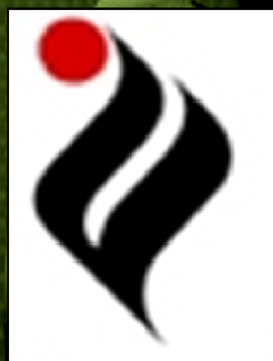
Molecular cloning and functional characterization of patatin gene promoters from potato cultivars

A thesis
submitted in fulfillment of the requirements
for the award of the degree of

Doctor of Philosophy
in
Biotechnology



By

Raghavendra Aminedi
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Department of Biotechnology and Environmental Sciences

THAPAR UNIVERSITY
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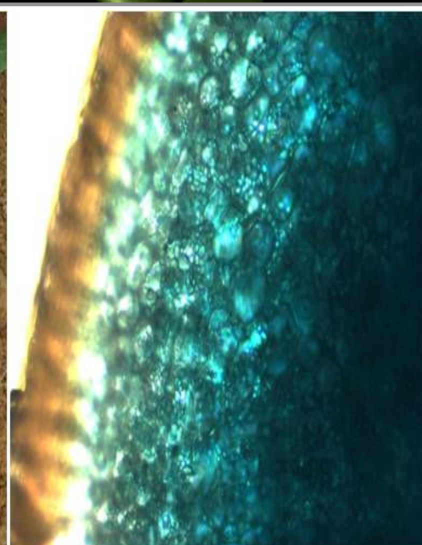
PUNJAB - INDIA

February 2013



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CERTIFICATE

This is to certify that the thesis entitled, "**Molecular cloning and functional characterization of patatin gene promoters from potato cultivars**" which is submitted by Mr. Raghavendra Aminedi, in fulfillment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

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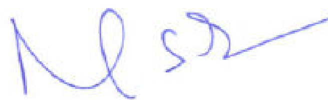

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DECLARATION

I, hereby declare that the work which is being presented in the thesis, "**Molecular cloning and functional characterization of patatin gene promoters from potato cultivars**" submitted by me for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. N. Das, Associate Professor, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

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(Raghavendra Aminedi)

Date: 01/10/2013 (03)

Place: TU, Patiala

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(Raghavendra Aminedi)

Place: Thapar University, Patiala

Date: 22 February 2013

List of Abbreviations

Name	Abbreviation
Amp	Ampicillin
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
CaMV 35S	Cauliflower mosaic virus 35S
CPRI	Central Potato Research Institute
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
DTT	Dithiothreitol
EBI	European Bioinformatics Institute
EDTA	Ethylenediamine-tetra acetic acid
Suc	Sucrose
GA_3	Gibberillic acid
GBSS	Granule-bound starch synthase
Glc	Glucose
GUS	Glucuronidase
IAA	Indole-3-acetic acid
IPTG	Isopropyl-b-thiogalactoside
Kan	Kanamycin
kb	Kilo base
kJ	Kilo joule
L	Litre
LA	Luria agar
LB	Luria broth
M	Molar
mg	Milligram
mg g^{-1}	Milligram per gram
mg L^{-1}	Milligram per liter
mg min^{-1}	Milligram per minute
mg mL^{-1}	Milligram per milliliter
µg	Microgram
µg mL^{-1}	Microgram per milliliter
min	Minute
mL	Milliliter
mM	Millimolar
MS	Murashige and Skoog

Contd..

NCBI	National Centre for Biotechnology Information
nm	Nanometer
nM	Nanomolar
O.D.	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
pI	Isoelectric point
pmoles	Picomoles
RNA	Ribonucleic acid
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
SE	Standard Error
SDS	Sodium dodecyl sulfate
STET	Sucrose Tris EDTA Triton X100
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
Tris	Tris-(hydroxymethyl-) aminomethane
TSS	Transcription start site
UTR	Untranslated region
V	Volt
v/v	Volume per volume
w/v	Weight per volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
YEM	Yeast extract mannitol

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Dedicated to my Parents

Introduction

Review of Literature
&
Objectives

1. Introduction

Potato (*Solanum tuberosum* L.), a member of the *Solanaceae* family, is the world's most important non-grain food crop with a high nutritive value. Moreover, the importance of this crop is also increasing in the nonfood industries. Some other economically important members of this family include tomato, pepper, aubergine (eggplant), petunia and tobacco. Potato comes only after wheat and rice, with a worldwide production of 330 million tons in 2009 (http://faostat.fao.org/). It is predicted that tuber and root crops will play an important role in feeding the developing world in the coming decades. For example, there is an annual average increase of 4.5 million tons per year in case of potato, exceeding those estimated for rice and wheat. Currently, India ranks third in terms of area of potato cultivation, and second in terms of production (around 34.39 million tons) of potato in 2009 (http://www.fao.org). The demand for quality potato is rapidly increasing because of increasing population, urbanization, tourism, consumption of processed products, and various nonfood uses. Food and Agriculture Organization (FAO) of the United Nations has acknowledged potato as the central for global food security in order to fight global poverty and hunger.

Currently, cultivation of potato globally is facing many newer challenges in the form of rapidly depleting agricultural land, deteriorating production environment due to climate changes, various abiotic and biotic stresses, and also postharvest preservation and consequent losses. Potato suffers from acute inbreeding depression, and is susceptible to many devastating viral, bacterial, fungal, nematodes and many other soil-borne pathogens (http://cpri.ernet.in; Bevan 1991). In order to overcome many of the challenges as stated above, classical breeding approaches for production of suitable potato varieties have become instrumental to the researchers across the world for the last more than half a century.

However, because of some inherent limitations of conventional breeding approaches, molecular breeding strategies are being adopted for addressing such challenges. Globally, the major thrust of potato research is to ensure further improvement of this horticulture crop both in terms of quality and quantity. Therefore, the potato crop became an important system for studying the various aspects of plant growth and development at morphological, cellular, physiological, biochemical, genetic and molecular levels. For example, the process of tuber initiation and development, dormancy and sprouting constitute the important phases of potato life cycle (Ewing et al. 1992). Biochemical differentiation during tuber formation is a very significant process as compared to the other somatic tissues of the potato, since the major aspects include the accumulation of large amounts of starch, and a set of relatively abundant proteins such as patatin and various proteinase inhibitors (Bevan 1991). A large number of genes are known to be expressed during the transition from stolons to tubers (Bachem et al. 2000). Most of the highly upregulated gene functions correspond to developmentally regulated biosynthesis of starch, proteinase inhibitors and patatin (Prat et al. 1990; Genova et al. 2011). Potato tubers proved to be very useful systems for studying the spatio-temporal nature of tuber-specific/developmentally regulated genes, and functional characterization of their promoters through transgenic means, and expression of useful transgene (s) as well. In other words, there is an exciting prospect of using potato tubers as 'factories' for the production of novel proteins and other commercially useful products i.e. plant molecular pharming/farming (Zhu et al. 2008; Obembe et al. 2011; Bansal et al. 2012). Most potato cultivars are autotetraploid, highly heterozygous having vast genetic resources. Therefore, different potato cultivars/clones have become quite attractive for isolation and characterization of many useful genes, more particularly developmentally regulated genes,

their regulation and functions which are relevant in both basic and applied research. The subsequent sections briefly cover some of the salient aspects of the potato crop.

1.1. About the potato crop

Morphological features: The potato, an herbaceous annual plant can grow up to 20-40 inches (50 to 100 cm) high. The ends of its underground stems or 'stolons', may enlarge greatly to form a few to more than 20 tubers of variable shapes and sizes. The skin of potato tuber varies in color from brownish white to deep purple. Its flesh normally ranges in color from white to yellow but it also may be purple. The tubers bear lateral buds (eyes) that grow into new plants when the conditions are favorable for growth. Leaves are alternate and irregularly pinnate compound. They bear white, pink, red, blue, or purple flowers with yellow stamens. Flowers are pentamerous, actinomorphic and perfect having sympetalous colored corollas. After flowering, some varieties of potato plants produce small green fruits, each containing up to 300 true seeds also called "true seed" or "botanical seed". Potato fruit contains large amounts of the toxic alkaloid solanine and is therefore not suitable for consumption.

Taxonomy: The genus *Solanum* consists of approximately 2000 members. Out of this, only about 160 wild and seven cultivated species are able to form tubers. The most common cultivated species of potato i.e., *tuberosum* is a hybrid between the diploid species *S. stentotomum* and the diploid weed *S. sparsipilum* with subsequent chromosome doubling (Ramanna and Hermsen 1979). The haploid potato genome consists of 12 chromosomes accounting approximately 840 million base pairs, making it a medium-sized plant genome (Genova et al. 2011). The potato has a series of ploidy levels, based on a haploid number of 12, ranging from diploid (2n=2x=24) to hexaploid (2n=6x=72) including triploids, tetraploids,

and pentaploids (Spooner et al. 2005). Most potato cultivars are autotetraploid (2n=4x=48) and display a high degree of heterozygosity, and suffer acute inbreeding depression.

Mode of propagation: The potato tuber, a modified stem, serves both as a storage organ and a vegetative propagation system unlike other major food crops such as cereals which are propagated through seeds i.e. products of sexual hybridization. The potato tubers meant for propagation are termed as 'seed tubers' or 'seed potatoes'. The main advantage is that a good potato clone can be maintained with a high degree of genetic purity. It is often colloquial to call a potato plant as a clone, because it is advanced through the generations by clonal propagation, another term of asexual or vegetative propagation. The disadvantage is that many deadly viruses and seed borne pathogens are progressively accumulated in the tubers and carried over repeated multiplications resulting in the gradual degeneration of a clone. For this reason, successful potato cultivation and production depends upon the availability of disease-free high quality seed tubers. This is mostly important in tropical and subtropical warm climates. For example, in the Indian subcontinent, there is an abundance of various vectors such as aphids, mites, thrips, white flies, and others responsible for virus transmission causing a huge loss to potato production. Therefore, safe agricultural practices are required for combating such menace. In addition to tubers, a potato plant can also be propagated through botanical seeds, which are known as 'true potato seeds' (TPS). In areas where seed potato production is not feasible or economic, TPS is an alternative means of propagation. Potato production through TPS cannot only reduce the production cost, but also increase the net profit of the farmers. However, TPS technology is presently not full proof for a large scale commercial exploitation due to one or other agro-technical as well as techno-economical problems such as difficulty in producing potatoes that was uniform in shape, color, size and

performance (as a requirement in food industry). Instead of producing a genetically identical clone as in the case of using seed tubers, each plant grown from open TPS is usually genetically different.

Nutrient profile: In a potato tuber, about 70-80 % is water and the rest comprises of dry matter. The major part of potato tuber consists of a storage tissue known as parenchyma. The major reserve materials stored inside this tissue are complex carbohydrates, starch grains, and proteins. Again starch is the major component of the dry matter accounting for approximately 70 % of the total solids. As compared to the cereals such as rice and wheat, the potato crop can be readily distinguished because of its higher capacity to produce dry matter, which is about 47.6 kg/ hectare/day. The approximate raw material composition of a potato tuber is as follows: the dry matter (~20 %), starch (13-16 %), total sugars (0-2 %), protein (2 %), fibre (0.5 %), lipids (0.1 %), vitamin A (trace amount/100 g fresh weight), vitamin C (31 mg/100 g fresh weight), minerals (trace amount), ash (1-1.5 %), and glycoalkaloids (<1mg/100 g fresh weight) as an antinutritional factor. The composition of the raw materials clearly suggests that the potato is a highly nutritious, digestible, and wholesome food (http://cpri.ernet.in). The levels of harmful glycoalkaloids are within the permissible limit of human consumption. The major enzymes present in potato tuber are amylase, tyrosinase, phosphorylase, catalase, aldehydrase, polyphenal oxidase, phosphatase and peroxidase.

1.2. Tuber biology in brief

Tuberization-a complex morpho-physiological process: Tuberization in potato is a complex developmental process influenced by a number of factors such as photoperiod, temperature, nitrogen nutrition, and endogenous levels of the phytohormones (Aksenova et al. 2012). This

process is accompanied with the expression of a large number of both constitutive and developmentally regulated genes which are involved in various housekeeping functions, storage starch biosynthesis, and accumulation of large amounts of different soluble proteins (Prat et al. 1990; Bevan 1991; Kloosterman et al. 2005; Agrawal et al. 2008). The tuberization process is favored by long nights (short photoperiods), cool night temperatures, low levels of nitrogen fertilizers, and 'physiological age" of the seed tuber. In fact, tuberization is the result of the activities of several interdependent processes which culminate in tuberization: for example stolon initiation, elongation, subapical swelling, cell division and the induction of specific proteins are a few of the contributing processes.

The cortical and pith cells, which are the bulk of the cells in mature tubers, are modified for the purposes of starch and storage protein accumulation. Many factors influence the initiation and subsequent growth of tubers. One of the best characterized is day length and the role of phytochrome. Like in most tuberiferous species, potato tuberization requires short days (SD); however, various potato subspecies and cultivars differ considerably in the extent of tuberization dependence on the day length. This is related to their origin and breeding strategies (Chailakhyan 1984). For example, potato cultivars from the subspecies *tuberosum* can produce tubers in the wide range of day lengths whereas the wild potato forms and plants from the subspecies *andigena* transit to tuberization only after induction by SD. However, the extent of photoperiodic response varies between the different potato genotypes (Ewing, 1995). In the light, stolons do not develop, but instead the axillary bud develops as a leafy shoot in the presence of high concentrations of sucrose and cytokinin. Conversely, stolons can develop in the dark in the absence of exogenous sucrose and cytokinins. Thus darkness is a crucial factor during stolon differentiation, whereas sucrose and cytokinins are the factors involved in

tuber initiation on stolons (Gopal et al.1997; Naik and Sarkar 1997; Gopal et al. 1998; Naik et al.1998). The requirement for high levels of sucrose during *in vitro* tuberization suggests that it also plays an important role in the process of tuber induction. There are many reports in the literature describing the importance of different phytohormones such as auxins, cytokinins, jasmonic acid and related compounds, or abscisic acid (ABA) during tuberization. However, one clear and significant observation was that GA levels declined during tuber induction (Hannapel et al. 1985). Potassium ion influences the effect of nitrogen levels on the rate of photoassimilate partitioning as suggested by *in vitro* tuberization studies (Sarkar and Naik 1998).

Some advances on in vitro tuberization aspects: The major difficulties of the seed potatoes arise due to in-borne seed pathogens and progressive accumulation of deadly viruses and microbial pathogens particularly in tropical and sub tropical regions. This causes gradual loss of good potato clones. Moreover, large-scale production of quality seed tubers, their refrigerated storage and transport to the far-off places are expensive propositions. Optimization of production levels and resistance to biotic and abiotic stresses are key objectives of global potato breeding. Therefore, *in vitro* production of disease-free microtubers is quite promising for successful potato cultivation. *In vitro* grown tubers i.e. microtubers, are miniature tubers (around 5 mm diameter) produced under tuber inducing conditions. Some of the major applications of microtubers include germplasm conservation and exchange (Estrada et al. 1986; Vecchio et al. 1994), sources for disease-free stock materials (Rosell et al. 1987), storage for longer period (Kwiatkowski et al. 1988; Naik and Sarkar 1997), ease of handling and transport as compared to microplants and normal tubers (Dodds 1988). Moreover, microtubers can be used as an explant during genetic transformation

studies (Gordon and William 1993). Microtubers proved to be useful systems for studying the expression of tuber-specific/developmentally regulated genes, and functional characterization of their promoters through transgenic means, and expression of useful transgene (s) as well (Visser et al. 1991; Naumkina et al. 2007; Zhu et al. 2008).

In potato, *in vitro* tuberization process is influenced by various parameters such as nutritional factors, growth regulating substances, temperature, photoperiod, and the genotypes of the cultivars. Wang and Hu (1982) first reported mass microtuberization *in vitro*. Since then many laboratories studied this process in MS medium supplemented with different plant growth regulators such as cytokinins (namely BAP, Kn, 2-iP), chlorocholine chloride (CCC), abscisic acid (Coleman et al. 2001; Donnelly et al. 2003). Moreover, the effects of sucrose, inorganic nitrogen, potassium, triazoles, coumarin, jasmonic acid, organic acids, photoperiod, temperature, and genotype have also been reported in the literature (Yu et al. 2000; Coleman et al. 2001; Donnelly et al. 2003; Zhang et al. 2006a; Zhang et al. 2006b; Dobranszki et al. 2008). In most of the studies, BAP was predominantly used for microtuber induction (Palmer and Smith 1969; Wang and Hu 1982; Hussey and Stacey 1984; Zhang et al. 2005; Sarkar et al. 2006). Garner and Blake (1989) reported that microtubers could also be produced *in vitro* on media free of growth regulating substances. Multiple signaling pathways are believed to be operational for controlling various aspects of tuberization process in potato (Sarkar 2008).

Considerable progress has been made on the Indian potato cultivars with regard to microtuberization aspects. Most of these cultivars are high-yielding and commercially important. They are consumed as vegetables, and some of them have processing attributes. These cultivars vary in their maturation period, tuber morphology, disease-resistance, genetic makeup, and adaptability to different agro-climatic zones in the country. Moreover, some of

the potato cultivars are also popular in the neighboring Asian countries. Studies on microtuberization were carried out in a few Indian potato cultivars covering mainly the following aspects: such as their selection for agronomic characters, field performance of the harvested microtubers produced under varying photoperiods; effect of light-induced greening during microtuber storage and their subsequent field performance (Gopal et al. 1997; Naik and Sarkar 1997; Gopal et al. 1998; Naik et al. 1998); role of sucrose and abscisic acid on different potato genotypes (Gopal et al. 2004, Sharma et al. 2011). Moreover, the role of inorganic nitrogen and potassium, different organic acids, and the antagonizing effects of cytokinins on the action of jasmonates during *in vitro* tuber induction were also studied (Sarkar and Naik 1998; Sharma et al. 2004; Sarkar et al. 2006). Out of the various factors studied, sucrose is an important signal molecule that regulates tuber formation *in vitro* by altering the GA level of the developing stolons and tubers (Xu et al. 1998). The importance of sucrose for tuberization *in vitro* has also been reported by other researchers (Wang and Hu, 1982; Garner and Blake, 1989; Donnelly et al. 2003). Sucrose at higher concentration is believed to play a key role in tuber induction. At increased levels of sugars, many sink-associated gene functions responsible for the synthesis of storage compounds are up-regulated (Morikami et al. 2005). For example, expression of patatin, a major tuber protein, could be readily induced in the cultured explants of potato leaves and stems at relatively high sucrose concentration (Wenzler et al. 1989).

1.3. Major biochemical changes during tuber development

Starch biosynthesis: Biosynthesis of large amount of storage starch (consists of approximately 25-30 % amylose and 70-80 % amylopectin), and the accumulation of a set of relatively abundant proteins are two major biochemical changes during tuber formation unlike other

somatic tissues of the potato. As starch is the predominant storage molecule in tubers, the activities of enzymes involved in starch deposition are developmentally regulated. Although the pathways leading to starch accumulation as granules in storage organs are not fully understood, evidence suggests that the pathway involving ADP-glucose predominates, with hexose sugars being imported into the amyloplast. The various enzymes required for starch biosynthesis in the amyloplasts during tuber development are ADP-glucose pyrophosphorylase, starch synthases (SS), starch-branching enzymes (SBEs), and starch debranching enzymes (DBEs) (Han et al. 2007; Zeeman et al. 2010). Starch synthase has several isoforms in the plastid (Dry et al. 1992): one isoform, granule-bound starch synthase I [GBSS, starch granule-bound ADP (UDP) glucose: α-1,4-D-glucan 4-α-glucosyl-transferase, EC 2.4.1.21], a protein of nearly 60 kDa, is bound exclusively to the starch granule and responsible for the biosynthesis of amylose fraction of starch in higher plants, and also contributes to the elongation of amylopectin chains. The rate of starch synthesis is directly correlated with the levels of granule-bound starch synthase. The ratio of amylose to amylopectin has profound influence on the physicochemical properties of the starch (Denyer et al. 2001). ADP-glucose pyrophosphorylase, one of the well-studied enzymes involved in starch metabolism, which is inhibited by the inorganic ions and stimulated by 3-phosphoglycerate. Interestingly, the activity of this enzyme increases approximately 20-fold during the early stages of tuber formation, indicating its important role in starch metabolism (Sowokinos 1976).

Biosynthesis of the tuber proteins: Protein composition changes dramatically during stolon-tuber transition resulting in the accumulation of a few abundant proteins. The most abundant protein in the tuber refers to a glycoprotein called patatin. Some of the other proteins present

in considerable amount are proteinase inhibitors (Sanchez-Serrano et al. 1986; Stiekema et al. 1988; Keil et al. 1989), proteins that are known to accumulate in other organs upon wounding (Graham et al. 1986; Cleveland et al. 1987; Keil et al. 1989; Johnson and Ryan 1990). Apparently, none of the tuber proteins studied so far is actually involved in tuberization. Most likely, these proteins have a function as a storage protein and/or in the plant defense system. Collectively, all these proteins account for more than 50 % of the tuber proteins. Some important attributes of patatin are described in the next section.

Patatin-the most abundant tuber protein: In potato tuber, patatin refers to ~40 kDa glycoprotein encoded by a large multigene family comprising of two major classes i.e. class I and class II. Patatin constitutes up to 40% of the total soluble proteins and are mainly localized in the plant cell vacuoles (Racusen and Foote 1980; Park et al. 1983). Class I patatin genes are predominantly expressed in tuber (Pikaard et al. 1987; Rocha-Sosa et al. 1989; Wenzler et al. 1989); while class II members are expressed in certain cell types of tuber and roots (Koster-Trpfer et al. 1989) but at very low level as compared to the class I members. Although patatin represents major tuber protein, unlike other storage proteins, it possesses lipid acyl hydrolase (LAH) and wax synthase activity (Dennis and Galliard 1974; Racusen 1985). Probably, patatin is also involved in plant defense. The content of patatin gradually increases as tuberization proceeds, reaching maximum levels when the tubers are ready to harvest. Thereafter, patatin levels decrease as the tubers age in storage and start to sprout. The various attributes such as tissue specificity of patatin genes, presence of patatin in high amounts in the potato tubers and its developmentally regulated biosynthesis suggest some similarities with storage proteins as found in other plant tissues. During the last three decades,

considerable progress has been made on patatin at biochemical, genetic and molecular levels which are categorically described in literature review section.

1.4. Global trend of the research activities in potato

Proper understanding of the different phases of growth and development involved in the potato life cycle is quite challenging. For example, morphologically, potato tubers are modified stems as mentioned earlier. Many endogenous and exogenous factors influence the initiation and subsequent growth of the tubers. Tuberization process i.e. stolon to tuber transition is still an enigma; since this process involves the activity of several interdependent complex processes. Efforts are still being made by the researchers in unraveling the underlying molecular mechanisms involved in the various phases of the tuberization process. In fact, this is quite a fascinating area to plant biologists. The potato is recognized as a priority crop in many countries. Conventional breeding strategies have become major thrust of potato research during the last many decades. The objective is to generate potato varieties suitable for varying agro-climatic zones of many countries. Apart from traditional breeding, molecular breeding strategies are also rapidly emerging. The purpose of these important strategies is to ensure and optimize sufficient production of quality tubers, value addition, resistance to various biotic and abiotic stresses, and proper post harvest storage without affecting the quality. All these facets are meant for improving this crop. Therefore, the potato has become an attractive system of research in terms of basic and applied aspects. Moreover, potato cultivars/clones provide vast genetic resources that could be explored for isolating different genes. This crop could be employed in various biotechnological applications. Currently, the primary focus of applied research is on disease control, manipulation of storage starch metabolism and improving nutritional qualities. Potato plants suffer from a variety of viral,

bacterial, nematode and fungal diseases which hugely affect the tuber yield and quality. Examples of some viral pathogens are potato viruses X and Y. *Streptomyces scabies* and *Erwinia carotovora* are bacterial pathogens which cause serious losses to the tubers. *Phythophthora infestans,* a deadly fungal pathogen, causes late blight disease in potato (Bevan 1991; Alex et al. 2012). Moreover, various physiological responses of the potato cultivars and their overall performance in the field and greenhouse under different stresses became the focus areas of research activities (Liu et al. 2005; Liu et al. 2006; Liu et al. 2008; Jensen et al. 2009). In the recent years, there is a growing demand for the processed potato products. The tuber dry matter content (22-23 %), the level of reducing sugars (less than 250 mg/100 g FW) and polyphenols (~30 mg/100 g FW) are some of the important parameters for selecting a potato variety for processing (Technical Bulletin No. 50, CPRI, 1999). But the production of quality tubers with processing attributes is not possible round the year. Therefore, we need to preserve them for certain time period particularly during summer. Apart from production under field conditions, there are also challenges during postharvest storage. Since storage at low temperatures leads to the undesirable 'cold-induced sweetening' in the potato tubers compromising its processing attributes and consumer acceptance (Isherwood 1973; Pollock and Rees 1975; Richardson et al. 1990; Zrenner et al. 1996). Many efforts are being made in many laboratories for addressing the aforesaid issues by adopting facile molecular techniques i.e. mostly transgenic approaches.

Recently, considerable advancements have been made in the area of structural genomics and bioinformatics. The knowledge would help in developing reliable molecular markers for qualitative and quantitative traits in potato. Functional genomics approaches are being adopted for gene discovery for targeted traits like late blight durable resistance, heat tolerance,

high temperature tuberization, better water and nutrient use efficiency. The proteomics and metabolomics approaches are quite helpful in deciphering basic aspects on photosynthesis, partitioning of photo-assimilates, starch metabolism, carotenoid and flavonoid synthesis, storage protein quality, and processing quality (Vision 2030; http://cpri.ernet.in). The recent significant advancement in the genome sequence project is an important step forward in the area of potato research. This huge exercise helps in predicting quite a large number of gene functions through annotations. In fact, the potato genome sequence would provide a platform not only for understanding structure and functions of different genes but also for the desired genetic improvement (through molecular breeding approaches) of this world's most important non-grain food crop. Eventually, the availability of the annotated data from the potato genome sequence project would help in characterizing the different germplasm collections based on the allelic variants. The data would assist the breeders for exploiting further the genetic potential of potato (Visser et al. 2009; Genova et al. 2011).

A large number of genes are known to be expressed during the transition from stolons to tubers. Most of the highly upregulated gene functions correspond to developmentally regulated biosynthesis of starch, proteinase inhibitors and patatin (Prat et al. 1990; Genova et al. 2011). Therefore, the diverse genetic resources of various potato genotypes are being explored for the isolation of different genes to study their structure and functions. Potato systems are also being employed efficiently for studying the spatio-temporal nature of tuber-specific/developmentally regulated genes, functional characterization of their promoters through transgenic means, and expression of useful transgene (s) as well (Rocha-Sosa et al. 1989; Zhu et al. 2008; Bansal et al. 2012). Many useful carbohydrates, novel proteins and metabolites could be produced in potato i.e. 'plant molecular farming' where potato tubers

could be used as ‘factories’ or ’bioreactors’ (Schwall et al 2000; Hellwege et al. 2000; Cairns 2003; Jobling 2004; Andersonn et al. 2006; Obembe et al. 2011).

It is commonly observed that the gene promoters specific to RNA Polymerase II show much more variations in sequence, and have a variety of *cis*-regulatory sequence motifs (basal promoter elements, enhancers/silencers) that occur both in the upstream and downstream regions of a gene relative to its transcription start site (TSS), and can act in either orientation. These sequence motifs provide the sites for binding the basal transcription factors, positive and/or negative transcription regulators. All these factors can act directly or indirectly through DNA-protein and/or protein-protein interactions which is evident in the published reports and rapidly expanding database dealing with various structural features of DNA, a large number of diverse *cis*-regulatory sequence motifs and their cognate transcription factors corresponding to many plant genes. In this context, many tissue-specific, inducible and developmentally-regulated potato genes became very attractive molecular tools to the researchers for understanding the molecular mechanisms involved in their expression and regulation in different potato tissues and cell types. Such types of studies provide insights with regard to their organization in the context of chromatin, sequence, expression pattern, major transcriptional regulation and importantly identification of *cis*-regulatory sequence motifs and the cognate transcription factors (Berk 1999; Kornberg 1999; Buratowski 2000; Li et al. 2002; Prasad et al. 2012). Apart from identifying the molecular mechanisms involved in gene transcription, one of the related areas of interest is in understanding posttranscriptional processing and mRNA stability in plants (Bailey-Serres et al. 1999; Gu et al. 1999; Gutierrez et al. 1999; Hunt et al. 2000; Das Gupta et al. 2001; Eckardt 2002).

Currently, class I patatin genes in particular became very suitable molecular tools in the area of plant molecular biology with regard to the regulation of gene expression studies, and many biotechnological applications. Moreover, the importance of patatin during tuber development in potato is well recognized during the last few decades (Rocha-Sosa et al. 1989; Wenzler et al. 1989; Jefferson et al. 1990; Stupar et al. 2006). The objectives of this study are on the patatin genes in potato, the next section deals with mainly the overall advancements in this area at various levels.

2. Review of Literature

2.1. Patatin-the most abundant tuber protein in potato

Some salient biochemical attributes of patatin: In potato, tuberization refers to a complex developmental process. Apart from storage starch biosynthesis, accumulation of large amounts of different soluble proteins also denotes a major biochemical differentiation. The major component of this set of proteins is patatin, a 40 kDa glycoprotein, accounting upto approximately 40 % of the total soluble protein in the potato tuber (Racusen and Foote 1980; Paiva et al. 1982). Some of the other proteins in the tubers present in significant amounts are type II proteinase inhibitors as mentioned earlier. All these proteins are also believed to play roles in the plant defense. As shown by EM-immunocytochemistry, patatin is mainly localized in the vacuoles of potato tubers (Sonnewald et al. 1989). The precursor protein contains a signal peptide (Kirschner and Hahn 1986), which allows the polypeptide to enter the lumen of the endoplasmic reticulum (Blobel 1980). The protein becomes N-glycosylated and the glycans are further modified to complex-glycans (Sonnewald et al. 1989). Patatin shows extensive charge heterogeneity and differ

between potato cultivars. However, the isoforms of patatin appeared immunologically identical both within and between cultivars (Park et al. 1983). Unlike most other storage proteins, it possesses enzymatic activity, i.e. lipid acyl hydrolase activity and wax synthase activity (Dennis and Galliard 1974; Racusen 1985). Apart from enzymatic functions, patatin also exhibits both plant defense and antioxidant activities (Strickland et al. 1995; Liu et al. 2003; Rydel et al. 2003; Shewry 2003; Sharma et al. 2004). Apart from biological functions, patatin adds nutritive value to potato for human consumption and other purposes.

2.2. Advances on patatin research at genetic and molecular levels

Major classes of patatin multicopy gene family and their expression patterns: In potato, patatin is encoded by a large multigene family with an estimated copy number of 10 to 18 per haploid genome, depending on the cultivar genotype (Twell and Ooms 1988). Genetic mapping studies indicated that all the patatin genes mapped to a single locus on chromosome 8 (Ganal et al. 1991). Sequence analyses revealed that the patatin coding regions are highly homologous with one another and most patatin proteins are immunologically and biochemically indistinguishable (Park et al. 1983; Mignery et al. 1984; Pots et al. 1999). However, based on the sequence divergence in the 5′-flanking regions (promoters) and 5′-UTR, the patatin gene family in potato was divided into two major classes: class I and class II. An insertion of a ~22-bp in the 5′-UTR is distinctly present in the members of class II gene family (Mignery et al. 1984). The core promoter regions i.e.the sequences approximately 87 bp upstream of TSS are highly homologous between the class I and class II members. Interestingly, the sequences further upstream of -87 differ markedly between the two classes (Rosahl et al. 1986; Mignery et al. 1988). The two classes of patatin genes are present in

approximately equal numbers in the potato genome. But they are known to vary with regard to their expression patterns in potato tissues such as tubers and roots. Class I patatin genes are highly expressed in the tubers and tuberized stolons but show very low expression in the leaves, stems and roots; while class II patatin genes are expressed at low levels only in certain cell types of tubers and root tips. All these findings clearly indicate that the class I patatin genes are primarily responsible for the accumulation of patatin proteins in the potato tuber (Pikaard et al. 1987; Rocha-Sosa et al. 1989; Wenzler et al. 1989; Koster-Trpfer et al. 1989; Liu et al. 1991). However, patatin could be expressed in petioles and stems of potato plants induced for tuberization upon removal of tubers and stolons (Paiva et al. 1983). In addition patatin accumulates to considerable levels in leaves of potato plantlets growing under axenic conditions on media supplied with high levels of sucrose (Rocha-Sosa et al. 1989, Wenzler et al. 1989). In these cases, induction of patatin expression is independent of the morphological differentiation process of tuberization. The accumulation of patatin could be inhibited by gibberellic acid (GA_3) either in whole plants or in the induced stem cuttings by providing right kind of stimuli (Hannapel et al. 1985).

Structure and function of the patatin genes: Considerable progress has been made on the patatin genes with regard to their structures and functions mostly covering the following the following aspects: organization in the potato genome, sequence features, expression patterns, functional characterization of the 5′-flanking regions, identification of some *cis*-regulatory sequence motifs/transcription factors, heterologous expression under the patatin promoters as evident from the published reports. The patatin genes, members of class I family in particular, became attractive molecular tools to the researchers because of the following reasons: a) patatin is one of the major soluble proteins expressed in the potato tubers; b) unlike most

other storage proteins, it serves dual functions in potato i.e. lipid acyl hydrolase (LAH) and wax synthase activitiy; c) encoded by a multigene family comprising of two major classes: class I and class II, having approximately 10 to 18 copies per haploid genome which are likely to be under varying transcriptional control i.e. differential expression in the potato tissues/cell-types; d) respon- siveness of the class I patatin genes in particular to various environmental and metabolic signals. Several patatin genomic clones (Bevan et al. 1986; Rosahl et al. 1986; Pikaard et al. 1986; Twell and Ooms 1988; Mignery et al. 1988; Rocha-Sosa et al. 1989; Liu et al. 1991; Stupar et al. 2006) and cDNA clones (Mignery et al. 1984; Nakamura et al. 1986; Stiekema et al. 1988) have been isolated and their promoters and the respective coding regions were characterized by sequencing. The genomic and cDNA cloning studies revealed the structural features of the 5′-flanking regions and exon/intron organization of the patatin genes as described in the following sections.

Mignery et al. (1984) isolated two cDNA clones pGM01 and pGM203 from the potato cultivar Superior. The cDNAs are highly homologous having 98% sequence identity. Each of the cDNAs was found to encode a protein consisted of 386 amino acids including an N-terminal 23-amino acid signal sequence. Sequence comparison towards the N-terminal regions of the purified patatin revealed that the above two cDNA clones corresponded to the two major species of patatin in potato. They also predicted some salient features of the 23-amino acid signal sequence such as presence of a lysine residue close to the initiating methionine, a stretch of ten hydrophobic amino acids in the middle, and an alanine residue at the junction with the amino-terminus of the mature protein. It was demonstrated that the patatin cDNA strongly hybridized to RNA from developing tubers and not to RNA from stems or leaves of potato. Rosahl et al. (1986) isolated and sequenced nearly a full-length

cDNA clone (pcT58), and a genomic DNA fragment of 5.3 kb (pgT5) containing the entire RNA coding part and ~0.8 kb 5'-upstream region. The transcription start site was determined by S1-mapping experiments. An open reading frame of 1,158 nucleotides (equivalent to 386 amino acids) was found in both the cDNA and the genomic clone. In the genomic sequence the ORF was found to be interrupted by six introns. The size of the introns was found to vary from 88 to 816 bp. All six exon-intron junction sequences of the patatin gene obeyed the GT/AG rule for the 5' and 3' ends of introns (Breathnach and Chambon 1981). A TATA box was found 25 nucleotides upstream of the transcription initiation site, and the sequence CAAACT was at position -60. In the 3' region, the consensus sequence AATAAA was found 13-18 nucleotides in front of the poly-A-addition site. Similar type of exon/intron organization and other sequence features were found in the class I patatin gene, λpat21 (~4.0 kb) as reported by Bevan et al. (1986). Nuclease mapping defined the 5' end of the mRNA approximately 45 bp upstream of the initiation codon. The coding region of λpat2l contained no codons for AsnXSer/Thr, the canonical glucosylation site (Sharon and Lis 1979).

