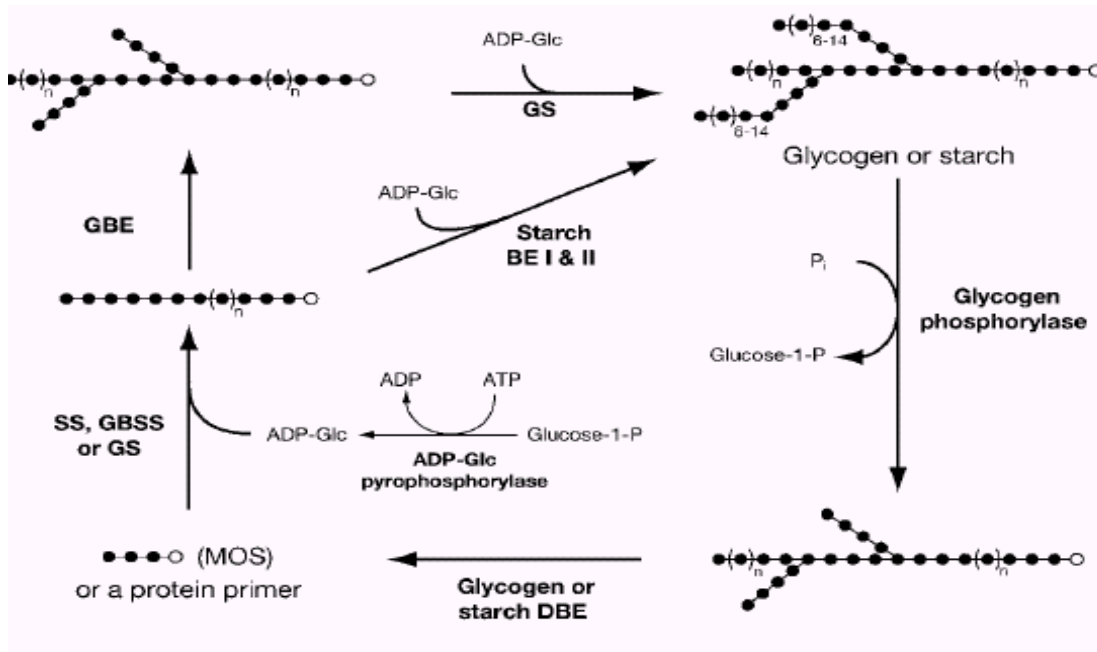
**Signal transduction pathway in tuber development:** The signal transduction pathway(s) controlling tuber induction and development is only beginning to be elucidated, and there is good evidence that shows the involvement of phytochrome in the response to photoperiod. Photoperiod is also known to affect the production and export of Sucrose, another signaling molecule. There is also evidence that indicates the involvement of  $Ca^{2+}$ /Calmodulin at some stage downstream in the transduction pathway, which is not surprising since they have been shown to be involved in at least one phytochrome signal transduction pathway. But, the identification of a tuber-inducing signal remains an elusive goal.



**Environmental factors and signaling molecules affecting the induction of tuberization. The transduction pathway still has to be defined.**

## STARCH BIOSYNTHESIS IN TUBERS

Starch is synthesized in chloroplasts of green plant tissues as well as amyloplasts, the starch storing organelles present in cells of seeds, roots and tubers, as a stable product of photosynthesis. The pathway is schematically shown below:

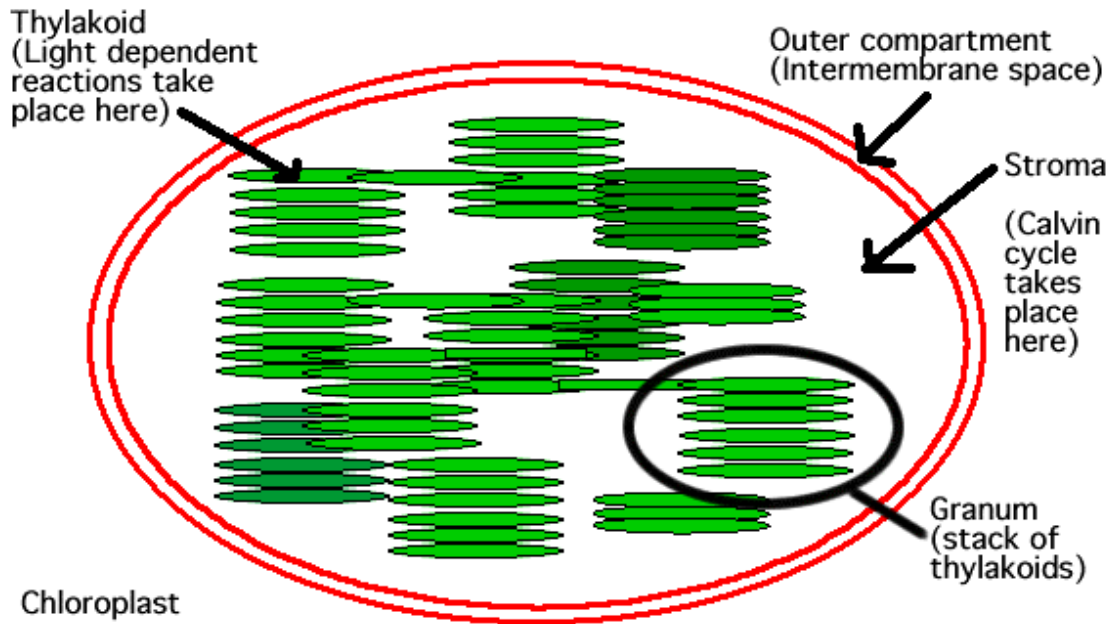


### Starch Biosynthesis pathway

Starch synthesis involves three key enzymes and their isoforms:

- (i) **ADP-Glucose Pyrophosphorylase (AGPase):** It catalyses the production of ADP-Glucose from Glucose-1-Phosphate and ATP. AGPase catalyses the rate-limiting step in starch biosynthesis and it is an allosteric enzyme with its activity modulated by 3-PGA (activator) and  $P_i$  (inhibitor).
- (ii) **Starch Synthase (SS):** Starch Synthase catalyses the elongation of glucan chains through the introduction of  $\alpha$ -(1 $\rightarrow$ 4) glucosidic linkages between the incoming glucose residues of ADP-Glucose and the growing glucan chains, at their non-reducing ends.

(iii) **Starch Branching Enzyme (SBE):** Starch branching enzyme introduces  $\alpha$ -(1 $\rightarrow$ 6) glucosidic bonds to form branched polysaccharides.



**Ultrastructure of a chloroplast site of starch biosynthesis in plants**

## **STARCH STRUCTURE**

Starch is produced as granules in most plants cells and is referred to as “native” in this particular granular state. Native starches from different botanical sources vary widely in structure and composition, but all granules consist of two major molecular components, amylose (20-30%) and amylopectin (70-80%), both of which are polymers of  $\alpha$ -D-glucose units in the  ${}^4C_1$  conformation. In amylose, these are linked - (1  $\rightarrow$  4)-, with the ring oxygen atoms all on the same side, whereas in amylopectin about one residue in every twenty is also linked -(1  $\rightarrow$  6)- forming branch-points.

## **ROLE OF GBSS IN STARCH BIOSYNTHESIS**

As discussed earlier, Starch Synthase (SS) enzyme is responsible for the extension of glucan chains at the non-reducing end of starch primer during starch biosynthesis. However, several isoforms of starch biosynthesis have been identified which contribute in their own unique way towards the structural organization of the starch granule. So far, following isoforms of starch synthase are known:

- **Granule-Bound Starch Synthase (GBSS)**
- **Starch Synthase I (SSI)**
- **Starch Synthase II (SSII)**
- **Starch Synthase III (SSIII)**

Out of the above four, GBSS is exclusively bound to the starch granule, whereas the other three enzymes may be located partially or entirely in the stroma (soluble phase) of chloroplast.

Tuber initiation and development together involve many genes pertaining to carbohydrate metabolism, protein metabolism and others. Some of these genes are constitutive in nature and many of them are expressed during only tuber development. Molecular mechanism pertaining to tuber- specific gene expression is an attractive area of research today. Since, if we can study and isolate tuber specific promoters could be used in driving foreign gene expression only in tubers.

## **2. REVIEW OF LITERATURE**

### **POTATO TUBER DEVELOPMENT**

The switch from stolon to tuber in a potato plant is a highly complex and poorly understood process. A multitude of factors are known to promote and inhibit tuberization, but how all these factors act together to regulate tuberization is not known. Nevertheless, factors like high nitrogen levels and high temperature are known to inhibit tuberization, whereas high irradiance and high sucrose levels are known to promote it.

Evidence is accumulating which indicate that a signal transduction pathway mediated by phytochrome and involving  $Ca^{2+}$ /Calmodulin controls tuber induction in potato, but the details await further investigations (Jackson *et al.*, 1999).

### **GENETIC MANIPULATION OF STARCH: RECENT DEVELOPMENTS & FUTURE PROSPECTS**

A number of recent developments have demonstrated the feasibility of production of novel starches with remarkable characteristics, which make them suitable for industrial manufacturing processes and also impart them properties hitherto unknown in native plant starches. Some of the properties of starch which are of concern are: nutrition quality improvement, amylose/amylopectin content, increasing starch quantity, altered granule structure, etc.

Amylose-free (*Waxy*) starches, which are produced by eliminating GBSS enzyme responsible for amylose biosynthesis, gelatinize easily, yielding clear pastes. Waxy wheat starch is used as a stabilizer and thickener in food products and as an emulsifier for salad dressings.