Based on the fused rocket immunoelectrophoresis, Pikaard et al. (1987) detected that patatin in roots was immunologically different from that of tuber. As revealed by Western blot analyses, root patatin was found to have a different molecular weight distribution as compared to the tuber patatin isoforms; whereas, immunoprecipitation of the *in vitro* translation products showed that the patatin precursors had similar molecular weight in both tissues. What they proposed post-translational processing could differ between the tubers and roots. Northern blots showed that tuber and root patatin mRNAs were of similar size, but the level of tuber patatin transcripts were about 100-fold more abundant than roots. S1 nuclease and

primer extension mapping suggested that the class II patatin transcripts as expressed in roots represented to be a subset of patatin transcripts expressed in the tubers (since both class I and II patatin transcripts were found in the tuber). They also demonstrated that the class II patatin mRNAs differed from class I transcripts by the presence of a 22 nucleotide insertion just upstream of the initiation codon. All these data indicated the differential expression of the patatin multigene family in tubers and roots.

Functional characterization of the 5′-flanking regions (promoters) of class I patatin genes: The modular organization of the 5′-flanking regions (promoters) of a few class I patatin genes were studied. The chimeric genetic constructs were made for the purpose of functional characterization as described in this section. Twell and Ooms (1987) isolated a member of the class I patatin family (~3.8 kb) from potato cultivar Desiree. This DNA fragment contained the promoter and 5' flanking DNA of the patatin gene, and used in making a chimeric gene i.e. patatin-bacterial chloramphenicol acetyltransferase (CAT) with a polyadenylation/termination sequences of the nopaline synthase gene *(nos).* This chimeric construct was used for genetic transformation of the same potato cultivar. The regenerated transformed potato plants showed CAT expression in the tubers, but not in leaves, stems or roots under *in vitro* conditions.

In order to assess the promoter activities the 5′-flanking regions of a few class I patatin genes were genetically fused to to the β-glucuronidase gene (GUS gene), and the chimeric gene expression was studied in different potato tissues. Rocha-Sosa et al. (1989) isolated the B33 clone (X14483), a class I member, having 5′-flanking sequence of size ~1.5 kb from the cv. Berolina, and studied the expression of B33 promoter-GUS gene in the potato cultivar Desiree (mostly used as a reference cultivar) under greenhouse and *in vitro* conditions. Under greenhouse condition, the GUS expression was more prominent in the tuber sections

containing parenchymatic cells but not in the peripheral phellem as evident from histochemical GUS staining. Fluorometric GUS assay further revealed that the GUS activities in the tubers were ~90, 150, and 1500-fold higher as compared to stem, root and leaf, respectively indicating high degree of tuber-specificity of this class I patatin gene promoter. A comparative sequence analysis of the 5'-upstream sequence of the B33 patatin gene revealed a high degree of homology to both class I and class II members up to -87 position, whereas further upstream of this up to approximately -1.7 kb, high degree of sequence identity was only found between the members of class I family (Bevan et al.1986; Mignery et al. 1988). The presence of two 208-bp direct repeats and three subrepeats therein were found to be distinctive features of B33 gene. The study of the regulation patatin genes appeared to be complicated by the fact that the tetraploid genome of potato carried approx. 40-60 patatin genes. It was well-conceived notion that the presence of relatively large number of patatin genes of either class in the potato genome could carry different *cis*-acting regulatory sequences, and therefore responsive to different environmental, metabolic and developmental signals. In fact, the observed differences in pattern and level of expression between class I and class II patatin genes supported this assumption.

Likewise, the 5′-flanking region (~2.5 kb) of another class I patatin genomic clone, PS20 was transcriptionally fused to GUS gene, and its expression in the transgenic potato tissues were studied both in field grown and under *in vitro* conditions by Wenzler et al. (1989). Their objective was to compare the GUS activities in the various organs of transgenic potato plants before and after tuberization under field conditions. Histochemical GUS staining showed that the patatin expression was uniformly distributed in the parenchymatic cells of the tuber, but could not be detected in the outer layers i.e. periderm or phellem cells. They also made some

interesting observations based on the fluorometric GUS assay data. Before tuberization, only trace levels of GUS activity were found in the extracts derived from leaves, roots, stems, and stolons. After tuberization, as consistent with the other published reports, extracts from the tubers contained high levels of GUS activity i.e. approx. 3000-fold higher levels than those seen in the stolon tips before tuberization. The level of GUS activity in the tubers containing the class I patatin-GUS construct was approximately 50 times higher than the transgenic tubers containing the class II patatin-GUS construct (Twell and Ooms 1987). Significantly, high level of GUS expression was also noted in the stolons of tuberizing potato plants (however, approx. 10-30 % of the level as seen in the tubers). Moreover, the GUS activities in the leaf and stem extracts were found to be 2-3 times higher as compared to the respective tissues from non-tuberized plants. These findings were consistent with the earlier report by Paiva et al. (1983), where they also found high levels of patatin protein in the tubers, at varying levels in the stolons attached to developing tubers, but significantly low in the stolons of non-tuberizing plants. With regard to GUS expression pattern, it was found to be maximum in the leaves present in the middle part of the plant after tuberization but uniformly low in the leaf extracts of plants before tuberization. On the contrary, the levels in stem extracts were highest from the basal region of the shoot both before and after tuberization. Again, these observations were consistent with the results obtained by Paiva et al. (1983) on patatin protein accumulation in different potato organs from plants grown under greenhouse conditions. This difference in the GUS activities was quite consistent with the difference in the steady state levels of class I and class II mRNAs in tubers as monitored by the S1 nuclease protection experiments.

Liu et al. (1991) reported the isolation of two class I patatin genes, B24 (~2.4 kb), pgT16 (~5kb) from the potato cultivar Berolina and a monohaploid cultivar AM 80/5793. The expression analyses were carried out in the tissues of transgenic potato plants containing the respective promoter (~1.9 kb of B24 and ~3.3 kb of pgT16)-GUS gene fusions. The GUS activity was found to be negligible in all the tissues of non-tuberizing potato plants including the stolon tips grown under greenhouse conditions. After tuber induction, GUS expression was noted predominantly in the starch containing parenchyamtic cells of the potato tuber, and also in the tuberizing stolons and stems but not in roots. The overall GUS expression of pgT16 was found to be 6-7 fold higher as compared to B24 indicating the former as a very strong promoter. All these published reports clearly suggested that the formation of storage organs i.e. tubers in the potato was found to be always associated with a high level of patatin expression. Sucrose is produced in leaves from the photosynthetic assimilates, acts as major transport form of sugar in the plants, and also as a precursor of storage starch synthesis in the tubers i.e. sink tissues. It was commonly believed that sucrose was involved in the signal transduction that led to changes in gene expression such as high level expression of the class I patatin genes in the potato tubers. Moreover, the varying levels of patatin transcripts in various potato tissues could be reasonably correlated with distribution of sucrose in those tissues.

Responsiveness of the class I gene promoters to sucrose and other signals: Although the class I patatin gene promoters were found to drive could drive predominant expression in the tubers, they were not strictly tuber-specific; since expression of the chimeric gene under these promoters could be highly induced in the cultured explants of leaves and stems at higher sucrose concentrations. However, significant patatin accumulation was also observed in the

other potato tissues under a variety of conditions. For example, Paiva et al. (1983) observed that patatin expression could be correlated with the formation of starch, a major storage carbohydrate in the potato tubers. Sucrose produced in leaves by photosynthesis is a precursor of starch synthesis and could thus be involved in the signal transduction. Rocha-Sosa et al. (1989) also studied the expression of the same chimeric gene as mentioned earlier in leaves of the transgenic potato plants grown in MS medium containing high (7 %) or low (2 %) levels of sucrose, and assayed the GUS activity after two weeks. High sucrose concentration had a prominent effect in leaf (~100-fold increase) in terms of expression of the chimeric gene as evident from both histochemical and flurometric GUS assay. Interestingly, the staining was seen in both spongy and palisade mesophyll cells, epidermal cells, but rarely detectable in different types of hair cells as well as in guard cells surrounding the stomatal openings. It was demonstrated *in vitro* that induction was specific for sucrose at an optimal concentration of 300-500 mM, while other sugars such as glucose and fructose were not effective (Jefferson et al. 1990; Wenzler et al. 1989b), as they observed high level of sucrose-inducible expression in the cultured potato leaf and stem at higher sucrose concentrations.

Wenzler et al. (1989) used chimeric class I patatin-GUS construct for generating transgenic potato plants. For this, they used 2.5 kb 5′-flanking region of the patatin genomic clone, PS20. They observed that the chimeric gene expression was not at significant levels in the extracts obtained from leaves or stems of either plants grown in pots or in plantlets produced *in vitro.* However, it could be induced to be expressed at high levels (approx. 2000-4000-fold) in either internodal stem segments or leaf explants by placing them on medium containing elevated levels of sucrose i.e. at 300 to 400 mM concentration. The GUS activity level in the root extracts from these plantlets was significantly higher than those of untransformed

plantlets. Although this activity was only 3% of that found in microtubers, but it was significantly greater than that seen in the roots of whole plants grown in potting medium. The GUS activity level in 10-35 mg microtubers produced from axillary buds of nodal-stem segments were surprisingly comparable to those seen in 20-50 g tubers from the same patatin-GUS transgenic plants grown in pots. The levels of GUS activity seen in extracts from these explants after three weeks incubation on high levels of sucrose were comparable to or greater than those seen in tuber extracts. The accumulation of high level of GUS activity in the tubers was accompanied by the accumulation of massive amounts of starch, patatin, and the other major tuber proteins. They also tested the ability of several sugars to induce GUS activity in the cultured leaf and stem explants. Sucrose was the most effective inducer mostly in many tissues, whereas, glucose and fructose could induce expression of the patatin-GUS gene depending on the tissue types. Interestingly, glucose appeared to be more effective in stem explants, while fructose in leaf explants.

In-depth molecular studies on heterologous gene expression are important areas of research in plant molecular biology. Efforts were also made in some laboratories in understanding the activities of the patatin promoters of both classes in the heterologous system under varying conditions. Martin et al. (1997) examined the function of the chimeric class I promoter-GUS and and class II promoter-GUS in the heterologous system such as *Arabidopsis thaliana*. Their studies indicated that the transcriptional regulation of patatin genes was conserved, such as sucrose responsiveness as observed in potato, in the transgenic *Arabidopsis.* It was demonstrated that class I promoter activity in roots was dependent on the supply of nitrogen, sugars and amino acids. In contrast, the class II promoter was found to be constitutively active in root tips and hydatodes. Through mutagenesis of the homozygous transgenic *Arabidopsis*

line, a few mutants were isolated that showed reduced sucrose responses. Moreover, some mutants showed modified expression patterns in the root tips. In other words, in such mutants either quantitative or qualitative GUS expression patterns were affected.

Naumkina et al. (2007) worked on a class I patatin promoter namely B33 promoter as mentioned earlier. In potato, the activity of this promoter could be induced in the organs other than tubers by sucrose or light. They compared the activity of this promoter fused with the reporter gene in B33::*GUS* transgenic *Arabidopsis* (*Arabidopsis thaliana* L.) plants and used the same DNA construct in potato. In *Arabidopsis* seedlings, it was shown that B33 promoter manifested a tissue-specificity and inducibility in a different manner than what was seen in potato. In noninduced *Arabidopsis* seedlings, B33 promoter was found to be mostly active in the roots; whereas, after sucrose treatment it became most active in the cotyledons. 10 mM sucrose concentration was sufficient in triggering a manifold activation of this promoter in the intact seedlings. The degree of B33 promoter induction by sucrose was found to be strictly organ-specific, and increased in the following sequence: root < hypocotyl < cotyledons. Interestingly 150–200 mM sucrose enhanced B33 promoter activity in cotyledons by 200 to 300 times, i.e., the overall induction was much stronger than in potato organs. Glucose and fructose were less effective than sucrose. The phytohormones such as gibberellins, auxins, and cytokinins that influence tuber formation in potato did not affect significantly B33 promoter activity in *Arabidopsis*. It was also noticed that a lag period of approx. 6 hours preceded sucrose-induced B33 promoter activation which suggested that the patatin promoter was not the primary target for the sucrose signal in *Arabidopsis*. The quantitative examination of heterologous expression of patatin class I promoter further clarified some of its basic functional characteristics. All these data clearly substantiated a view that class I patatin genes

are not strictly ‘tuber-specific’, as they could respond to different environmental and metabolic signals.

Studies on the cis-regulatory sequence motifs and trans-acting factors: It is likely that various characteristic sequence features and diverse *cis*-regulatory sequence motifs (i.e. enhancers/ silencers) in the 5′-flanking regions of patatin genes responsible for differential gene expression in different cell-types/tissues. Considerable progress has been made on the functional characterization of a few class I patatin promoters which helped in defining some *cis*-regulatory sequence motifs and their cognate *trans*-acting factors responsible for differential transcriptional regulation. In other words, all these features helped to know their roles in the observed patterns of gene expression (Jefferson et al. 1990; Liu et al. 1990; Grierson et al. 1994; Kim et al. 1994; Zourelidou et al. 2002).

Jefferson et al. (1990) conducted a series of promoter deletions of the 5′ flanking regions of λpat21 inorder to define the regions of the patatin promoter that conferred tuber specific expression and sucrose inducibility. Sequences between - 40 and - 400 bp and between -400 and -957 bp of the transcriptional start site were able to confer tuber-specific expression on a heterologous truncated promoter. The activities of the promoters *in vitro* revealed that the minimum promoter necessary for sucrose responsiveness and tuber specificity extended - 369 bp from the cap site. A longer promoter of - 674 bp contained a quantitative element that stimulated GUS expression on both low and high levels of sucrose at least ten fold. As the length of the promoter increased, expression in the tuber became more specific compared to all other organs tested, such that the -369 bp and the -674 bp promoters expressed approximately 50 % of their GUS activity in tubers, and the -2164 and -3500 bp promoters expressed 95 % of their activity in the tubers.

The *cis*-acting elements affecting tuber-specificity and sucrose-inducibility were identified through deletion analysis of class I patatin gene B33, in transgenic potato plants by Liu et al. 1990. A putative tuber specific element was located downstream from position -195. Nuclear proteins present in leaf and tuber extracts bind specifically to a conserved AT rich motif present within this region. A DNA fragment located between -183 and -143, including the binding site, however, was not able to enhance the expression of a truncated 35S promoter from cauliflower mosaic virus. Independent positive elements that contributed to a 100-fold increase relative to the basic tuber-specific element were located between: -228 and -195, -736 and -509, -930 and -736, -1512 and -951. Sucrose inducibility was controlled by sequences downstream of position -228. They concluded that the tuber-specific and sucrose-inducible elements are in close proximity.

Further studies on patatin gene promoters revealed that a highly conserved 100-bp region containing two repeat domains referred to as the ‘A-box’ and the ‘B-box’, which was critical for the transcriptional regulation of class I patatin gene expression (Grierson et al. 1994). Several lines of evidences suggested the crucial roles of the A- and the B-boxes in conferring tuber specificity and sucrose-inducibility. Since deletions within the A- or the B-box negatively affected both tuber-specific and sucrose-inducible expression of the patatin promoter.

Kim et al. (1994) performed *in vitro* protein-binding studies to characterize the *trans*-acting regulatory elements of a class I patatin gene, PS20. By employing electrophoretic mobility shift assays (EMSA), they identified nuclear proteins that interact specifically with the proximal portion of the class I patatin promoter that is required for tuber-specific and sucrose-inducible expression. The factors were detected in nuclear extracts prepared from potato

tubers and sucrose-induced leaves, but not in extracts from leaves of normal potato plants. By DNase I footprinting, they could localize four putative transcription factor-binding sites. Competitive EMSA was employed to show that the same protein factor binds to at least two of the sites, boxes D and M.

Zourelidou et al. (2002) mapped the B-box promoter element and confirmed that a 10-bp motif within the B-box was important for tuber-specific and sucrose-inducible gene expression. The regulatory protein which was believed to bind the B-box was subsequently identified as ‘Storekeeper’; a new class of DNA binding protein which was thought to be a regulator of patatin gene expression, possibly acting as a transcription factor.

Recent advances on differential transcription of the patatin multigene family: The high similarities between the patatin gene copies particularly in the coding regions as mentioned earlier have made it difficult to study the individual members of this multicopy gene family by the traditional molecular techniques such as gel blotting and isozyme analysis. Stupar et al. (2006) attempted to better define the patatin multicopy gene family in terms of gene structure, organization, and expression profile of different patatin genes during tuber development. In eukaryotes, it is known that RNA Polymerase II-mediated transcription is a complex multistep process that involves opening the local chromatin structures which are in the immediate vicinity of the promoter, transcription initiation, promoter clearance and elongation. Most of these processes are known to involve various *cis*-regulatory sequence motifs and their cognate transcription factors which act through DNA-protein and/or protein-protein interactions. Stupar et al. (2006) made a significant contribution with regard to patatin gene expression (both class I and class II) in potato in the context of local chromatin structures. They demonstrated the dramatic increase of patatin gene expression during stolon to tuber transition

coincided with an increase of histone H4 lysine acetylation. Moreover, they monitored a group of patatin gene transcripts, designated patatin gene group A, which was found to be the most abundant group during all stages of tuber development. Interestingly, the other patatin gene groups, with a 48-bp insertion in the 3′-untranslated region, were not expressed in the stolons, but displayed a gradual increase in expression level following the initiation of tuberization process. Stupar et al. (2006) also reported the correlation between the 3′-UTR structure of patatin genes and their differential expression during tuber development. Reasonably, they concluded that the patatin gene copies in the chromosome exhibited alterations with regard to overall chromatin state that could be responsible for differential transcriptional regulation during the developmental transition from stolons into tubers.

2.3. Rationale behind the present study

The overall structures, various sequence features, in-depth understanding of spatio-temporal nature of expression, biological roles of different plant genes are important prerequisites for both basic and applied aspects of research. In fact, these are very important aspects of modern plant molecular biology and plant/agricultural biotechnology. Such efforts lead to isolation and functional characterization of different plant gene promoters and assessing their strengths under varying conditions. Since, they serve as important molecular tools to the researchers. In the area of plant/crop biotechnology, particularly in the crop improvement programmes through molecular breeding, various binary genetic constructs are frequently used for the purpose of genetic transformation of the desired plants/crops. Usually for targeted gene expression, various tissue-specific promoters are often used. Therefore, efforts are being made in many laboratories for exploring different plant genetic resources during the last many decades (Kumar et al. 1998; Mandaokar et al. 2000; Rajam et al. 2007; Saeed et al. 2008).

According to the National Biotechnology Development strategy, Government of India one of the mandates is to explore our own indigenous plant genetic resources for various biotechnological applications.

There are a number of high-yielding Indian potato cultivars including some processing varieties suitable to different agro-climatic zones of the Indian subcontinent. These potato cultivars were released through conventional breeding programme by Central Potato Research Institute (CPRI) Shimla during the last more than five decades. All these potato cultivars vary with regard to their overall genetic make-up, maturation time, crop yield, disease resistance, tuber dry matter and reducing sugar contents, and also the extent of cold-induced sweetening during storage at low temperatures. For further improvement of these heterozygous and tetraploid potato cultivars, advanced molecular breeding needs to be facilitated instead of conventional breeding approaches, since the latter processes are time consuming, and relatively difficult for many desirable traits. As we know, in potato, the process of tuber development involves a very complex, physiological, cellular, biochemical and molecular steps and associated with a vast number of gene functions; where quite a large number of genes are developmentally regulated. Therefore, isolation, characterization, genetic manipulation, and use of different tuber-specific, developmentally regulated genes are some priority areas of current potato research. In this context, different potato cultivars/clones with their rich genetic resources have become quite attractive systems to the plant molecular biologists.

Literature survey clearly indicated that there was no considerable progress on isolation and characterization of the developmentally regulated genes in the Indian potato cultivars. Therefore, apart from molecular cloning studies, it is equally important to study the biological

role of the different tuber-specific genes involved under different developmental stages of tuberization. Here, the major aim was to explore the genetic resources of some of the high-yielding popular Indian cultivars for studying mainly class I patatin genes at molecular level. In potato, patatins are encoded by a relatively large multigene family. Multiple-allelism is also common for many potato genes controlling different metabolic pathways and morphological aspects. The presence of a relatively large number of patatin genes in the potato genome indicates that the different members of this multigene family are likely to have varying 5′-flanking sequences with characteristic sequence features and diverse *cis*-regulatory sequence motifs i.e. enhancers/ silencers. All these features probably explain why the members of patatin genes respond to different environmental, metabolic and developmental signals and show differential gene expression in different cell-types/tissues. Literature survey also revealed that only a few class I and class II patatin genes were studied at biochemical, genetic and molecular levels as compared to its relatively large family size in the potato genome. Therefore, many more functional patatin genes belonging to either of the classes are yet to be isolated and characterized from the potato cultivars/clones. This could help in thorough understanding of the expression patterns, regulation, overall biological role of the individual members of this important multigene family, and some of the molecular events involved in the complex process of tuberization in particular. Till to date, there was no report available on isolation of patatin genes, characterization of their promoter regions, and their regulations under varying conditions in these potato cultivars. Moreover, isolation of the 5′-flanking regions of patatin genes will eventually lead to the identification of various *cis*-regulatory elements and their cognate *trans*-acting factors essential for tuber-specific expression as well as expression in different tissues of potato and in other plants. Such

exercises will be quite useful in getting useful and efficient promoters for targeted gene expression, an important biotechnological application in the areas of crop improvement and 'molecular farming'. Keeping all these points in view, the following objectives were framed for this thesis work.

3. Objectives

- **Molecular cloning of patatin gene promoters from potato cultivars**
- **Functional characterization to assess the efficiency and tuber-specificity of patatin gene promoters**
- **To identify putative *cis*-acting elements in patatin gene promoters**

Materials and Methods

4. Materials and Methods

4.1. Materials

Potato germplasm

The various potato cultivars such as Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Chandra-mukhi (KCM), Kufri Jyoti (KJ), Kufri Ashoka (AS) and Kufri Pukhraj (PR) (for convenience the names are abbreviated as shown within parenthesis) were procured from Central Potato Research Institute (CPRI), Shimla, India and are routinely maintained on MS basal medium in our laboratory.

Chemicals/Biochemicals

The chemicals required were purchased from Sisco Research Laboratory Pvt. Ltd. Mumbai, Qualigens Fine Chemicals, Merck, CDH Pvt. Ltd., New Delhi and HiMedia Laboratories, Mumbai. Various enzymes used were purchased from Bangalore Genei Pvt. Ltd., Bangalore and Amersham Biosciences Ltd., Hongkong. The oligonucleotide primers as used in this study were synthesized by Bangalore Genei Pvt. Ltd., Bangalore. All salts and additives were purchased from HiMedia Labs Limited, India and growth hormones from Sigma chemicals, USA. The gel extraction Qiagen Kit was purchased from Genetix. Glasswares and Plasticwares were purchased from Borosil and Tarsons Products Pvt. Ltd.

Establishment of disease-free potato germplasm

The Indian potato cultivars namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chandramukhi, Kufri Jyoti, Kufri Ashoka, and Kufri Pukhraj vary with regard to their genetic makeup, maturation time and growth in different agro-climatic zones of the Indian subcontinent. The cultivars Kufri Chandramukhi and Kufri Ashoka are early maturing,

whereas the remaining cultivars are medium maturing. All these cultivars along with Desiree (a late maturing exotic cultivar) were routinely micropropagated on MS basal medium in our laboratory under controlled conditions (16 h light/8 h dark, 25-27ºC, 70 % relative humidity) for four to five weeks. The micropropagated potato plantlets of the potato cultivars were used for genomic DNA isolation. The potato cultivar Kufri Chipsona-1 (a processing cultivar) was used in the present study for *Agrobacterium*-mediated genetic transformation.

Bacterial strains and plasmids

E. coli DH5α: supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 *rel*A1

E.coli DH5α strain was maintained on Luria agar medium, whereas, the *E.coli* DH5α transformants harbouring pUC19 plasmid vector were maintained on Luria agar medium containing 50 μg mL^{-1} of ampicillin.

Agrobacterium tumefaciens (LBA4404) strain: LBA4404 (Ach5 pTiAch5) Sm/Sp(R) in the virulence plasmid (from Tn904); all T-DNA of pTiAch5 eliminated in pAL4404 (Hoekema et al. 1983). LBA4404 strain was maintained on YEM medium containing rifampicin (15 μg mL^{-1}) and streptomycin (50 μg mL^{-1}). During triparental mating, *E. coli* pRK2013 and *Agrobacterium tumefaciens* LBA4404 strains were used as helper and recipient, respectively.

pUC19 plasmid vector: pUC19 (GenBank Accession No: X02514) is a commonly used plasmid cloning vector in *E.coli* (Yanisch-Perron et al. 1985). It is a high copy number plasmid with a size of 2686 bp carrying 54-bp multiple cloning site that contains unique sites for a number of different restriction endonucleases. It carries an antibiotic resistance gene (*Amp*^{r}) as a selection marker.

pBI121-a *Ti*-plasmid based vector: pBI121, a binary vector most commonly used for *Agrobacterium*-mediated genetic transformation of plants. It acts as a shuttle vector between *E. coli* and *A. tumefaciens* (containing both *E. coli* and *A. tumefaciens* specific *ori* of replication) having a size of ~14 kb (GenBank Accession No AF485783, Chen et al. 2003). The T-DNA cassette of this vector contains *nptII* gene (kanamycin resistant) and a constitutive promoter CaMV 35S transcriptionally fused with the *uidA* reporter gene which encodes β-glucuronidase (GUS). In this study, this binary vector was used as a control, and also in making different class I patatin-GUS gene fusions.

Oligonucleotide primers

The following oligonucleotide primers were used, and the details are described below:

The primers based on class I patatin gene sequences: The following oligonucleotide primers were designed based on the available potato class I patatin gene sequence in the database (GenBank Accessions X87216 and X60396). The different forward primers as used in this study were: PT-F074, 5′-TAATTGACCGGAGACTATAC-3′ (corresponding to the bases 74-93, GenBank Acc. No. X87216); PT-F610, 5′-TTCTTATCAATTCTGACGTG-3′ (correspond-ding to the bases 610-629) and PT-F1B, 5′-TAGATCTTACTTCTATTGGC-3′ (corresponding to the bases 1-20, GenBank Acc. No. X60396). All these three forward primers were designed from different regions of upstream sequences of the patatin genes. The upstream forward primer, PT-F074 and the downstream primer, PT-F610 surrounding the core promoter regions could facilitate in studying different lengths of patatin promoter and to see the divergence in the upstream sequences and/or conserved regions surrounding the core promoter regions of various patatin isoforms. In order to explore the possibility of isolating many different members, the third forward primer, PT-F1B was designed from a different member of

class I patatin gene (Acc. No. X60396). Likewise, one of the reverse primers PT-R1478, 5′-CGTAGCACATGTTGAACTAG-3′ (complementary to the bases 1459-1478, GenBank Acc. No. X87216) corresponds to the coding region that spans C-terminus of the signal peptide and the first amino acid of mature patatin protein. The purpose was to see whether the sequences are conserved in the coding region in patatin gene family members. In fact, the coding regions of the members of a multigene family usually remain highly conserved. Another purpose was to ensure that the 5′-flanking sequences as isolated in this study correspond to the class I patatin gene family. In fact, the reverse primer, PT-R1478 was designed based on this notion. In order to specifically amplify the 5′-flanking sequence of class I patatin genes followed by directional cloning in to pBI121 vector, another reverse primer, PT1-RV (5′-CGGGATCCCGTGCTTTGAGCATATAAC AAG-3′), surrounding the putative TSS along with a hooking of *Bam*HI site was designed based on the Accession No X87216. This extra site helped in generating chimeric class I patatin-GUS fusions by replacing the existing CaMV 35S promoter in pBI121.

The primers based on pBI121 vector sequence: The following primers were designed based on the available pBI121 binary vector sequence in the database (GenBank Accession No. AF485783): The CaMV 35S promoter-specific primers: Forward primer, AF35-01 (5'-ATTCAAATAGAGGACCTAAC-3') corresponding to the bases 5291-5310 and reverse primer, BR3502 (5'-CCGTGTTCTCTCCAAATGA-3') complementary to the 5791-5809 bases of the sequence. GUS gene-specific primers: Forward primer, US-F5832 (5'-TGGTCAGTCCCTTATGTTAC-3') corresponding to the bases 5832-5851 and two reverse primers, US-R6747 (5'-CAAGTCCGCATCTTCATGAC-3') complementary to the

bases 6728-6747, and US-R7719 (5'-TTCGAGCTCGGTAGCAATTC-3') complementary to the 7700-7719 bases of the sequence.

4.2. Methods

4.2.1. Isolation of genomic DNA from the potato cultivars

The plant materials (~ 0.1 g to 2.0 g) were used for total DNA isolation based on the protocol described by Kumari et al. (2012). One of the isolation steps involved potassium acetate to remove carbohydrates and other bulky impurities. The steps involved in the isolation are briefly given here: ~ 2.0 g of plant material was ground to fine powder in the presence of liquid nitrogen and transferred to a conical flask containing 15 mL of extraction buffer (50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 250 mM NaCl, 15 % sucrose) pre-incubated at 65°C. The contents were mixed well and incubated at 65°C for 20 min with intermittent gentle shaking. 5.0 mL of 5.0 M potassium acetate solution was added, mixed vigorously and incubated further on ice for 20 min and then centrifuged at 4000 x g, 4°C for 20 min. The supernatant was filtered through two layers of fine muslin cloth and 0.70 volume of isopropanol was added, mixed gently and incubated at -20°C for 4-5 hrs. The DNA was harvested by centrifugation at 10000 x g, 4°C for 15 min. The crude DNA pellet was washed with ice cold 70% ethanol, air dried and suspended in 500 μL of TE buffer [10 mM Tris-HCl (pH 8.0) and 1.0 mM EDTA (pH 8.0)]. For further purification of DNA, DNase-free RNase treatment was carried out followed by solvent extraction twice using a mixture of phenol:chloroform: isoamyl alcohol (25:24:1), followed by DNA precipitation using 0.1 vol of 3.0 M sodium acetate (pH 5.5) and 2.0 vol of ethanol and the DNA was finally recovered by dissolving in 200-250 μL of TE buffer. For 0.1 to 0.2 g plant materials, the same protocol

was adopted by scaling down the necessary steps accordingly. The quality and quantity of the potato DNA samples were initially checked spectrophotometrically by measuring the A_{260}/A_{280} ratio and absorbance at 260 nm, respectively.

4.2.2. Agarose gel electrophoresis

Agarose gel electrophoresis was performed using standard method as described by Sambrook et al. (1989). 0.8 % agarose gel was made in 0.5X TBE/1X TAE buffer to which ethidium bromide dye was added (working conc. 0.5-1.0 μg mL^{-1}). The DNA samples were loaded and electrophoresis was carried out at 5-8 V cm^{-1}. The DNA bands were visualized under UV light.

4.2.3. Restriction digestion of DNA

Routinely restriction digestion was carried out in a reaction volume of 20 μL for 3-4 hrs at specified temperatures. The ingredients of a typical restriction digestion reaction included: DNA sample (0.5 -1.0 μg), restriction enzyme (1-5 U μL^{-1}), specific buffer (1X), BSA (1X) and the final volume was adjusted with sterile double distilled water.

4.2.4. Polymerase Chain Reaction

PCR is an iterative process, consisting of three cycling parameters, heat denaturation of DNA template, annealing of oligonucleotide primers to single stranded DNA templates, and extension of the annealed primers by a thermostable DNA polymerase. The PCR reaction was set in a 50 μL reaction volume and the composition was as follows: 5μL 10X PCR buffer, 0.5-1.0 μg template DNA, 20 pmoles forward primer, 20 pmoles reverse primer, 2.5 μL of 2.5 mM dNTP mix, 1.0 μL (1-3 U μL^{-1}) *Taq* DNA polymerase and finally the volume was made up to 50 μL with sterile double distilled water. After initial denaturation at 94ºC for

1 min 30 s, the thermal cycling parameters were: denaturation at 94ºC for 1 min, annealing at 55ºC for 2 min; polymerization at 72ºC for 1-3 min for 30 cycles followed by final extension at 72ºC for 5 min.

4.2.5. Klenow enzyme treatment

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 along with 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. The Klenow treatment was set in a 40 μL reaction volume. The reaction mixture includes: 25 μL of amplified DNA products, 4 μL 10X Klenow enzyme buffer, 1 μL dNTP-mix and 1-2 U μL^{-1} of Klenow enzyme. The reaction was carried out at 28°C for 40 min and then terminated by incubating at 65°C for 5-7 min.

4.2.6. Purification of DNA fragments

For purification of the desired DNA bands from the agarose gel, a commercial DNA purification kit was used. QIAEX II agarose gel extraction protocol was followed as per the manufacturer's instructions. For this purpose, DNA samples were run on 0.8 % agarose in 1X TAE buffer along with a control lane. The control lane was excised with the help of a sterile scalpel and visualized on UV-transilluminator. The position of DNA bands of interest was marked and then the corresponding bands were excised from sample lanes without UV exposure. The gel slices were weighed separately in 1.5 mL microfuge tube. Three volumes of Buffer QX1 was added to one volume of gel. QIAEX II solution was resuspended by vortexing for 30 seconds and 25 μL was added to each sample. In order to solubilize the agarose, the samples were incubated at 50°C for 10 min with intermittent vortexing after

every 2 min. This was done to keep QIAEX II in suspension. The samples were centrifuged at 14000 rpm for 30 seconds and the supernatant was discarded. The pellet was washed once with 500 μL of Buffer QX1 followed by 500 μL of PE Buffer twice. The pellet was air dried until it appeared powdery white. 7 μL of sterile water and 7 μL of TE buffer were added to the pellet and resuspended by vortexing. It was incubated at 50°C for 10 min and then centrifuged at 14000 rpm for 30 seconds. The supernatant containing the purified DNA was carefully transferred into a clean microfuge tube. The above two steps were repeated to increase the yield. Finally, the eluates were combined and 2.5 μL was loaded on 0.8 % agarose gel in order to check the yield.

4.2.7. Ligation reaction

A ligation reaction was set up in order to ligate the insert into vector, using the enzyme T4 DNA ligase. The enzyme catalyzes the formation of phosphodiester bond between the juxtaposed 5'-phosphate and 3'-OH termini in the duplex DNA. The Ligation reaction was carried out in a 15 μL reaction volume with following components: 0.3-0.5 μg vector DNA, 0.5-1.0 μg insert, 1.5 μL 10X buffer and 5 U μL^{-1} T4 DNA ligase. T4 DNA ligase buffer containing PEG 8000 was used in case of blunt end ligation. The reaction was set at 15°C for cohesive end ligation, and at 21°C for blunt end ligation for a period of 6-8 hrs.