Freeze-thaw stability is one of the most desirable properties of starch for use by food industry in food products that are stored at low temperatures. Recently, a remarkably freeze-thaw stable potato starch was produced by Antisense inhibition of three genes for enzymes, namely Starch synthase II, Starch synthase III and granule-bound starch synthase (Jobling *et al.*, 2002).

The key regulatory enzyme of starch biosynthesis, AGPase, has been the focus of investigations to increase starch content in plants. A 30% increase in starch content was observed in transgenic potato expressing an *E. coli* AGPase that is insensitive to regulation by  $P_i$ , an inhibitor of AGPase (Stark *et al.*, 1992).

Potato starch has the highest level of phosphate among commercial starches, which is partly responsible for its high swelling power and stable-paste properties. The enzyme responsible for incorporation of phosphate residues in potato starch has been recently identified as  $\alpha$ -glucan water dikinase (GWD). Antisense inhibition of the gene that encodes this enzyme results in a starch that has low phosphate content and viscosity, an increase in apparent amylose and altered amylopectin structure (Lorberth *et al.*, 1998).

## **STARCH SYNTHESIS IN PLANTS**

Starch biosynthesis is a very complicated and incompletely characterized process. Furthermore, the understanding of the relationships between starch composition, starch granule structure and the functional properties of starch is incomplete. So far, the use of molecular biological techniques has provided significant contributions to our understanding of starch biosynthesis (Davis *et al.*, 2003).

Much of our understanding of starch biosynthesis has come from analysis of natural mutants of maize, rice, barley, potato and pea. In addition, the study of the unicellular alga *Chlamydomonas reinhardtii* has also provided significant insights in this regard. Recently, studies have been initiated on model plant *Arabidopsis thaliana* to understand starch biosynthesis for several reasons, the most prominent being the short time period over which starch synthesis by various enzymes can be monitored, and that the rate of starch synthesis in leaves can be controlled by altering the irradiance and accurately measuring it by supplying  $^{14}\text{CO}_2$  (Zeeman *et al.*, 2002). Through these studies, the involvement of various enzymes in starch biosynthesis has been well documented.

As explained before, ADP- Glucose Pyrophosphorylase (AGPase) catalyses the formation of ADP-Glucose, the precursor for starch synthesis, from Glucose-1-phosphate and ATP. Since it is regulated allosterically, AGPase is often referred to as a rate-limiting step in starch biosynthesis. AGPase exists as a tetrameric protein composed of two small and two large subunits, which are encoded by distinct genes. The small subunits are responsible for catalytic activity and the large subunits for allosteric regulation. This observation is substantiated by sequence conservation of two subunits, and by mutant analysis.

Starch Synthases catalyse the extension of glucan chains at their non-reducing ends. Multiple isoforms of starch synthases have been found in almost all plant tissues studied to date, from green algae to monocots. SSI, SSII, and SSIII are involved in

amylopectin biosynthesis, although the roles for each enzyme class have not been clearly established. In contrast to GBSSI, which is active only when bound to starch granule, it is not clear whether SSI, SSII and SSIII are functional enzymes when bound to the starch granule, or simply become trapped inside the starch granule as it grows.

The role of SSI in starch biosynthesis remains unclear, as no mutations in this gene, or gene suppression experiments leading to a reduction in SSI activity, have been reported. The failure to recover mutations in model plants such as maize suggests that the contribution of the enzyme to starch biosynthesis is either so large that mutants are effectively lethal or so subtle that the mutations are not picked up by visual screens of seed size or kernel appearance (Ball and Morell, 2003).

SSII was identified by mutation at *RUG5* locus of pea. The effect of the mutation upon the starch of the embryo is dramatic; Although content is barely affected, starch granule is grossly misshapen, and the distribution of chain lengths in the amylopectin is very different from wild-type starch, in that there are more very short chains (<15 glucose units) and very long chains. It appears that SSII is specifically responsible for the elongation of very short chains that form the basis of the clusters within amylopectin.

SSIII is also involved in amylopectin biosynthesis, as demonstrated by mutant analysis. SSIII mutants have been isolated in *Chlamydomonas* and maize, where there is a major impact on the synthesis of amylopectin, resulting in amylopectin with modified chain-length distribution and decreased starch synthesis.

Starch branching enzymes (SBEs) introduce  $\alpha$ -(1 $\rightarrow$ 6) glucosidic bonds to form branched polysaccharides. Several isoforms of SBE have been identified in developing storage tissues of higher plants. The existence of so many isoforms raises the possibility that different forms create chains of different lengths or branch points at different frequencies. All isoforms of SBE have been put into two classes A and B, based on their primary sequence. It has been suggested that type A isoforms participate in the synthesis of shorter glucan chains that lie wholly within clusters, whereas type B isoforms participate in the synthesis of long and intermediate length chains that will span clusters.

Apart from the above enzymes, there are other enzymes also which play an important role in starch biosynthesis, which include starch debranching enzyme,  $\alpha$ -glucan water dikinase (GWD), disproportionating enzyme (D-enzyme), etc.

Starch biosynthesis, despite such advances, remains an unsolved mystery, with several of its aspects remaining unknown and unexplained. A major unresolved question

of starch biosynthesis consists of the priming of both polysaccharide synthesis and granule formation. These processes may or may not be entirely independent. For efficient manipulation of starch biosynthesis in plants, further characterization of the enzymes participating in the process needs to be done. Determining the contribution of each enzyme to amylose, amylopectin and granule structure has been made difficult, because there is a strong interdependence between the enzymes involved. Also, the relationship between starch biosynthesis gene expression and the structure of amylose, amylopectin and the starch granule needs to be investigated.

### **GRANULE-BOUND STARCH SYNTHASE (GBSS)**

GBSS is responsible for the biosynthesis of amylose fraction of starch in higher plants. So far, two isoforms of GBSS have been identified: GBSSI and GBSSII. GBSSI is responsible for amylose biosynthesis in endosperm, whereas GBSSII synthesizes amylose in tissues other than endosperm, as in pericarp, aleurone layer, and embryo. GBSS gene from potato contains 13 introns, the first of which is located in the untranslated leader. The GBSS promoter contains a G-Box-like sequence. The gene also has a transit peptide sequence of 77 amino acids, required for routing of the precursor to the plastids (van der Leij *et al.*, 1991).

Based on Southern blotting analysis and PCR, 8 alleles of GBSS gene have been identified, which were grouped into 4 classes, distinguishable by southern blotting analysis. The major difference between the alleles identified was the absence or presence of a 140-bp fragment at a site 0.5 kb upstream of the ATG start codon of the gene for GBSSI (van de Wal *et al.*, 2001).

GBSS protein has a molecular weight of 58-60 kDa. It differs from other starch synthases in that it is exclusively bound to the starch granule. Among the starch synthases found in the plastid stroma of higher plants, starch synthase II is also found bound to the starch granules in significant amounts. Comparison of potato GBSSI and SSII primary amino acid sequences revealed a strong similarity over a core region of  $\approx$  60 kDa. The core also shows similarity to all other known starch synthases and the bacterial glycogen synthases. It includes an N-terminal KTGGL motif, thought to be required for binding of ADP/ADP-Glucose, and a second, very similar motif, known as KTGGL-look-alike motif close to the C-terminus. GBSS and SSII differ in several characteristics, e.g. affinity for ADP-glucose, thermo-sensitivity, effect of citrate, etc. It has been suggested that the C-terminal region of GBSS is responsible for conferring most of the specific properties associated with it (Edwards *et al.*, 1999).

The fact that GBSS is responsible for amylose biosynthesis came from the analysis of *waxy* mutants of wheat, maize and potato. Also, antisense RNA inhibition of GBSS by *A. rhizogenes* – mediated transformation in potato plants also showed a correlation between reduction in GBSS activity & formation and deposition of amylose (Kuipers *et al.*, 1994).

It has been postulated that the synthesis of amylose by GBSS may be more due to spatial distribution of the enzyme inside the starch granule rather than a specific property. It is suggested that due to being buried deep inside the starch granule, SBE is not able to act upon the product of GBSS, which, thus, remains linear and unbranched (Martin & Smith, 1995).

Two models have been proposed for amylose biosynthesis by GBSS in higher plants:

**MOS – Elongation Model:** According to this model, GBSSI synthesizes amylose by elongating malto-oligosaccharides (MOS) of 2-7 glucose units. Evidence for this mechanism has come from mutant analysis and studies involving radiolabelled maltose. Also, the granules are permeable to molecules upto approximately 1 kDa in size, roughly the size of maltoheptaose

**Amylopectin Cleavage Model:** Experiments on green alga *Chlamydomonas reinhardtii* revealed another mechanism of amylose biosynthesis by GBSSI. In this model, GBSSI elongates amylopectin to create extremely long chains. These amylose-sized chains are then cleaved from amylopectin by an as yet unidentified hydrolase activity. But, experiments with pea embryos could not substantiate this model. But, *Chlamydomonas* could elongate MOS to form amylose. Thus, in *Chlamydomonas*, amylose can be generated *in vitro* by two different mechanisms whilst in higher plants, amylose synthesis from MOS only has been demonstrated (Denyer *et al.*, 2001).

## **GBSS GENE PROMOTER**

Several studies have attempted to characterize the GBSS gene promoter and look for any *cis*-acting elements in it that may confer the promoter its tissue-specificity. Chimaeric genes of promoter sequences from the potato gene encoding GBSS and the  $\beta$ -glucuronidase (GUS) reporter gene were used to study GBSS expression and regulation. Analysis of stable transformants revealed that GBSS promoter sequence of 0.4 kb was sufficient to result in tissue-dependent GUS expression, and levels in stably

transformed microtubers exceeded levels in corresponding leaves by many orders of magnitude (van der Steege *et al.*, 1992).

Also, Antisense RNA-mediated inhibition of transgenic potato plants was achieved by putting the gene under the constitutive CaMV 35S promoter and GBSS gene promoter. Expression of the antisense GBSS gene from the GBSS promoter resulted in a higher stability of inhibition in tubers of field-grown plants as compared to expression from the CaMV 35S promoter. The antisense effect was observed to increase during tuber growth (Kuipers *et al.*, 1994). In a similar study, eleven antisense RNA constructs, derived from the full-length GBSS cDNA, the genomic GBSS coding region (gDNA), or fragments of each of these sequences, were analyzed for their inhibitory effect. Complete antisense inhibition was achieved in clones carrying the antisense construct in which full length GBSS cDNA was driven by GBSS promoter (Kuipers *et al.*, 1995).

Transgenic plant as bioreactor has been used to produce recombinant proteins for medicinal purposes, including mammalian antibodies, blood substitutes and vaccines. As the demand for biopharmaceuticals is expected to increase, transgenic plants have the potential to provide virtually unlimited quantities of proteins for use as tools in both human health care and the bioscience.

Being able to use plants as chemical factories utilizing the sun's energy, rather than fossil fuels, is one dream of biotechnologists genetically modifying crops to produce industrial feedstocks. They hope these will be environmentally friendly solutions to the demands for biodegradable plastics and the production of designer oils and starches. The use of GM potatoes with a modified starch profile is the most advanced application of GM in the field. The Swedish company involved, Amylogene, is applying for consent to grow these potatoes in Europe, and this could be granted in 2004. These are unlikely to be grown in the UK as starch potatoes are grown in Eastern Europe, the Nordic countries, Germany, Belgium and France. The high amylopectin starch extracted will be used in the pulp and paper industry.

## AIM OF THE PRESENT SYUDY

Potato (*Solanum tuberosum* L.) is one of the major food crops of the world. It is a rich source of starch in human diet. It ranks fourth in global production after maize, rice and wheat. Keeping in view its importance as a global food crop, genetic engineering of starch biosynthesis is an attractive and challenging goal in plant biotechnology. Several transgenic potato lines have been created with useful traits hitherto unknown in wild-type plants. These include potatoes with higher starch content, increased pest resistance, high and low amylose content, increased protein content, etc.

Another promising area of research is the use of potato tuber as a "Bioreactor", that is removing the starch in tubers and tricking them to produce novel compounds or biomolecules which are very tedious to produce in conventional bioreactors which employ micro-organisms like *E. coli* and yeast. Examples include production of vaccines, hormones, fructans, growth factors, etc.

However, for efficient genetic manipulation of potato to produce novel organic compounds in potatoes, we need to understand the structure and function of tuber-specific genes along with functional characterisation of their promoters. The gene promoter for granule-bound starch synthase (GBSS) is one of them. Keeping in view the above objective, the aim of the present study is to carry out molecular cloning and functional characterisation of GBSS gene promoter from Indian potato cultivars, which are suitable to our agro-climatic conditions. These gene promoters after functional characterisation and assessment of their strength can be subsequently used to drive heterologous gene expression in plants.

Indian potato cultivars differ markedly regarding their starch content. So, it is evident that these cultivars show varietal differences in gene expression, especially regarding enzymes related to starch biosynthesis. Starch biosynthesis involves many different isoforms of starch synthase, and these cultivars are expected to show some differences regarding the expression profile of these isoforms. Also, the genotype of a plant is dramatically affected by the environment. Previous reports of cloning of GBSS gene and its corresponding promoter have been reported from other potato varieties, most notably the European variety *Desiree*, but GBSS gene promoter has not been cloned from any Indian potato cultivar. So, we are interested in the cloning and characterisation of GBSS gene promoters from potato cultivars suitable to our agro-climatic conditions.

### **3. MATERIALS AND METHODS**

#### **3.1 MATERIALS**

##### **3.1.1 PROCUREMENT OF POTATO GERMPLASMS AND OTHER MATERIALS**

The germplasms of various potato cultivars such as Kufri Chandramukhi, Kufri Chipsona-1, Kufri Chipsona-2 and Kufri Jyoti were routinely maintained in our laboratory.

Various enzymes and fine chemicals used for molecular biology techniques were purchased from Bangalore Genei Pvt. Ltd., Bangalore, Amersham Biosciences, Hong Kong and MBI Fermentas.

The routinely used chemicals were bought from Himedia Laboratories Pvt. Ltd., Mumbai and Sisco Research Laboratories Pvt. Ltd., Mumbai.

Glasswares and Plastic wares were bought from Borosil and Tarsons Products Pvt. Ltd.

##### **3.1.2 BACTERIAL STRAINS AND PLASMIDS**

a) *E. coli* DH5 $\alpha$ : *supE44*  $\Delta$ *lacU169* ( $\Phi$ 80 *lacZ*  $\Delta$ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1s*

b) pUC19: GenBank Accession No. **X02514** (2,686 bp) [Yanisch-Perron *et al.*, 1985]

The above *E. coli* strain and pUC19 plasmid were procured from Bangalore Genei Pvt. Ltd. and routinely maintained in the laboratory. *E. coli* DH5 $\alpha$  strain was maintained on Luria-Agar Medium, whereas, *E. coli* DH5 $\alpha$  transformed with pUC19 plasmid was maintained on Luria-Agar medium containing Ampicillin.

##### **3.1.3 MEDIA USED**

###### **Luria-Bertani (LB) Medium:**

Yeast Extract	:	0.5% (w/v)
Tryptone	:	1.0% (w/v)
NaCl	:	1.0% (w/v)
Agar	:	1.5% (w/v)

For preparing LA-Ampicillin medium, ampicillin was added to the LA medium at the working concentration of 50  $\mu$ g/ml, after autoclaving.

### 3.1.4 BUFFERS USED

#### Gel Loading Buffer (5X):

Sucrose	:	35% (w/v)
EDTA	:	50.0 mM (pH 8.0)
Bromophenol Blue	:	0.2% (w/v)

#### STET Buffer:

Sucrose	:	8.0% (w/v)
Triton X-100	:	0.5% (w/v)
EDTA	:	50 mM (pH 8.0)
Tris-HCl	:	10.0 mM (pH 8.0)

The volume was made up with sterile, double-distilled water.

#### Solution I :

Glucose	:	50 mM
EDTA	:	10.0 mM (pH 8.0)
Tris-HCl	:	25 mM (pH 8.0)

#### Solution II:

NaOH	:	0.2 N
SDS	:	1% (w/v)

#### Solution III:

Potassium acetate	:	3 M
-------------------	---	-----

#### TBE Buffer (5X):

Tris Basis	:	54 g
Boric Acid	:	28 g
EDTA	:	3.8 g

The pH of the buffer was set at 8.0 and the volume was made up with sterile, double-distilled water.

**TE Buffer (1X):**

Tris-HCl	:	10.0 mM (pH 8.0)
EDTA	:	1.0 mM (pH 8.0)

**3.1.5 ENZYMES USED****3.1.5.1 Restriction Enzymes:**

Various hexacutter restriction enzymes such as *EcoRI*, *BamHI*, *HindIII* and *SmaI* were used in this study. Restriction digestion was carried out in buffer supplied by the manufacturer. Depending upon specific enzyme, reaction was carried out at appropriate temperature and BSA was added as required.

**3.1.5.2 Other Enzymes:****Ribonuclease A** (procured from SRL)

Stock Solution	:	10 mg/ml
Working Solution	:	10-15 µg/ml

DNase-free RNase A was prepared as follows:

In a buffer containing 10mM Tris-HCl (pH 8.0) and 15 mM NaCl RNase was added, the solution was boiled for 10 minutes, followed by slow cooling, after which, it was dispensed into aliquots and stored at -20°C for subsequent use.

**Lysozyme** (procured from SRL)

Stock Solution	:	10 mg/ml
Working Solution	:	100-200 µg/ml

Working solution was freshly prepared when required.

**T4 DNA Ligase** (procured from MBI Fermentas)

Stock Solution	:	400 U/µl
Working Solution	:	40 U/µl

The enzyme was diluted using dilution buffer as provided by the manufacturer.

**Klenow Fragment of *E. coli* DNA Polymerase I** (procured from MBI Fermentas)

Stock Solution	:	5 U/µl
Working Solution	:	2 U per 50 µl of reaction volume

Klenow enzyme treatment was carried out in the buffer as supplied by the manufacturer.

### 3.1.6 OTHERS

#### **X-Gal (5-Bromo-4-Chloro-3-Indolyl- $\beta$ -D-Galactoside)**

Stock Solution : 20 mg/ml

Working Solution : 20  $\mu$ g/ml

It was prepared by dissolving the required amount in N, N-dimethyl formamide. It was prepared in a fresh, sterile, microfuge tube. The tube was wrapped by aluminium foil as it is light-sensitive and was stored at -20°C.

#### **IPTG (IsoPropyl Thio- $\beta$ -D-Galactoside)**

Stock solution : 100 mg/ml

Working solution : 100  $\mu$ g/ml

It was prepared in double-distilled, sterile water when required.