4.2.8. Genetic transformation of *E. coli* DH5α with plasmid vectors

E.coli DH5α was transformed with various DNA samples using the standard $CaCl_2$ method (Mandel and Higa 1970). Briefly, the steps are given here. A single bacterial colony was inoculated in 25 mL of Luria Broth and incubated at 125 rpm at 37°C for overnight. A small aliquot of overnight grown culture was used to re-inoculate 25 mL of fresh Luria broth and

then incubated at 37°C with shaking to obtain an O.D. around 0.4-0.6 at 590 nm. The culture was kept on ice to arrest the cell growth. Cell pellet was recovered by centrifuging the cells at 6500 rpm for 10 minutes. The pellet was resuspended properly in 10 mL of ice-cold 100 mM $CaCl_2$. The cells were recovered by centrifugation at 6500 rpm for 10 minutes. The pellet was resuspended in 1.0 mL of ice-cold 100 mM $CaCl_2$ and kept in ice for 2½ hours.

Around 100 µL of the competent cell suspension was dispensed in sterile microfuge tubes and kept at 0°C. 6-7 µL of ligation mix was added to each tube containing competent cell suspension, mixed well and kept at 4°C for 30 minutes. Heat shock was performed at 42°C for 2 minutes, followed by the addition of 1 mL LB and incubation at 37°C for 1½ hour. Centrifugation was carried out at 6500 rpm for 6-7 min and 600-800 µL of supernatant was discarded to concentrate the cells. The pellet was resuspended and 100 µL of the above transformed cell suspension was plated on LA medium containing ampicillin (50 µg mL^{-1}) with X-Gal and IPTG. The plates were incubated at 37°C for 16-18 hours. The transformants were further analyzed on the basis of blue/white colour selection in the case of pUC19 vector.

4.2.9. Isolation of plasmid DNA

Plasmid isolation was carried out by the following methods:

Boiling method: Plasmid isolation in mini scale was carried out by boiling prep method as described by Holmes and Quigley (1981). In this process bacterial transformant colonies were inoculated aseptically in 4.5 mL LB containing ampicillin in test tubes. The culture was incubated at 37°C/120 rpm for overnight. Cells were harvested from 1.5 mL overnight grown culture in microfuge tubes. The pellet was loosened by vortexing, followed by resuspension in 800 µL of STET buffer. 30 µL of lysozyme was added to the bacterial suspension and mixed well. Each microfuge tube containing cell suspension was kept in boiling water bath for

1.5 min. After cooling down to room temperature high speed centrifugation (12,000 rpm) was carried out for 15 min. After removing the pellet, 2.0 µL of RNase solution was added to the supernatant to remove the contaminating RNA. After incubation at 37°C for 45 min equal volume of phenol: chloroform was added, mixed for 5-7 min and centrifugation was performed at 10,000 rpm for 10 min. To the upper aqueous layer, $1/10^{th}$ volume of 3M sodium acetate (CH_3COONa) and equal volume of isopropanol was added and incubated at 4°C for 45 min for the precipitation of plasmid DNA. Then the tubes were centrifuged at 12,000 rpm for 15 min. The DNA pellet was washed with chilled 70 % ethanol to ensure the removal of excess salts and other impurities. Finally, DNA pellet was air dried at room temperature and dissolved in 20-30 µL of TE buffer.

Alkali lysis method: This method was essentially same as described by Birnboim and Doly (1979) having the following steps in mini scale preparation. Transformant *E. coli* colony was inoculated in 25 mL LB containing either ampicillin or kanamycin (working conc. of 50 µg mL^{-1}). The culture was incubated at 37ºC overnight with shaking at 120 rpm. 1.5 mL overnight grown culture was centrifuged and the supernatant was decanted. 200 µL of Solution I was added followed by addition of 30 µL lysozyme (stock 10 mg mL^{-1}) and mixed well. Then 400 µL of Solution II was added and mixed gently by inverting the tubes. Then 300 µL of Solution III was added and mixed well till curdy white precipitate was formed. The tubes were kept in ice for 30 min by high speed centrifugation for 15 min. The supernatant was transferred to a sterile microfuge tube and 2.0 µL of DNase free RNAse (10 mg mL^{-1}) was added, mixed and incubated at 37ºC for 30 min. Extraction was done with equal volume of phenol and chloroform mixture (1:1) followed by equal volume of chloroform. Upper aqueous layer was transferred to a sterile microfuge tube followed by the addition of equal

volume of isopropanol. Kept at 4ºC for 20 min, and centrifuged at 12,000 rpm for 15 min. Supernatant was discarded and the pellet was washed with 70 % ethanol. The pellet was air-dried and dissolved in 30 μL of TE buffer and stored at -20ºC.

4.2.10. Sequence analyses

The nucleotide sequences of 1454-bp *St*PM01, 1441-bp *St*PM01, 2747-bp *St*PN02, 2332-bp *St*PK01, and 874-bp *St*PK03 were analyzed carefully by the NCBI BLAST tools. The deduced amino acid sequence was predicted by the open reading frame (ORF) finder available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). DNA Dynamo software (http://www.bluetractorsoft ware. co.uk/) was used for G+C content analysis. Isochore plots were generated by another EMBL-EBI sequence analysis tool (http://www.ebi.ac.uk/Tools/ emboss/cpgplot/). Multiple sequence alignment was done using ClustalW2, an EMBL-EBI sequence analysis tool with its default parameters (http://www. ebi.ac.uk/Tools/) and Multalin software (Corpet 1988) (http://www.multalin. toulouse.inra. fr/multalin/) using both Class I and Class II patatin gene sequences for predicting the consensus sequences. The putative *cis*-elements from different regions of Class I patatin promoters were extracted *in silico* by PLACE (Higo et al. 1999) (http://www.dna. affrc.go.jp/PLACE/) and Plant PAN (Chang et al. 2008) (http://plantpan.mbc.nctu.edu.tw) databases. Moreover, efforts were made to see the sequence identity of some sequence features with the well-characterized published *cis*-regulatory elements. In order to generate phylogenetic tree, multiple sequence alignment was done first by the MultAlin software, followed by the neighbor-joining method using MEGA 5.0 software (Saitou and Nei 1987; Tamura et al. 2011). For the above purpose, a total of 19 patatin gene sequences from different potato cultivars/clones were collected. Seventeen of them belonged to class I:

Solanum tuberosum cv. Kufri Chandramukhi (JX124230), cv. Kufri Chandramukhi (JX124231), cv. Berolina (X60396), cv. Zhongshu No. 4 (JQ341191), cv. unknown (AY485645), cv. K. Chipsona-1(JX124229), cv. AM 80/5793 (X60399), cv. Superior (M18881), cv. Superior (M18882), cv. Katahdin haploid USW1 (DQ274179), cv. unknown (AY778964), cv. Unknown (GQ352473), cv. Berolina (X14483), cv. Kufri Jyoti (JX124227), cv. Kufri Chipsona -1 (JX124228), cv. Maris Piper (X03956), cv. Dongnong-303 (X87216), and the remaining two were from class II patatin gene family: *Solanum tuberosum* cv. AM80/5793 (X60397), and cv. AM80/5793 (X60398)

4.2.11. Genetic transformation of *Agrobacterium* strain LBA4404

Transformation by triparental mating: In triparental mating, the donor strain (*E. coli* harboring *Ti* plasmid with gene of interest) was mated with conjugal helper strain (*E. coli* harboring broad host range plasmid pRK2013) and a recipient *Agrobacterium* strain LBA4404 (harboring *vir* plasmid). The *Ti* plasmid in *E. coli* was mobilized to recipient *Agrobacterium* strain due to mobilization function of pRK2013 (broad host range plasmid). After mating, *A. tumefaciens* strains harboring the engineered plant transformation vector (*Ti* plasmid with gene of interest) were selected by growth in the presence of antibiotics for which resistance is provided by genetic markers unique to those recipient *Agrobacterium* and *Ti* plasmid vector (*Ti* plasmid with gene of interest). The steps involved were: the recipient *Agrobacterium tumefaciens* strain LBA4404 was grown on Luria agar medium containing rifampicin (15 µg mL^{-1}) at 28ºC. The donor *E. coli* strain harboring engineered Ti plasmid and conjugal helper *E. coli* strain (pRK2013) were grown on Luria agar medium containing kanamycin (50 µg mL^{-1}) at 37ºC. A single colony of each freshly grown strain was patched separately close to each other on Luria agar plates. The three patches were mixed with a

sterile loop and the plates were incubated at 28ºC for 24 hrs. A small portion of triparental patch was picked with the help of a sterile loop and serially diluted in 0.9% saline. An aliquot of 100 µL serially diluted cell suspension was spread on Luria agar plate containing rifampicin (15 µg mL^{-1}) and kanamycin (50 µg mL^{-1}) and incubated at 28ºC. Single colonies of transformed *Agrobacterium* were streaked on YEM medium containing rifampicin (15 µg mL^{-1}) and kanamycin (50 µg mL^{-1}) for further use.

Electroporation: *Agrobacterium tumefaciens* strain LBA4404 was grown in 25 mL of YEM broth for overnight at 28ºC/160 rpm. The overnight grown bacterial culture was transferred to prechilled 30 mL oak ridge centrifuge tube and the bacterial cells were pelleted by centrifugation at 6500 rpm for 10 min at 4°C. The cell pellet was washed thrice with ice cold 10% glycerol (10-15 mL). Finally, the cell pellet was resuspended in the mixture of 30 µL of 1M sorbitol and 90 µL of 10 % glycerol. Electrocompetent cells were kept in ice for immediate use or stored at -20ºC for further use. To the aliquot of electrocompetent cells (20 µL) in ice cold microfuge tube, plasmid DNA (1.0-3.0 µg) was added and mixed well. The electrocompetent cell mixture was transferred to the electroporator cuvette. The cuvette was placed in the chamber slide, and the electric pulse was given. Immediately after the pulse, the cuvette was removed from the chamber and YEM broth was added in to the cuvette to transfer the cells to test tube containing 1 mL of YEM broth and incubated for 3-5 hrs at 28°C at 160 rpm. The *Agrobacterium* transformants were selected by spreading 100 µL of concentrated (centrifuged at 6000 rpm for 10 min) cells on YEM (rif^{15} + kan^{50}) plates.

4.2.12. *Agrobacterium*-mediated genetic transformation of potato

The well characterized *Agrobacterium* strain LBA4404 transformants (corresponding to individual genetic constructs) was used for co-cultivation. The single colony of transformant

strain was grown in YEM broth for 24 hrs up to 0.4-0.5 O.D and then 1.0 mL of culture was diluted with 10 mL of MS basal medium. Inter-nodal stem segments of five to six weeks old potato plantlets (Kufri Chipsona-1), grown in MS medium, were co-cultivated with diluted culture for 20 min, blot dried on sterile filter paper, placed horizontally on MS basal medium and incubated in dark for 48 hrs in growth room. Internodal stem segments were washed with cefotaxime (250 mg L^{-1}) and shifted to the selective shoot regeneration medium (MS medium containing Zeatin 2.5 mg L^{-1}, GA_3 3.0 mg L^{-1}, IAA 0.1 mg L^{-1} supplemented with kanamycin 80 mg L^{-1} and cefotaxime 250 mg L^{-1}) for regeneration and primary selection of the transgenic potato lines. The initial shoots were further transferred to the rooting media i.e., MS medium supplemented with IAA (0.1 mg L^{-1}), kanamycin (80 mg L^{-1}) and cefotaxime (250 mg L^{-1}), to obtain complete potato plantlets. Around ten independent transgenic potato lines corresponding to each binary genetic construct were screened by PCR approach using different sets of gene-specific primer pairs.

4.2.13. Production of potato mini tubers

After proper hardening and acclimatization, the selected tissue culture raised transgenic and non-transgenic potato plantlets (including control and other cultivars) were grown in polybags for mini tuber production in the restricted experimental plots. Mini tubers were harvested from the cultivars as well as from transgenic lines corresponding to each genetic construct after three and a half month of cultivation in the field (Mid of November to the end of February). After harvesting, the tubers were properly cleaned with tap water and air dried. Weight of the tubers and other morphological features were noted. The explants corresponding to both control and transgenic potato lines of different genetic constructs were collected to perform histochemical and fluorometric GUS assay.

4.2.14. Histochemical GUS staining of different potato tissues

For GUS histochemical staining, free hand thin sections were made from different explants i.e. tuber, leaf, stem, root and stolon of potato plants corresponding to the control plantlets (non transformed), individual transgenic lines corresponding to pBI121, and different class I patatin-GUS constructs. β-glucuronidase (GUS) assay was carried out using the protocol essentially as described by Jefferson et al. (1987). The steps involved were briefly outlined as follows: The hand free tissue sections were fixed in a fixative consisting of 4% paraformaldehyde in 0.1 M phosphate buffer, followed by incubation with 1 mM of X-Gluc substrate solution (1 mg 5-bromo-4-chloro-3-indolyl β-D-Glucuronide (X-Gluc) in 0.1 mL methanol, 1 mL 2x buffer, 20 μL 0.1 M potassium ferrocyanide, 20 μL 0.1 M potassium ferricyanide, 10 μL 10 % (w/v) solution of Triton X-100, 0.85 mL.water) and incubated at 37^0C for overnight. The stained tissue sections were washed with 70% ethanol to remove excess chlorophyll from the tissues. The tissue section was mounted on the slide and examined under bright field microscopy.

4.2.15. Fluorometric GUS assay in different potato tissues

Quantitative analysis of the class I patatin-GUS gene fusions along with pBI121 and non-transformed (control) potato plants was carried out through Fluorometric GUS assay. The strength of the promoter is directly correlated to the amount of GUS enzyme produced. For GUS assay, different tissues i.e. tuber, leaf, stem, root and stolon etc. were collected from the control plantlets, individual transgenic lines corresponding to pBI121 and different class I patatin-GUS constructs. The pBI121 transgenic potato lines acted as reference control to confirm and compare the constitutive nature of the promoter with that of different class I patatin-GUS binary constructs as generated in this study. To quantitate the GUS enzyme

present in the different tissues, GUS enzyme was isolated from different tissues i.e. harvested tubers, leaf, stem, root and stolon of independent transgenic lines corresponding to each construct. Tissue samples were homogenized in GUS extraction buffer (50 mM Na_3PO_4, pH 7.0, 10 mM β-mercaptoethanol, 1 mM Na_2EDTA, 0.1 % sodium lauryl sarcosine, 0.1 % Triton X 100) followed by centrifugation at 15000 rpm for 5 min. The soluble protein fractions were taken to determine GUS activities. GUS activity was determined from the rate of 4-methyl umbelliferone (4-MU) produced from 4-methyl umbelliferyl glucuronide (4-MUG) as the substrate as described by Jefferson et al. (1987). The assay mixture consisted of 5 µL of enzyme extract and 1 mM 4-MUG in 500 µl of GUS extraction buffer. Incubation was carried out for 18-24 h at 37°C in a sterile eppendorff tube. Aliquot of 100 µL were taken and the reaction was stopped by the addition of 0.9 ml of 0.2 M Na_2CO_3. Fluorescence was measured with excitation at 365 nm and emission at 467 nm on a Turner Biosystems spectrofluorimeter. The fluorimeter was calibrated with different standards of 4-MU dissolved in 0.2 M Na_2CO_3 ranging from 5 nM to 500 nM.

4.2.16. Protein estimation by Bradford method

The Bradford protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs (Bradford 1976). The soluble proteins of the GUS enzyme extracts from different tissues of control and transgenic lines of each construct were quantified based on the standard curve made by using different dilutions of BSA stock (1 mg mL^{-1}) 20 µg, 40 µg, 60 µg, 80 µg and 100 µg. The steps involved were outlined as: 30 µL of the tissue extract was made up to 100 µL with sterile water. Then 5 mL of Bradford reagent was added

and incubated at room temperature for 5-10 min. Absorbance was recorded at 595 nm spectrophotometrically.

4.2.17. *In vitro* tuberization

In vitro microtuberization was optimized for some high-yielding Indian potato cultivars (2n=4x=48) namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Ashoka, and Kufri Pukhraj procured from Central Potato Research Institute (CPRI), Shimla, India. In order to study the sucrose inducible nature of various class I patatin-GUS fusions in transgenic potato lines, microtubers were produced i*n vitro* from the transgenic and control potato plantlets using different concentrations of sucrose (3 %, 6 %, and 9 % w/v) in the liquid MS medium under complete darkness. The microtuberization protocol was adopted as follows: Aseptically grown potato microplants (~3 weeks old) were used to produce nodal cuttings (usually 3 to 4 nodes per explant) for subsequent culturing in liquid MS medium supplemented with 30 g L^{-1} sucrose (~10 explants per bottle containing 25 mL). The liquid cultures were incubated for further three weeks under controlled conditions (16 h light/8 h dark, 25±2^{0}C, 70 % relative humidity) to produce proper healthy plantlets. The residual liquid medium from the culture bottles was replaced with ~40 mL of the microtuber induction media having varying concentrations of sucrose (3 %, 6 %, and 9 % w/v), followed by incubation at 20^{0}C in complete darkness. After four weeks, healthy microtubers (30-60 mg) were harvested. The stem, leaf and roots (~30-70 mg) were collected from the potato plantlets grown in the semi-solid MS medium having varying sucrose concentrations as stated above. *In vitro* tuberization experiment was carried out in triplicate for each media formulation. The histochemical and fluorometric GUS assay were performed in different tissues as described earlier.

Sr. No.	**Chemicals/Biochemicals**	**Stock conc. (mg mL^{-1})**	**Working conc. (µg mL^{-1})**	**Solvent used**
1.	Ethidium bromide	5	0.5-1.0	Sterile water
2.	X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside)	20	20 (30 µL per plate)	N, N-dimethyl Formamide
3.	IPTG (Isopropyl thio-β-D-galactoside)	100	100 (8 µL per plate)	Sterile water
4.	Ampicillin	50	50	Sterile water
5.	Kanamycin	50	50	Sterile water
6.	Rifampicin	15	15	Methanol
7.	Streptomycin	50	50	Sterile water
8.	Cefotaxime	250	250	Sterile water

Sr. No.	Buffers	Composition and preparation
1.	0.5 M Tris-HCl (pH 8.0)	For 100 mL stock, 6.05 g of Tris base was dissolved in 50 mL water and pH was adjusted with 6.0 N HCl and made up volume with water and autoclaved
2.	0.5 M EDTA (pH 8.0)	For 100 mL stock, 18.6 g of sodium salt of EDTA was dissolved in 50 mL water and pH was adjusted with concentrated NaOH and made up volume with water and autoclaved
3.	3.0 M Sodium acetate (pH 5.5)	For 50 mL stock, 12.3 g of Sodium acetate was dissolved in water and adjust the pH with glacial acetic acid and final volume 50 mL
4.	0.5 M Sodium acetate (pH 4.7)	For 50 mL stock, 2.05 g of Sodium acetate was dissolved in water and adjust the pH with glacial acetic acid and final volume 50 mL.
5.	5 M Potassium acetate	49 g of potassium acetate was dissolved in water and made final volume 100 mL and autoclave
6.	3 M Potassium acetate (pH4.8)	29.4 g of potassium acetate was dissolved in water and 11.5 mL of glacial acetate was added and made final volume 100 mL and autoclave
7.	0.5 M $MgCl_2$	10.2 g of $MgCl_2$ was dissolved in sterile water and final volume 100 mL
8.	1.0 M Sorbitol	18.2 g of sorbitol was dissolved in sterile water and final volume 100 mL
9.	0.5 M KCl	3.73 g of KCl was dissolved in sterile water and final volume 100 mL
10.	0.5 M Sucrose	17.1 g of sucrose was dissolved in sterile water and final volume 100 mL
11.	*Solutions used for plasmid isolation by alkali lysis method*	
	Solution I:	Glucose 50 mM; Tris-HCl 25 mM (pH 8.0); EDTA 10 mM (pH 8.0)
	Solution II:	NaOH 0.2 N; SDS 1.0 %
	Solution III:	Potassium acetate (3M)
12.	The buffer for plasmid isolation by boiling method (STET)	8.0 % (w/v) Sucrose, 0.5 % (w/v) Triton X 100, 50 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0) Volume was made up by double distilled water and autoclaved.
13.	STE Buffer	0.3 M NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0)
14.	Saline EDTA	0.15 M Sodium chloride, 0.1 M EDTA (pH 8.0)

Continued…

15.	TE Buffer (1X)	10.0 mM Tris-HCl (pH 8.0), 1.0 mM EDTA (pH 8.0) Volume was made up whit distilled water and autoclaved
16.	TBE Buffer (5X)	54 g L^{-1} Tris base, 28 g L^{-1} Boric acid, 3.8 g L^{-1} EDTA The pH of the buffer was set at 8.0. Volume was made up with distilled water and autoclaved
17.	TAE Buffer (5X)	24.2 g L^{-1} Tris-base, 5.7 mL L^{-1} Glacial acetic acid, 10 mL L^{-1} 0.5M EDTA (pH 8.0). Volume was made up by double distilled water and autoclaved.
18.	DNA Gel Loading Buffer (5X)	35 % (w/v) Sucrose or 40 % glycerol, 20.0 mM EDTA (pH 8.0), 0.1 % (w/v) Bromophenol blue Volume was made up with sterile water.
19.	DNA extraction buffer	50 mM Tris-HCl pH 8.0, 50 mM EDTA (pH 8.0), 250 mM NaCl, 15 % sucrose
20.	10 % SDS	10 g of SDS was dissolved in 70 mL sterile water and made volume to 100 mL
21.	0.2 M Phosphate Buffer	200 mM Phosphate buffer: Stock solutions of A: 200 mM $NaH_2PO_4.2H_2O$ and B: $Na_2HPO_4.2H_2O$ were prepared. 39 mL of stock solution A was mixed with 61 mL of stock solution B to attain pH 7.0.
22.	Bradford Reagent	Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95 % ethanol, add 100 mL 85 % (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use.
23.	*Buffers for Fluorometric GUS Assay*	
	GUS Extraction Buffer	50 mM Na_3PO_4, pH 7.0, 10 mM dithiothreitol (DTT), 1 mM Na2EDTA, 0.1 % Sodium Lauryl Sarcosine, 0.1 % Triton X 100. Total volume was made with double distilled water. (β-mercaptoethenol can be substituted for DTT)
	Assay Buffer	1 mM MUG in GUS extraction buffer (Dissolve 22 mg 4 Methyl umbelliferyl β-D glucuronide (MUG) in 50 mL GUS extraction buffer in a 50 mL disposable polypropylene tube)
	Stop Buffer	0.2 M Na_2CO_3 (Dissolve 21.2 grams of Na_2CO_3 in deionized distilled water. Make up to 1 liter)
24.	*Buffers for Histochemical GUS staining*	
	2x Buffer	phosphate buffer pH 7.0 (made of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4)
	Fixative	4% formaldehyde, prepared fresh from paraformaldehyde, in 1x buffer
	X-gluc substrate solution	Dissolve 1 mg 5-bromo-4-chloro-3-indolyl β-D-Glucuronide (X-Gluc) in 0.1 mL methanol, add 1 mL 2x buffer, 20 µL 0.1 M potassium ferrocyanide, 20 µL 0.1 M potassium ferricyanide, 10 µL 10 % (w/v) solution of Triton X-100, and 0.85 mL.water. (Note: Filter sterilize the solution using 0.2 microfilter)

Restriction Enzymes

Various hexacutter and tetracutter restriction enzymes such as ***Eco*RI, *Bam*HI, *ECl*136II, *Sac*I, *Sma*I and *Hind*III** were used in this study. Restriction digestion was carried out in the buffer as supplied by the manufacturers. Depending on enzyme, reaction was carried out at appropriate temperature, and BSA added if required.

Description of some restriction enzymes used in this study:

Restriction enzymes	**Restriction site**	**Reaction temperature**
*Eco*R1	GAATTC	37ºC
*Bam*H1	GGATCC	37ºC
*Hind*III	AAGCTT	37ºC
*ECl136*II	GAGCTC	37ºC
*Sac*1	GAGCTC	37ºC
*Sma*1	CCCGGG	25ºC

Other Enzymes

Apart from restriction enzymes some other enzymes such as mentioned below: Several reactions were carried out using buffers as supplied by the manufacturers. Depending on enzyme, reaction was carried out at appropriate temperature.

Description of Different Enzymes:

Sr. No.	Various enzymes	Stock conc.	Working conc.	Other relevant details
1.	Lysozyme	10 mg mL^{-1}	300-400 µg mL^{-1}	Sterile water was used for stock preparation
2.	Ribonuclease A	10 mg mL^{-1}	10-20 µg mL^{-1}	Dissolved in 10 mM Tris-HCl (pH 8.0) and 15 mM NaCl. buffer and kept in boiling water bath for 10 min followed by slow cooling to room temperature and stored at -20 ºC for subsequent use
3.	T4 DNA Ligase	400 U $µL^{-1}$	~ 40 U $µL^{-1}$	The enzyme was diluted using dilution buffer as provided by the manufacturer.
4.	Klenow Fragment of *E. coli* DNA polymerase I	5 U $µL^{-1}$	2 U per 50 µL of reaction volume	Klenow enzyme treatment was carried out in the buffer as supplied by the manufacturer.
5.	*Taq* DNA Polymerase	3U $µL^{-1}$	3 U $µL^{-1}$	--

Various media formulations:

Sr. No.	Media	Composition
1.	Luria Bertani (LB) Medium	0.5 % (w/v) Yeast extract, 1.0 % (w/v) Tryptone, 1.0 % (w/v) NaCl, 1.5 % (w/v) Agar-Agar Volume was made up by single distilled water and autoclaved.
2.	YEM Medium	0.4 g L^{-1} Yeast extract, 10.0 g L^{-1} Mannitol, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.5 g $L^{-1}$$K_2HPO_4$, 0.1 g L^{-1} NaCl, 1.5 % Agar-Agar
3.	Shoot Regeneration medium	MS medium, Zeatin (2.5 mg L^{-1}), GA_3 (3.0 mg L^{-1}), IAA (0.1 mg L^{-1})
4.	Selective shoot regeneration medium	MS medium, Zeatin (2.5 mg L^{-1}), GA_3 (3.0 mg L^{-1}), IAA (0.1 mg L^{-1}), kanamycin (80 mg L^{-1}) and cefotaxime (250 mg L^{-1})
5.	Selective root inducing medium	MS medium, IAA (0.1 mg L^{-1}), kanamycin (80 mg L^{-1}) and cefotaxime (250 mg L^{-1})

Composition and Stock Preparations for Murashige and Skoog (MS) Basal Medium:

MS Major Salts:

S. No.	MS Major Salts	MS Basal conc. (mg L^{-1})	Amount required for 100X stock (g L^{-1})	Use of stock for 1L medium (mL)
1.	KNO_3	1900.0	190.0	10.0
2.	NH_4NO_3	1650.0	165.0	10.0
3.	$MgSO_4.7H_20$	370.0	37.0	10.0
4.	$CaCl_2.2H_2O$	440.0	44.0	10.0
5.	KH_2PO_4	170.0	17.0	10.0

Note: All the MS major salts stock solutions to be prepared separately.

MS Minor Salts:

S. No.	MS Minor Salts	MS Basal conc. (mg L^{-1})	Amount required for 1000X stock (g L^{-1})	Use of stock for 1L medium (mL)
1.	H_3BO_4	6.20	6.20	1.0
2.	$MnSO_4.4H_2O$	22.30	22.30	1.0
3.	$ZnSO_4.7H_2O$	8.60	8.60	1.0
4.	$Na_2MoO_4.2H_2O$	0.25	0.25	1.0
5.	$CuSO_4.5H_2O$	0.025	0.025	1.0
6.	$CoCl_2.6H_2O$	0.025	0.025	1.0
7.	KI	0.83	0.83	1.0

Note: The $Na_2MoO_4.2H_2O$ was added first followed by the addition of H_3BO_4 during preparation of minor salts stock solutions.

MS Vitamins:

S. No.	Name of Vitamins	MS Basal Conc. (mg L^{-1})	Amount required for 1000X stock (mg mL^{-1})	Use of stock for 1L medium (mL)
1.	Nicotinic Acid	0.5	0.5	1.0
2.	Pyridoxine HCl	0.5	0.5	1.0
3.	Thiamine HCl	0.1	0.1	1.0
4.	Glycine	2.0	2.0	1.0
5.	Myo-inositol	100.0	100.0	1.0

Note: All the MS vitamins stock solutions to be prepared separately.

S. No.	Name of Chemical	MS Basal Conc. (mg L^{-1})	Amount required for 1000X stock (mg mL^{-1})	Use of stock for 1L medium (mL)
1.	$Fe_2EDTA. 2H_2O$ (sodium salt)	30.0	30.0	1.0

Note: Preparation of MS basal medium included major salts, minor salts, vitamins, $Fe_2EDTA.2H_2O$, 3.0 % sucrose, 0.7-0.8 % agar agar. The pH of medium was adjusted to 5.8 using 0.01N HCl or 0.01N NaOH.

Various phytohormones:

Sr. No.	**Phytohormones**	**Stock conc** (mg mL^{-1})	**Working Conc** (mg L^{-1})	**Details of preparation**
1.	Zeatin	2.5	2.5	Zeatin was dissolved in 0.1 N HCl, heated gently and made to the volume by adding sterile water. The pH adjusted to ~5.0.
2.	IAA	2.0	0.1	Auxin such as IAA (Indole acetic acid) was dissolved in 0.1 N KOH, stirred gently and made up the volume by adding distilled water.
3.	2,4-D	2.0	2.0	2,4-D (2,4-dichlorophenoxy acetic acid) was dissolved in 0.1 N KOH, stirred gently and made up the volume by adding distilled water.
4.	Gibberellins (GA_3)	3.0	3.0	GA_3 was dissolved in 95% ethanol, stirred gently and made up to the volume by adding sterile water.

Results & Discussion

5. Results and Discussion

5. 1. First Objective: Molecular cloning of patatin gene promoters from potato cultivars

The initial focus of this study was on molecular cloning and characterization of some class I patatin genes having 5′-flanking regions (promoters) from the different Indian potato cultivars. The various steps involved are described in the following sections.

5.1.1. Establishment and maintenance of potato germplasm

Virus-free potato plantlets were raised through apical meristem culture by excising the apical dome and subcultured *in vitro* on MS medium (Murashige and Skoog 1962). We routinely maintain the pathogen-free germplasm of different Indian potato cultivars namely Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Chandramukhi (KCM), Kufri Jyoti (KJ), Kufri Pukhraj (PR), Kufri Ashoka (AS) and cultivar Desiree (De) which are suitable to our agro-climatic conditions. For convenience, the abbreviated forms of these cultivars as used in this thesis work are also mentioned within the parenthesis. The potato cultivars were routinely micropropagated in our laboratory under controlled conditions (16 h light/8 h dark, 25±2^0C, 70% relative humidity) on MS-Basal medium at 3-4 weeks interval. In this study, Kufri Chipsona-1, Kufri Jyoti and Kufri Chandramukhi (Fig. 1) were chosen for exploring their genetic resources. The cultivar Kufri Chipsona-1 was also used during *Agrobacterium*-mediated genetic transformation.

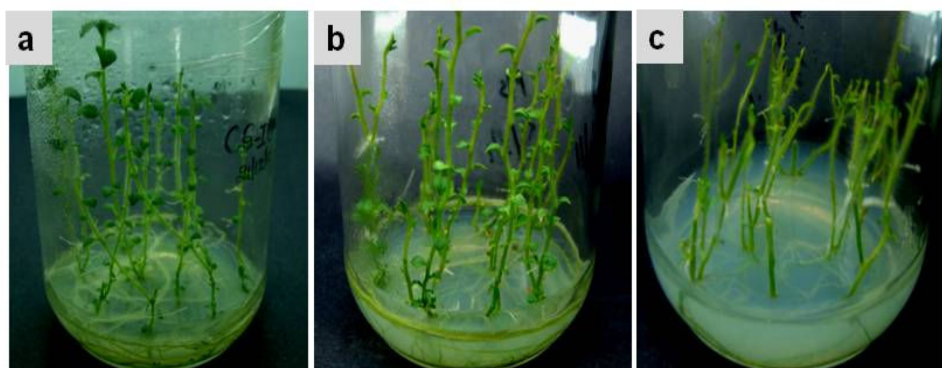

Fig. 1 Micropropagated potato plantlets of different potato cultivars cultured on MS basal medium **a** Kufri Chipsona 1, **b** Kufri Jyoti, **c** Kufri Chandramukhi

5.1.2. Isolation of total DNA from the potato cultivars

Total genomic DNA was isolated from the micropropagated plantlets of the potato cultivars namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chandramukhi and Kufri Jyoti based on a simple and efficient protocol as described by Kumari et al. (2012). The former two cultivars are processing varieties. One of the isolation steps involved potassium acetate in order to remove carbohydrates and other bulk impurities. The total DNA obtained by this method was found to be essentially free of inhibitory materials as checked by different restriction enzyme digestions. The yield of the DNA samples was determined spectrophotometrically by measuring the absorbance at 260 nm, and found to be in the range of 70 to 120 µg per gram of the plant material which was sufficient for most of the molecular biological techniques. The quality of the DNA was found to be considerably pure (i.e. A_{260}/A_{280} ~1.8). The genomic DNA preparations were also analyzed by agarose gel electrophoresis (Fig. 2).

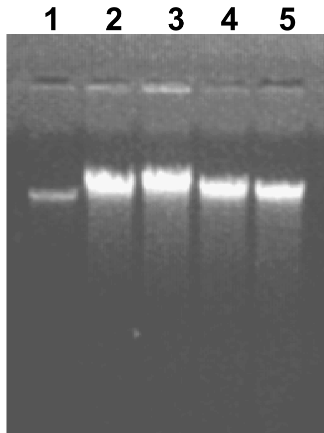

Fig. 2 Agarose gel analyses of total genomic DNA isolated from different potato cultivars. lane 1, Bacteriophage λ DNA; lane 2, Kufri Chipsona-1; lane 3, Kufri Chipsona-2; lane 4, Kufri Chandramukhi; and lane 5, Kufri Jyoti.

5.1.3. Molecular cloning of the partial class I patatin genes from the potato cultivars

Polymerase chain reactions: For isolation of class I patatin genes from some of the Indian potato cultivars, polymerase chain reaction (PCR) approach was adopted in this study. The aim was to amplify partial class I patatin genes having mostly the 5′ flanking regions (promoters) from the individual potato cultivars namely, Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chandramukhi, and Kufri Jyoti. For this purpose, PCR was carried out using the respective genomic DNA as template and the patatin gene-specific different primer pairs keeping only the reverse primer as common in all the cases. After initial denaturation at 94ºC for 1 min 30 s, the thermal cycling parameters were: denaturation at 94ºC for 1 min, annealing at 55ºC for 2 min; polymerization at 72ºC for 2 min or 1 min for 30 cycles followed by final extension at 72ºC for 5 min. The PCR products were then separated on 0.7% agarose gel to visualize the amplified DNA bands as shown in Fig. 3. With respect to the first primer pair i.e. PT-F074 and PT-R1478, the sizes of the prominent amplified DNA bands for the different cultivars were: CS-1 (~2.3 kb and ~2.0 kb), KCM (~1.4 kb), KJ (~2.7 kb), CS-2 (~1.4 kb) as shown in Fig. 3a. Here, ~1.4 kb DNA band was apparently found to be common for the potato cultivars KCM and CS-2. Likewise, corresponding to the second primer pair i.e. PT-F610

and PT-R1478, the sizes of the prominent amplified DNA bands for the different cultivars were: CS-1 (~2.0 kb and ~0.8 kb), KCM (~0.8 kb), KJ (~2.0 kb and ~1.3 kb), CS-2 (~0.8 kb) as shown in Fig. 3b. With respect to the third primer pair i.e. PTF1B and PT-R1478, the prominent DNA bands were: CS-2 (~1.4 kb) and KCM (~1.4 kb) as shown in Fig. 3c. Interestingly, there was no amplification in the cases of the potato cultvars, CS-1 and KJ.