### 3.2 METHODS

#### 3.2.1 DESIGNING OF OLIGONUCLEOTIDE PRIMERS

Based on the available genomic DNA sequence of the gene encoding granule-bound starch synthase (GBSS) protein in the GenBank database (Accession No. X58453), three primers, each a 20-mer, were designed and designated as follows:

**GB-F054**, the forward primer, was designed from the upstream promoter region of the GBSS gene, and corresponds to bases 54-73 of the above gene, having sequence as follows:

**5' – AAT GCA ACA GCA TCT TGT AC – 3'**

**GB-F514**, the second forward primer, was also designed from the promoter region of the GBSS gene, and corresponds to the bases 514-533 of the above gene, having sequence as follows:

**5' – AGA CAT AGG AAT GTC AAG TG – 3'**

**GB-R804**, the reverse primer, was designed from the region having putative transcription start site of the GBSS gene sequence, as it was expected to be conserved among different GBSS isoforms, and is complementary to the bases 783-804 of the GBSS gene, having sequence as follows:

**5' – CTT GTT GAG CTG TGT GAG TG – 3'**

During PCR, the above three oligonucleotide primers were used in two combinations, keeping reverse primer as common in both the cases. The combinations were as follows:

1) **GB-F054 & GB-R804 (First Primer Pair)**

2) **GB-F514 & GB-R804 (Second Primer Pair)**

**US-R6747**, a 20-mer, the reverse primer, was designed from the middle region of GUS gene sequence. It is complementary to the bases 6726-6747 of GUS gene, having the sequence as follows:

**5'-CAA GTC CGC ATC TTC ATG AC- 3'**

**Recombinant pUC19 clones as used in this study:**

pRN-GK01: Having 5'- flanking region of GBSS gene from the potato cultivar Kufri Chipsona-1 using the primer pair (GB-F054 +GB-R1382)

pRN-GM01: Having 5'- flanking region of GBSS gene from the potato cultivar Kufri Chandramukhi using the primer pair (GB-F054 +GB-R1382)

pRN-GM02 having 5'- flanking region of GBSS gene from the potato cultivar Kufri Chandramukhi using the primer pair (GB-F054 +GB-R1382)

### **3.2.2 POLYMERASE CHAIN REACTION (PCR):**

In this study, the total plant DNA isolated from the potato cultivars Kufri Chipsona-1 and Kufri Chandramukhi were used as templates and PCR was carried out using the above pairs of the oligonucleotide primers. PCR was carried out in 50µl reaction volume and various components were used as follows:

10X PCR Buffer (with 15 mM MgCl <sub>2</sub> )	- used at a concentration of 1X
Template DNA	- 0.1 to 1.0 µg
Forward Primer	- 10 pmoles
Reverse Primer	- 10 pmoles
dNTPs	- 25 µM
Sterile, D.D. Water	- to make up the volume 50 µl
<i>Taq</i> DNA Polymerase	- 3 U

The temperature cycling programs were used as follows:

**For First Primer Pair**

Initial Denaturation (Pre-PCR) -	94°C, 1 min 30 sec
Denaturation -	94°C, 1 min.
Annealing -	55°C, 2 min.
Polymerization -	72°C, 1 min.
Final Extension (Post- PCR) -	72°C, 5 min.

The reaction was carried out for 30 cycles.

**For Second Primer Pair**

Initial Denaturation (Pre-PCR) -	94°C, 1 min 30 sec
Denaturation -	94°C, 1 min.
Annealing -	55°C, 2 min.
Polymerization -	72°C, 1 min.
Final Extension (Post-PCR) -	72°C, 5 min.

The reaction was carried out for 30 cycles.

**3.2.3 PREPARATION OF PLASMID VECTOR FOR LIGATION:**

For cloning of amplified cloned GBSS promoter, plasmid pUC19 and pBI121 were used as the cloning vectors. The pUC19 was processed before ligation by digestion with *Sma*I, a hexacutter restriction enzyme that produces blunt-ended linearized vector.

The pBI121 was digested with HindIII followed by klenow enzyme treatment and followed by digestion with BamH1. Restriction digestion was set as described in next section.

**3.2.3.1 SETTING UP A RESTRICTION DIGESTION REACTION**

Restriction endonucleases are enzymes that recognize short DNA sequences and cleave double-stranded DNA at specific sites within or adjacent to the recognition sequences. Restriction digestion reaction was set up according to manufacturer's instructions and was usually carried out in a reaction volume of 15 µl for 3-4 hours at optimum temperatures, depending upon the restriction enzyme.

### **3.2.4 KLENOW ENZYME TREATMENT**

Amplified DNA products were precipitated with alcohol, the DNA pellet was dissolved in minimum volume of water. In the same tube, a 50 µl of reaction volume was set up by adding required amount of 10X Klenow enzyme buffer, dNTP-mix and finally 1-2 unit of Klenow enzyme was added. The reaction was carried out at room temperature for 30 to 40 minutes and then terminated by incubating in 65°C water-bath for 5-7 minutes. DNA was once extracted with equal volume of Phenol/Chloroform mixture (1:1). The aqueous phase was transferred to a clean, sterile microfuge tube, and precipitated with alcohol. The DNA pellet was washed with 70% ethanol, air-dried and finally dissolved with minimum volume of TE buffer.

### **3.2.5 RECOVERY OF DNA BANDS USING ELECTRO-ELUTION**

Electro-elution was performed for DNA purification. For this, dialysis tubes were used, which were sterilized and treated to make them free of any contaminating impurities. First, sterilized dialysis tubes were prepared as follows: Dialysis tubing was cut into pieces of required length, and washed with distilled water thoroughly. Then, these were boiled in 2% w/v sodium bicarbonate and 1mM EDTA for 10 min. The tubes were again washed thoroughly with distilled water to remove bicarbonate. Tubes were then boiled for a few minutes in distilled water. The beaker was then decanted, and filled with 200 ml of 1 mM EDTA, and the tubes were then boiled in 1 mM EDTA for 10 min. The tubes were then allowed to cool and then stored at 4°C.

For electro-elution, DNA samples were run in two lanes on 0.8% Agarose in 1X TAE buffer along with a control lane in which sample was also loaded. The gel was run for sufficient time and then, the control lane was cut with the help of a scalpel and visualized on a UV-transilluminator. The position of DNA band of interest was marked and then, corresponding bands from sample lanes were cut. Then, these bands were transferred to the sterilized dialysis tubes and immersed in 1X TAE buffer. These tubes were then immersed in 0.5% TBE gel. The gel was run for sufficiently long time and then, buffer containing the eluted DNA was transferred into a fresh, sterile microfuge tube. This electro-eluted DNA was used for subsequent ligation and cloning purposes.

### **3.2.6 SET UP OF LIGATION REACTION**

A ligation reaction was set up in order to ligate the linearized vector and the insert, using the enzyme T4 DNA Ligase. It catalyses the formation of phosphodiester

bond between the juxtaposed 5' phosphate and 3'- OH termini in the duplex DNA. It can join blunt (flush) ends as well as cohesive termini.

The components of a ligation reaction are as follows:

Vector DNA	- $\geq 0.3 \mu\text{g}$
Insert DNA	- 0.3 to 0.5 $\mu\text{g}$
T4 DNA Ligase Enzyme	- 1-10 U
T4 DNA Ligase Buffer	- Used at a concentration of 1X

The reaction volume was made up to 15  $\mu\text{l}$  and the reaction mixture was incubated at 21°C for 3 hours.

### **3.2.7 *E. coli* DH5 $\alpha$ TRANSFORMATION**

*E. coli* DH5 $\alpha$  was transformed with the ligation mix using the standard CaCl<sub>2</sub> method (Mandel and Higa, 1970). To prepare the competent cells, a single bacterial colony was inoculated in 20 ml of Luria Broth and incubated at 125 rpm at 37°C overnight. A small aliquot of overnight-grown culture was used to re-inoculate 20 ml of fresh Luria Broth and then incubated at 37°C with shaking grown up to an O.D. of around 0.4 at 590 nm. The culture was then cooled to 0°C in ice. Cell pellet was recovered by centrifuging the cells at 5000 rpm for 6 min. The pellet was resuspended well in 15 ml of ice-cold 0.1 M CaCl<sub>2</sub>. The cells were recovered by centrifugation at 5,000 rpm for 6 min. The pellet was resuspended in 1.0 ml of ice-cold 0.1 M CaCl<sub>2</sub> for at least 2 hours at 0°C. 100  $\mu\text{l}$  of the competent cell suspension was dispensed in sterile microfuge tubes and kept at 0°C. 2-3  $\mu\text{l}$  of ligation mix was added each tube containing competent cell suspension, mixed well and kept at 0°C for 30 min. Heat shock was given at 42°C for 2 min. to all the tubes, followed by addition of 1 ml LB and incubation at 37°C for 1 hr. 110  $\mu\text{l}$  of the above transformed cell suspension was plated on LA-Ampicillin medium containing X-Gal and IPTG. The plates were incubated at 37°C for 16-18 hrs. The transformants were further analyzed on the basis of Blue/White colour selection.