PCR data clearly suggested the genotypic variations between the potato cultivars as evident from the distinct variations of the amplified DNA band patterns with respect to each primer pair. In order to avoid non-specific amplifications, each PCR was carried out under high stringent condition (where annealing temperature was kept at 55^0C). Therefore, it is very likely that the prominent amplified DNA products corresponding to the individual potato cultivars are specific to the patatin genes. The sizes of these DNA bands were analyzed carefully; only a few of them were found to be consistent (i.e. of expected sizes) if compared with the available class I patatin gene sequences in the database according to the locations of the primer sequences. The sizes of a number of amplified DNA bands did not match with other known class I patatin sequences. This was possibly due to the sequence divergences in the farther upstream promoter regions of class I patatin genes in the Indian potato cultivars because of structural diversity of the patatin multicopy gene family. In fact, all these notions were considerably substantiated in this study as described categorically in the following sections. The PCR data specific to the second primer pair clearly suggest that the promoter regions around 0.7 kb upstream of the transcription start site are well conserved in the class I patatin genes in most of the potato cultivars. The PCR-amplified products specific to the cultivar KJ showed significant variations if compared with the other cultivars.

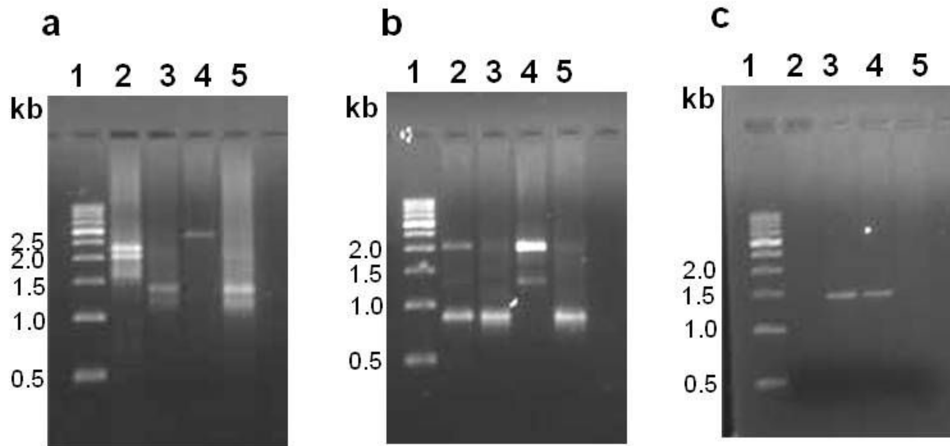

Fig. 3 PCR amplified DNA products of class I patatin genes by using different combination of gene specific primer pairs. **a** Amplified DNA products from different potato cultivars using the primer pair PT-F074 and PT-R1478. lane 1, 500 bp DNA ladder; lane 2, cv. Kufri Chipsona-1; lane 3, cv. Kufri Chandramukhi; lane 4, cv. Kufri Jyoti; lane 5, cv. Kufri Chipsona 2; **b** Amplified DNA products from different potato cultivars using the primer pair PT-F610 and PT-R1478. lane 1, 500 bp DNA ladder; lane 2, cv. Kufri Chipsona-1; lane 3, cv. Kufri Chandramukhi; lane 4, cv. Kufri Jyoti; lane 5, cv. Kufri Chipsona 2; **c** Amplified DNA products from different potato cultivars using the primer pair PT-F1B and PT-R1478. lane 1, 500 bp DNA ladder; lane 2, cv. Kufri Chipsona-1; lane 3, cv. Kufri Chipsona 2; lane 4, Kufri Chandramukhi; lane 5, cv. Kufri Jyoti.

Molecular cloning: For the purpose of molecular cloning, only a few PCR-amplified DNA products were chosen in this study covering mainly three potato cultivars: i) CS-1 (~2.3 kb), KCM (~1.4 kb), and KJ (~2.7 kb) specific to the first primer pair; ii) CS-1 (~0.8 kb), corresponding to the second primer pair; and iii) KCM (~1.4 kb) corresponding to the third primer pair. The PCR-amplified DNA bands were polished with Klenow enzyme, purified and then cloned into the *Sma*I site of pUC19 vector. All these cloned DNA fragments were finally sequenced in both the directions by commercial company Bangalore Genei, Bangalore. The NCBI BLAST search tool of all these sequences showed considerable sequence identity with the available class I patatin gene sequences in the databases. The cloned partial class I patatin genes having mostly the 5′-flanking regions were designated as follows:

- *St*PM01 (1454 bp), specific to the first primer pair and the cv. KCM
- *St*PM02 (1441 bp), specific to the third primer pair and the cv. KCM
- *St*PN02 (2747 bp), specific to the first primer pair and the cv. KJ
- *St*PK01 (2332 bp), specific to the first primer pair and the cv. CS-1
- *St*PK03 (874 bp), specific to the second primer pair and the cv. CS-1

All the above sequence information were submitted to the NCBI database: *St*PM01 (GenBank Acc. No. **JX124230**); *St*PM02 (GenBank Acc. No. **JX124231**); *St*PN02 (GenBank Acc. No. **JX124227**); *St*PK01 (GenBank Acc. No. **JX124228**); *St*PK03 (GenBank Acc. No. **JX124229**). The major part of each sequence refers to the region upstream of the predicted transcription start site (TSS). All these clones were quite relevant to the present study since they were used during functional characterization for assessing the promoter activities.

5.1.4. Sequence analyses and comparison of the partial class I patatin genes

The nucleotide sequences of 1454-bp *St*PM01, 1441-bp *St*PM02, 2747-bp *St*PN02, 2332-bp *St*PK01, and 874-bp *St*PK03 were examined carefully with the help of the NCBI BLAST tools. Each sequence consists of a major 5′-flanking (promoter) region and the remaining portion refers to only a part of first exon. Sequence analyses and comparison data are presented in two sections depending mostly on the source of isolation, length of the sequences. For example, the partial class I patatin genes, *St*PM01 and *St*PM02 having comparable sequence lengths correspond to the potato cultivar Kufri Chandramukhi were described in the first instance. Similarly, the remaining class I patatin genes as obtained in this study were described in the later section.

5.1.4.1. Sequence analyses and comparison of StPM01 and StPM02

The NCBI Blast search analyses: The nucleotide sequences of *St*PM01 and *St*PM02 were analyzed by NCBI BLAST tools. The 1454-bp *St*PM01 and the 1441-bp *St*PM02 consisted of 1336-bp and 1323-bp 5′-flanking (promoter) regions upstream of their predicted TSS, respectively; and the remaining portions referred to only a part of first exon, and found to be identical as shown in Fig. 4. Here, the available partial coding region included the N-terminal 23-amino acid vacuolar targeting signal peptide and only the first amino acid of the mature protein. The 1336-bp 5′-flanking region of *St*PM01 shared ~98% sequence identity with the corresponding region of *St*PM02 along with a few major and minor gaps (the query coverage being ~95%). The overall variation between these two sequences were mostly due to insertions/deletions rather than cumulative point mutations. However, the major conspicuous difference was due to the absence of a 69 bp region in *St*PM02 corresponding to the bases 1042-1110 of *St*PM01. Interestingly, we also noticed the presence or absence of such distinguishing sequence feature in the 5′-flanking regions of other available class I members in the database. Such type of observation was not documented in the earlier published reports. The NCBI Blast search further revealed that the 1336 bp 5′-flanking region of *St*PM01 showed varying sequence identities with the class I patatin genes as isolated from other potato cultivars, such as, 98% (JX124229, cv. Kufri Chipsona 1; JQ341191, cv. Zhongshu No. 4; AY485645, unknown cultivar), 97% (JX124228, cv. Kufri Chipsona 1; X03956, cv. Maris piper, Bevan et al.1986; M18880, cv. Desiree, Mignery et al. 1988), 96% (X60396, cv. Berolina, Liu et al. 1991; X87216, cv. Dongnong 303), 94% (GU168944, cv. Longshu 3), 93% (JX124227, cv. Kufri Jyoti), 91% (X14483, cv. Berolina, Rocha-Sosa et al. 1989), and 90% (X60399, cv. AM 80/5793, Liu et al. 1991). During Blast search, a wide range of query

coverage (i.e. around 99 to 50%) could also be noticed for the above sequences. All these data clearly indicated considerable sequence divergence in the upstream 5′-flanking regions of the class I patatin gene family members. Apart from insertions/deletions at different locations, significant mismatches were also found in the respective aligned regions with the query sequence. Sequence analysis data clearly showed that none of the class I patatin genes under study, was found to be identical with any other patatin gene family members reported to date.

Multiple sequence alignment of the partial patatin genes: For direct comparison with some known members of class I and class II patatin gene family, multiple sequence alignments were carried out. This exercise revealed the extent of sequence identity and divergence between the different members. Moreover, this helped in detecting the important sequence features and putative *cis*-regulatory sequence motifs in the patatin genes. For multiple sequence alignment, a total of four partial patatin gene sequences were chosen that included three partial class I patatin gene sequences namely *St*PM01 and *St*PM02 of this study, and the clone B33 (GenBank Acc. No. X14483), and one member of class II family i.e. the clone LPOT23 (GenBank Acc. No. X07030) as shown in Fig. 4. During this alignment, some minor manual adjustments were also made that provided a clear view for examining sequence identities, nature and locations of the divergences, and other salient features in the above patatin gene sequences of both classes. Importantly, it also helped in detecting the common putative *cis*-regulatory sequence motifs. Upstream of TSS, 79 bp region of both the classes appeared to be highly conserved, but they were found to vary abruptly in the further upstream regions which were consistent with earlier reports. However, significant sequence divergence could be found if compared between the class I family members.

```
StPM01                                                                                 TATTG--------ACCGGAGACTATACCATA   23
StPM02  TAGATCTTA-CTTCTATTGGCTTTGTTTTATTATCATTTTTCACACTCTGTGGTTTAGTAATTG--------ACCGGAGACTATACCATA   81
   B33  TAAACTTTAACTTATGTTAAATTTGTAATGATAAAATTTTTA-----TTGTAAATTAAAAATTACTTATAAAATTGGGCATTATAACATA  129
        .. .  ... ... . ..   .....  .  ..  ......       ...   ...  .***           *::**::*:****:****

                                                                     CCTTTT
StPM01  T-AAGGCCTAATCA-------------------------------------CAACTTAGTCTCCTTTTTTATTTTTTCTTTATTT----   70
StPM02  T-AAGGCCTAATCA-------------------------------------CAACTTAGTCTCCTTTTTTATTTTTTCTTTATTT----  128
   B33  TGAAAGACAAATTGTGTTACATATTTTACTTTTGACTTTAATATGAATATTTCAATTTAAATCATTGTTTTATTTTCTCTTTCTTTTTAC  219
        * **:*:*:***::                                     ***:***:::::*:*********:*****:***

                CCAAT/RTTTTTR              GATA                 GTAC/YACT        RTTTTTR
StPM01  ------AGGAGACCAATTTTTAA---------------AGATATGAAC----------TTGGTACT---------ATTTTTA------TG  114
StPM02  ------AGGAGACCAATTTTTAA---------------AGATATGAAC----------TTGGTACT---------ATTTTTA------TG  172
   B33  AGGTATAAAAGGTGAAAATTGAAGCAAGATTGATTGCAAGCTATGTGTCACCACGTTATTGATACTTTGGAAGAAATTTTTACTTATATG  309
              *::**:::**::**:**               **:****:::          ***:****         *******      **
                    RTTTTTR                      YACT             CCAAT/E-box
StPM01  GTTTTATGT----GTGGTTTTTA---TGTTCT--TTTGTTCATTTTG-CACTTATAACTTTAGAGACAATTGATGTTGTCCCTGAT---T  191
StPM02  GTTTTATGT----GTGGTTTTTA---TGTTCT--TTTGTTCATTTTG-CACTTATAACTTTAGAGACAATTGATGTTGTCCCTGAT---T  249
   B33  TCTTTGTTTAGGAGTAATATTTGATATGTTTTAGTTAGATTTCTTGTCATTTATG-CTTTAGT-ATAATTTAGTTATTTTTATTATAT  397
        ::***:*:*    **::*:***:    ****:*  **:*:*::*:*** **:****::******::*:****:::***:*:::*::*   *
                          GATA                     CCTTTT               YACT
StPM01  GAAC-TAAATAATTAGCGAC-GATATAGTTTTGTCGGTAGTAATAACCTTTTTAGTGACAAAACA--TACTATTAACTACAAAAAAAGT-  276
StPM02  GAAC-TAAATAATTAGCGAC-GATATAGTTTTGTCGGTAGTAATAACCTTTTTAGTGACAAAACA--TACTATTAACTACAAAAAAAGT-  334
   B33  GATCATGGGTGAATTTGATACAAATATTTTTGTCATTA--AATAAATTAATTTATCACAACTTGATTACTTCAGTGACAAAAAATGTA  485
        **:* *:::*:*:*:::**: :*:***:*******::**::*****::*::**::*:****::::  ****:*:*:::********:**

                 YACT                                     E-box                      E-box
StPM01  ---------TACACTTTTT-----ATGACAAATAATA------AATTCGTCACAAATGT------------TTATG----CATTTGGGGA  330
StPM02  ---------TACACTTTTT-----ATGACAAATAATA------AATTCGTCACAAATGT------------TTATG----CATTTGGGGA  388
   B33  TTGTCGTAGTACCCTTTTTTGTTGAATATGAATAATTTTTTTATTTTGTGACAATTGTAATTGTCACTACTTATGATAATATTTAGTGA  575
                 ***:******     *::*::******:      *:**:**:****:***            *****    :****:*:**

                                             ATATT               GTAC/YACT          YACT/LRE
StPM01  CG-ATTTTTC---------------TTTTTGTGGT--TAATATATTAGTTTTAGCGACGAAGTACTAAATCGTCTTTGTATACTTTGAGT  402
StPM02  CG-ATTTTTC---------------TATTTGTAGT--TAATATATTAGTTTTAGCGACGAAGTACTAAATCGTCTTTGTATACTTTGAGT  460
   B33  CATATATGTCGTCGGTAAAAGCAAACACTTTCAGTGACAAAATAATAGATTTAATCACAAAATTATTAACCTTTTTATAATAATAAATT  665
        *: **:*:**                :·:**:::·**  :**:***:***:****:::**:**::*:**:*:*:***:**::::*::*:*

           ACGT                      RTTTTTR                   RTTTTTR
StPM01  GACACACGTTTAGTGACGACTAATTGACGAA--ATTTTTGTCGT-CACACAAA---ATTTTTAGTGACGAAACATGATTTATAGATGGCG  486
StPM02  GACACACGTTTAGTGACGACTAATTGACGAA--ATTTTTGTCGT-CACACAAA---ATTTTTAGTGACGAAACATGATTTATAGATGGCG  544
   B33  TATCCCTAATTTAT-ACATTTAAG-GACAAAGTATTTTTTTATATATAAAAAATAGTCTTTAGTGACGAT-CGTAGTGT-TGAGTCTAG  751
        :*::*::::**::*:**:::***::***:**  ******:*::* :*:*:***   :*:**********::*:*::*:*:*:::*:::*
                                                              YACT
StPM01  AAATTAT----TTGTCCCTC--ATAATCTAATTTGGTGTAG--TGATCATTACT------CCTTTGTTTGT--------TTTATTTGTCA  554
StPM02  AAATTAT----TTGTCCCTC--ATAATCTAATTTGGTGTAG--TGATCATTACT------CCTTTGTTTGT--------TTTATTTGTCA  612
   B33  AAATCATAATGTTGAATCTAGAAAAATCTCATGCAGTGTAAAATAAACCTCAAAAAGGACGTTCAGTCCATAGAGGGGGTGTATGTGACA  841
        ****:**    ***:::**:  *:*****:**:::*****:  *:*:*:*:*::      ::*:::*:::*         *:***:**:**

                                         CCAAT        ACGTG                             ATATT
StPM01  TGTTAGT-TCATTAAAAAAAAAAATCTCTTCTTATCAAT----TCTGACGTGTTTAATA-TCATAAG----ATTAAAAA-ATATTT-TAA  632
StPM02  TGTTAGT-TCATTAAAAAAAAAAATCTCTTCTTATCAAT----TCTGACGTGTTTAATA-TCATAAG----ATTAAAA--ATATTT-TAA  689
   B33  CCCCAACCTCAGCAAAAGAAAACCTCCCTTC--AACAAGGACATTTGCGGTGCTAAACAATTCAAGTCTCATCACACATATATTTATTA  929
        ::::*::: ***::****:****::**:****::*:***:     *:**::***:*:**:* *:::***     **:*:*:▪ ****** *:*

StPM01  TATATCTTTAATTTAAAACCACAAAGT--------------TCAAATT-TCTTCGT----------TAAC--TTAATTTGTCAAATCAGG  695
StPM02  TATATCTTTAATTTAAAACCACAAAGT--------------TCAAATT-TCTTCGT----------TAAC--TTAATTTGTCAAATCAGG  752
   B33  TATAATACTAATAAAGAATAGAAAAGGAAAGGTAAACATCATTAAATCGTCTTTGTATATTTTTAGTGACAACTGATTGACGAAATCTTT 1019
        ****::::****::*:**:::::****              *:****: ****:**           *:**  :*:***::::*****:::
                                                            TATTCT                      B-BOX/CC
StPM01  CTC---AAAGATCGTTTTTCATATCGGAA-------------TGAGGATTTTATTTATTCTTTTAAAAATAAAGAGGTGTTGAGCTAAAC  769
StPM02  CTC---AAAGATCGTTTTTCATATCGGAA-------------TGAGGATTTTATTTATTCTTTTAAAAATAAAGAGGTGGTGAGCTAAAC  826
   B33  TTCGTCACACAAAATTTTTAGTGACGAAACATGATTTATAGATGATGAAATTATTTGTCCCTCATAATCTAA---TTTGTTGTAGTGATC 1106
        :**    *:*:*:::*****::*::**:**             ***:**::******:*:*:::**:::***:::::**▪**:::*:*:*

        AAT    TSSR2(B-box)/E-box
StPM01  AA--TTTCAAATCTCATCTCACATATGGGGTCAG-CCACAAAAATAAA----------------------GAACGGTTGGAACGGATCTA  834
StPM02  AA--TTTCAAATCTCATCACACATATGGGGTCAG-C-ACAAAAATAAA----------------------GAACGGTTGGAACGGATCTA  890
   B33  ATTACTCCTTTGTTTGTTTTATTTGTCATGTTAGTCCATTAAAAAAAAATATCTCTCTTCTTATGTACGTGAATGGTTGGAACGGATCTA 1196
        *:  :*:*::::*::*:▪:*::*:*:::**:** *▪*::****:***                       ***:****************

           TSSR2(A-box)/SURE-2/SURE-1/ATAGAA       GTAC                                  E-box
StPM01  TTATATAATACTAATAAAGAATAGAAAAAGGAAAGTGAGTGAGGTACGAGGGAGAGAATCTGTTTAATATCAGAGTCGATCATGTGTCAG  924
StPM02  TTATATAATACTAATAAAGAATAGGAAAAGGAAAGTGAGTGAGGTGCGAGGGAGAGAATTTGTTTAATATCAGAGTCGATCATGTGTCAG  980
   B33  TTATATAATACTAATAAAGAATAGAAAAAGGAAAGTGAGTGAGGTTCGAGGGAGAGAATCTGTTTAATATCAGAGTCGATCATGTGTCAA 1286
        ************************▪******************** *************▪******************************:

               GATA    LRE    E-box         CCAAT   I-box/GATA                      AGAAA
StPM01  TTTTATTGATATGACTTTGACTTCAACTGAGTTTAAGCAATTTTGATAAGGCGAGGAAAATCACAGTGCTGAATCTAGAAAAATCTCATA 1014
StPM02  TTTTATCGATATGATTCTGACTTCAACTGAGTTTAAGCAATTCTGATAAGGCGGAGAAAATCATAGTGCTGAGTCTAGAAAAATCTCATG 1070
   B33  TTTTATCGATATGACCCTAACTTCAACTGAGTTTAACCAATTCCGATAAGGCGAGAAATATCATAGTATTGAGTCTAGAAAAATCTCATG 1376
        ******.*******▪:.*:*****************:*****.:*********▪▪:**:****.***::***.*****************.
```

Fig. 4 contd..

```
              GATA                ACGT                GGGCGG                                 AGAAA
StPM01    CAGTGTGAGATAAATCTCAACAAAAACGTTGAGTCCATAGAGGGGGT-GTATGTGACACCC-AACCTCAGCAAAAGAAAACCTCCCCTCA 1102
StPM02    CAGTGTGAGATAAACCTCAACAAGAA---------------------------------------------------------------- 1097
   B33    TAGTGTGGGGTAAACCTCAGCAAGGACGTTGAGTCCATAGAGGGGGGTGTATGTGACACCCCAACCTCAGCAAAAGAAAACCTCCCCTCA 1466
          :******:*:****·****:***·:*▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪ ▪▪▪▪▪▪▪▪▪▪▪▪▪ ▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪

             E-box    B-BOX/CCAAT                CTATAWAWA/ATATT CTATAWAWA/SURE-2 SURE-1/ATAGAA
StPM01    AGAAGGACATTTGCGGTGCTAAACAATTTCAAGTCTCATCACACATATATATT----ATATAATACTAATAAAGAATAGAAAAAGGAAAG 1188
StPM02    -------CATTTGCGGTGCTAAACAATTTCAAGTGTTATCACACATATATATT----ATATATTACTAATAAAGAATAGAAAAAGGAAAG 1175
   B33    AGAAGGACATTTGCGGTGCTAAACAATTTCAAGTCTCATCACACATATATATATATTATATAATACTAATAAATAATAGAAAAAGGAAAG 1556
          ▪▪▪▪▪▪▪**************************▪*▪***************:    *****▪**********:***************

              YACT      E-box     TGHAAARK       GATA                          YACT
StPM01    GTAAACATCACTAATGACAGTTGCGGTGCAAAGTGAGTGAGATAATAAACATCAGTAATAGACATCACTAACTTTTATTGGTTATGTCAA 1278
StPM02    GTAAACATCACTAATGACAGTTGCGGTGCAAAGTGAGTGAGATAATAAACATCACTAATAGACATCACTAACTTTTATTGGTTATGTCAA 1265
   B33    GTAAACATCACTAACGACAGTTGCGGTGCAAACTGAGTGAGGTAATAAACATCA-------------CTAACTTTTATTGGTTATGTCAA 1633
LPOT23                                                                   258 AACTTTTATTGGTTATGTCAA  278
          **************:*****************:********:************ ::::::::::::**♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦

             AAAAAAATTTC           ACGTG CTATAWAWA           ▼                                 YAC
StPM01    ACTCAAAATAAAATTTCTCAACTTGTTTACGTGCCTATATATACCATGCTTGTTATATGCTCAAAGCACC--AACAAAATTTAAAAACAC 1366
StPM02    ACTCAAAATAAAACTTCTCAACTTGTTTACGTGCCTATATATACCATGCTTGTTATATGCTCAAAGCACC--AACAAAATTTAAAAACAC 1353
   B33    ACTCAAAGTAAAATTTCTCAACTTGTTTACGTGCCTATATATACCATGCTTGTTATATGCTCAAAGCACC--AACAAAATTTAAAAACAC 1721
LPOT23    ACTCAAAATAAAATTTCTCAACTAGTTTACGTGACTATATATACCATGCTTGTTATATGTTCAAAGCACCCAAAAAAAAATTAAATACAC  368
          ♦♦♦♦♦♦♦:♦♦♦♦♦▪♦♦♦♦♦♦♦♦♦*♦♦♦♦♦♦♦♦♦*♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦*♦♦♦♦♦♦♦♦♦♦  ♦♦*♦♦♦♦*♦♦♦♦♦*♦♦♦♦

          T/LRE                      E-box/TGHAAARK    YACT                              GATA/ATATT  YAC
StPM01    TTTGA---------------------ACATTTGCAAAATGGCAACTACTAAATCTTTTTAATTTTATTTTTTATGATATTAGCAACTAC 1435
StPM02    TTTGA---------------------ACATTTGCAAAATGGCAACTACTAAATCCTTTTTAATTTTATTTTTTATGATATTAGCAACTAC 1422
   B33    TTTGA---------------------ACATTTGCAAAATGGCAACTACTAAAACTTTTTAATTTTATTTTTTATGATATTAGCAACTAC 1790
LPOT23    TTTGAGATATTAGTTTTTATTAATTATAATCTGCAAAATGGCAACTACTAAATATTTTACAATTTTAATTTTTATGATGTTAGCAACTAC  458
          ♦♦♦♦♦                     **♦♦*♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦:*▪♦♦♦♦*♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦*♦♦♦♦♦♦♦♦♦♦♦

PStPM01                                             M  A  T  T  K  S  F  L  I  L  F  F  M  I  L  A  T  T   18
PStPM02                                             M  A  T  T  K  S  F  L  I  L  F  F  M  I  L  A  T  T   18
   PB33                                             M  A  T  T  K  T  F  L  I  L  F  F  M  I  L  A  T  T   18
PLPOT23                                             M  A  T  T  K  Y  F  T  I  L  I  F  M  M  L  A  T  T   18
                                                    =  =  =  =  =     =     =  =     =  =     =  =  =  =

          T       E-box
StPM01    TAGTTCAACATGTGCTACG 1454
StPM02    TAGTTCAACATGTGCTACG 1441
   B33    TAGTTCAACATGTGCTAAG 1809
LPOT23    TAGTTCAACATTTGCT     474
          ♦♦♦♦♦♦♦♦♦♦♦*♦♦♦♦***

                       ↓
PStPM01   S  S  T  C  A  T     24
PStPM02   S  S  T  C  A  T     24
PB33      S  S  T  C  A  K     24
PLPOT23   S  S  T  F  A        23
          =  =  =     =
```

Fig. 4 Multiple sequence alignment of the nucleotide and deduced amino acid sequences of four patatin genes (first three from class I and the remaining one from class II) from the potato cultivars (for the GenBank Accession numbers see the text; numbering of the bases is kept same as the respective sequences appear in the GenBank database). Single-letter code is used for the amino acids. This alignment is based on ClustalW2 tool. Dashes in the nucleotide sequences indicate gaps arising during alignment. '▼' denotes putative transcription start site (TSS). The downward arrow indicates the cleavage site of the vacuolar transit peptide. The basal promoter elements and other *cis*-regulatory sequence motifs are overlined in *St*PM01 sequence (most of them are common in other sequences as well); the consensus sequence motifs are also shown at the respective places. The 208-bp long repeats are highlighted in B33 sequence (whereas, the subrepeats at three places are underlined). The symbols ♦, ∗, :, ▪, and · denote the conserved nucleotides between the four, first three, first two, first and third, and second and third sequences, respectively; whereas, the symbol = denotes the conserved amino acids between the corresponding four partial deduced patatin sequences.

If *St*PM01 and *St*PM02 sequences were compared individually with the corresponding B33 upstream promoter regions, sequence divergence appeared to be more prominent in terms of mostly insertions in the latter at a number of locations. This sequence comparison also revealed only a few mismatches around 500 bp region upstream of TSS in B33 as compared to the large number of mismatches in its further upstream regions. Rocha-Sosa et al. (1989) had shown the presence of long direct repeats of 208 nucleotides (also containing subrepeats) in B33 (Fig. 4). One of the repeats of B33 (bases 1355-1566) in the proximity of TSS appeared to be highly conserved in *St*PM01; but it was only partially conserved in the corresponding region of *St*PM02 as it lacked the 69 bp region as mentioned earlier. Both *St*PM01 and *St*PM02 differed significantly corresponding to the other direct repeat sequence of B33 (bases 763 to 970), although the former two sequences were nearly identical in this region. A 37 bp AT rich motif (subrepeats) was found to occur at three places i.e. at position 925, 1195, and 1520 in the B33 promoter region. In both *St*PM01 and *St*PM02 promoter regions, such type of nearly identical sequence motifs could be found corresponding to the latter two places of B33. Although the subrepeat at position 925 of B33 was not well conserved in *St*PM01 and *St*PM02, but the corresponding regions were AT rich in the latter two sequences.

The available regions downstream of TSS were nearly identical between the two classes, except one minor i.e. 2 bp, and a distinguishing 21 bp insertions in the 5′-UTR of the class II member. These features were consistent with the earlier reports. The context of the translation initiation codon i.e. TGCAAA<u>ATG</u>GCA appeared to be identical between the members of both the classes, and mostly complied to the plant consensus, TAAACA<u>ATG</u>GCT (Joshi 1987). The available partial coding regions of *St*PM01 and *St*PM02 were found to be identical; but variations could be found at two places, S6T (conservative) and T24K (non-conservative) if

compared with B33. If compared with the class II member, LPOT23 mostly non-conservative substitutions were found at four places, S6Y, F11I, I14M, and C22F. As the N-terminal 23-amino acid refers to the vacuolar transit peptide; such type of amino acid changes between the members of patatin class may have some biochemical significance.

G+C content analysis of patatin gene family members: The overall G+C content of the promoter and the transcribed regions of *St*PM01 were found to be 30% and 27%, respectively; and the values for the corresponding regions of *St*PM02 were 29% and 28%, respectively. For the class I patatin genes from other potato cultivars, the corresponding values were found to be ~28%, and 28-30%, respectively. Moreover, the isochore plots as generated by using EBI tools further revealed some interesting sequence features both in the class I and class II members as shown in Fig. 5. *St*PM01 and *St*PM02 showed almost similar patterns (Fig. 5a, b) except the 69-bp insertion in the former sequence. In case of B33, the isochore plot revealed considerable segment-wise variations in the upstream of TSS (Fig. 5 c). Immediately upstream of TSS, around 300 bp region showed relatively less G+C content in the class I members. But the overall variations were more prominent in case of the class II member e.g. pgT4 as shown in Fig. 5d. All these sequence features particularly in the 5′-flanking regions of the patatin genes are likely to have impact on the overall functionality of the individual promoters as they can influence the secondary structures of DNA, chromatin structures, and complex interactions with various transcription factors.

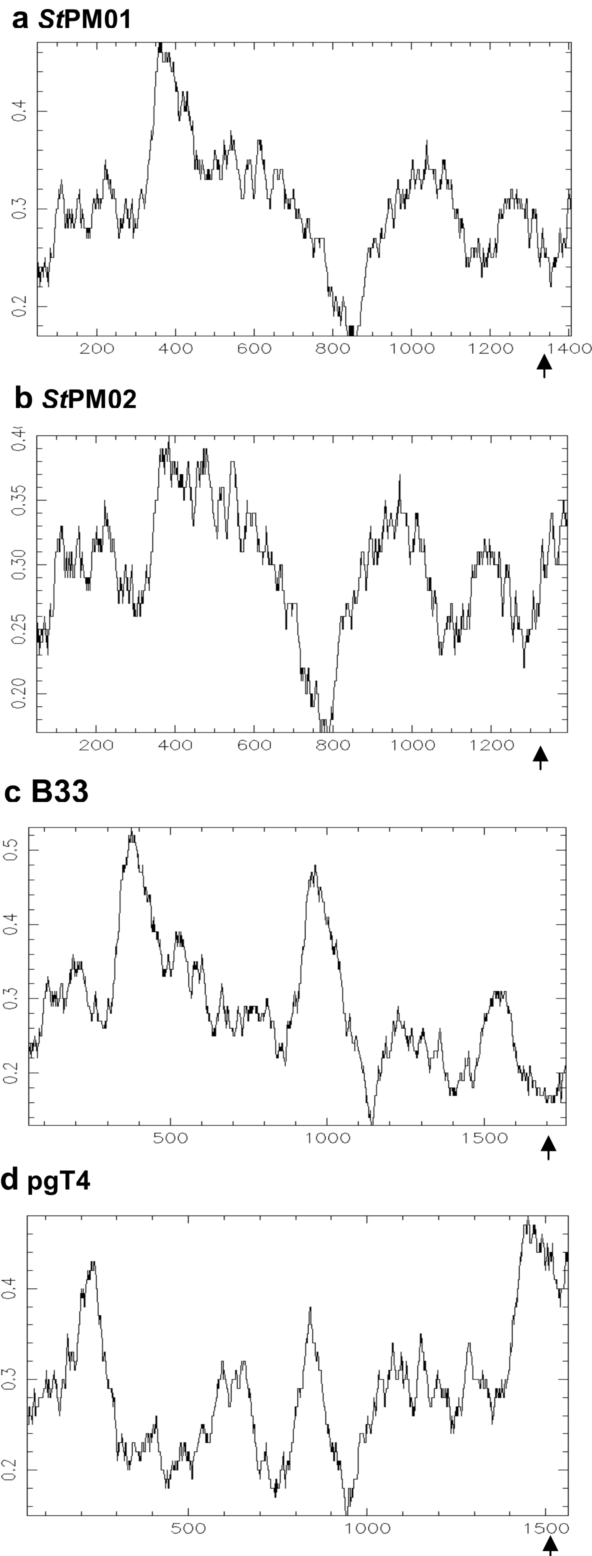

Fig. 5 G+C percent analyses of partial class I (a, b, and c) and class II (d) patatin genes having mostly the promoter regions from potato by isochore plots generated through EBI sequence analysis tools (EMBOSS). The vertical axis in each figure refers to G+C percent, and the horizontal axis indicates base position of the respective DNA sequence. Predicted transcription start site (TSS) in each isochore plot is shown by upward arrow: **a** 1454 nts *St*PM01 (TSS at 1337), **b** 1441 nts *St*PM02 (TSS at 1324), **c** 1809 nts B33 (TSS at 1700), **d** 1611 nts pgT4, GenBank Acc No. X60397 (TSS at 1526)

5.1.4.2. Sequence analyses and comparison of StPN02 and StPK01

The NCBI Blast search analyses: The nucleotide sequences of *St*PN02 and *St*PK01 were analyzed by NCBI BLAST tools. The 2747-bp *St*PN02 and the 2332-bp *St*PK01 consisted of 2629 bp and 2214 bp 5′-flanking (promoter) regions upstream of their predicted TSS, respectively; and the remaining portions referred to only a part of first exon as shown in Fig. 6. The exon part in each sequence contained only a small coding region for 24 amino acids comprising of 23-amino acid N-terminal vacuolar targeting signal peptide and the first amino acid of the mature protein. Only two mismatches of nucleotides at positions 2706 and 2709 led to two non-conservative substitutions i.e. I11F and V12F. The 2629-bp 5′-flanking region of *St*PN02 shared ~93% sequence identity with the corresponding region of *St*PK01 (with a few major and minor gaps in their aligned regions, and the query coverage being ~83%) indicating considerable sequence divergence between them. As evident in Fig. 6, the major conspicuous difference between the two genes was the insertion of a 487 bp region i.e. the bases 1917-2403 of *St*PN02. Such a large insertion was found to be mostly uncommon in the 5′-flanking regions of the available class I members. A highly conserved 37-bp direct repeats were found at positions 1677 and 2458 of StPN02 and the corresponding region of *St*PK01. Some other salient sequence features are described in the next sections. The 2629 bp 5′-flanking region of *St*PN02 was found to be close (i.e. 99% sequence identity) to a gene copy in the potato cultivar Katahdin haploid USW1 (DQ274179, Stupar et al. 2006); however, significant sequence divergence was noticed if compared with some known class I patatin genes from other potato cultivars, such as, 96% (X60399, AM 80/5793, Liu et al. 1991;

X87216, cv. Dongnong 303), 95% (JQ341191, cv. Zhongshu No. 4), 94% (JX124231, cv. Kufri Chandramukhi; and M18882, cv. Superior, Mignery et al. 1988), 93% (X03956, cv. Maris piper, Bevan et al.1986; JX124230, cv. Kufri Chandramukhi; X60396, cv. Berolina, Liu et al. 1991; M18881, cv. Superior, Mignery et al. 1988), 92% (GQ352473, AY485645, unknown cultivars). Moreover, a wide range of query coverages (i.e. around 100 to 37%) were noticed during the Blast search. Apart from insertions/deletions at different locations, nucleotide mismatches could be found in the aligned regions. All these data clearly showed considerable sequence divergence in the 5′-flanking regions of the class I members. None of the class I patatin genes under study, was found to be identical with any other patatin gene family members reported to date.