### **3.2.8 MINIPREP ISOLATION OF PLASMID DNA**

Plasmids were isolated by rapid boiling method (Holmes & Quigley, 1981). Transformed bacterial colonies were inoculated aseptically in 5 ml Luria Broth containing Ampicillin in test tubes. The culture was incubated overnight at 37°C at 125 rpm. Cells were harvested from 1.5 ml overnight grown culture in microfuge tube. The dried pellet was vortexed briefly prior to resuspension in 800  $\mu\text{l}$  of STET buffer. 20  $\mu\text{l}$  of lysozyme was added to the bacterial suspension and mixed well. Then, it was kept in boiling water

bath for 1.5 minutes. After cooling down to room temperature, high-speed centrifugation (12,000 rpm) was carried out for 15 minutes. The clean supernatant was transferred into sterile and clean microfuge tube followed by addition of DNase-free RNase solution. After incubation at 37°C for 45 minutes, solvent extraction was carried out by using equal volume of phenol/chloroform mixture (1:1) followed by centrifugation at 10,000 rpm for 10 minutes. The clean upper aqueous layer was transferred to a fresh microfuge tube, made the solution 0.3 M with respect to sodium acetate, and then added equal volume of isopropanol and mixed well. After incubation at 4°C for 30 minutes, centrifugation was done at 10,000 rpm for 10 min. The DNA pellet obtained was washed with chilled 70% ethanol to ensure the removal of excess salts and centrifuged as above. Finally, DNA pellet was dissolved in minimum volume of TE buffer.

#### **MINIPREP ISOLATION OF pBI121 PLASMID DNA**

Plasmids were isolated by alkalysis method. Bacterial transformed colonies were inoculated aseptically in 5 ml Luria Broth containing Kanamycin in test tubes. The culture was incubated overnight at 37°C at 140 rpm. Cells were harvested from 1.5 ml overnight grown culture in microfuge tube. The dried pellet was vortexed briefly prior to resuspension in 200 µl of Solution I. 35 µl of lysozyme was added to the bacterial suspension and mixed well. Then, 400 µl of Solution II was added and mixed properly and kept in ice for 5 min. The solution becomes clear. 300 µl of Solution III was added to it and incubated in ice for 15-45 min. After, high-speed centrifugation (12,000 rpm) was carried out for 15 minutes. The clean supernatant was transferred into sterile and clean microfuge tube followed by addition of DNase-free RNase solution. After incubation at 37°C for 45 minutes, solvent extraction was carried out by using equal volume of phenol/chloroform mixture (1:1) followed by centrifugation at 8,000 rpm for 10 minutes. The clean upper aqueous layer was transferred to a fresh microfuge tube, made the solution 0.3 M with respect to sodium acetate, and then added equal volume of isopropanol and mixed well. After incubation at 4°C for 30 minutes, centrifugation was done at 12,000 rpm for 15 min. The DNA pellet obtained was washed with chilled 70% ethanol to ensure the removal of excess salts and centrifuged as above. Finally, DNA pellet was dissolved in minimum volume of TE buffer.

#### **3.2.9 ANALYSIS OF THE CLONED INSERTS**

Plasmid DNA isolated from white transformant colonies was further analyzed for screening of recombinant pUC19 and pBI121 clones. For this, restriction digestion of

isolated plasmid DNA was done with *EcoRI* and *BamHI*. Also, the recombinant clones were used as template to carry out PCR using the corresponding primer pairs. To further check whether the clones are GBSS-specific, PCR was carried out using the clones separately as template with both the primer pairs, with the forward primer located internally with respect to the clones and GBSS specific primers and GUS specific primers to see the intactness of GBSS promoters in pBI121 clones.

## **4. RESULTS AND DISCUSSION**

Study of regulation of gene expression is one of the thrust areas of modern molecular biology. We know that each cell of a multicellular organism contains full complement of the genetic material, but during development, these cells become specialized in order to perform a specific function. This process of specialization is known as differentiation, and is brought about by spatio-temporal regulation of gene expression during development and afterwards. Literature review revealed that GBSS gene promoter exhibits tissue-specificity, and is specifically expressed in potato tubers. It is thus, an amenable target for directing heterologous gene expression in potato tubers. Keeping in view the importance of this gene promoter, the present thesis work mainly deals with molecular cloning and characterisation studies on granule-bound starch synthase (GBSS) gene promoter from potato cultivars. The results obtained in this study are given in the following sections:

### **4.1 DESIGNING OF PRIMERS:**

The genomic DNA sequence corresponding to the GBSS protein from potato is available in the GenBank database (Accession No. X58453). The sequence is 4663 bp long. The sequence is for the GBSS gene found in potato. GBSS gene is found at *AMF* locus of the 8th chromosome of potato. The transcription start site is from 796th base pair and so, bases 1 to 795 correspond to the promoter region of GBSS gene. The transit peptide is of 77 amino acids and is coded by bases 1074 to 1304. The transit peptide is used for targeting the protein to the plastid. Primer designing should be such that there are minimum chances of non-specific amplification. Considering the factors that the GC content should be preferably greater than or equal to 50% and that there should not be repeats of any particular base, three primers (each 20-mer) specific for GBSS protein were designed. The forward primer, designated as **GB-F054**, corresponds to the bases 54-73 of the gene sequence and having the following sequence.

**5' – AAT GCA ACA GCA TCT TGT AC – 3'**

**GB-F514**, the second forward primer, corresponds to the bases 514-533 of the gene sequence and having the following sequence.

**5' – AGA CAT AGG AAT GTC AAG TG – 3'**

**GB-R804**, the reverse primer, was complementary to the bases 783-804 of the gene sequence having sequence as follows:

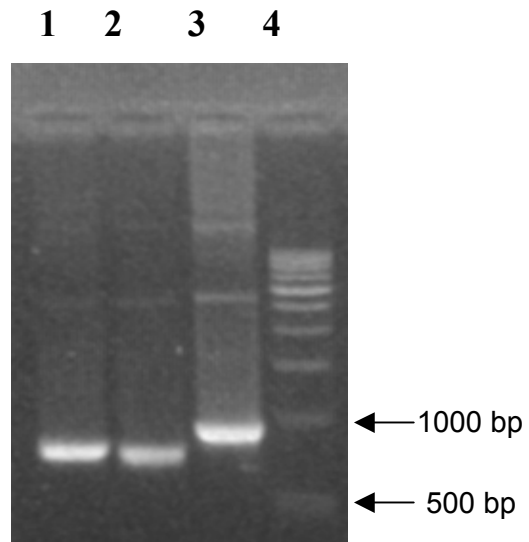
**5' – CTT GTT GAG CTG TGT GAG TG – 3'**

Some features of the GenBank Sequence of GBSS, which was used for the designing of Oligonucleotide primers, are as follows:

The rationale behind the designing of oligonucleotide primers is discussed below: three oligonucleotide primers were designed, two forward and one reverse. First forward primer, GB-F054, was designed from the promoter region, i.e. upstream from the transcription start site. Second forward primer, GB-F514, was designed from the promoter region of the GBSS gene, but it had more proximity to the transcription start site (+1 site). Usually, it has been observed that the sequences proximal to the +1 site and TATA box are fairly conserved, but as we go upstream from TATA box, there is an increased sequence divergence, and many regulatory regions conferring tissue-specificity and other attributes regarding expression to the gene are present here. The reverse primer, GB-R804, was designed from the region having the putative transcription start site of the GBSS gene promoter.

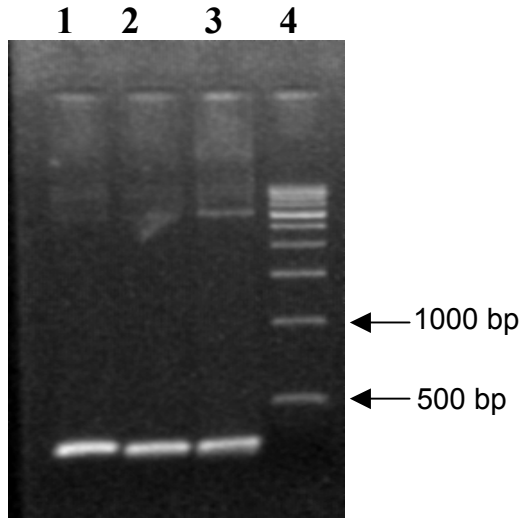
#### **4.2 POLYMERASE CHAIN REACTION (PCR):**

Four sets of PCR reactions were carried out using following primer pairs: GB-F054 & GB-R804 (First Primer pair); GB-F514 & GB-R804 (Second Primer Pair) and pRN-GK01, pRN-GM01 and pRN-GM02 pUC19 clones as templates respectively. Temperature cycling was done as follows: For both the primer pairs- 1 cycle at 94°C for 1 minute (Pre-PCR), 30 cycles of (94°C for 1 minute, 55°C for 2 minutes and 72°C for 1 minute) and the last cycle at 72°C for 5 minutes (Post-PCR). The PCR products were resolved in 0.7% Agarose gel containing ethidium bromide and DNA bands were visualized. In case of primer pair GB-F054 and GB-R804 and pRN-GK01, pRN-GM01 as template, the size of amplified DNA fragment was found to be approximately 0.75 kb whereas using pRN-GM02 as template, the size of amplified DNA fragment was found to be approximately 0.85 kb. the single amplified DNA of size approximately 0.3 kb was obtained using 2<sup>nd</sup> set of primer and pRN-GK01, pRN-GM01 and as template as shown in **Fig.1 & Fig.2**.



**Fig.1. PCR-amplified DNA products using first primer pair  
(GB-F054 & GB-R804)**

Lane 1, PCR using pRN-GK01 as template; Lane 2, PCR using pRN-GM01 as template;  
Lane 3, PCR using pRN-GM02 as template; Lane 4, 500 bp DNA Ladder



**Fig.2. PCR-amplified GBSS gene promoter DNA products using second primer pair (GB-F514 & GB-R804)**

Lane 1, pRN-GK01 as template; Lane 2, pRN-GM01 as template; Lane 3, pRN-GM02 as template; Lane 4, 500 bp DNA Ladder

The size of PCR- amplified fragments as shown in Fig.1 and Fig. 2 are consistent as expected. It may be noted here that the region containing TATA box appears to be fairly conserved in the above clones. The sequence microheterogeneity could be further revealed only by sequencing.

#### **4.3 PREPARATION OF LINEARISED PLASMID VECTOR:**

In this study, pUC19, a high-copy plasmid vector, was used for molecular cloning. First, transformation of *E. coli* DH5 $\alpha$  strain was carried out with pUC19 and then the plasmid was isolated in mini-scale. Approximately 2-3  $\mu$ g of pUC19 DNA was digested completely with hexacutter *Sma*I that produced blunt-ended termini. The completion of the digestion was further checked through agarose gel electrophoresis using a proper control. The linearized pUC19 was purified by electroelution.

#### **4.4 KLENOW TREATMENT OF PCR- AMPLIFIED PRODUCTS:**

Prior to ligation, the termini of the target DNA fragment and the vector should be compatible. Generally, the PCR-amplified DNA products are not truly blunt-ended as the enzyme *Taq* DNA polymerase has a tendency to add an extra 'A' residue at the 3' end of both the strands. Moreover, the PCR-amplified DNA may have 3' recessed termini. As a polishing step here, *Klenow* treatment served two purposes: firstly, removal of the extra

'A' residue at the 3' ends; secondly, filling up the recessed 3' termini in the amplified DNA products.

#### **4.5 LIGATION, TRANSFORMATION & SELECTION OF TRANSFORMANTS:**

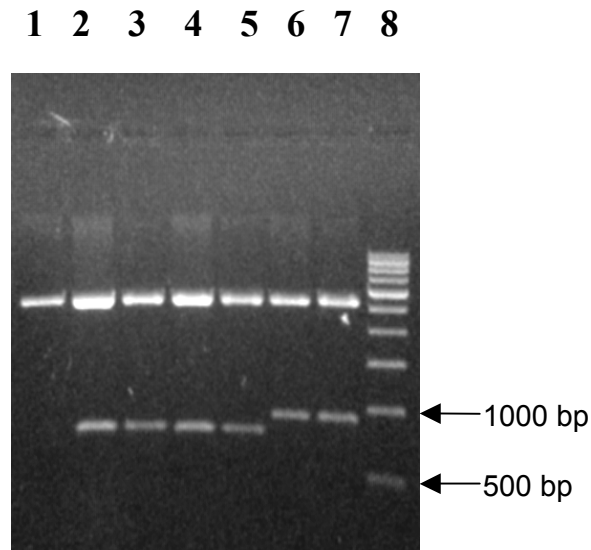
Typical ligation reaction was carried out using polished PCR-amplified DNA bands and *Sma* I- treated pUC19. To improve the efficiency of ligation, the reaction was carried out at 21°C and PEG 8000 (PolyEthylene Glycol) was included in the ligation buffer. Ligation reaction was carried out in three sets:

- (i) PCR-amplified DNA fragment (size ~ 0.75 kb) obtained by using first primer pair and the template pRN-GK01
- (ii) PCR-amplified DNA fragment (size ~ 0.75 kb) obtained by using first primer pair and the template pRN-GM01
- (iii) PCR-amplified DNA fragment (size ~ 0.85 kb) obtained by using first primer pair and the template pRN-GM02

Each ligation mix was individually used to transform competent *E. coli* DH5 $\alpha$  strain as described in section 3.2.7 and then plated on LA-Ampicillin plates containing X-Gal and IPTG. The transformants were then screened by  $\alpha$ -complementation (Blue/White colony selection). The number of white colonies appeared to vary among different ligation mixtures. Around 25 to 30 white transformant colonies were obtained from each set of ligation mix as discussed earlier and each white colony was further purified by streaking it to single colony.

#### **4.6 ISOLATION AND RESTRICTION ANALYSES OF RECOMBINANT CLONES:**

Plasmid DNA was isolated on mini-scale from each white transformant colony. The recombinant pUC19 clones having pRN-GK01 based PCR-amplified DNA fragments with first primer pair are designated as P-1 set of clones. Similarly, recombinant pUC19 clones corresponding to PCR-amplified pRN-GM01 & pRN-GM02 DNA fragments with first primer pair were designated as P-2 & P-3 clones. A few selective clones from each set were digested with the enzyme *Eco*RI & BamH1 separately and together to check the presence of DNA insert (**Fig. 3**). The number in the parenthesis denotes the specific clone of each set.



**Fig. 3. Restriction analyses of the recombinant pUC-19 clones**

Lane 1, pUC19 digested with *Eco*RI; Lane 2, P-1(#9) digested with *Eco*RI & BamH1; Lane 3, P-1(#13) digested with *Eco*RI & BamH1; Lane 4, P-2(#2) digested with *Eco*RI & BamH1; Lane 5, P-2(#4) digested with *Eco*RI & BamH1; Lane 6, P-3(#8) digested with *Eco*RI & BamH1; Lane 7, P-3(#12) digested with *Eco*RI & BamH1; Lane 8, 500 bp DNA ladder

To check the intactness of the above cloned inserts PCR was carried out using the corresponding set of primers. The size of the amplified DNA in each case exactly matched with the PCR-generated DNA fragment as obtained using the original clones pRN-GK01, pRN-GM01 & pRN-GM02 as templates. The insert in the data is not shown.

#### **4.7 ORIENTATION OF THE INSERT IN THE RECOMBINANT CLONES:**

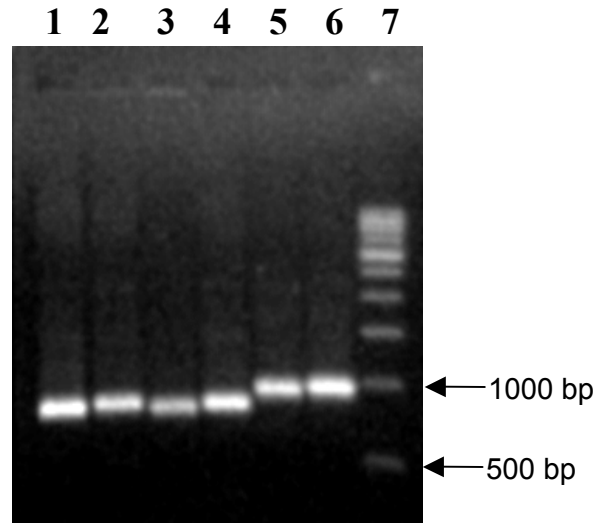
To further characterize these recombinant clones PCR was carried out to check the orientation of insert in the pUC19 clones. All the selected clones from P-1, P-2 & P-3 sets were amplified using the following primer pairs:

First set of primer pair: UP-F361 + GB-R804

Second set of primer pair: GB-F054 + GB-R804

Here, UP-F361 is vector specific primers and GB-F054 & GB-R804 are gene specific primer. So, the clone which is amplified in first set of primer, has insert in forward orientation and similarly, the clone which is amplified in second set of primer has insert in reverse orientation. The clones are analyzed though PCR using both the following

primers: UP-F361 and GB-F054 along with gene specific reverse primer as shown in **Fig.4**. Here, we have selected the following clones, having insert in forward orientation: P-1 (#13), P-2 (#4) & P-3(#8)



**Fig.4. Checking of the orientation of insert in the recombinant clones**

Lane 1, PCR amplified P-1 (#13) clone using UP-F361 + GB-R804; Lane 2, PCR amplified P-1 (#13) clone using GB-F054 +UP-R486; Lane 3, PCR amplified P-2 (#4) clone using UP-F361 + GB-R804; Lane 4, PCR amplified P-2 (#4) clone using GB-F054 +UP-R486; Lane 5, PCR amplified P-3(#8) clone using UP-F361 + GB-R804; Lane 6, PCR amplified P-3(#8) clone using GB-F054 +UP-R486; Lane 7, 500 bp DNA ladder

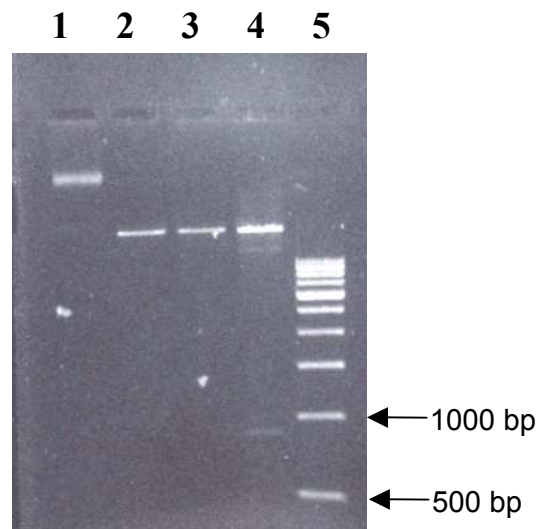
#### **4.8 ELECTROELUTION OF INSERTS:**

Our objective was to clone the GBSS gene specific promoters in Ti- plasmid based vector system for subsequent functional characterization. In this study, pBI121 (a Ti- plasmid based vector was used). It is a large plasmid (size~ 14.0 kb). It contains CaMV gene promoter alongwith GUS reporter gene downstream to it. It also has gene for kanamycin resistance. So, our plan is to substitute CaMV gene promoter with GBSS gene promoter properly without disturbing the function of other relevant genes of the vector.