Multiple sequence alignment of the partial patatin genes: For multiple sequence alignment, a total of four partial patatin gene sequences from potato i.e. *St*PN02 and *St*PK01 of this study, λpat21 (GenBank Acc. No. X03956), and one class II member, LPOT23 (GenBank Acc. No. X07030) as shown in Fig. 6. For close inspection and comparison between the sequences, a few minor manual adjustments were made in this alignment. Upstream of TSS, a 79 bp region appeared to be nearly identical in both the classes. However, in the further upstream regions, significant sequence divergence could be noticed not only between the two classes but within the members of the same class. For example, the 5′-flanking regions of *St*PN02, *St*PK01, and λpat21 were the glaring examples as they were found to vary significantly in terms of major and minor insertions/deletions and nucleotide mismatches. More categorically, *St*PN02 appeared to be more distinct in terms of a major insertion, and deletions at some locations;

and comparison between *St*PK01 and λpat21 revealed only minor insertions/deletions and some mismatches. In other words, the latter two sequences were found to be more close to each other. Interestingly, the major conspicuous 487-bp insertion as evident in *St*PN02 was found to occur in the highly conserved region of the class I members. The available regions downstream of TSS were nearly identical between the two classes, except one minor i.e. 2 bp, and a distinguishing 21 bp insertions in the 5′-UTR of the class II member. This observation was consistent with the earlier published reports. The context of translation initiation codon i.e. ATG appeared to be identical between the two classes i.e. TGCAAA<u>ATG</u>GCA, which was close to the plant consensus, TAAACA<u>ATG</u>GCT (Joshi 1987). The available partial coding regions of *St*PK01 and λpat21 were found to be identical but two mismatches could be detected in *St*PN02 as mentioned earlier. Comparison between *St*PN02 and the class II member revealed a few more mismatches that led to amino acid substitutions (mostly non-conservative) at five places i.e. S6Y, L8T, V12F, I14M, and C22F. Such type of changes between the members of class I and class II might have some functional significance in terms of vacuolar targeting and subsequent proteolytic cleavage.

```
          TTGAC  GAGAC                                       CCTTTT                   GAGAC/CCAAT/CANNTG
StPN02  TAATTGACCGGAGACTATACCATATAAGGCCTAATCACAACTTAGTCCCCTTTTTTATTTCTTCTTTATTTAGGAGACCAATTGTTAAAG   90
StPK01  TAATTGACCGGAGACTATACCATATAAGGCCTAATCACAACTTAGTCTTCTTTTTTATTTTTTCTTTATTTAGAAGACCAATTGTTAAAA   90
λpat21  TAATTGACCGGAGACTATACCATATAAGGCCTAATCACAACTTAGTCTTCTTTTTTATTTTTTCTTTATGTAGAAGACCAATTGTTAAAA  147
        ***********************************************..***********.********:***.***************.

                         RTTTTTR             RTTTTTR                                         RTT
StPN02  ATATGAACTTGGTATTATTTTTATGGGTTTATGTGGGGTTTTTATGTTCTTTTGTTCATTTTGCACTTATAATTTTACTGAATTGCAGTT  180
StPK01  ATATAAACTTGGTACTATTTTTATGGTTTTATGTGGGGGTTTTATGTTCTTTTGTTCATTTTGCACTTATAATTTTACTGAATTGCAGTT  180
λpat21  ATATGAACTTGGTACTATTTTTATGGTTTTATGTGGGGTTTTTATGTTCTTTTGTTCATTTTGCACTTATAATTTTACTGAATTGCAGTT  237
        ****•*********.***********:***********•***************************************************
        TTTR
StPN02  TTTACATTATGTTTTAATAGTGAGCAGTTTCATGAATGATGAAGTTTATGTTGCCATATAGAGTAGTTAGTGATGATATACTTCATAAAC  270
StPK01  TTTACATTATGTTTTAATAGTTAGCAGTTTCATGAATGATGAAGTTTATGTTGCCATATAGAGTAGTTTGTGATGATATACTTCATAAAC  270
λpat21  TTTACAATATGTTTTAATAGTTAGCAGTTTCATGAATGATGAAGTTTATGTTGCCATATAGAGTAGTTTGTGATGATATACTTCAAAAAC  327
        ******:**************.**********************************************.****************:****

                                GRWAAW                                                 CANNTG GAG
StPN02  TTCCACTTATGTTAAATTTGTAATGATAAAATTTA----TATTGTAAATTAAAAATTACTTATAAAACTAGGCATTACAACATATGAGAG  356
StPK01  TTTCACTTATGTTAAATTTGTAATGATAAAATTTAGTTATATTGTAAATCAAAAATTACTTATAAAATTGGGCATTACAACATATGAAAG  360
λpat21  TTTCACTTATGTTAAATTTGTAATGATAAAATTTAATTATATTGTAAATCAAAAATTACTTATAAAATTGGGCATTACAACATATGAAAG  417
        **.********************************  ...***********.*****************.*.*****************.**

        AC                        TTGAC CCAAT       CCAAT
StPN02  ACAAATTGTGTTACATATTTTACTTTTGACTCCAATATGAATATCTCAATTTAAATCTTTGTTTTATTTTCTCTTTGTTTTCACAGGTAT  446
StPK01  ACAAATTGTGTTACATATTTTACTTTTGACTCCAATATGAATATCTCGATTTAAATCTTTGTTTTATTTTCTCTTTCTTTTTACAGGTAT  450
λpat21  ACAAATTGTGTTACATATTTTACTTTTGACTCCAATATGAATATCTCAATTTAAATCTTTGTTTTATTTTCTCTTTCTTTTTACAGGTAT  507
        ***********************************************•********************************.********
                                                                                  RTTTTTR
StPN02  AAAAGGTGAGAATTGAAGCAAGATTGATTGCAAGCTATGTGTCACC---------------------GAATTTTTACTTATATGTCTTTG  515
StPK01  AAAAGGTGAGAATTGAAGCAAGATTGATTGCAAGCTATGTGTCACCACATTATTGATACGTTGGAAGGAATTTTTACTTATATGTCTTTG  540
λpat21  AAAAGGTGAGAATTGAAGCAAGAT-GATTGCAAGCTATGTGTCACCACATTATTGATACGTTGGAAGGAATTTTTACTTATATGTTTTTG  596
        ************************:*********************.....................******************:****
                                                                                                 TA
StPN02  TTTAGGAGTAATATCTGATATATTTTAGTTAGATTTTTTTGTCATTGGACATATTTTACTTTTATTTAAGGAGTTTGTAATGAGATATTA  605
StPK01  TGTAGGAGTAATTATTGATATATTTTATTTAGATTTTTTTGTCATTGGACATATTTTACTTTTATTTAAGGAGTTTGTAATGAGATGTTA  630
λpat21  TGTAGGAGTAATTTTTGATATATTTTAGTTAGATTCTTTTGTCATTGGACATATTTTACTTTTATTTAAGGAGTTTGTAATGAGATATTA  686
        *.**********.•.************•*******:*****************************************************•***
        TTCT                 RTTTTTR                                      RTTTTTR
StPN02  TTCTTTAGTATTATTTTAGTTATTTTTATTATATGATCATGGGTGAATTTTGATACAAATATTTTTGTCATTAAATAAATTAATTCATCA  695
StPK01  TTCTTTAGTATAATTTAAGTTATTTTTATTATATGATCATGGGTGAATTTTGATACAAATATTTTTGTCATTAAATAAATTAATTCATCA  720
λpat21  TTCTTTAGTATAATTTAAGTTATTTTTATTATATGATCATGGGTGAATTTTGATACAAATATTTTTGTCATTAAATAAATTAATTCATCA  776
        ***********.****.*************************************************************************
                                                                                               CCAA
StPN02  CAACTTGATTACTTTCAGTGACAAAAAAATGTATTGTCGTAGTACCCTTTATTGTTGAATATGAATAATTT---TTTTTATTTTGTGACAA  782
StPK01  CAACTTGATTACTTTCAGTGACAAAAAAATGTATTATCGTAGTACCCTTTATTGTTAAATATGAATACTTTTTATTTTTATTTTGTGACAA  810
λpat21  CAACTTGATTACTTTGCATGACAAAAAAATGTATTATCGTAGTACCCTTTATTGTTAAATATGAATACTTTTTATTTTTATTTTGTGACAA  866
        ***************:::****************.*********************.*************...******************

        T/CANNTG           GRWAAW         CCAAT           GRWAAW                         GRWAAW
StPN02  TTGTAATTGTCACTACTTATGATAATATTTAGTGACAATATATGTCGTCGGTAAAAGCAACACTTTTAGTGACAAAATGATAAATTTAAT  872
StPK01  TTGTAATTGTCACTACTTATGATAATATTTAGTGACAATA-ATGTCGTCGGTAAAAGCAACACTTTTAGTGACAAAATGATAAATTTAAT  899
λpat21  TTGTAATTGTCACTACTTATGATAATATTTAGTGACAATATATGTCGTCAGTAAAAGCAACACTTTTAGTGACAAAATGATAAATTTAAT  956
        ****************************************•*********:***************************************

                  CCTTTT/CTATAWAWA
StPN02  CACAAAATTATTAACCTTTTTTATAATAATAAATATGTCCCTAATTTATACATTTAAGGACAAAATATTTTTTTGTATATAAAAATAGTC  962
StPK01  CACAAAATTATTAACTTTTTTTATAATAATAAATTTGTCCCTAATTTATACATTTAAGGACAAAATATTTTTTTGTAAATAAAAATAGTC  989
λpat21  CACAAAATTATTAACCTTTTTTATAATAATAAATTTGTCCCTAATTTATACATTTAAGGAAAAAATATTTTTTTGTAAATAAAAACAGTC 1046
        ***************•******************.************************:***************.********:****

                                                                    GAGAC/CCAAT/CANNTG
StPN02  TTTAGTGACGATATTATATTTTTTCAACTACGAAATACATACAACTTTAGAGACAATTGATGTTGTCCCTGATTGAACTAAAAAATTAGC 1052
StPK01  TTTAGTGACGATATTATATCTTTTCAACTACGAAACACATATAACTTTAGAGACAATTGATGTTGTCCCTGATTGAACTAAATAATTAGC 1079
λpat21  TTTAGTGACGATATTATATCTTTTCAACTATGAAA-ACATACA-CTTTAGAGACA-TTGATGTTGTCCCTGATTGAACTAAATAATTAGC 1133
        *******************.***********:****  *****•*:***********:***************************.*******

                                        CCTTTT    GRWAAW
StPN02  GACGATATAGTTTTGTCGG-TAG-TAATAACCTTTTTAGTGATAAAACATACTATTAACTACAAAAAAAG-TTACACTTTTTATGACAAA 1139
StPK01  GACGATATAATTTTGTCGG-TAG-TAATAACCTTTCTAGTGACAAAACATACTATTAACTACAAAAAAAG-TTACACTTTTTATGACAAA 1166
λpat21  GACGATATAGTTTTGTCGGGTAGGTAATAACCTTTTTAGTGACAAAACATACTATTAACTACAAAAAAAGGTTACACTTTTTATGACAA- 1222
        *********•********* *** ***********•******.************************** *****************:

                  CANNTG   CANNTG
StPN02  TAATAAATTCGTCACAAATGTTTATGCATTTGGGGACGATTTTTCTTTTTGTATTTAATA--TATTAGTTTTAGCGACGAAGCACTATAT 1227
StPK01  TAATAAATTCGTCACAAATGTTTATGCATTTGGAGACGATTTTTCTTTTTGTAGTTAATGCTTATTAGTTTTAGCGACGAAGCACTAAAT 1256
λpat21  TAATAAATTCGTCACAAATGTTTATGCATTTGGGGACGATTTTTCTTTTTGTAGTTAATGCGTATTAGTTTTAGCGACGAAGCACTAAAT 1312
        *********************************•*******************.*****.. ***************************.**
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Fig. 6 contd..

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                                                           TTGAC                                  RTTTTTR
StPN02  CGTCTTTGTATATTTGAGTGACACG-GTTTAGTGACGACTGATTGACGAAATTTTTT-CGTCACACAAAATTTTTAGTGACGAAACATG 1315
StPK01  CGTCTTTGTATACTTTGAGTGACACACGTTTAGTGACAACTGATTGACGATATTTTTTTCGTCTCACAAAATTTTTAATGACGAAACACG 1346
λpat21  CGTCTTTGTATACTTTGAGTGACACACGTTTAGTGATGACTGATTGACGAAATTTTTTTCGTCTCACAAAATTTTTAGTGACGAAACATG 1402
        ************.************..*********:•***********•*******.****.*************•**********•*

StPN02  ATTTATAGATGACGAAATTATTTGTCCCTCATAATCTAATTTGTTGTAGTGATCATTACTCCTTTGTTTGTTTTATTTGTCATGTTAGTT 1405
StPK01  ATTTATAGATGACGAAATTATTTGTCCCTCATAATCTAATTTGTTGTAGTGATCATTACTCCTTTGTTTGTTTTATTTGTCATGTTAGTT 1436
λpat21  ATTTATAGATGACGAA-TTATTTGTCCCTCATAATCTAATTTGCTGTAGTGATCATTACTCCTTTGTTTGTTTTATTTGTCATGTTAGTT 1491
        ****************:**************************:**********************************************

            GRWAAW               CCAAT     ACGTG                TAAAG
StPN02  CATTAAAAAGAAAAT--CTCTCTTCTTATCAATTCTGACGTGTTTAATATCATAAGATTAAAGAATATTTAAATATATCTTTAATTTAAA 1493
StPK01  GATCAAAAAAAAAAAAACTCTCTTCTTATCAATTCTGACGTGTTTAATATCATAAGATTAAAAAATATTTTAATATATCTTTAATTTAAA 1526
λpat21  CATTAAAAAAAAAAT--CTCTCTTCTTATCAATTCTGACGTGTTTAATATCATAAGATTAAAAAATATTTTAATATATCTTTAATTTAAA 1579
        •**•*****.****•  *********************************************.***************************
                                                                                           TATTC
StPN02  ACCACAAAGTTCAAATTTCTTCGTTAACTTAATTTGTCAAATCAGGCTCAAAGATCGTTTTTCATATCGGAATGAGGATTTTATTTATTC 1583
StPK01  CCCACAAAGTTTAAATTTCTTAGTTAACTTAATTTGTCAAATCAGGCTCAAAGATCGTTTTTCATATCGGAATGAGGATTTTATTTATTC 1616
λpat21  GCCACAAAATTTAAATTTCTTCGTTAACATAATTTGTCAAATCAGGCTCAAAGATCGTTTTTCATATCGGAATGAGGATTTTATTTATTC 1669
         *******:**.*********•******:*************************************************************

        T        TAAAG          GCTAAACAAT/CCAAT/TSSR2(B-box)/CANNTG            TAAAG
StPN02  TTTTAAAAATAAAGAGGTGGTGAGCTAAACAATTTCAAATCTCATCACACATATGGGGTCAGCCACAAAAATAAAGAACGGTTGGAACGG 1673
StPK01  TTTTAAAAATAAAGAGGTGTTGAGCTAAACAATTTCAAATCTCATCACACTTATGGGGTCAGCCACAAAAATAAAGAACGGTTGGAACGG 1706
λpat21  TTTTAAAAATAAAGAGGTGTTGAGCTAAACAATTTCAAATCTCATCACACATATGGGGTCAGCCACAAAAATAAAGAACGGTTGGAACGG 1759
        *******************.******************************•***************************************

            CTATAWAWA/SURE-2/TSSR2(A-box)/SURE-1/ATAGAA
StPN02  ATCTATTATATAATACTAATAAAGAATAGAAAAAGGAAAGTGAGTGAGGTGCGAGGGAGAGAATTTGTTTAATATCAAATTCGG------ 1757
StPK01  ATCTATTATATAATACTAATAAAGAATAGAAAAAGGAAAGTGAGTGAGGTGCGAGGGAGAGAATCTGTTTAATATCAGAGTCGATCATGT 1796
λpat21  ATCTATTATATAATACTAATAAAGAATAGAAAAAGGAAAGTGAGTGAGGTGCGAGGGAGAGAATCTGTTTACTATCAGAGTCGATCATGT 1849
        ****************************************************************.******:*****.*.***......

                                CANNTG        CCAAT   GATAAG                                GRWAAW
StPN02  ------------------CTCTGACTTCAACTGAGTTTAGGCAATTCTGATAAGGCGGGGAAAATCATAGTGCTGAG-TCTAGAAAAAT 1827
StPK01  GTCAGTTTTATCGATATGACTTTGACTTCAACTGAGTTTAAGCAATTCTGATAAGGCGAGAAAAATCACAGTGTTGAA-TCTAGAAAAAT 1885
λpat21  GTCAGTTTTATCGATATGACTCTGATTTCAACTGAGTTTAAGCAATTCTGATAAGGCGAGGAAAATCACAGTGCTGAAATCTAGAAAAAT 1939
        ..................**•***:**************.****************.*•*******.****•***. ***********
                  GRWAAW
StPN02  CTCATGTAGTGTGAGATAAACCTCAACAAGGACGGTGAGTCCATAGA-GGGGTGTATGTGACACCCCAACCTCAACAAAAGAAAACCTCC 1916
StPK01  CTTATACAGTGTGAGATAAATCTCAACAAAAACGTTGAGTCCATAGAAGGGGGTGTATGTGACACCCCAACCTCAACAAAAGAAAACCTCC 1975
λpat21  CTCATA--GTGTGAGATAAGTCTCAACAAAAACGTTGAGTCCATAGAGGGGGGTGTATGTGACACCCCAACCTCAGCAAAAGAAAACCTCC 2027
        **•**. :***********:.********..***.************ *************************:***************

                                                                          GRWAAW
StPN02  tttcaacaaggacattgggtccatagaggggatgtatgtgacatcataacttaagataaaaatgtaagaattattattaattatgtctta 2006

StPN02  cttatggcccaagtttacttgtaacccaagtaataccataaataatatttaataaggaatagatctcgtccgtacattggttacttgatg 2096

         ACGT                                                                    TATTCT  CCAAT
StPN02  gacgtaccggattaagtcataacctgtataaattggtcctccctccacccattagggttaccacatattctctacaataattccatcgct 2186

                      TAAAG                   CCAAT
StPN02  gacggttgtggtaacataaagttagggaaaggaggtagaaaccaatttacgaccaacgcttccgcttctctctgtgatgatgtcttcctc 2276

                                                                                  ACGT          CANNT
StPN02  atcaggtatgtgcccctaacgatctctatgtaagattatcgtgttcaagatcctgatattatgtattaaaacgtatttaatcttacatat 2366

        G               GRWAAW                            CANNTG    GCTAAACAAT/CCAAT            CTAT
StPN02  ggtagattgtttgagcggataatcttcatatacctccCCTCAACAAGGACATTTGCGGTGCTAAACAATTTCAAGTCTCATCACACATAT 2456
StPK01                                1976 CCTCAAGAAGGACATTTGCGGTGCTAAACAATTTCAAGTCTCATCACACATAT 2028
λpat21                                2028 CCTCAAGAAGGACATTTGCGGTGCTAAACAATTTCAAGTCTCATCACACATAT 2080
                                           *****************************************************

        AWAWA/CTATAWAWA/SURE-2/TSSR2(A-box)/SURE-1/ATAGAA      CANNTG    TGHAAARK      G
StPN02  ATATTATATAATACTAATAAAGAATAGAAAAAGGAAAGGTAAACATCACTAATGACAGTTGCGGTGCAAAGTGAGTGAG----------- 2535
StPK01  ATATTATATAATACTAATAAAGAATAGAAAAAGGAAAGGTAAACATCACTAATGACAGTTGCGGTGCAAAGTGAGTGAGATAATAAACAT 2118
λpat21  ATATTATATAATACTAATAAAGAATAGAAAAAGGAAAGGTAAACATCACTAATGACAGTTGCGGTGCAAAGTGAGTGAGATAATAAACAT 2170
        ******************************************************************************...........

          RWAAW                                                                 ACGTG CTATAWAWA
StPN02  --GTAATAAACATCACTAACTTTTATTGGTTATGTCAAACTCAAAATAAAATTTCTCAACTTGTTTACGTGCCTATATATACCATGCTTG 2623
StPK01  CAGTAATAGACATCACTAACTTTTATTGGTTATGTCAAACTCAAAATAAAATTTCTCAACTTGTTTACGTGCCTATATATACCATGCTTG 2208
λpat21  CAGTAATAGACATCACTAACTTTTATTGGTTATGTCAAACTCAAAATAAAATTTCTCAACTTGTTTACGTGCCTATATATACCATGCTTG 2260
LPOT23              258 AACTTTTATTGGTTATGTCAAACTCAAAATAAAATTTCTCAACTAGTTTACGTGACTATATATACCATGCTTG  330
        ..******.********♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦*♦♦♦♦♦♦♦♦♦*♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦
```

Fig. 6 contd..

```
             ▼                          ACTTTG                       CANNTG/TGHAAARK
StPN02   TTATATGCTCAAAGCACC--AACAAAATTTAAAAACACTTTGA---------------------ACATTTGCAAAATGGCAACTACTAAA 2690
StPK01   TTATATGCTCAAAGCACC--AACAAAATTTAAAAACACTTTGA---------------------ACATTTGCAAAATGGCAACTACTAAA 2275
λpat21   TTATATGCTCAAAGCACC--AACAAAATTTAAAAACAATTTGA---------------------ACATTTGCAAAATGGCAACTACTAAA 2327
LPOT23   TTATATGTTCAAAGCACCCAAAAAAAAATTAAATACACTTTGAGATATTAGTTTTTATTAATTATAATCTGCAAAATGGCAACTACTAAA  420
         ♦♦♦♦♦♦♦*♦♦♦♦♦♦♦♦♦♦  ♦♦*♦♦♦♦*♦♦♦♦♦*♦♦♦:♦♦♦♦♦                     **♦♦*♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦

PStPN02                                                                          M  A  T  T  K    05
PStPK01                                                                          M  A  T  T  K    05
Pλpat21                                                                          M  A  T  T  K    05
PLPOT23                                                                          M  A  T  T  K    05

                                                        CANNTG
StPN02   TCTTTTTTAATTTTAATTGTTATGATATTAGCAACTACTAGTTCAACATGTGCTACG 2747
StPK01   TCTTTTTTAATTTTATTTTTTATGATATTAGCAACTACTAGTTCAACATGTGCTACG 2332
λpat21   TCTTTTTTAATTTTATTTTTTATGATATTAGCAACTACTAGTTCAACATGTGCTACG 2384
LPOT23   TATTTTACAATTTTAATTTTTATGATGTTAGCAACTACTAGTTCAACATTTGCT     474
         ♦*♦♦♦♦**♦♦♦♦♦♦♦·♦♦·♦♦♦♦♦♦♦*♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦♦*♦♦♦♦***

                                                          ↓
PStPN02 S  F  L  I  L  I  V  M  I  L  A  T  T  S  S  T  C  A  T     24
PStPK01 S  F  L  I  L  F  F  M  I  L  A  T  T  S  S  T  C  A  T     24
Pλpat21 S  F  L  I  L  F  F  M  I  L  A  T  T  S  S  T  C  A  T     24
PLPOT23 Y  F  T  I  L  I  F  M  M  L  A  T  T  S  S  T  F  A        23
```

Fig. 6 Multiple sequence alignment of the nucleotide and deduced amino acid sequences of four patatin genes (first three refer to class I and the remaining one refers to class II) from the potato cultivars (see the text for GenBank Accession numbers; numbering of the bases of the respective sequences was kept unaltered as they appear in the GenBank database). Single-letter code is used for the amino acids. This alignment is based on ClustalW2 tool. Dashes in the nucleotide sequences indicate gaps arising during alignment. '▼' denotes putative transcription start site (TSS). The downward arrow indicates the cleavage site of the vacuolar transit peptide. The basal promoter elements and the other predicted *cis*-regulatory sequence motifs are overlined in *St*PN02 sequence (most of them were common in other sequences as well); the consensus sequence motifs are also shown at the respective places. In *St*PN02, the major insertion of 487-bp is shown by lower case letters. Two direct repeats of 37-bp in *St*PN02, and a unique 21-bp in the 5′-UTR of LPOT23 are underlined. The symbols ♦, ∗, **:** and · denote the conserved nucleotides between the four, first three, first and second, second and third sequences, respectively; whereas, the conserved amino acids in the partial patatin sequences are highlighted.

G+C content analyses of patatin gene family members: The overall G+C content of the promoter and the transcribed regions of *St*PN02 were found to be 29% and 28%, respectively; but the corresponding values were found to be identical i.e. 27% in case of *St*PK01. For other class I patatin genes, the values were found to be ~28%, and 28-30%, respectively. The isochore plots (i.e. G+C content in windows over a DNA sequence) using EBI tools revealed some interesting sequence features mostly in 5′-flanking regions of the patatin genes as shown in Fig. 7. Unlike *St*PN02, *St*PK01 and λpat21 showed almost similar patterns because of significant sequence divergence in the former (Fig. 7a-c).

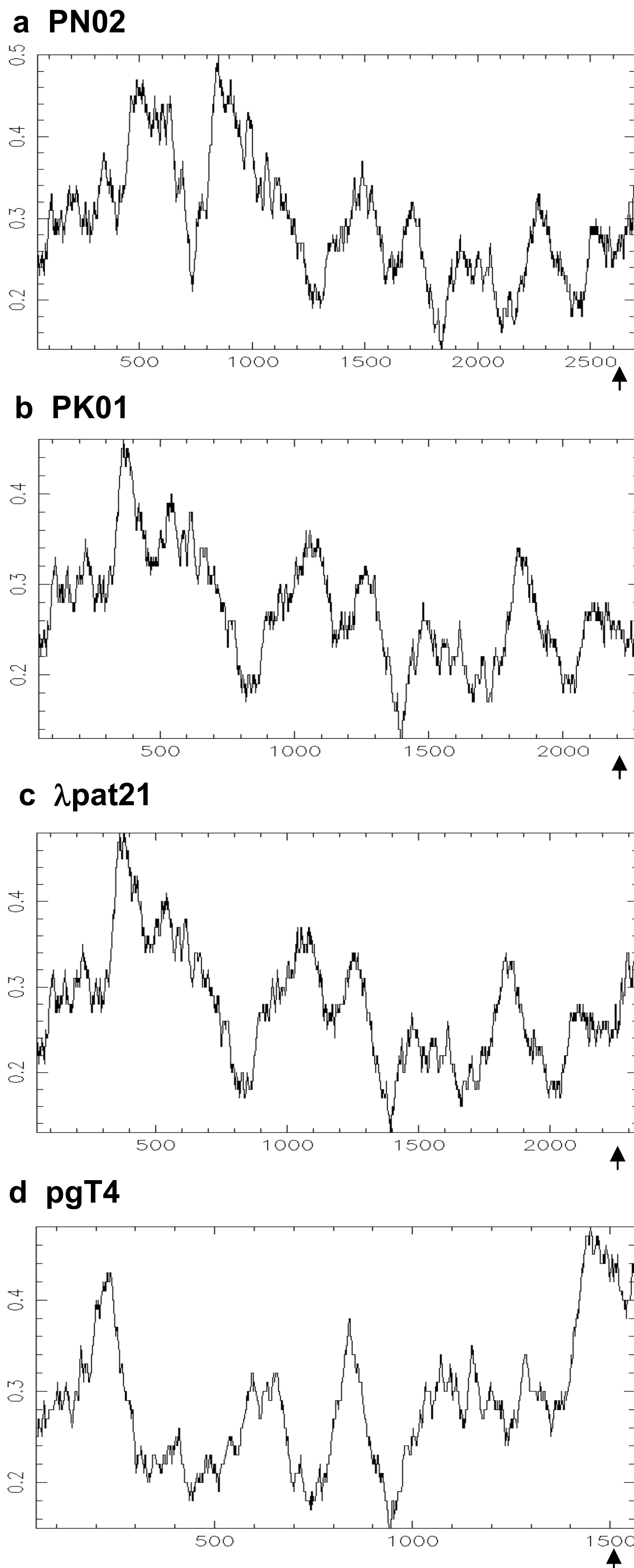

Fig. 7 **G**+C percent analyses of partial class I (**a-c**) and class II (**d**) patatin genes having mostly the promoter regions from potato by isochore plots. The vertical axis in each figure refers to G+C percent, and the horizontal axis indicates base position of the respective DNA sequence. Predicted transcription start site (TSS) in each isochore plot is shown by upward arrow: **a** 2747nts of *St*PN02 (TSS at 2630), **b** 2332 nts of *St*PK01 (TSS at 2215), **c** 2384 nts of λpat21 (TSS at 2267), **d** 1611 nts of pgT4, GenBank Acc No. X60397 (TSS at 1526)

In the class I members, around 900 bp upstream of TSS showed relatively less G+C content with more or less similar isochore plots. The overall variations were more prominent in case of the class II member, pgT4 (Fig. 7d). All these sequence features particularly, in the 5′-flanking regions are likely to influence the overall DNA secondary structures, chromatin organization, complex interplay with various transcription factors, and hence the overall activities of the individual promoters in different cell types/tissues.

5.1.4.3. Phylogenetic relationship of patatin gene family members

A phylogenetic tree was generated based on the 19 patatin gene sequences (corresponding to the 2747-bp *St*PN02) to know the evolutionary relationship between the patatin gene family members from different potato cultivars/clones (Fig. 8).

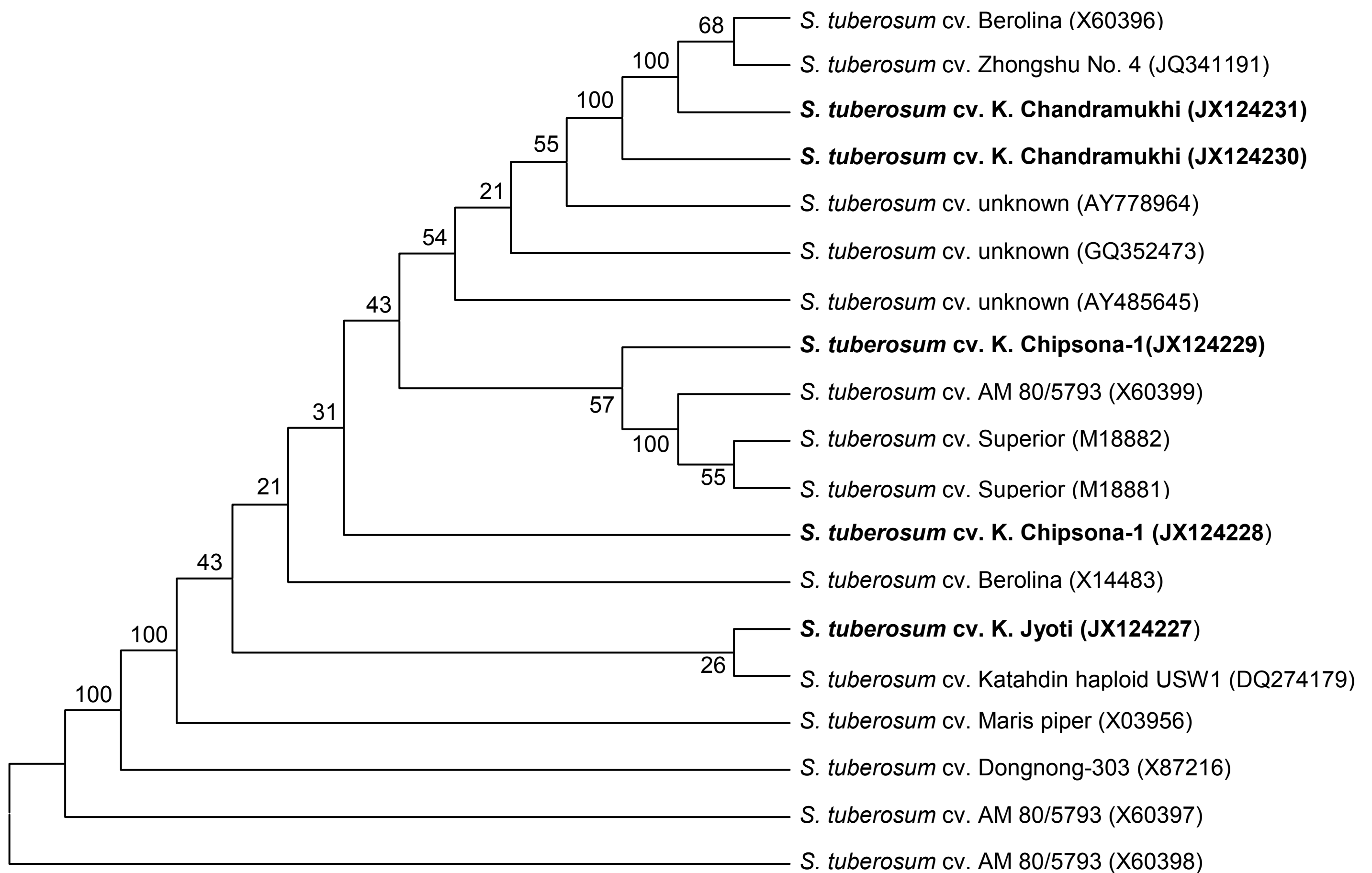

Fig. 8

Fig. 8 A phylogenetic tree is based on the 19 (seventeen from class I and two from class II) partial patatin gene sequences corresponding to different potato cultivars/breeding lines (refer to the figure for the source and GenBank Accession Number for each gene). The sequences from the nucleotide database were selected based on NCBI blast search using *St*PN02 sequence of this study. Multiple sequence alignment was done using *MultAlin* software. The phylogenetic tree was generated based on this alignment using MEGA5 software by the Neighbour-joining method (Tamura et al. 2011). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Five class I patatin genes of this study i.e. *St*PN02 (JX124227) and *St*PK01 (JX1242228), *St*PK03 (JX124228), *St*PM01 (JX124230), and *St*PM02 (JX124231) occupied at different positions in the phylogenetic tree are shown in bold.