For this purpose, P-1(#13), P-2(#4) & P-3(#8) clones were digested with *Ecl136II* & *BamH1* together and the inserts from the respective clones were electroeluted. The isolated inserts were used for subsequent cloning in pBI121 vector.

#### 4.9 PREPARATION OF pBI121 VECTOR:

pBI121 DNA was isolated in small scale from *E. coli* transformants. Approximately 2-3 µg of pBI121 DNA was digested completely with *HindIII* that produced cohesive termini. The linearized pBI121 was checked by running agarose gel electrophoresis using suitable control followed by filling of 5'-overhangs with Klenow enzyme. Solvent extraction was carried out to purify the vector, then precipitated with ethanol further dissolved in minimum volume of TE buffer. The linearized vector was digested with *BamH1* enzyme to release CaMV 35S promoter. And the pBI121 vector devoid of CaMV 35S promoter was isolated for further cloning studies. Restriction analyses of pBI121 vector were shown in **Fig.5**.



**Fig.5 Restriction analyses of pBI121 vector**

Lane 1, pBI121 vector; Lane 2, pBI121 digested with *HindIII*; Lane 3, pBI121 digested with *BamH1*; Lane 4, pBI121 digested with *HindIII* & *BamH1* together; Lane 5, 500 bp DNA ladder.

#### 4.10 CLONING STUDIES USING pBI121 VECTOR:

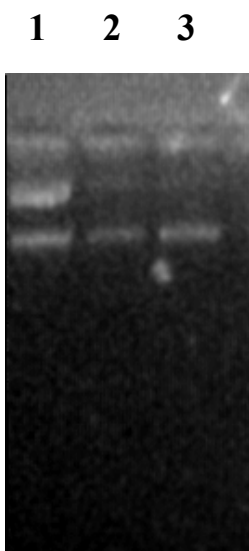
Ligation reaction was carried out in three sets using the following inserts and the processed pBI121 vector( as described in the previous section)

- (i) DNA insert from P-1(#13) clone
- (ii) DNA insert from P-2(#4) clone

(iii) DNA insert from P-3(#8) clone

Each ligation mix was separately used to transform competent *E. coli* DH5 $\alpha$  strain as described in section 3.2.7 and then plated on LA-Kanamycin plates. The number of colonies appeared to vary among different ligation mixtures. Around 5 to 10 transformant colonies were obtained from each set of ligation mix as discussed earlier and each colony was further purified by streaking it to single colony. Plasmid DNA was isolated on mini-scale from each transformant colony. The recombinant pBI121 clones corresponding to the insert from P-1(#13) clone was designated as P-A clones. Similarly, recombinant pBI121 clones corresponding to the inserts released from P-2(#4) & P-3(#8) clones were designated as P-B & P-C clones respectively.

In this study, only P-B (#1 & #2) clones as shown in the **Fig.6** were used for the further characterization of checking the orientation of GBSS- promoter with respect to the GUS gene.



**Fig.6: Agarose gel electrophoresis of pBI121 recombinant clones**

Lane 1, pBI121 vector; Lane 2, P-B (#1); Lane 3, P-B(#2)

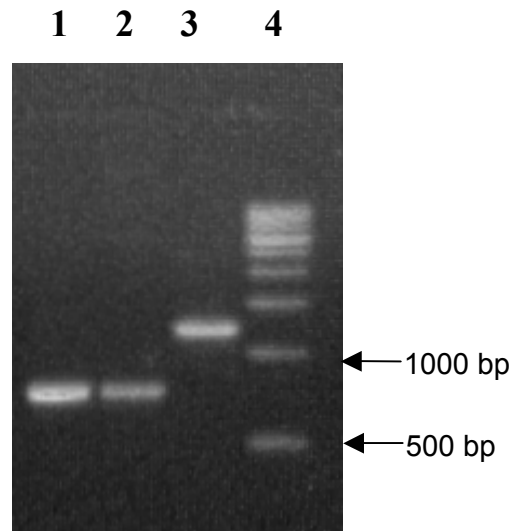
These clones were characterized by PCR technique. All these clones were amplified with two sets of primers.

First set of primer : GB-F054 + GB-R804

Second set of primer : GB-F514 + US-R6747

The purpose of using the primer pair GB-F054 & GB-R804 is to check the intactness of the GBSS promoter derived insert in recombinant pBI121 whereas the primer pair GB-F514 & US-R6747 helps to find out the orientation of the GBSS promoter with

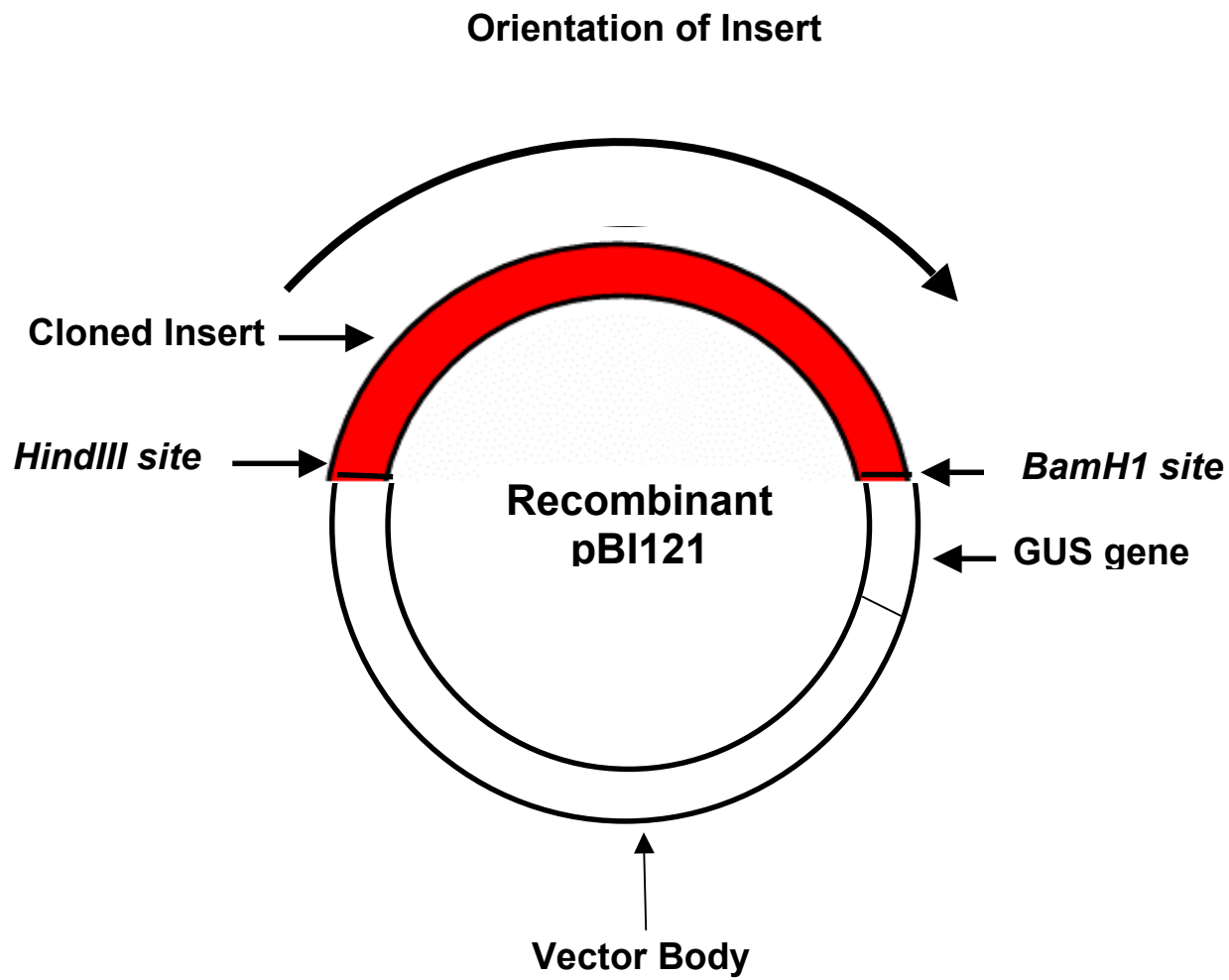
respect to the GUS reporter gene. The size of the PCR amplified DNA fragment are consistent as expected as shown in **Fig.7**



**Fig.7 Characterization of P-B(#1) clone by PCR**

Lane 1, PCR amplified P-B(#1) using primers(GB-F054 + GB-R804); Lane 2, PCR amplified pRN-GM01 using primers (GB-F054 + GB-R804); Lane 3, PCR amplified P-B2(#1) clone using primers( GB-F514 + US-R6747); Lane 4, 500 bp DNA ladder.

Here the P-B (#1) clone (pBI121-based) as constructed in this study are specific to GBSS gene promoter (derived from the potato cultivar Kufri Chandramukhi) that represent only a part of the gene, containing its regulatory region. This clone is to be used for transformation of *Agrobacterium tumefaciens* for subsequent transfer into potato plants. Biochemical assay of GUS gene product would only reveal the efficacy of this tuber-specific promoter. Similarly further attempts are required in making pBI121-based genetic constructs using GBSS-specific promoters isolated from other potato cultivars.



**Fig. 8. Schematic Representation of recombinant clones  
(P-B clone)**

## 5. SUMMARY

Various experimental steps as adopted in this study are briefly discussed below:

- ◆ Three oligonucleotide primers were designed (GB-F054, GB-F514 and GB-R804, each a 20-mer) based on the GBSS gene sequence available in GenBank database (Accession No. X58453). The forward primer, GB-F054 was designed from the upstream promoter region. Another forward primer, GB-F514 was designed from the promoter region, proximal to the TATA box. The reverse primer, GB-R804 was designed from the region consisting of putative transcription start site.
- ◆ Polymerase Chain reaction (PCR) was carried out using pRN-GK01, pRN-GM01 & pRN-GM02 clones as template and first set of primers (GB-F054 and GB-R804). The size of the amplified DNA was ~ 0.75 kb in both the templates pRN-GK01 and pRN-GM01 whereas it was ~ 0.85 kb in case of the template pRN-GM02. The above recombinant plasmids (used as templates) refer to recombinant pUC19 having larger DNA fragments comprising of 5'-flanking region along with coding region of transit peptide sequence as isolated earlier.
- ◆ Similarly, further PCR was carried out using pRN-GK01, pRN-GM01 & pRN-GM02 clones as template and second set of primers (GB-F514 and GB-R804). Here, it was found that only a similar sized fragment (~ 0.30 kb) was amplified for all these clones. So, we may infer that the region containing TATA box amplified by the second primer pair is conserved among different isoforms of GBSS.
- ◆ In this study, all the PCR-amplified DNA fragments were polished by treating with *Klenow* fragment of *E. coli* DNA Polymerase I, for cloning into *SmaI* site of pUC19 vector. The intactness of the cloned inserts was checked by restriction analyses and PCR.
- ◆ The pRN-GK01, pRN-GM01 & pRN-GM02 derived subclones as obtained in this study were designated as P-1, P-2 & P-3 set of clones. The orientation of the inserts was checked properly.
- ◆ The above sub clones were digested with *Ecl136II* & *BamH1* together. The size of the released inserts was ~ 0.75 kb for both P-1 & P-2 set of clones whereas ~0.85 kb for P-3 set of clones.

- ◆ For making genetic construct in Ti-plasmid based vector, pBI121 was digested with *Hind*III filled with klenow and followed by digestion with *Bam*H1 in order to generate blunt end and 5'-cohesive termini.
  - ◆ Finally GBSS specific DNA inserts were ligated into the processed pBI121 vector. Here the potato cultivar Kufri Chandramukhi derived GBSS specific-promoter cloned in pBI121 designated as P-B set of clones. The intactness of the promoter and its orientation with respect to GUS gene was also checked by PCR.
  - ◆ Now the Ti-plasmid based vector is ready for further functional characterization of the GBSS gene promoter.
-

## 6. REFERENCES

- Ball, S., Guan, H.P., James, M., Myers, A., Keeling, P., Mouille, G., Buléon, A., Colonna, P. and Preiss, J. (1996) From glycogen to amylopectin: a model for the biogenesis of the plant starch granule. *Cell*, 86: 349-352
- Ball, S.G. and Morell, M.K. (2003) From Bacterial Glycogen to Starch: Understanding the Biogenesis of the Plant Starch Granule. *Annual Review of Plant Biology*, 54: 207-233
- Davis, J.P., Supatcharee, N., Khandelwal, R.N. and Chibbar, R.N. (2003) Synthesis of novel starches *in planta*: Opportunities and Challenges. *Starch/Stärke*, 55: 107-120
- Denyer, K., Johnson, P., Zeeman, S. and Smith, A.M. (2001) The control of amylose synthesis. *Journal of Plant Physiology*, 158: 479-487
- Denyer, K., Waite, D., Edwards, A., Martin, C. and Smith, A.M. (1999) Interaction with amylopectin influences the ability of granule-bound starch synthase I to elongate malto-oligosaccharides. *The Biochemical Journal*, 342: 647-653
- Edwards, A., Borthakur, A., Bornemann, S., Venail, J., Denyer, K., Waite, D., Fulton, D., Smith, A. and Martin, C. (1999) Specificity of starch synthase isoforms from potato. *European Journal of Biochemistry*, 266: 724-736
- Fernie, A.R., and Willmitzer, L. (2001) Molecular and biochemical triggers of potato tuber development. *Plant Physiology*, 127:1459-1465
- Flipse, E., Straatman-Engelen, I., Kuipers, A.G., Jacobsen, E. and Visser, R.G. (1996) GBSS T-DNA inserts giving partial complementation of the amylose-free potato mutant can also cause co-suppression of the endogenous GBSS gene in a wild-type background. *Plant Molecular Biology*, 31: 731-739
- Fulton, D.C., Edwards, A., Pilling, E., Robinson, H.L., Fahy, B., Seale, R., Kato, L., Donald, A.M., Geigenberger, P., Martin, C. and Smith, A.M. (2002) Role of granule-bound starch synthase in determination of amylopectin structure and starch granule morphology in potato. *Journal of Biological Chemistry*, 277: 10834-10841

- Gaur, P.C., Naik, P.S., Kaushik, S.K. and Chakrabarti, S.K. (1999) Indian Potato varieties. *CPRI Technical Bulletin*, 51: 14-24
- Holmes, D.S. and Quigley, M. (1981) A rapid boiling method for preparation of bacterial plasmids. *Analytical Biochemistry*, 114: 193- 197
- Jackson, S.D. (1999) Multiple signaling pathways control tuber induction in potato. *Plant Physiology*, 119: 1-8
- Jobling, S. (2004) Improving starch for food and industrial applications. *Current Opinion in Plant Biology*, 7: 210-218
- Jobling, S.A., Westcott, R.J., Tayal, A., Jeffcoat, R. and Schwall, G.P. (2002) Production of a freeze-thaw-stable potato starch by Antisense inhibition of three starch synthase genes. *Nature Biotechnology*, 20: 295-299
- Kuipers, A.G., Soppe, W.J., Jacobsen, E. and Visser, R.G. (1994) Field evaluation of transgenic potato expressing an Antisense granule-bound starch synthase gene: increase of the antisense effect during tuber growth. *Plant Molecular Biology*, 26: 1759-1773
- Kuipers, A.G., Soppe, W.J., Jacobsen, E. and Visser, R.G. (1995) Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene expression in potato. *Molecular and General Genetics*, 246: 745-755
- Kuipers, A.J., Jacobsen, E. and Visser, R.G.F. (1994) Formation and deposition of amylose in the potato tuber starch granule are affected by the reduction of granule-bound starch synthase gene expression. *Plant Cell*, 6: 43-52
- Lorberth, R., Ritte, G., Willmitzer, L. and Kossmann, J. (1998) Inhibition of a starch-granule-bound protein leads to modified starch and repression of cold sweetening. *Nature Biotechnology*, 16: 473-477
- Mandel, M. and Higa, A. (1970) Calcium-dependent bacteriophage DNA infection. *Journal of Molecular Biology*, 53: 159-162
- Martin, C. and Smith, A.M. (1995) Starch Biosynthesis. *Plant Cell*, 7: 971-985
- Mason-Gamer, R.J., Weil, C.F. and Kellogg, E.A. (1998) Granule-bound starch synthase: structure, function and phylogenetic utility. *Molecular Biology and Evolution*, 15: 1658-1673

- Ramanna, M.S., and Hermsen, J.G. (1979) Genome relationships in tuber-bearing Solanums. In *The Biology and Taxonomy of the Solanaceae*, ed. Hawkes, J.G., Lester, R.N. and Skelding, A.D. Academic Press, London, 647.
- Smith, A.M., Denyer, K. and Martin, C. (1997) The synthesis of the starch granule. *Annual Review of Plant Physiology and Plant Molecular Biology*, 48: 67-87
- Smith, A.M., Denyer, K., and Martin, C.R. (1995) What controls the amount and structure of starch in storage organs? *Plant Physiology*, 107: 673-677
- Stark, D.M., Timmerman, K., Barry, G.F., Preiss, J., and Kishore, G.M. (1992) Regulation of the amount of starch in plant tissues by ADP glucose Pyrophosphorylase. *Science*, 258: 287-292
- Van de wal, M.H.B.J., Jacobsen, E. and Visser, R.G.F. (2001) Multiple allelism as a control mechanism in metabolic pathways: GBSSI allelic composition affects the activity of granule-bound starch synthase I and starch composition in potato. *Molecular Genetics and Genomics*, 265: 1011-1021
- Van der Leij, F.R., Visser, R.G., Ponstein, A.S., Jacobsen, E. and Feenstra, W.J. (1991) Sequence of the structural gene for granule-bound starch synthase of potato (*Solanum tuberosum* L.) and evidence for a single point mutation in the amf allele. *Molecular and General Genetics*, 228: 240-248
- Van der steege, G., Nieboer, M., Swaving, J. and Tempelaar, M.J. (1992) Potato granule-bound starch synthase promoter-controlled GUS expression: regulation of expression after transient and stable transformation. *Plant Molecular Biology*, 20: 19-30
- Wattebled, F., Buléon, A., Bouchet, B., Ral, J.P., Liénard, L., Delvallé, D., Binderup, K., Dauvillée, D., Ball, S. and D'Hulst, C. (2002) Granule-bound starch synthase I: A major enzyme involved in the biogenesis of B-crystallites in starch granules. *European Journal of Biochemistry*, 269: 3810-3820
- Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, 33: 103-119
- Zeeman, S.C, Tiessen, A., Pilling, E., Kato, K.L., Donald, A.M. and Smith, A.M. (2002) Starch synthesis in *Arabidopsis*. Granule synthesis, composition, and structure. *Plant Physiology*, 129: 516-529