For this purpose, multiple sequence alignment was done by the MultAlin software, followed by generation of the phylogenetic tree using MEGA 5.0 software by the Neighbor-Joining method (with bootstrap consensus). Seventeen of them belonged to class I, and the remaining two (GenBank Accessions X60397, X60398) from class II patatin gene family. In terms of branching patterns, the class I and the class II members were readily distinguishable. *St*PM01 and *St*PM02 of this study were found to constitute a nested clade. The remaining class I patatin genes, *St*PN02 *St*PK01 and *St*PK03 occupied distinct positions in a phylogenetic tree.

5.1.5. Concluding remarks

Designing of the oligonucleotide primers and the PCR approaches worked well in obtaining five new partial class I patatin genes having mostly the 5′-flanking regions from some of the Indian potato cultivars: (i) *St*PM01 and *St*PM02 from the potato cultivar Kufri Chandramukhi, (ii) *St*PK01 and *St*PK03 from the potato cultivar Kufri Chipsona-1, and (iii) *St*PN02 from Kufri Jyoti. The sequence information was submitted to the NCBI GenBank database under the Accession numbers JX124227 (*St*PN02), JX124228 (*St*PK01), JX124229 (*St*PK03), JX124230 (*St*PM01), and JX124231 (*St*PM02). Careful sequence analysis by NCBI Blast

Tools revealed that none of the class I patatin genes as isolated in the present study were identical to the class I patatin genes reported to date. The potato cultivars under study were found to vary significantly with regard to the PCR-amplified DNA products suggesting clearly the genotypic variations between them. Patatin is encoded by a relatively large multicopy gene family occurred as a cluster in specific chromosome, therefore it is very likely that the overall organization and some sequence featrures of this gene family could vary between these cultivars as evident from the PCR data of this study. Employing different combination of the gene-specific primers, similar molecular approaches could be further adopted in isolating more members of this gene family by exploring the genetic resources of different potato cultivars/clones. With regard to gene isolation, the strategies as adopted in this study were relatively less arduous as compared to the conventional procedures.

Multiple sequence alignments explicitly showed the extent and locations of the significant similarities/ divergences within and between the members of the major classes of patatin gene family. These two classes were found to vary significantly with regard to their 5′-UTRs and the upstream 5′-flanking regions. However, considerable sequence divergence could also be noticed if compared between the class I members. For example, the major conspicuous difference between *St*PM01 and *St*PM02 was due to the absence of a 69 bp region in *St*PM02 corresponding to the bases 1042-1110 of *St*PM01. Presence or absence of such distinguishing sequence feature could also be noticed in the 5′-flanking regions of other available class I members. Likewise, the major conspicuous difference between *St*PN02 and *St*PK01 was due to the insertion of a 487 bp region i.e. the bases 1917-2403 (i.e. located 226 bp upstream of the predicted TSS) of *St*PN02 which made it very distinct, since such a major insertion was mostly uncommon in the class I members as mentioned earlier. Moreover, a highly conserved

37-bp direct repeats were found at nucleotide positions 1677 and 2458 of *St*PN02 and also in the corresponding regions of *St*PK01. A number of point mutations could also be seen mostly in their farther upstream regions. The isochore plots (i.e. G+C content in windows over a DNA sequence) using EBI tools revealed some interesting sequence features (similarities/ dissimilarities) mostly in 5′-flanking regions of the patatin genes of this study and the available class I members (such as B33 and λpat21) and the class II member, pgT4. Functional genes are found predominantly in the GC-rich isochore classes. To know evolutionary relation, a phylogenetic tree was generated. Importantly, the patatin genes of this study occupied distinct positions in the phylogenetic tree.

All the sequence features particularly in the 5′-flanking regions of the patatin genes are likely to have impact on the overall functionality of the individual promoters as they can influence the secondary structures of DNA, chromatin structures, and complex interactions with various transcription factors. The next 'Results and Discussion' section deals with the functional characterization of the 5′-flanking regions of the patatin genes through transgenic approaches.

5.2. Second Objective: Functional characterization to assess the efficiency and tuber-specificity of patatin gene promoters

Apart from successful molecular cloning of some class I patatin genes from the Indian potato cultivars, functional characterization of the respective 5′-flanking regions (promoters) of the patatin constituted a considerable part of the present study. The various steps starting from making the Ti-plasmid based genetic constructs to the generation of transgenic potato plants for assessing the promoter activities are categorically described in the following sections.

5.2.1. Patatin:GUS binary vector construction

The respective 5′-flanking regions (upstream of the putative TSS) of the class I patatin genes, were used in making several patatin-GUS binary genetic constructs (Fig. 9b-e) based on the Ti-plasmid vector pBI121. The binary vector pBI121 consists of CaMV 35S-GUS fusion (Chen et al. 2003, Acc No AF485783). The same binary vector, pBI121 was also used as a control for potato transformation (Fig. 9a) to see the constitutive expression of the GUS gene.

The steps in making the class I patatin:GUS binary constructs are given below:

The 1454-bp *St*PM01 and 1441-bp *St*PM02 consisted of 1336 bp and 1323 bp 5′-flanking regions upstream of TSS, respectively. Likewise, the 2332-bp *St*PK01 consisted of 2214 bp 5′-flanking region upstream of TSS. All these 5′-flanking regions were used for functional characterization i.e. assessing the respective promoter activities. However, in the case of the 2747-bp *St*PN02 (having 2629 bp 5′-flanking regions upstream of TSS), only a portion i.e. from 1426 to 2640 bp was first subcloned into pUC19, designated as *St*PN03 which was subsequently used for functional characterization. The following steps were adopted for making a total of four Ti-plasmid based binary genetic constructs: (i) the 1347 bp (1-1347 bp) promoter region having predicted TSS at position 1337 was amplified using the *St*PM01 clone as template, and the primer pair PT-F074 and PT1-RV; (ii) the 1334 bp (1-1334 bp) promoter region having predicted TSS at position 1324 was amplified using the *St*PM02 clone as template, and the other primer pair PT-F1B and PT1-RV; (iii) the 1215 bp region (1426-2640 of *St*PN02) having predicted TSS at position 2630 was amplified using the *St*PN02 clone as template, and the primer pair PT-F610 and PT1-RV; and (iv) the 2225 bp region (1-2225 bp) having predicted TSS at position 2215 was amplified using the *St*PK01 clone as template, and the primer pair PT-F074 and PT1-RV. The respective amplified DNA fragments were

cloned into the *Sma*I-*Bam*HI site of pUC19 vector. The respective inserts were then released as *Ecl*136II-*Bam*HI fragments, and cloned into the upstream of GUS gene in pBI121 binary vector by replacing the constitutive CaMV 35S promoter. Prior to ligation, in order to remove the CaMV35S promoter from pBI121, it was first digested with *Hind*III, treated with Klenow enzyme for filling the 3′-recessed termini, and then digested with *Bam*HI. The resulting binary vectors having *St*PM01 promoter-GUS, *St*PM02 promoter-GUS, *St*PN03 promoter-GUS, *St*PK01 promoter-GUS gene fusions were designated as pPT1-RN01, pPT1-RN02, pPT1-RN03 and pPT1-RN04, respectively.

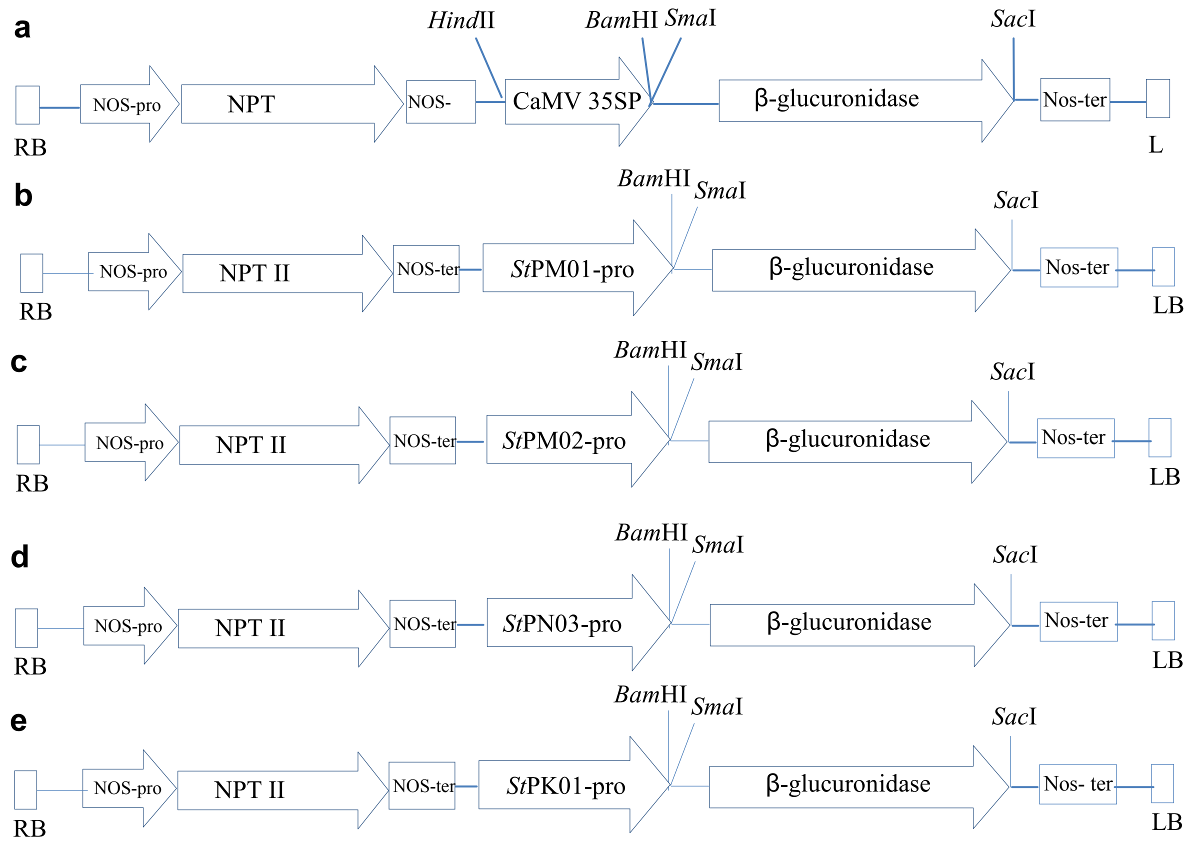

Fig. 9 Schematic diagrams of the T-DNA regions of the binary vectors: **a** pBI121, **b** pPT1-RN01, and **c** pPT1-RN02, **d** pPT1-RN03, and **e** pPT1-RN04 are shown (drawn not to scale). RB right border; LB left border; Nos-pro the *nos* promoter; Nos-ter the *nos* terminator; NPT II neomycin phosphotransferase; CaMV 35SP the

Legend to the Fig. 9 contd…

CaMV 35S promoter; *St*PM01-pro the promoter of *St*PM01 gene; *St*PM02-pro the promoter of *St*PM02 gene; *St*PN03-pro the promoter of *St*PN02 gene; StPK01-pro the promoter of *St*PK01 gene; different restriction sites are also indicated in the diagrams.

The orientation and intactness of the respective class I patatin promoters relative to the GUS in each binary genetic construct were checked by PCR using the promoter and GUS-specific primers (data not shown).

5.2.2. Genetic transformation of *Agrobacterium tumefaciens* LBA4404

Each of the Ti-plasmid based genetic constructs was used to transform *Agrobacterium tumefaciens (*LBA4404) strain by either of the following techniques: electroporation, triparental mating. The *Agrobacterium* transformants were selected on a medium containing rifampcin (15 µg/ml) and kanamycin (50 µg/ml). Ti plasmid DNA was isolated again from the respective *Agrobacterium* transformants for checking the intactness and the orientation of the genetic constructs.

5.2.3. *Agrobacterium*-mediated genetic transformation of potato

The internodal stem segments of the micropropagated potato plantlets of the cultivar Kufri Chipsona-1 (a processing variety) were co-cultivated with the individual *Agrobacterium* transformants harbouring class I patatin-GUS fusions using the modified protocol as reported by Beaujean et al. (1998) with some minor modifications. Initial screening of the putative transgenic potato lines (around 20-25 lines) corresponding to each genetic construct was carried out by the following molecular technique. Total DNA was isolated from each of the independent transgenic lines and intactness of the transferred DNA into potato system was checked by PCR using different combinations of gene-specific primers. A good number of

regenerated plantlets corresponding to each binary genetic construct showed PCR amplification as expected indicating stable integration of the transgene(s) into potato genome. The regeneration of multiple shoots corresponding to pBI121 and class-I patatin-GUS fusions are shown in the Fig.10a-e.

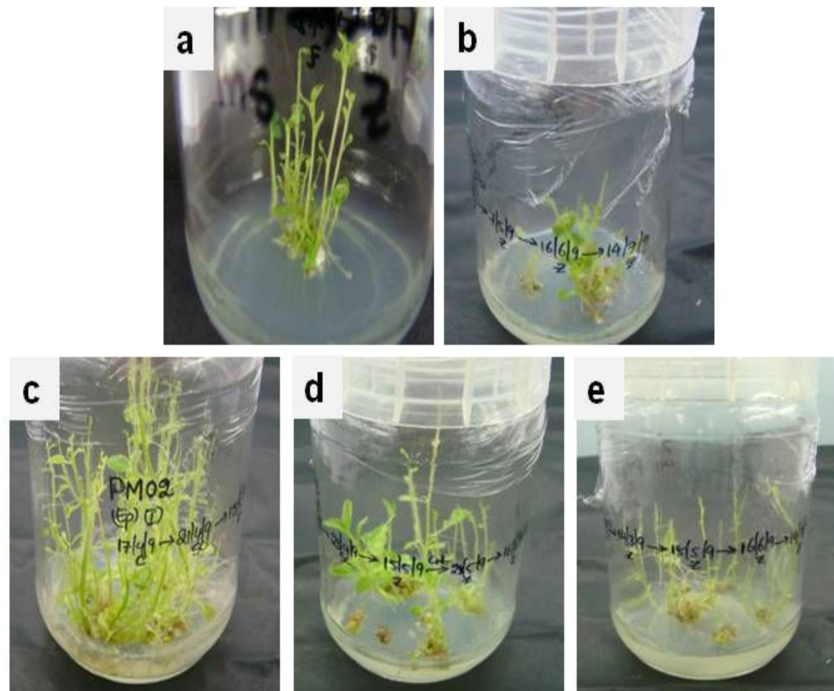

Fig. 10 Multiple shoot regeneration from internodal stem segments of cv. Kufri Chipsona-1 transformed with pBI121 (CaMV 35S-GUS fusion) and different class I patatin-GUS fusions. (a) pBI121, (b) *St*PM01, (c) *St*PM02, (d) *St*PN03, and (e) *St*PK01

5.2.4. Production of minitubers from transgenic potato lines

After proper hardening and acclimatization, the transgenic potato plantlets corresponding to each binary construct were grown in polybags under field conditions for production of minitubers in the restricted experimental plots as shown in Fig. 11. The visual examination of the transgenic plants and the tubers revealed no conspicuous changes with regard to the overall

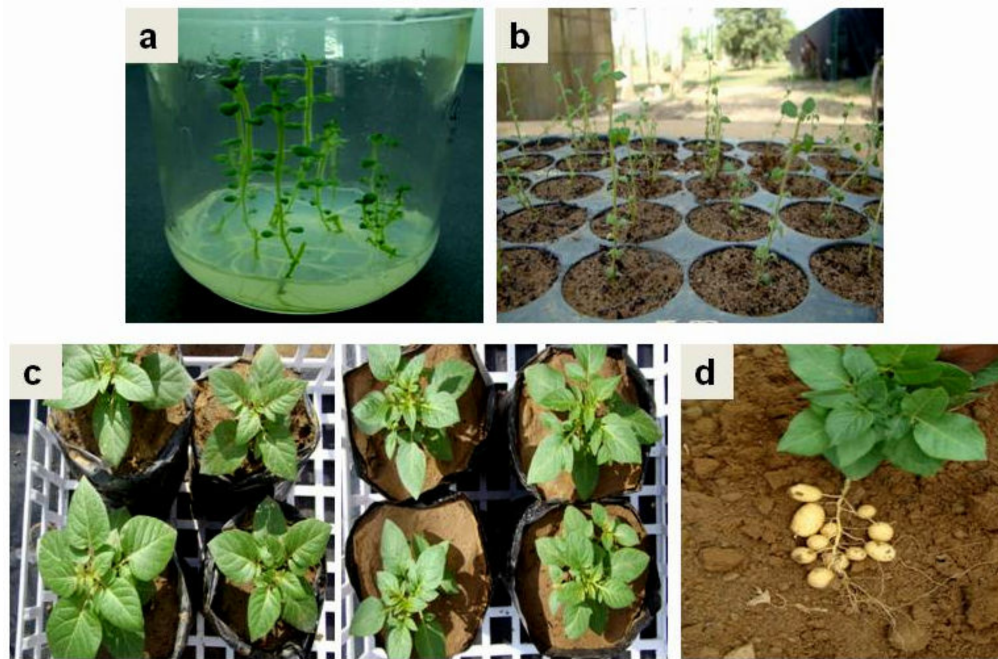

Fig. 11 Hardening and Acclimatization of transgenic potato lines corresponding to a class I patatin-GUS construct (StPM01 promoter-GUS gene) **a** micropropagated transgenic potato plantlets, **b** transgenic potato lines planted in portrays, **c** healthy transgenic potato plants growing in the polypags in a restricted environmental plot, **d** transgenic minitubers attached to the plants during harvest

plant growth and morphology, tuber size, yield and texture as compared to the control (i.e. nontransgenic plants). Potato minitubers were harvested after ~10 weeks.

After harvesting, the minitubers were properly cleaned with tap water and air dried. The presence of transgene(s) and its orientation and intactness were further checked by PCR in the harvested potato minitubers. Here the PCR data corresponding to only one chimeric genetic construct i.e. *St*PK01 promoter-GUS (the respective binary vector is pPT1-RN04) is provided (Fig. 12). For this purpose, genomic DNA was isolated from the tubers corresponding to the six independent transgenic lines. As expected, the size of the PCR product was ~3.1 kb for each transgenic potato line. Likewise, PCR was carried out for the transgenic tubers specific to the other chimeric genetic constructs (data not shown).

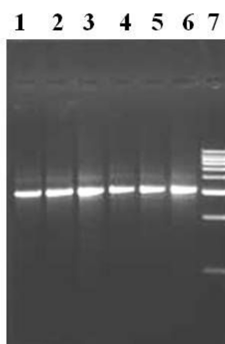

Fig. 12 Checking the presence of *St*PK01 promoter-GUS chimeric genes in the six transgenic potato lines using promoter-specific forward and GUS-specific reverse primers, PT-F074 and US-R6747, respectively. Lanes 1-6, PCR products (~3.1 kb) corresponding to six independent transgenic potato lines (T1-T6); lane 7, 1 kb DNA ladder

5.2.5. Histochemical GUS assay

For GUS histochemical staining, free hand thin sections were made from different explants i.e. tuber, leaf, stem, root and stolon of potato plants corresponding to the control plantlets (non transformed), individual transgenic lines corresponding to pBI121 and different class I patatin-GUS constructs. β-glucuronidase (GUS) assay was carried out using the protocol essentially as described by Jefferson et al. (1987). Histochemical studies clearly indicated that none of the tissues of control non-transformed potato plants showed GUS staining (Fig.13a-e). The constitutive CaMV 35S promoter driven GUS expression was found in all the tissues, but prominent in the medulla and perimedullary regions of tuber, vascular bundles of stem as compared to leaves and roots indicating the constitutive nature of the CaMV 35S promoter (Fig. 13f-e).

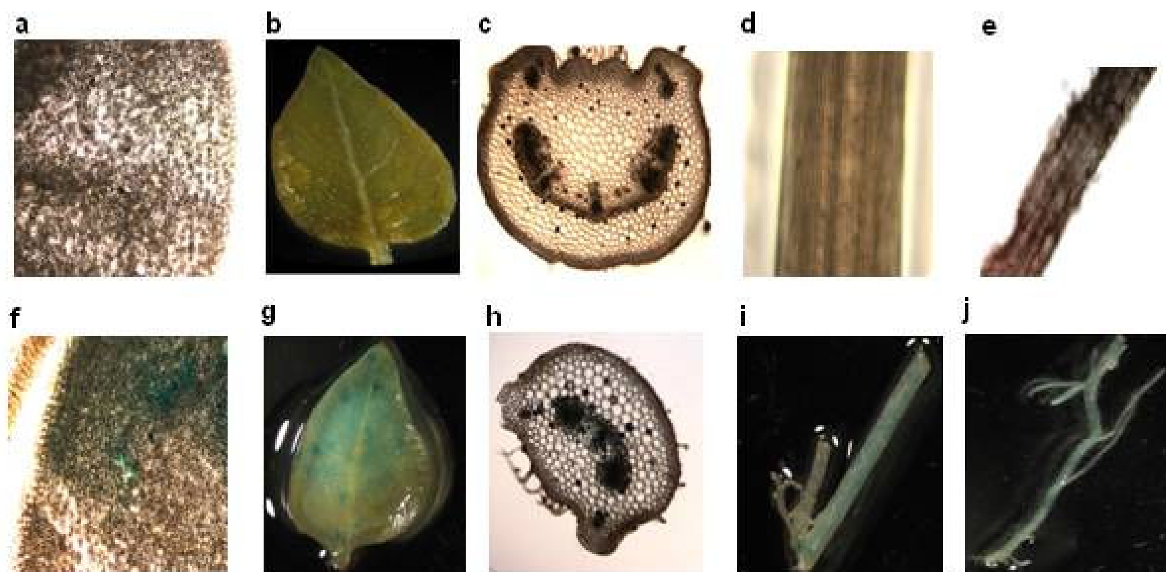

Fig. 13 Histochemical GUS staining patterns in the different tissues of transgenic and non transgenic plants of the potato cultivar Kufri Chipsona-1. In each row, the order of tissues/tissue sections was kept same from left to right i.e. tuber section, leaf, stem section, stolon, and root. **a-e** non transgenic (control) potato plant, **f-j** transgenic potato plant (CaMV 35S promoter-GUS fusion)

In the cases of the different class I patatin promoter-GUS fusions, the GUS expression was more prominent in the parenchymatic cells of the cortical and medullary regions of tubers but not evident in the periderm, and to some extent in tuberizing stolons but was found to be absent or insignificant in leaves and roots, indicating the tuber-specificity of the promoters under study (Fig. 14).

5.2.6. Fluorometric GUS assay

The strength of a promoter is directly correlated to the amount of the GUS enzyme produced in the tissue(s) of a transgenic plant. GUS enzyme was extracted by homogenizing the tissue samples of different explants i.e. tuber, leaf, stem, root and stolon of potato plants corresponding to the control plantlets (non transformed), individual transgenic lines corresponding to the different class I patatin-GUS constructs as generated in this study along with pBI121. GUS activity was determined from the rate of 4-methylumbelliferone (4-MU) produced with 4-methylumbelliferyl glucuronide (4-MUG) as substrate and as per the protocol described by Jefferson et al. (1987). GUS activities of the different class I patatin promoters of

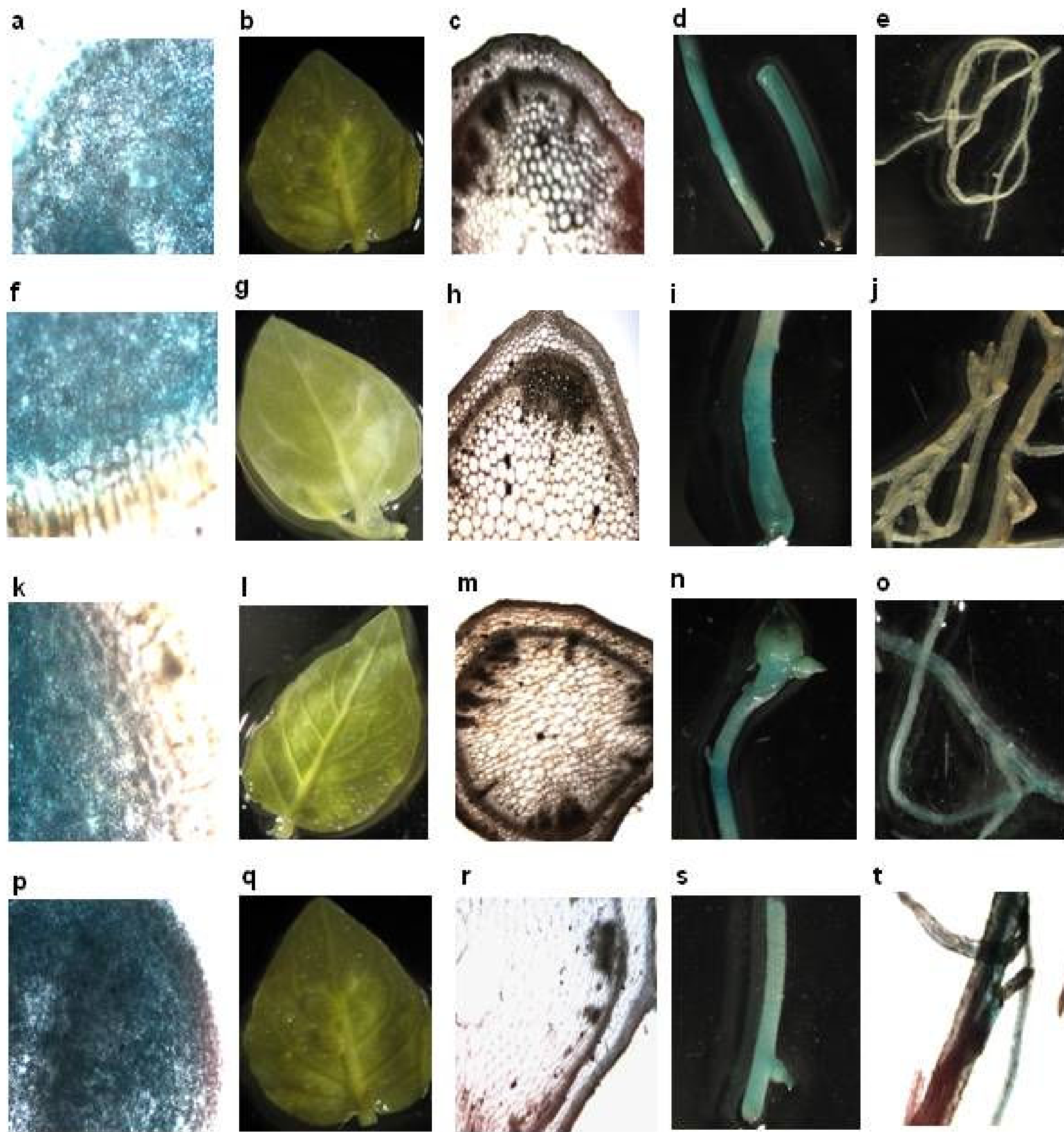

Fig. 14 Histochemical GUS staining patterns in the different tissues of transgenic potato plants harbouring class I-patatin-GUS chimeric genes corresponding to the cultivar Kufri Chipsona-1. In each row, the order of tissues/tissue sections was kept same from left to right i.e. tuber section, leaf, stem section, stolon, and root. **a-e** transgenic potato plant (*St*PM01 promoter-GUS fusion), **f-j** transgenic potato plant (*St*PM02 promoter-GUS fusion), **k-o** transgenic potato plant (*St*PN03 promoter-GUS fusion), **p-t** transgenic potato plant (*St*PK01 promoter-GUS fusion)

the study were found to be maximum in the tubers as compared to the stolon, stem, root and leaves of transgenic potato plants indicating clearly the tuber-specificity of these promoters. As the GUS activities were found to vary considerably between the independent transgenic potato lines corresponding to each binary genetic construct, the GUS activity data were presented in terms of their mean values ±SE (n=10). As shown in Table 1, corresponding to the *St*PM01 promoter-GUS fusion, the GUS activity in the tubers was found to be 7611.37±1393.83 pmol 4-MU/mg protein/min; whereas, the values were 1798.95±128.45, 46.22±10.99, 32.08±9.57, 6.26±1.61 pmol 4-MU/mg protein/min in the cases of stolons, stems, roots, and leaves, respectively.

Table 1 Fluorometric GUS assay in the different tissues of transgenic potato lines corresponding to the chimeric *St*PM01 promoter-GUS genetic construct

Transgenic potato lines	Tuber	Stolon	Stem	Root	Leaf
RN1-1	6287.13	2419.05	135.11	29.22	3.94
RN1-2	3128.93	1276.15	23.76	67.0	2.42
RN1-3	2568.01	1656.89	15.40	5.43	2.01
RN1-4	16785.29	2221.53	68.90	86.13	19.33
RN1-5	4052.39	1598.23	33.80	15.57	2.73
RN1-6	7592.36	1797.52	27.80	35.25	2.94
RN1-7	8658.56	2062.09	38.55	18.62	6.45
RN1-8	12024.68	1969.11	50.23	10.89	10.50
RN1-9	5255.43	1884.67	25.18	45.60	4.67
RN1-10	9761.23	1104.32	43.50	87.10	7.68
Mean	7611.37± 1393.83	1798.95± 128.45	46.22± 10.99	32.08± 9.57	6.26± 1.61
Control	0.97	0.79	0.83	0.65	0.23

GUS activities are expressed in 4-MU pmol/ mg protein/min

Control refers to the potato cultivar.Kufri Chipsona 1 (non-transformed)

Here, the value in the tubers was found to be ~4-fold higher as compared to the stolons; whereas, ~165, ~237, and ~1216-fold higher than in stems, roots, and leaves, respectively. The GUS activity data for the *St*PM02 promoter-GUS fusion was shown in Table 2. In this case, the value in the tubers was found to be 2032.50±437.62 pmol 4-MU/mg protein/min; whereas the

values were 738.85±100.19, 27.00±6.78, 14.00±3.67, 4.27±1.44 pmol 4-MU/mg protein/min in the stolons, stems, roots, and leaves, respectively.

Table 2 Fluorometric GUS assay in the different tissues of transgenic potato lines corresponding to the chimeric *St*PM02 promoter-GUS genetic construct

Transgenic potato lines	Tuber	Stolon	Stem	Root	Leaf
RN2-1	2134.10	916.19	39.59	12.44	3.19
RN2-2	3149.80	928.75	41.16	14.25	4.52
RN2-3	1250.75	797.17	17.23	10.34	2.07
RN2-4	1868.57	856.43	28.00	11.62	2.12
RN2-5	4062.16	1215.72	50.40	26.44	9.37
RN2-6	1050.79	496.89	10.24	7.83	1.75
RN2-7	4368.97	1024.57	66.11	41.70	15.60
RN2-8	957.15	528.76	5.48	4.93	1.55
RN2-9	1131.96	458.52	8.72	6.82	1.82
RN2-10	350.26	165.54	3.68	3.68	0.78
Mean	2032.50± 437.62	738.85± 100.19	27.00± 6.78	14.00± 3.67	4.27± 1.44
Control	0.97	0.79	0.83	0.65	0.23

GUS activities are expressed in 4-MU pmol/ mg protein/min

Control refers to the potato cultivar Kufri Chipsona 1 (non-transformed)

Here, the value in the tubers was found to be ~3-fold higher as compared to the stolons; whereas, ~76, ~147, ~482-fold higher than in stems, roots, and leaves, respectively. As shown in Table 3, corresponding to the *St*PN03 promoter-GUS fusion (i.e. pPT1-RN03) the value in the transgenic tubers was found to be 948.07±179.72 pmol 4-MU/mg protein/min; whereas the values were 459.51±77.95, 17.90±2.99, 9.77±2.16, 3.51±1.04 pmol 4-MU/mg protein/min in the stolons, stems, roots, and leaves, respectively. Here, the value in the tubers was found to be ~2-fold higher as compared to the stolons; whereas, ~57, ~93, ~270-fold higher than in stems, roots, and leaves, respectively. Qualitatively, similar trend of GUS expression was also

noted in case of the *St*PK01 promoter-GUS fusion (i.e. pPT1-RN04). The GUS activity in the transgenic tubers was found to be 6838.76±1364.27 pmol 4-MU/mg protein/min; whereas, the values were 1429.90±162.14, 40.87±10.52, 28.55±6.60, 10.01±2.19 pmol 4-MU/mg protein/min in the cases of stolons, stems, roots, and leaves, respectively as shown in Table 4. Here, the value in the tubers was found to be ~4.5-fold higher as compared to the stolons; whereas, ~167, ~239, and ~683-fold higher than in stems, roots, and leaves, respectively.

Table 3 Fluorometric GUS assay in the different tissues of transgenic potato lines corresponding to the chimeric *St*PN03 promoter-GUS genetic construct

Transgenic potato lines	Tuber	Stolon	Stem	Root	Leaf
RN3-1	1328.50	646.98	25.23	11.59	4.57
RN3-2	253.26	150.76	3.46	4.52	1.32
RN3-3	576.83	325.57	12.81	7.22	1.59
RN3-4	1846.64	900.23	32.57	26.13	10.52
RN3-5	213.77	188.99	8.68	2.19	1.40
RN3-6	1134.38	445.52	22.68	6.82	2.61
RN3-7	775.54	375.19	15.64	8.51	1.56
RN3-8	1586.43	750.24	28.80	15.54	6.82
RN3-9	1279.17	536.47	16.69	9.54	3.67
RN3-10	486.11	275.15	10.97	5.70	1.08
Mean	948.07± 179.72	459.51± 77.95	17.90± 2.99	9.77± 2.16	3.51± 1.04
Control	0.97	0.79	0.83	0.65	0.23

GUS activities are expressed in 4-MU pmol/ mg protein/min

Table 4 Fluorometric GUS assay in the different tissues of transgenic potato lines corresponding to the chimeric *St*PK01 promoter-GUS genetic construct

Transgenic potato lines	Tuber	Stolon	Stem	Root	Leaf
RN4-1	10021.68	1856.68	55.88	43.18	9.96
RN4-2	8925.89	1512.48	44.36	31.07	15.5
RN4-3	4682.16	1375.47	18.82	15.66	7.68
RN4-4	1786.53	728.74	10.07	9.56	2.69
RN4-5	12084.90	2328.52	86.57	55.28	17.33
RN4-6	6587.49	1224.19	27.56	18.86	6.52
RN4-7	2526.47	865.51	15.49	10.49	4.48
RN4-8	13362.73	1958.74	105.78	68.36	23.9
RN4-9	1024.65	950.63	7.94	5.17	1.56
RN4-10	7385.10	1498.11	36.25	27.70	10.56
Mean	6838.76± 1364.27	1429.90± 162.14	40.87± 10.52	28.55± 6.60	10.01± 2.19
Control	0.97	0.79	0.83	0.65	0.23

GUS activities are expressed in 4-MU pmol/ mg protein/min

Control in Tables 3 & 4 refers to the potato cultivar.Kufri Chipsona 1 (non-transformed)

For all the class I patatin gene promoters under study, the GUS expression was found to be maximum in the case of tubers followed by stolons, stem, roots, and leaves. However, these promoters showed significant variations in terms of the overall GUS expression levels. The strengths of the promoters were found to be in the order *St*PM01 > *St*PK01 > *St*PM02 > *St*PN03, respectively. All these observations i.e. with regard to the strong tuber-specificity, difference in the qualitative and quantitative nature of GUS gene expression under different class I patatin gene promoters were quiet consistent with the earlier reports. In fact, the *St*PM01 and *St*PK01 promoters of this study could now be recognized as one of the strong class I patatin gene promoters if compared with the published functional characterization data

corresponding to the 5′-flanking regions of a few other genomic clones of this class, such as B33 (Rocha-Sosa et al. 1989), pgT16 and B24 (Liu et al. 1991), and PS20 (Wenzler et al. 1989). In the case of the CaMV 35S-GUS fusion, the levels of GUS activity in the extracts of the tubers and the other tissues of the independent transgenic potato lines were found to be more or less uniform except in the stem where the level of expression was low. The fluorometric GUS assay data in the different tissues of the control potato plants were found to be insignificant.

Statistical analyses on the promoter activities: Statistical analyses were performed using student t test to show the significant differences in the GUS activities between the individual promoters. Here, the differences in the GUS activities in the tubers are shown in Fig. 15. The significant differences between the promoter activities in different potato tissues were also noted as indicated by their respective 'p' values (Table 5).

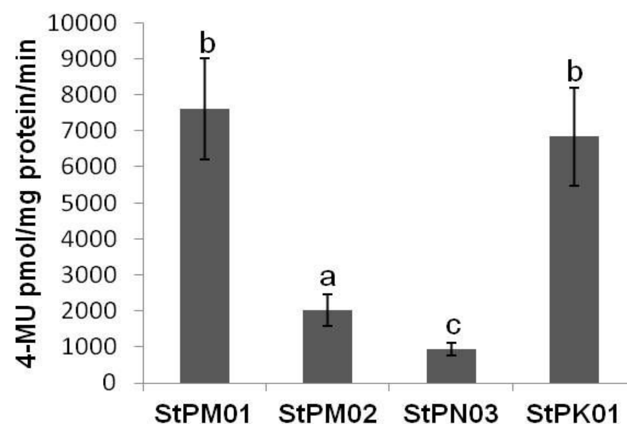

Fig. 15 GUS activities in the tuber extract corresponding to different Class I patatin promoters. Columns represent the mean±SE. $p \leq 0.05$, significantly different from each other and are shown by different small letters. Bars represented with common small letters are nonsignificant. Results are representative of ten independent transformed potato lines.

Table 5 The 'p' values corresponding to the Class I patatin promoter activities in different potato tissues

Class I patatin promoter	Tuber	Stolon	Stem	Root	Leaf
*St*PM01 X *St*PM02	0.0013	0.0001	0.1586*	0.0207	0.3693*
*St*PM01 X *St*PN03	0.0002	0.0001	0.0243	0.0068	0.1371*
*St*PM01 X *St*PK01	0.600*	0.0913*	0.7463*	0.3568*	0.2857*
*St*PM02 X *St*PN03	0.0300	0.0413	0.2406*	0.3608*	0.5823*
*St*PM02 X *St*PK01	0.0030	0.0019	0.2785*	0.0626*	0.0732*
*St*PN03 X *St*PK01	0.0004	0.0001	0.0500	0.0137	0.0238

The p values derived from mean±SE (n=10). The statistical analyses refer to the differences between the promoters. P values i.e. $p \leq 0.05$ represent statistical significant difference. Values with '*' are non significant.

5.2.7. *In vitro* tuberization in potato

Microtubers, an outcome of the microtuberization process are miniature tubers produced under tuber inducing conditions *in vitro*. *In vitro* tuberization process in potato is influenced by various parameters such as nutritional factors, growth regulating substances, temperature, photoperiod, and the genotypes of the cultivars. In most of the studies, BAP was predominantly used for microtuber induction. It is commonly believed that cytokinins had strong promotive effects on tuberization, and constitutes major part of the tuberization stimulus, either alone or in combination with other substances. Moreover, the effects of sucrose, inorganic nitrogen, potassium, triazoles, coumarin, jasmonic acid, organic acids, photoperiod, temperature, and genotype have also been reported in the literature. Microtubers proved to be very useful systems for studying the spatio-temporal nature of tuber-specific/developmentally regulated gene expression, and functional characterization of their promoters. Keeping this in view, with proper media formulation the i*n vitro* tuberization technique was adopted as shown in Fig. 16.

Fig. 16 Various steps of *in vitro* tuberization in potato (here the cultivar was Kufri Chipsona-1)

This technique was routinely employed for studying sucrose responsiveness of the class I patatin gene promoters under study as described in the following section.

5.2.8. Sucrose responsiveness of the chimeric class I patatin promoter-GUS genes

It is known that the expression of patatin gene in potato is subject to a variety of developmental, environmental, and metabolic signals. Rocha-Sosa et al. (1989) and Wenzler et al. (1989) clearly demonstrated high sucrose inducibility of a few class I patatin gene promoters in the axenically grown potato plantlets under varying sucrose concentrations. Keeping this in view, a total of five independent transgenic lines for each chimeric gene of this study were examined under varying sucrose concentrations (such as 3 %, 6 % and 9 % w/v). *In vitro* grown microtubers along with stems and leaves of the potato plantlets were taken for determination of GUS activity. Here only the fluorometric GUS assay data were provided.

The mean GUS activity values of *St*PM01 and *St*PM02 promoter-GUS fusions were shown in Table 6. For *St*PM01 promoter-GUS fusion, at 3% sucrose, the value in the microtubers was found to be ~52 and ~345-fold higher as compared to stems, and leaves, respectively; in case of 6% sucrose, the value in the microtubers was found to be ~2 and ~6-fold higher as compared to stems, and leaves, respectively; whereas at 9% sucrose, the value in the microtubers was found to be only ~1.2 and ~2-fold higher as compared to stems, and leaves, respectively.

Table 6 Induction of class I patatin promoter-GUS chimeric gene expression in different tissues of the transgenic potato plantlets under varying sucrose concentrations

Sucrose Conc. (% w/v)	Microtuber	Stem	Leaf
*St*PM01 pro-GUS			
3	2993.90±29.04	57.51±0.98	8.68±1.68
6	4770.43±43.21	2915.25±6.50	766.32±3.60
9	5626.11±62.37	4757.16±11.13	2648.87±5.40
*St*PM02 pro-GUS			
3	824.58±19.23	34.85±0.48	5.36±0.62
6	1037.15±25.86	1326.35±3.08	315.93±2.40
9	1324.78±45.14	1952.36±7.23	856.67±5.20

GUS activities are mean values ± SE (n=5) expressed in 4-MU pmol/mg protein/min

By increasing the sucrose concentration from 3 to 9% in the tuber induction medium, the level of GUS activity in the tubers was increased by nearly 2-fold; whereas, in the stem and leaf the values were abruptly increased i.e. by ~83 and ~305-fold, respectively. In case of *St*PM02 promoter-GUS fusion, at 3% sucrose, the value in the microtubers was found to be ~24 and ~154-fold higher as compared to stems, and leaves, respectively; in case of 6% sucrose, interestingly the value in the microtubers was found even less than in the stem, but more than 3-fold higher than in leaves; in case of 9% sucrose, the value in the microtubers was found to be ~1.5-fold less and ~1.5-fold higher as compared to stems, and leaves, respectively. By increasing the sucrose concentration from 3 to 9% in the tuber induction medium, the level of GUS activity in the tubers was increased by 1.6-fold; whereas, in the stem and leaf the values were increased by ~56 and ~160-fold, respectively. In case of the *St*PM02 promoter, sucrose

induction in the stem was found to be even more prominent as compared to the tubers at higher sucrose concentrations. As observed under field conditions, it was again substantiated that the *St*PM01 promoter was significantly stronger than the *St*PM02 promoter. Similarly, the mean GUS activity values *St*PN03 and *St*PK01 promoter-GUS fusion were shown in Table 7.

Table 7 Induction of class I patatin promoter-GUS chimeric gene expression in different tissues of the transgenic potato plantlets under varying sucrose concentrations

Sucrose Conc. (% w/v)	Microtuber	Stem	Leaf
*St*PN03 pro-GUS			
3	378.94±16.68	22.61±1.08	3.14±0.86
6	454.72±30.78	786.55±4.32	168.59±3.60
9	530.51±38.52	1028.23±9.68	458.29±6.48
*St*PK01 pro-GUS			
3	2458.39±35.14	48.73±1.62	7.52±0.95
6	3857.46±46.62	2168.25±4.36	640.62±2.69
9	4386.12±58.49	3685.94±9.21	2106.72±7.23

GUS activities are mean values ± SE (n=5) expressed in 4-MU pmol/mg protein/min

In case of *St*PN03 promoter-GUS fusion, the level of GUS activity in the microtuber extracts corresponding to 3 % sucrose was found to be ~17 and ~120-fold higher as compared to stems, and leaves, respectively. In case of 6 % sucrose, the value in the tuber extract was found to be even less than in the stem, but 2.5-fold higher as compared to leaves; whereas at 9 % sucrose, the value in the tuber extract was found to be comparable to stem and slightly (~1.1-fold) higher than leaves, respectively. By increasing the sucrose concentration from 3 to 9 % in the tuber induction medium, the level of GUS activity in the tubers was increased by 1.4-fold;

whereas, in the stem and leaf the values were increased by ~45 and ~146-fold, respectively. In case of the *St*PN03 promoter, sucrose induction was found to be even more prominent in the stems as compared to the tubers at higher sucrose concentrations. In case of *St*PK01 promoter-GUS fusion, the level of GUS activity in the microtuber extracts corresponding to 3 % sucrose was found to be ~50 and ~327-fold higher as compared to stems, and leaves, respectively. In case of 6 % sucrose, the value in the tuber extract was found to be ~1.8 and ~6-fold higher as compared to stems, and leaves, respectively; and at 9 % sucrose, the value in the tuber extract was found to be only ~1.2 and ~2-fold higher as compared to stems, and leaves, respectively. By increasing the sucrose concentration from 3 to 9 % in the tuber induction medium, the level of GUS activity in the tubers was increased by nearly 1.8-fold; whereas, in the stem and leaf the values were increased by ~75 and ~280-fold, respectively. As observed under field conditions, it was again substantiated that the *St*PK01 promoter was significantly stronger than *St*PN03 promoter.

All these data clearly indicated a commonality between the promoters under study i.e. prominent sucrose inducibility in the stem and leaf as compared to tubers. Broadly we can say that at elevated sucrose concentrations, GUS activity levels in the tuber and stem appeared to be almost comparable for the patatin promoters. It seems that there might be some common molecular interplay involved in transcriptional regulation of these chimeric genes both in the tuber and stem at higher sucrose concentration which needs to be understood further. In plants, the most abundant form of transport sugar is sucrose which is most likely exported from the leaf by transferring it to the apoplast, and then by active loading into the phloem. Upon arrival in potato tubers, sucrose is mainly converted to starch. Several enzymes are involved in the biosynthesis of starch, i.e. ADP-glucose pyrophosphorylase, starch synthases and the

branching enzyme. Regulation of starch synthesis is exerted at the level of ADP-glucose pyrophosphorylase. A marked and rapid increase in ADP-glucose pyrophosphorylase (AGP) activity coincides with the initiation and very early development of potato tubers, showing a good correlation between enzyme activity and capacity of starch synthesis. The inhibition of AGPase activity in tubers by antisense AGPase expression resulted in significantly decreased levels of starch, as would be predicted, and to a concomitant increase in sucrose, but also led to a marked decrease in the accumulation of mRNA and protein for patatin (Muller-Rober et al. 1992). One explanation for this observation is that products subsequent to AGPase activity are involved in signaling patatin transcription, or that the flux of metabolites from sucrose to starch is detected and signals patatin transcription. Previous studies have shown that exogenous sucrose, and to a lesser extent fructose and glucose, can activate patatin transcription in excised stem and leaf tissue (Jefferson et al. 1990; Wenzler et al. 1989). In all cases, the accumulation of tuber specific proteins closely correlates with starch formation. It is conceivable that the availability of starch or one of its precursors acts as a signal, not only to initiate the morphological changes typical for tuberization but also for the activation of the 'tuber-specific genes'. Expression in leaves is closely related to whether these organs are currently sink tissues, which depends on carbohydrate imported from other regions of the plant. Removal of potato tubers, for example, results in the accumulation of patatin and starch in above-ground stems and petioles where they do not normally occur (Paiva et al. 1983). In the absence of the normal storage tissue, therefore, other vegetative tissues assume a storage role. In a similar way, elevated levels of the major transport sugar sucrose, results in an increase of the transcripts in leaf and petiole. These results suggest that the accumulation of these proteins is regulated by the immediate need for storage rather than strict developmental control. However,

the detailed signal transduction pathway involved in sucrose inducibility of patatin gene is still obscure.For the CaMV 35S-GUS fusion, the GUS activity levels did not show any significant changes in the potato tissues at varying sucrose concentrations. In the case of non-transformed control potato plants, the respective GUS activity values were found to be insignificant. As evident in the earlier reports, the observed patterns of the sucrose inducibility of different chimeric patatin-GUS fusions were in accordance with the earlier reports. For example, Wenzler et al. (1989) used chimeric class I patatin (2.5 kb of the genomic clone PS20)-GUS construct and observed high level of sucrose-inducible expression in the cultured transgenic potato stem and leaf explant, and concluded that the above promoter was not strictly tuber-specific. Likewise, Rocha-Sosa et al. (1989) also studied the expression levels of B33 promoter-GUS chimeric gene in leaves of the transgenic potato plants kept in MS medium containing high (7 %) or low (2 %) levels of sucrose and assayed the GUS activity after two weeks; and demonstrated that high sucrose concentration had a prominent effect in leaf (spongy and palisade mesophyll cells, epidermal cells) in terms of expression (~100-fold increase) of the chimeric gene. Histochemical studies also showed intense GUS staining with increased sucrose concentrations for all the class I patatin promoters under study. The expression of class I patatin genes is subject to a variety of developmental, environmental, and metabolic signals as evident in the earlier reports. The functional characterization under both field and *in vitro* conditions suggests that the variations as evident in the 5′-flanking regions of *St*PM01, *St*PM02, *St*PN03 and *St*PK01 could probably influence their overall functionality that could be resolved by further in-depth molecular studies.

5.2.9. Concluding remarks

A total of four chimeric class I patatin-GUS binary genetic constructs, designated as pPT1-RN01 (*St*PM01-GUS fusion), pPT1-RN02 (*St*PM02-GUS fusion), pPT1-RN03 (*St*PN03-GUS fusion), pPT1-RN01 (*St*PM01-GUS fusion) were generated using the pBI121 vector. The strength of these promoter activities were assessed under heterozygous and polyploid background of a potato cultivar, Kufri Chipsona-1. Histochemical GUS staining clearly indicated that more or less similar expression patterns could be found for the genes under study. Patatin promoter-driven GUS expression was found to be more prominent in the parenchymatic cells of the cortical and medullary regions (hence they could be recognized as tuber-specific promoters) but not evident in the periderm of the tuber sections, and also prominent in the tuberizing stolons. On the contrary, the GUS expression was found to be absent or insignificant both in leaves and roots. However, very low level of GUS staining could be detected in the vascular bundles of stems. In the case of CaMV 35S promoter, GUS expression could be detected in all the tissues such as medulla and perimedullary regions of the tuber, leaf, vascular bundles of the stem, stolon and root, indicating its constitutive nature of driving gene expression. As expected, none of the tissues of non-transformed potato plants (control) showed GUS staining. To know the level of GUS activities, fluorometric GUS assays were carried out in the different tissues of field-grown transgenic and non-transgenic potato plants such as tuber, stolon, stem, leaf and root. The GUS activity levels for the different promoters were found to be common, and in the following order: tuber > stolon > stem > root > leaf. However, the GUS activity levels corresponding to the individual chimeric

class I patatin promoter-GUS fusions in the different tissues were found to be different. All these data are provided clearly in the earlier sections. In the case of the CaMV 35S-GUS fusion, the levels of GUS activity in the extracts of the tubers and the other tissues of the independent transgenic potato lines were found to be more or less uniform except in the stem where the level of expression was low. The fluorometric GUS assay data in the different tissues of the control potato plants were found to be insignificant. *In vitro* studies under varying sucrose concentrations clearly indicated that all the class I patatin genes under study are highly sucrose inducible particularly in the stems and leaves. In this context, it is to be noted that the overall activity levels of the individual class I promoters were significantly higher under field conditions. It is possible that more complex molecular interplay under field conditions may be stimulatory in enhancing level of transcription under these promoters. As expected, the GUS activity levels did not show any significant changes in the potato tissues at varying sucrose concentrations in the case of CaMV 35S-GUS fusion. This study also helped in comparing relative strengths of the promoters. For example, the *St*PM01 and *St*PK01 promoters were found to be significantly stronger than the *St*PM02 and *St*PN03 promoters under both field and axenic conditions. In fact, the *St*PM01 and *St*PK01 promoters could now be categorized as strong class I patatin gene promoters if compared with the published data for other class I patatin genes.

5.3. Third Objective: To identify putative *cis*-acting elements in patatin gene promoters

Apart from functional characterization of the 5′-flanking (promoter) regions of the class I patatin genes as elaborated under second objective, efforts were also made in examining the sequences carefully for detection of the putative *cis*-regulatory sequence motifs. Since all these sequence motifs are known to play crucial roles in conferring tissue specificity and inducible nature of gene expression. A number of diverse putative *cis*-regulatory motifs mostly in the 5′-flanking regions of the class I patatin genes were predicted in this study. Most of these sequence motifs as found in the class I patatin genes were not documented in the earlier reports. The putative *cis*-regulatory elements of the individual sequences are described in the following sections. These elements can also be clearly seen in the multiple sequence alignments as shown in the Fig. 4 and Fig. 6.

5.3.1. Detection of putative *cis*-regulatory sequence motifs in *St*PM01 and *St*PM02

It is known that *cis*-regulatory sequence motifs (basal promoter elements and enhancers/ silencers) could be present both in the upstream and downstream regions of a gene relative to its TSS, and they may act in either orientation. The entire *St*PM01 sequence was carefully examined for detection of putative *cis*-regulatory sequence motifs. Most of these sequence motifs were identical/nearly identical to the well-characterized *cis*-regulatory sequence motifs reported in the literature. Based on *St*PM01 sequence as a reference, the details of the putative *cis*-regulatory motifs specific to its (+) strand are provided in the Table 8. With the help of sequence alignment as shown in Fig. 4, the common and unique sequence motifs could also

be predicted in *St*PM02, B33, and LPOT23 as well. In case of B33, the common sequence motifs were mostly confined to the ~400 bp region upstream of TSS. The common basal promoter elements, such as TATA-box and ACGT core element were found in the proximal promoter regions of both the classes. Apart from these core promoter elements, a few more *cis*-regulatory sequence motifs could also be detected in the basal promoter regions such as ACGTG (ABRE), AAAAAATTTC (HSE), YACT-element. Even in the downstream of the TSS, a few *cis*-motifs could be found, such as YACT element, LRE, E-boxes, -300 element, GATA-core, ATATT (root motif); most of these motifs were found to be common in both the classes. Around 500 bp region upstream of the core promoter of the class I members contained a number of diverse putative *cis*-regulatory motifs which were known to be associated with plants in relation to regulation of tissue-specific expression, light and sucrose responsiveness, transcription enhancers, abiotic stresses and other attributes. More specifically, some of them were tuber-specific sucrose responsive elements such as TSSR2 (B-box) and TSSR2 (A-box), sucrose response elements such as SURE-1 and SURE-2, Box II, E-boxes, I-box, B-box, CCAAT-boxes, GATA-boxes, CURE element, pollen regulatory elements, LRE, -300 element. A few more TATA-box like sequences could also be detected in this region. Interestingly, some of the *cis*-motifs were found to occur in an overlapping manner. The distinguishing 69 bp of *St*PM01 contained ACGT-core, GC-box like sequence, pollen regulatory elements which were also common in B33. The further available upstream regions of *St*PM01 and *St*PM02 were mostly identical, and found to contain the following *cis*-motifs such as E-boxes, ABRE, ACGT-core, CCAAT-boxes, GATA-boxes, pyrimidine

Table 8 Putative *cis*-regulatory sequence motifs found in the promoter and downstream regions of class I patatin gene (*St*PM01 sequence was used as reference for indicating the positions, also see Fig. 4)

cis-regulatory sequence motif	Consensus	Motifs detected	Motif position	Reference
TATA-box	CTATAWAWA	CTATATATA, TTATATAAT ATATATATT, TTATATAAT	1313, 1156, 1147, 835	Forde et al. 1985; Joshi 1987
CCAAT-box	CCAAT	ACAAT, GCAAT, ACAAT, TCAAT, ACAAT, CCAAT	1125, 961, 768, 588, 170, 77	Forde et al. 1985
GC-box core	GGGCGG	GGGGGT	1056	Okada et al. 2005
ABRE/ACGT element	ACGTG ACGT	ACGTG ACGT	1307, 597 1307, 1040, 597, 408	Nakashima et al. 2006 Simpson et al. 2003
E-box	CANNTG	CAATTG, CAAATG, CATTTG, CATATG, CATGTG, CAACTG, CAGTTG	1444 ,1373, 1206 , 1110, 948, 915, 789, 321, 309, 171	Stalberg et al. 1996
SEF4 binding site	RTTTTTR	ATTTTTA, ATTTTTG,	453, 434, 127, 106, 80	Allen et al. 1989
Sucrose Response Elements (SUREs): SURE-1/SURE-2	AATAGAAAA/ AATACTAAT	AATAGAAAA/ AATACTAAT	1173, 854 841, 1160	Grierson et al. 1994
TSSR2 (B-box)	ATTTAATCTCT-ACTAAAA	TTTCAAATCTCATCTCACA	772	Butler & Hannapel 2012
TSSR2 (A-box)	AATTCAAGTTA-GAATTGAAAAA	AATACTAATAAAGAATAG-AAAAA	841	
B-BOX SITE	GCTAAACAAT	GCTAAACAAT	1120, 763	Zourelidou et al. 2002
Pyrimidine box	CCTTTT	CCTTTT	236, 48	Morita et al. 1998
Box II	ATAGAA	ATAGAA	1174, 855	Kapoor & Sugiura 1999
LRP element	TATTCT	TATTCT	735	Thum et al. 2001
LRE (T box)	ACTTTG	ACTTTG	1365, 938, 394	Chan et al. 2001
-300 element	TGHAAARK	TGCAAAGT, TGCAAAAT	1215, 1377	Thomas & Flavell 1990
GATA box	GATA	GATA	1421, 1229, 1023, 969, 932, 211, 89	Lam and Chua 1989
I box	GATAAG	GATAAG	969	Donald & Cashmore 1990
GT-1 binding site/ GT-1 core	GRWAAW	GAAAAA, GGAAAA, GAAAAT, GATAAA, GGTAAA, GATAAT, GGTTAA	347, 858, 979, 980, 1002, 1023, 1177, 1188, 1229	Villain et al. 1996
HSE	AAAAAATTTC	AATAAAATTTC	1285	Pastuglia et al. 1997
CACT	YACT	CACT, TACT	1433, 1391, 1254, 1197, and also at 10 more places	Gowik et al. 2004
CURE	GTAC	GTAC	878, 374, 101	Quinn & Merchant 1995
Pollen regulatory Element	AGAAA	AGAAA	1176, 1087, 1001, 857	Bate and Twell 1998
Root motif	ATATT	ATATT	1422, 1151, 624, 354	Elmayan & Tepfer 1995
W box	TTGAC	TTGAC	941, 426, 3	Eulgem et al. 2000

boxes, LRP elements, YACT elements, SEF4 binding sites, CURE element, root-motif; but the corresponding upstream region of B33 showed significant sequence divergence, except a few common *cis*-motifs. Yanagisawa and Schmidt (1999) reported that Dof proteins were a family of transcription factors associated with diverse promoters of plant-specific genes. AAAG sequence was regarded as the recognition core of Dof proteins. Such sequence motifs could be detected at a number of places both in the (+/-) strands mostly in the 5′ flanking regions of patatin genes. Some of the *cis*-motifs as detected in this study were also found in the (-) strand. Even a few more *cis*-motifs could also be detected (data not shown).

5.3.2. Detection of putative *cis*-regulatory sequence motifs in *St*PN02 and *St*PK01

The *St*PN02 sequence was also carefully examined for detection of the putative *cis*-regulatory sequence motifs. As mentioned previously, in this case also most of these sequence motifs were identical/nearly identical to a number of well-characterized *cis*-regulatory sequence motifs as reported in the literature. The *St*PN02 sequence was used as a reference, and the details of the diverse *cis*-regulatory motifs along with their positions with reference to its (+) strand are provided in the Table 9. Moreover, the sequence alignment as shown in Fig. 6 helped in identifying some common and unique *cis*-sequence motifs present in other patatin genes of both classes such as *St*PK01, λpat21, and LPOT23.

Table 9 Putative *cis*-regulatory sequence motifs found in the promoter and downstream regions of class I patatin gene (*St*PN02 sequence was used as reference for indicating the positions, see Fig. 6)

cis-regulatory sequence motif	Consensus	Motifs detected	Motif position	Reference
TATA-box	CTATAWAWA	CTATATATA, TTATATAAT, ATATATATT, TTATATAAT, TTATAATAAT	2606, 2460, 2453, 1679, 893	Forde et al. 1985; Joshi 1987
CCAAT-box	CCAAT	ACAAT, CCAAT , ACAAT, GCAAT, ACAAT, TCAAT, ACAAT, TCAAT, CCAAT	2431, 2228, 2170, 1780,1612, 1432, 1015, 817, 779, 402, 388, 78	Forde et al. 1985
ABRE/ ACGT element	ACGTG/ ACGT	ACGTG/ ACGT	2600, 1441/ 2600, 2347, 2098, 1441	Nakashima et al. 2006; Simpson et al. 2003
Sucrose Response Elements (SUREs): SURE-1/SURE-2	AATAGAAAA/ AATACTAAT	AATAGAAAA/ AATACTAAT	2479, 1698/ 2466, 1685	Grierson et al. 1994
TSSR2 (B-box)	ATTTAATCTCT-ACTAAAA	TTTCAAATCTCATCTCACA	1616	Butler & Hannapel 2012
TSSR2 (A-box)	AATTCAAGTTA-GAATTGAAAAA	AATACTAATAAAGAATAG-AAAAA	1685	
SURE core	GAGAC	GAGAC	1012, 354, 74, 11	Maruyama-Nakashita et al. 2005
B-box element	GCTAAACAAT	GCTAAACAAT	2426, 1607	Zourelidou et al. 2002
E-box	CANNTG	CATGTG, CATTTG, CAGTTG, CATTTG, CATATG, CAACTG, CATATG, CATTTG, CAAATG, CAATTG	2737, 2666, 2512, 2416, 2362, 1767, 1633, 1166, 1154, 1016, 780, 347, 79	Stalberg et al. 1996
LRP element	TATTCT	TATTCT	2162, 1579, 604	Thum et al. 2001
'-300 element'	TGHAAARK	TGCAAAAT, TGCAAAGT	2670, 2521	Thomas & Flavell 1990
GATA box	GATA	GATA	2714, 2384, 2331, 1971, 1842, 1788, 1091, 1056, 972, 861, 803, 657, 599, 532, 295, 255, 90	Lam & Chua 1989
I box	GATAAG	GATAAG	1788	Donald & Cashmore 1990
GT-1 binding site/ GT-1 core	GRWAAW	GGTAAT, GGTAAA, GAAAAA, GATAAT, GATAAA, GAAAAA, GAAAAT, GGAAAA, GAAAAA, GAAAAT, GATAAA, GGTAAA, GATAAT, GATAAA	2535, 2494, 2483, 2384, 1971, 1842, 1821, 1799, 1798, 1702, 1415, 1091, 861, 832, 803, 295	Villain et al. 1996
Box II	ATAGAA	ATAGAA	2480, 1699	Kapoor & sugiura 1999
Pyrimidine box	CCTTTT	CCTTTT	1081, 887, 49	Morita et al. 1998
Root motif	ATATT	ATATT	2715, 2457, 2332, 2161, 2051, 1468, 1237, 1199, 978, 973, 937, 807, 664, 600, 566, 535, 371, 305	Elmayan &Tepfer 1995
SEF4 binding site	RTTTTTR	ATTTTTA, ATTTTTG, ATTTT-TA, GTTTTTA, ATTTTTA	1296, 666, 627, 495, 178, 128, 107	Allen et al. 1989
TAAAG motif	TAAAG	TAAAG	2474, 2203, 1693, 1655, 1593, 1462, 86	Plesch et al. 2001
T box	ACTTTG	ACTTTG	2658	Chan et al. 2001
W box	TTGAC	TTGAC	1270, 382, 04	Eulgem et al. 2000

The common basal promoter elements (which usually occurred around 100 bp upstream of TSS), such as TATA-box and ACGT core element were found in the proximity of the predicted TSS in both class I and class II members. Interestingly, a few more TATA-box like sequences could also be found in the further upstream regions of the class I members. A few more *cis*-sequence motifs such as ACGTG (ABRE), GRWAAW (GT-1 core element) could also be found in the basal promoter regions. Close inspection of the further upstream regions of the class I patatin genes also revealed a number of diverse putative *cis*-regulatory motifs associated with different plant gene functions in relation to the cell/tissue-specific transcriptional regulation, light and sucrose responsiveness, transcription enhancers, abiotic stresses and other environmental and metabolic signals. Some of these important sequence motifs were tuber-specific sucrose responsive elements such as TSSR2 (B-box) and TSSR2 (A-box), Sucrose response elements such as SURE-1 and SURE-2, Box II, E-boxes, I-box, B-box, CCAAT-boxes, GATA-boxes, CURE element, pollen regulatory elements, LRE, '-300 element', pyrimidine box, SEF4 binding site (enhancers). In some locations, the sequence motifs were found to occur in overlapping manner and/or in tandem. The major 487-bp insertion in *St*PN02 was found to contain ACGT-core, CCAAT-box, LRP element, GT-1 core, E-box, TAAAG motif, and others. In the downstream of the TSS, a few common *cis*-motifs such as E-box, '-300 element', T-box could be found in both classes. Some of these *cis*-motifs could also be detected in the (-) strand. AAAG sequence motif could also be detected at a number of places both in the (+/-) strands mostly in the 5′ flanking regions of the patatin genes. Likewise, a few more *cis*-motifs could also be detected (data not shown).

It is commonly observed that the gene promoters specific to RNA Polymerase II show much more variations in sequence, and have a variety of *cis*-regulatory sequence motifs (basal

promoter elements, enhancers/silencers) that occur both in the upstream and downstream regions of a gene relative to its TSS, and can act in either orientation. These sequence motifs provide the sites for binding the basal transcription factors, positive and/or negative transcription regulators. In fact, RNA Polymerase II-mediated transcription is a complex multistep process that involves opening the chromatin structures (i.e. changes in nucleosomal structure and organization in the local chromatin structures which are in the immediate vicinity of the promoter), transcription initiation, promoter clearance and elongation. All these factors can act directly or indirectly through DNA-protein and/or protein-protein interactions which is evident in the published reports and rapidly expanding database dealing with various structural features of DNA, a large number of diverse *cis*-regulatory sequence motifs and their cognate transcription factors corresponding to many plant genes (Yadav et al. 2005; Sreenivasulu et al. 2007; Khong et al. 2008; Assuncao et al. 2010; Krishnaswamy et al. 2011; Ramegouda et al. 2012). In this context, there was some progress on the expression of patatin genes. Stupar et al. (2006) demonstrated the dramatic increase of patatin gene expression during stolon to tuber transition coincided with an increase of histone H4 lysine acetylation. It is very likely that some of the putative *cis*-regulatory sequence motifs as detected in the patatin gene sequences could be involved in the tuber specificity i.e. under developmental regulation, and also in the transcriptional regulation under varying environmental and metabolic signals. Since all the patatin promoters under study were found to be predominantly tuber-specific. They are highly sucrose inducible. Moreover, the strength of each promoter was found to vary between field and *in vitro* conditions as described previously.

5.3.3. Concluding remarks

Most of steps in transcription of the protein-coding genes involve various *cis*-regulatory sequence motifs and their cognate transcription factors as identified in many plant genes. As a preliminary characterization, a number of diverse putative *cis*-regulatory sequence motifs were predicted in this study. This exercise will be quite useful in designing experiments through genetic manipulation for further in-depth molecular studies on the class I patatin gene promoters under study; which will eventually help in unraveling the molecular mechanisms involved in their transcriptional regulation in different cell/tissue types, and under varying environmental and metabolic signals.

6. Major outcomes of this thesis work

- Molecular cloning strategies through PCR led to the isolation of a total of five partial class I patatin genes having mostly the 5′-flanking (promoter regions) regions from the Indian potato cultivars for the first time.
- A thorough sequence analyses showed significant variations particularly in the upstream of their core promoter regions. The observed sequence variations were mainly due to some major insertions/deletions apart from cumulative point mutations to a lesser extent. None of them were found identical to the class I patatin gene members reported to date.
- A total of four chimeric patatin promoter-GUS genetic constructs were generated.
- Transgenic potato lines were generated using a processing potato cultivar, Kufri Chipsona-1 (having heterozygous and polyploidy background) through *Agrobacterium*-mediated genetic transformation.
- Under field conditions, all the class I patatin promoters under study showed very high level of GUS expression in the tubers as compared to the other tissues of potato such as leaves, root, stem, and stolon as evident from histochemical and fluorometric GUS assays, indicating their high degree of tuber-specificity.
- Under *in vitro* conditions, these class I patatin promoters were found to be highly sucrose inducible particularly in the stems and leaves.
- Some of the class I patatin promoters of this study were found to be very strong if compared with some other promoters of this class reported in the literature. Therefore, such promoters could be useful molecular tools to the researchers for both basic and applied research.
- As a preliminary characterization, a number of diverse putative *cis*-regulatory sequence motifs were predicted. Most of them were not documented in the earlier reports. This exercise will be quite useful for in-depth molecular studies in unraveling the molecular mechanisms involved in the transcriptional regulation of the class I patatin genes.

7. Summary

In order to fulfill the objectives of this thesis work, a number of different experiments were carried out. All the results were summarized and provided below sequentially:

- The genetic resources of some of the Indian potato cultivars namely Kufri Chipsona-1, Kufri Chandramukhi and Kufri Jyoti were explored in the present study for isolation of a few partial class I patatin genes. For this purpose, good quality (A_{260}/A_{280} ~1.8) genomic DNA was isolated from the individual cultivars by a simple and efficient method. The DNA yield was found be in the range of ~70 to 120 µg per gram of the plant materials which was suitable for the molecular biological techniques.
- For isolation of the partial class I patatin genes, PCR approach was employed successfully using the individual potato genomic DNA as template, and class I patatin gene-specific oligonucleotide primers.
- A total of five partial class I patatin genes mostly having the 5′-flanking (promoter) regions were isolated from the potato cultivars. These genes were designated as *St*PM01 and *St*PM02 (source: cv. Kufri Chandramukhi); *St*PK01 and *St*PK03 (source: cv. Kufri Chipsona-1); and *St*PN02 (source: cv. Kufri Jyoti). The sequence information were submitted to the NCBI GenBank database under the Accession Numbers JX124227 (*St*PN02), JX124228 (*St*PK01), JX124229 (*St*PK03), JX124230 (*St*PM01), and JX124231 (*St*PM02), respectively. All these patatin gene sequences with regard to the Indian potato cultivars in particular were reported for the first time.
- Multiple sequence alignment using the class I patatin genes under study and the other available class I and class II patatin members revealed significant sequence divergence between them, particularly in the farther upstream promoter regions. These variations

were mainly due to large insertion/deletions as evident from the presence of a 69-bp insertion in *St*PM01, and a major insertion of 487-bp in *St*PN02 apart from the cumulative point mutations. Such distinct sequence features were found to be mostly uncommon in other class I patatin gene sequences. Moreover, some of these features could be readily used in dividing the class I patatin genes into further subclasses.

- To assess the promoter activities, a total of four chimeric class I patatin-GUS constructs were made using the 5′ flanking regions of *St*PM01, *St*PM02, *St*PN03 (derivative of *St*PN02), *St*PK01 fragments by replacing the CaMV 35S promoter in the binary vector pBI121; and the resulting binary vectors were designated as pPT1-RN01, pPT1-RN02, pPT1-RN03, and pPT1-RN04, respectively.
- *Agrobacterium tumefaciens* strain (LBA4404) was transformed with each of the above vectors by triparental mating procedure.
- Each of the *Agrobacterium* transformants was co-cultivated with the internodal stem segments of the potato cv. Kufri Chipsona-1, followed by the selection of the independent transgenic lines on selective shoot inducing medium. The orientation and the intactness of the transgene (s) were checked through PCR using the respective gene-specific primers. A number of independent transgenic potato lines were generated corresponding to each chimeric genetic construct.
- The strength and the tissue/cell-specificity of the individual chimeric genes were assessed under both field and *in vitro* conditions. Under field conditions, the class I patatin promoter driven GUS expression was found to be predominant in the tuber (medulla and perimedullary regions) as compared to the other tissues such as stolon, stem, root, and leaf as evident from the Histochemical and Fluorometric GUS data,

indicating their high tuber-specificity. In the case of the CaMV 35S promoter, GUS expression could be detected in all the tissues indicating its constitutive nature of driving gene expression. As expected, none of the tissues of non-transformed potato plants (control) showed GUS staining.

- Fluorometric GUS assays further revealed that the GUS expression patterns for all the chimeric class I promoter-GUS genetic fusions were found to be in the order tuber > stolon > stem > root > leaf. The level of GUS expression was found to be very high as compared to the other tissues in potato. For example, in case of the *St*PM01 promoter-GUS fusion, the GUS activity level in the tubers was found to be 7611.37 pmol 4-MU/mg protein/min; whereas, the values were 1798.95, 46.22, 32.08, 6.26 pmol 4-MU/mg protein/min in the cases of stolons, stems, roots, and leaves, respectively. Similar trend was also observed for the other class I patatin promoter-GUS fusions of this study. Based on the Fluorometric GUS data, the class I patatin promoters under study could be arranged in the following order according to their strengths: *St*PM01 > *St*PK01 > *St*PM02 > *St*PN03.
- *In vitro* studies under varying sucrose concentrations (i.e. 3, 6, and 9 % w/v) clearly indicated that all the class I patatin promoters were highly sucrose inducible in both stems and leaves. For example, in the case of *St*PM01 promoter-GUS fusion, by increasing the sucrose concentration from 3 to 9% in the tuber induction medium, the level of GUS activity in the tubers was increased by nearly 2-fold; whereas, in the stems and leaves the values were abruptly increased i.e. by ~83 and ~305-fold, respectively.

- The overall activity of each class I patatin promoter was found to be significantly higher under field conditions. This could be possibly due to more complex molecular interplay prevailing under field conditions. Some of the molecular circuitry may play stimulatory roles in enhancing the level of transcription under these promoters.
- The promoters of *St*PM01 and *St*PK01 were found to be significantly stronger as compared to the *St*PM02 and *St*PN03 promoters. In fact, the *St*PM01 and *St*PK01 promoters could now be recognized as strong class I patatin gene promoters if compared with the published data.
- A number of diverse putative *cis*-regulatory sequence motifs were predicted by careful sequence analyses. Most of them were not documented in the earlier reports. Although this exercise refers to only a preliminary characterization, but it will be quite useful in pursuing further in-depth molecular studies to understand the molecular mechanisms involved in the transcriptional regulation of the class I patatin genes.
- The various molecular strategies as adopted in this study will be useful in exploring the genetic resources of other potato cultivars/clones for isolation of many more patatin genes. Moreover, the class I patatin gene promoters under study would be valuable molecular tools to the researchers for both basic and applied aspects of research.

References

8. References

Agrawal L, Chakraborty S, Jaiswal DK, Gupta S, Datta A, Chakraborty N (2008) Comparative Proteomics of Tuber Induction, Development and Maturation Reveal the Complexity of Tuberization Process in Potato (*Solanum tuberosum* L.). J Proteome Res 7:3803–3817

Aksenova NP, Konstantinova TN, Golyanovskaya SA, Sergeeva LI, Romanov GA (2012) Hormonal Regulation of Tuber Formation in Potato Plants. Russ J Plant Physl 59:451–466

Allen RD, Bernier F, Lessard PA, Beachy RN (1989) Nuclear factors interact with a soybean beta-conglycinin enhancer. Plant Cell 1:623–631

Andersonn M, Melander M, Pojmark P, Larsson H, Bulow L, Hofvander P (2006) Targeted gene suppression by RNA interference: an efficient method for production of high-amylose potato lines. J Biotechnol 123:137–148

Artschwager E (1924) Studies on the potato tuber. J Agric Res 27:800–835

Assunção AGL, Herrero E, Lin YF, Huettel B, Talukdar S, Smaczniak C, Immink RGH, van Eldik M, Fiers M, Schat H, Aarts MGM (2010) The Arabidopsis thaliana transcription factors bZIP19 and bZIP23 regulate the adaptation to zinc deficiency. Proc Natl Acad Sci 107:10296–10301

Axel C, Zannini E, Coffey A, Guo J, Waters DM, Arendt EK (2012) Ecofriendly control of potato late blight causative agent and the potential role of lactic acid bacteria: a review. Appl Microbiol Biotechnol 96:37–48

Bachem CWB, van der Hoeven R, Lucker J, Oomen R, Casarini E, Jacobsen E, Visser RGF (2000) Functional Genomic Analysis of Potato Tuber Life_Cycle. Potato Res 43:297–312

Bansal A, Kumari V, Taneja D, Sayal R, Das N (2012) Molecular cloning and characterization of granule-bound starch synthase I (GBSSI) alleles from potato and sequence analysis for detection of *cis*-regulatory motifs. Plant Cell Tiss Organ Cult 109:247–261

Bailey-Serres J, Rochaix JD, Wassenegger M, Filipowicz W (1999) Plants, their organelles, viruses and transgenes reveal the mechanisms and relevance of post-transcriptional processes. EMBO J 18:5153−5158

Bate N, Twell D (1998) Functional architecture of a late pollen promoter: pollen specific transcription is developmentally regulated by multiple stage-specific and co-dependent activator elements. Plant Mol Biol 37:859–869

Beaujean A, Sangwan RS, Lecardonne A, Sangwan-Norreel BS (1998) *Agrobacterium*-mediated transformation of three economically important potato cultivars using sliced intermodal explants: an efficient protocol of transformation. J Exp Bot 49:1589–1595

Berk AJ (1999) Activation of RNA polymerase II transcription. Curr Opin Cell Biol 11:330−335

Bevan M (1991) Gene activity during tuber formation in potato (*Solanum tuberosum*). In: Grierson D (ed) Developmental regulation of plant gene expression pp. 75–93

Bevan M, Barker R, Goldsbrough A, Jarvis M, Kavanagh T, Iturriaga G (1986) The structure and transcription start site of a major potato tuber protein. Nucleic Acids Res 14:4625–4638

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Bradford MM (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein–Dye Binding. Anal Biochem 72:248–254

Breathnach R, Chambon P (1981) Organization and expression of eucaryotic split genes coding for proteins. Ann Rev Biochem 50:349–383

Buratowski S (2000) Snashots of RNA polymerase II transcription initiation. Curr Opin Cell Biol 12:320–325

Butler NM, Hannapel DJ (2012) Promoter activity of polypyrimidine tract-binding protein genes of potato responds to environmental cues. Planta 236:1747–1755

Cairns AJ (2003) Fructan biosynthesis in transgenic plants. J Exp Bot 54:549–567

Chailakhyan MK (1984) Photoperiodic and Hormonal Regulation of Tuberization in Plants. Moscow: Nauka

Chan CS, Guo L, Shih MC (2001) Promoter analysis of the nuclear gene encoding the chloroplast glyceraldehyde-3-phosphate dehydrogenase B subunit of *Arabidopsis thaliana.* Plant Mol Biol 46:131–141

Chang WC, Lee TY, Huang HD, Huang HY, Pan RL (2008) "PlantPAN: Plant Promoter Analysis Navigator, for identifying combinatorial cis-regulatory elements with distance constraint in plant gene group". BMC Genomics 9:561

Chen PY, Wang CK, Soong SC, To KY (2003) Complete sequence of the binary vector pBI121 and its application in T-DNA insertion from transgenic plants. Mol Breeding 11:287−293

Cleveland TE, Thornburg RW, Ryan CA (1987) Molecular characterization of a wound-inducible inhibitor I gene from potato and the processing of its mRNA and protein. Plant Mol Biol 8:199−207

Coleman WK, Donnelly DJ, Coleman SE (2001) Potato microtubers as research tools: A review. Am J Potato Res 78:47−55

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16:10881–10890

Das Gupta J, Gu H, Schoenberg DR (2001) Position and sequence requirements for poly(A) length regulation by the poly(A) limiting element. RNA 7:1034−1042

Dennis S, Galliard T (1974) Wax ester formation catalysed by isoenzymes of lipolytic acyl hydrolase. Phytochemistry 13:2469−2473

Denyer K, Johnson P, Zeeman S, Smith AM (2001) The control of amylose synthesis. J Plant Physiol 158:479−487

Dobranszki J, Tabori KM, Hudak I (2008) *In Vitro* Tuberization in Hormone-Free Systems on Solidified Medium and Dormancy of Potato Microtubers. Fruit Veg Cereal Sci Biotech 2:82−94

Dodds JH (1988) Tissue culture technology: Practical application of sophisticated methods. Am Potato J 65:167−180

Donald RG, Cashmore AR (1990) Mutation of either G box or I box sequences profoundly affects expression from the Arabidopsis rbcS-1A promoter. EMBO J 9:1717–1726

Donnelly DJ, Coleman WK, Coleman SE (2003) Potato microtuber production and performance: a review. Am J Potato Res 80:103−115

Dry I, Smith AM, Edwards A, Bhattacharyya M, Dunn P, Martin C (1992) Characterization of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs. Plant J 2:193–202

Eckardt NA (2002) Alternative splicing and the control of flowering time. Plant Cell 14:743–747

Elmayan T, Tepfer M (1995) Evaluation in tobacco of the organ specificity and strength of the rolD promoter, domain A of the 35S promoter and the 35S2 promoter. Transgenic Res 4:388–396

Estrada R, Melendez GN, Dodds JH (1986) Induction of *in vitro* tubers in a broad range of potato genotypes. Plant Cell Tiss Org Cult 7:3−10

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci 5:1360–1385

Ewing EE (1995) The Role of Hormones in Potato (*Solanum tuberosum* L.) Tuberization. Plant Hormones. Physiology, Biochemistry and Molecular Biology, Davies PG, Ed., Dordrecht: Kluwer, pp. 698–724

Ewing EE, Struik PC (1992) Tuber formation in potato: induction, initiation, and growth, Horticultural Review, vol. 14, Wiley, New York, pp. 89–198

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evol 39:783–791

Forde B, Heyworth A, Pywell J, Kreis M (1985) Nucleotide sequence of a B1 hordein gene and the identification of possible upstream regulatory elements in endosperm storage protein genes from barley, wheat and maize. Nucleic Acids Res 13:7327–7339

Ganal MW, Bonierbale MW, Roeder MS, Park WD, Tanksley SD (1991) Genetic and physical mapping of the patatin genes in potato and tomato. Mol Gen Genet 225:501−509

Garner N, Blake J (1989) The induction and development of microtubers *in vitro* on media free of growth regulating substances. Ann Bot 63:663−674

Genova AD, Goverse A, Massa AN et al., The Potato Genome Consortium (2011) Genome sequence and analysis of the tuber crop potato. Nature 475:189–195

Gopal J, Chamail A, Sarkar D (2004) *In vitro* production of microtubers for conservation of potato germplasm: effect of genotype, abscisic acid, and sucrose. In Vitro Cell Dev Biol Plant 40:485−490

Gopal J, Minocha JL, Sidhu JS (1997) Comparative performance of potato crops raised from microtubers Induced in the dark versus microtubers induced in light. Potato Res 40:407−412

Gopal J, Minocha L, Dhaliwal HS (1998) Microtuberization in potato *(Solanum tuberosum* L). Plant Cell Rep 17:794−798

Gordon SW, William BR (1993) A modified method for routine *Agrobacterium* mediated transformation of *In vitro* grown potato microtubers. Plant Cell Rep 12:324−327

Gowik U, Burscheidt J, Akyildiz M, Schlue U, Koczor M, Streubel M, Westhoff P (2004) cis-Regulatory elements for mesophyll-specific gene expression in the C4 plant Flaveria trinervia, the promoter of the C4 phosphoenolpyruvate carboxylase gene. Plant Cell 16:1077–1090

Graham JS, Hall G, Pearce G, Ryan CA (1986) Regulation of proteinase inhibitors I and II mRNAs in leaves of wounded tomato plants. Planta 169:399–405

Grierson C, Du JS, de Torres-Zabala M, Beggs K, Smith C (1994) Separate *cis*-sequences and trans factors direct metabolic and developmental regulation of a potato tuber storage protein gene. Plant J 5: 815–826

Gu H, Das Gupta J, Schoenberg DR (1999) The poly(A)-limiting element is a conserved *cis*-acting sequence that regulates poly(A) tail length on nuclear pre-mRNAs. Proc Natl Acad Sci 96:8943−8948

Gutierrez RA, MacIntosh GC, Green PJ (1999) Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. Trends Plant Sci 4:429–438

Han Y, Gasic K, Sun F, Xu M, Korban SS (2007) A gene encoding starch branching enzyme I (SBEI) in apple (*Malus* x *domestica*, Rosaceae) and its phylogenetic relationship to *Sbe* genes from other angiosperms. Mol Phylogenet Evol 43:852–863

Hannapel DJ, Miller JC, Park WD (1985) Regulation of Potato Tuber Protein Accumulation by Gibberellic Acid. Plant Physiol 78:700–703

Hellwege EM, Czapla S, Jahnke A, Willmitzer L, Heyer AG (2000) Transgenic potato (*Solanum tuberosum*) tubers synthesize the full spectrum of inulin molecules naturally occurring in globe artichoke (*Cynara scolymus*) roots. Pro Natl Acad Sci 97:8699–8704

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. Nucleic Acids Res 27:297–300

Holmes DS, Quigley M (1981) A rapid boiling method for the preparation of bacterial plasmids Anal Biochem 114:193–197

http://cpri.ernet.in

http://faostat.fao.org/

http://www.fao.org

Hunt AG, Meeks LR, Forbes KP, Das Gupta J, Mogen BD (2000) Nuclear and chloroplast poly(A) polymerases from plants share a novel biochemical property. Biochem Biophys Res Commun 272:174−181

Hussey G, Stacey NJ (1984) Factor effecting the formation of *In vitro* tubers of potato (*Solanum tuberosum* L.). Ann Bot 53:565−78

Isherwood FA (1973) Starch-sugar interconversion in *Solanum tuberosum*. Phytochemistry 12: 2579−2591

Jefferson R, Goldsbrough A, Bevan M (1990) Transcriptional regulation of a patatin-1 gene in potato. Plant Mol Biol 14:995−1006

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–1907

Jobling S (2004) Improving starch for food and industrial applications. Curr Opin Plant Biol 7:210–218

Johnson R, Ryan CA (1990) Wound inducible potato inhibitor-II genes enhancement of expression by sucrose. Plant Mol Biol 14:527–536

Joshi CP (1987) An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucleic Acids Res 15:6643–6653

Kapoor S, Sugiura M (1999) Identification of two essential sequence elements in the nonconsensus type II PatpB-290 plastid promoter by using plastid transcription extracts from cultured tobacco BY-2 cells. Plant Cell 11:1799–1810

Keil M, Sanchez-Serrano JJ, Willmitzer L (1989) Both wound inducible and tuber-specific expression are mediated by the promoter of a single member of the proteinase inhibitor II gene family. EMBO J 8:1323–1330

Khong NG, Richaud F, Coudert Y, Pati PK, Santi C, Périn C, Breitler J-C, Meynard D, Vinh DN, Guiderdoni E, Gantet P (2008) Modeling rice by transcription factors. Biotechnol Genet Eng 25:381–404

Kim SY, May GD, Park WD (1994) Nuclear protein factors binding to a class I patatin promoter region are tuber-specific and sucrose-inducible. Plant Mol Biol 26:603–615

Kirschner B, Hahn H (1986) Patatin, a soluble protein of the potato *(Solanum tuberosum* L.) tuber is synthesized as a larger precursor. Planta 168:386–389

Kloosterman B, Vorst O, Hall RD, Visser RGF, Bachem CW (2005) Tuber on a chip: differential gene expression during potato tuber development. Plant Biotech J 3:505–519

Kornberg RD (1999) Eukaryotic transcriptional control. Trends Cell Biol 9:M46–M49

Koster-Trpfer M, Frommer WB, Rocha-Sosa M, Rosahl S, Schell J, Willmitzer L(1989) A class II patatin promoter is under developmental control in both transgenic potato and tobacco plants. Mol Gen Genet 219:390–396

Krishnaswamy S, Verma S, Rahman MH, Kav NNV (2011) Functional characterization of four APETALA2-family genes (*RAP2.6, RAP2.6L, DREB19 and DREB19*) in *Arabidopsis*. Plant Mol Biol 75:107–127.

Kumar PA, Mandaokar A, Sreenivasu K, Chakrabarti SK, Sharma SR, Bisaria S, Kaur S, Sharma RP (1998) Insect-resistant transgenic brinjal plants. Mol Breeding 4:33–37

Kumari V, Bansal A, Aminedi R, Taneja D, Das N (2012) Simplified extraction of good quality genomic DNA from a variety of plant materials. Afr J Biotechnol 11:6420–6427

Kwiatkowski S, Martin MW, Brown CR, Sluis CJ (1988) Serial microtuber formation as a long-term conservation method for *in vitro* potato germplasm. Am Potato J 65:369−375

Lam E, Chua NH (1989) ASF-2: A Factor That Binds to the Cauliflower Mosaic Virus 35s Promoter and a Conserved GATA Motif in *Cab* Promoters. Plant Cell 1:1147–1156

Li G, Hall TC, Holme-Davis R (2002) Plant chromatin: development and gene control. BioEssays 24:234–243

Liu F, Jensen CR, Shahanzari A, Anderson MN, Jacobsen S-E (2005) ABA regulated stomatal control and photosynthetic water use efficiency of potato (*Solanum tuberosum* L.) during progressive soil drying. Plant Sci 168:831–836

Liu F, Shahanzari A, Anderson MN, Jacobsen S-E, Jensen CR (2006) Physiological responses of potato (*Solanum tuberosum* L.) to partial root-zone drying: ABA signalling, leaf gas exchange, and water use efficiency. J Exp Bot 57:3727–3735

Liu F, Song R, Zhang X, Shahanzari A, Anderson MN, Plauborg F, Jacobsen S-E, Jensen CR (2008) Measurement and modelling of ABA signalling in potato (*Solanum tuberosum* L.) during partial root-zone drying. Environ Exp Bot 63:385–391

Jensen C, Liu F, Jacobsen S-E, Anderson M, Plauborg F (2009) Sustainable crop production under limited water supply. IOP Conference Series: Earth Environ Sci 6 (No 47)

Liu XJ, Prat S, Willmitzer, Frommer WB (1990) Cis regulatory elements directing tuber-specific and sucrose-inducible expression of a chimeric class I patatin promoter [GUS-gene fusion. Mol Gen Genet 223:401–406

Liu XY (1989) Developmental- and tissue specific expression of chimeric class I and class II patatin genes in transgenic potato plants. Ph.D. thesis, Free University of Berlin

Liu XY, Rocha-Sosa M, Hummel S, Willmitzer L, Frommer WB (1991) Adetailed study of the regulation and evolution of the two classes of patatin genes in *Solanum tuberosum* L. Plant Mol Biol 17:1139–1154

Liu YW, Han CH, Lee MH, Hsu FL, Hou WC (2003) Patatin, the tuber storage protein of potato (*Solanum tuberosum* L.), exhibits antioxidant activity *in vitro*. J Agric Food Chem 51:4389–4393

Mandaokar A, Goyal RK, Shukla A, Bhalla R, Chaurasia A, Reddy VS, Altosaar I, Sharma RP, Kumar PA (2000) Transgenic tomato plants resistant to fruit borer (Helicoverpa armigera Hubner). Crop Prot 19:307–312

Mandel M, Higa A (1970) Calcium-dependent bacteriophage DNA infection. J Mol Biol 53:159–162

Martin T, Hellmann H, Schmidt R, Willmitzer L, Frommer WB (1997) Identification of mutants in metabolically regulated gene expression. Plant J 11:53–62

Maruyama-Nakashita A, Nakamura Y, Watanabe-Takahashi A, Inoue E, Yamaya T, Takahashi H (2005) Identification of a novel cis-acting element conferring sulfur deficiency response in Arabidopsis roots. Plant J 42:305–314

Mignery GA, Pikaard CS, Hannapel DJ, Park WD (1984) Isolation and sequence analysis of cDNAs for the major potato tuber protein, patatin. Nucl Acids Res 12:7987–8000

Mignery GA, Pikaard CS, Park, WD (1988) Molecular characterization of the patatin multigene family of potato. Gene 62:27–44

Morikami A, Matsunaga R, Tanaka Y, Suzuki S, Mano S, Nakamura K (2005) Two cis-regulatory elements are involved in the sucrose-inducible expression of the sporamin gene promoter from sweet potato in transgenic tobacco. Mol Genet Gen 272:690–699

Morita A, Umemura T, Kuroyanagi M, Futsuhara Y, Perata P, Yamaguchi J (1998) Functional dissection of a sugar-repressed K-amylase gene (RAmy1A) promoter in rice embryos. FEBS Lett 423:81–85

Muller-Rober B, Sonnewald U, Willmitzer L (1992) Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. EMBO J. 11:1229–1238

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473−497

Naik PS, Sarkar D (1997) Influence of light-induced greening on storage of potato microtubers. Biol Plant 39:31−34

Naik PS, Sarkar D, Gaur PC (1998) Yield components of potato microtubers: *in vitro* production and field performance. Ann Appl Biol 113:91−99

Nakamura K, Hattori T, Asahi T (1986) Direct immunological identification of full length cDNA clones for plant protein without gene fusion to *E. coli* protein. FEBS Lett 198:16–20

Nakashima K, Fujita Y, Katsura K, Maruyama K, Narusaka Y, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Transcriptional regulation of ABI3- and ABA-responsive genes including RD29B and RD29A in seeds, germinating embryos, and seedlings of Arabidopsis. Plant Mol Biol 60:51–68

Naumkina EM, Bolyakina YP, Romanov GA (2007) Organ-Specificity and Inducibility of Patatin Class I Promoter from Potato in Transgenic Arabidopsis Plants. Russ J Plant Physl 54:350–359

Obembe OO, Popoola JO, Leelavathi S, Reddy SV (2011) Advances in plant molecular farming. Biotechnol Adv 29:210–222

Okada T, Bhalla PL, Singh MB (2005) Transcriptional activity of male gamete-specific histone gcH3 promoter in sperm cells of Lilium longiflorum. Plant Cell Physiol 46:797–802

Paiva E, Lister RM, Park WD (1982) Comparison of the protein in axillary bud tubers and underground stolon tubers in potato. Am Potato J 59:425–433

Paiva EP, Lister RM, Park WD (1983) Induction and accumulation of major tuber proteins of potato in stems and petiole. Plant Physiol 71:161–168

Palmer CE, Smith OE (1969) Cytokinins and tuber initiation in the potato Solanum tuberosum L. Nature 221:279−280

Park WD (1983) Tuber proteins of potato - a new and surprising molecular system. Plant Mol Biol Rep 1:61–66

Park WD, Blackwood C, Mignery GA, Hermodson MA, Lister RM (1983) Analysis of the heterogeneity of the 40000 molecular weight tuber glycoprotein of potatoes by immunological methods and by NH_2-terminal sequence analysis. Plant Physiol 71:156–160

Park WD, Hannapel DJ, Mignery GA, Pikaard CS (1985) Molecular approaches to the study of the major tuber proteins. In: Li PH (ed) Potato Physiology, Academic Press, Orlando, FL, pp. 261–278

Pastuglia M, Roby D, Dumas C, Cock JM (1997) Rapid induction by wounding and bacterial infection of an S gene family receptor like kinase gene in *Brassica oleracea*. Plant Cell 9:49–60

Peterson RL, Barker WG, Howarth MJ (1985) Development and structure of the tubers, in: P.H. Li, (Ed.), Potato Physiology, Academic Press, New York, pp. 123–152

Pikaard CS, Brusca JS, Hannapel DJ, Park WD (1987) The two classes of genes for the major potato tuber protein, patatin, are differentially expressed in tubers and roots. Nucleic Acids Res 15:1979–1994

Pikaard CS, Mignery GA, Ma DP, Stark VJ, Park WD (1986) Sequence of two apparent pseudogenes of the major tuber protein patatin. Nucleic Acids Res 14:5564–5566

Plesch G, Ehrhardt T, Mueller-Roeber B (2001) Involvement of TAAAG elements suggests a role for Dof transcription factors in guard cell-specific gene expression. Plant J 28:455–464

Pollock C, ap Rees T (1975) Activities of enzymes of sugar metabolism in cold-stored tubers of *Solanum tuberosum*. Phytochemistry 14:613–617

Pots AM, Gruppen H, van Diepenbeek R, van der Lee JJ, van Boekel MAJS, Wijngaards G, Voragen AGJ (1999) The effect of storage of whole potatoes of three varieties on the patatin and protease inhibitor content; a study using capillary electrophoresis and MALDI-TOF mass spectrometry. J Sci Food Agric 79:1557–1564

Prasad BRV, Kumar SV, Nandi A, Chattopadhyay S (2012) Functional interconnections of HY1 with MYC2 and HY5 in *Arabidopsis* seedling development. BMC Plant Biol 12:37

Prat S, Frommer WB, Hofgen R, Keil M, Kossmann J (1990) Gene expression during tuber development in potato plants. FEBS Lett 268:334–338

Quinn JM, Merchant S (1995) Two copper-responsive elements associated with the Chlamydomonas Cyc6 gene function as targets for transcriptional activators. Plant Cell 7:623–628

Racusen D (1985) Patatin and esterase in Desiree potato tuber. J Food Ind 9:361–367

Racusen D, Foote M (1980) A major soluble glycoprotein of potato tubers. J Food Biochem 4:43–52

Rajam MV, Chandola N, Saiprasad Goud P, Singh D, Kashyap V, Choudhary ML, Sihachakr D (2007) Thaumatin gene confers resistance to fungal pathogens as well as tolerance to abiotic stresses in transgenic tobacco plants. Biol Plant 51:135–141

Ramanna MS, Hermsen JG (1979) Genome relationships in tuber-bearing solanums. Biol and Taxonomy of the *solanaceae*. Ed. Hawkes JG, Lester RN, Skelding AD. Academic Press, London, 647

Ramegowda V, Senthil-Kumar M, Nataraja K, Reddy MK, Mysore KS, Udayakumar M (2012) Expression of a finger millet transcription factor, *EcNAC1*, in tobacco confers abiotic stress tolerance. *PLoS One* 7:e40397

Richardson DL, Davies HV, Ross HA, Mackay GR (1990) Invertase activity and its relation to hexose accumulation in potato tubers. J Exp Bot 41:95–99

Rocha-Sosa M, Sonnewald U, Frommer WB, Stratmann M, Schell J, Willmitzer L (1989) Both developmental and metabolic signals activate the promoter of a class I patatin gene. EMBO J 8:23–29

Rosahl S, Schmidt R, Schell J, Willmitzer L (1986) Isolation and characterization of a gene from *Solanum tuberosum* encoding patatin, the major storage protein of potato tubers. Mol Gen Genet 203:214–220

Rosell G, De–Bertoldi FG, Tizio R, De–Bertoldi FG (1987) *In vitro* mass tuberization as a contribution to potato micropropagation. Potato Res 30:111−116

Rydel TJ, Williams JM, Krieger E, Moshiri F, Stallings WC, Brown SM, Pershing JC, Purcell JP, Alibhai MF (2003) The crystal structure, mutagenesis, and activity studies reveal that patatin is a lipid acyl hydrolase with a Ser-Asp catalytic dyad. Biochemistry 42:6696–6708

Saeed H, Vodkin LO, Stromvik MV (2008) Promoters of the soybean seed lectin homologues Le2 and Le3 regulate gene expression in vegetative tissues in Arabidopsis. Plant Sci 175:868–876

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sanchez-Serrano JJ, Schmidt R, Schell J, Willmitzer L (1986) Nucleotide sequence of proteinase inhibitor II encoding cDNA of potato *(Solanum tuberosum)* and its mode of expression. Mol Gen Genet 203:15–20

Sarkar D (2008) The signal transduction pathways controlling in planta tuberization in potato: an emerging synthesis. Plant Cell Rep 27:1–8

Sarkar D, Naik PS (1998) Effect of inorganic nitrogen nutrition on cytokinin–induced potato microtuber production *in vitro*. Potato Res 41:211−217

Sarkar D, Pandey SK, Sharma S (2006) Cytokinins antagonize the jasmonates action on the regulation of potato (Solanum tuberosum) tuber formation in vitro. Plant Cell Tiss Organ Cult 87:285–295

Schwall GP, Safford R, Westcott RJ, Tayal A, Shi YC, Gidley MJ, Jobling AS (2000) Production of very high amylase potato starch by inhibition of SBE A and B. Nat Biotechnol 18:551–554

Sharma AK**,** Venkatasalam EP, Singh RK (2011) Micro-tuber production behaviour of some commercially important potato (*Solanum tuberosum*) cultivars. Indian J Agr Sci 81:1008–13

Sharma N, Gruszewski HA, Park SW, Holm DG, Vivanco JM (2004) Purification of an isoform of patatin with antimicrobial activity against *Phytophthora infestans*. Plant Physiol Biochem 42: 647–655

Sharma S, Chanemougasoundharam A, Sarkar D, and Pandey SK (2004) Carboxylic acids affect induction, development and quality of potato (Solanum tuberosum L.) microtubers grown in vitro from single-node explants. Plant Growth Regul 44:219–229

Sharon N, Lis H (1979) Comparative Biochemistry of Plant Glycoproteins. Biochem Soc Transactions 7:783–799

Simpson SD, Nakashima K, Narusaka Y, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Two different novel cis-acting elements of erd1, a clpA homologous Arabidopsis gene function in induction by dehydration stress and dark-induced senescence. Plant J 33:259–270

Sonnewald U, Sturm A, Chrispeels MJ, Willmitzer L (1989) Targeting and glycosylation of patatin the major potato tuber protein in leaves of transgenic tobacco. Planta 179:171–180

Sowokinos JR (1976) Pyrophosphorylases in Solanum tuberosum. L. Changes in ADP glucose and UDP glucose pyrophosphorylase activities associated with starch biosynthesis during tuberization, maturation, and storage of potatoes. Plant Physiol 57:63–68

Spooner DM, McLean K, Ramsay G, Waugh R, Bryal GJ (2005) A single domestication for potato based on multilocus amplified fragment length polymorphism genotyping. Proc Natl Acad Sci 102:14694–14699

Sreenivasulu N, Sopory SK, Kavi Kishor PB (2007) Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene 388:1–13

Stalberg K, Ellerstom M, Ezcurra I, Ablov S, Rask L (1996) Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein in transgenic Brassica napus seeds. Planta 199:515–519

Stiekema WJ, Heidekamp F, Dirkse WG, Beckum JV, Haan PD, Bosch CT, Louwerse JD (1988) Molecular cloning and analysis of four potato tuber mRNAs. Plant Mol Biol 11:255–269

Strickland JA, Orr GL, Walsh TA (1995) Inhibition of *Diabrotica* larval growth by patatin, the lipid acyl hydrolase from potato tubers, Plant Physiol 109:667–674

Stupar RM, Beaubien KA, Jin W, Song J, Lee M, Wu C, Zhang H, Han B, Jiang J (2006) Structural Diversity and Differential Transcription of the Patatin Multicopy Gene Family During Potato Tuber Development. Genetics 172:1263–1275

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5:Molecular evolutionary genetics analysis usingmaximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Thomas MS, Flavell RB (1990) Identification of an Enhancer Element for the Endosperm-Specific Expression of High Molecular Weight Glutenin. Plant Cell 2:1171–1180

Thum KE, Kim M, Christopher DA, Mullet JE (2001) Cryptochrome, Cryptochrome, and Phytochrome A Co-Activate the Chloroplast *psbD* Blue Light–Responsive Promoter. Plant Cell 13:2747–2760

Twell D, Ooms G (1987) The 5' flanking DNA of a patatin gene directs tuber specific expression of a chimaeric gene in potato. Plant Mol Biol 9:365–375

Twell D, Ooms G (1988) Structural diversity of the patatin gene family in potato cv. Desiree. Mol Gen Genet 212:325–336

Vecchio V, Ferraro SG, Pagano MT, Andrenelli L (1994) Effect of saccharose and CCC [(2-chloroethyl)trimethylammonium chloride] on *in vitro* production of microtubers of potato cultivars *(Solanum tuberosum).* Sementi Elette 5:63−68

Villain P, Mache R, Zhou DX (1996) The Mechanism of GT Element-mediated Cell Type-specific Transcriptional Control. The Journal of Biological Chemistry 51:32593–32598

Visser RGF, Bachem CWB, de Boer JM, Bryan GJ et al. (2009) Sequencing the Potato Genome: Outline and First Results to Come from the Elucidation of the Sequence of the World's Third Most Important Food Crop. Am J Pot Res 86:417–429

Visser RGF, Stolte A, Jacobsen E (1991) Expression of a chimaeric granule-bound starch synthase-GUS gene in transgenic potato plants. Plant Mol Biol 17:691–699

Wang JP, Bughrara S (2007) Monitoring of gene expression profiles and identification of candidate genes involved in drought tolerance in Festuca mairei. Mol Genet Genomics 277:571–587

Wang PJ, Hu CY (1982) *In vitro* mass tuberisation and virus-free seed potato production in Taiwan. Am Potato J 59:33−37

Wenzler H, Mignery G, Fisher L, Park W (1989) Sucrose regulated expression of a chimeric potato tuber gene in leaves of transgenic tobacco plants. Plant Mol Biol 13:347–354

Wenzler HC, Mignery GA, Fisher LM, Park WD (1989) Analysis of a chimeric class-I patatin-GUS gene in transgenic potato plants: high-level expression in tubers and sucrose-inducible expression in cultured leaf and stem explants. Plant Mol Biol 12:41–50

Willmitzer L (1988) The use of transgenic plants to study plant gene expression. Trends Genet 4:13–18

Xu X, van Lammeren AAM, Vermeer E, Vreugdenhil D (1998) The role of gibberellin, abscisic acid, and sucrose in the regulation of potato tuber formation in vitro. Plant Physiol 117:575–584

Yadav V, Mallappa C, Gangappa SN, Bhatia S, Chattopadhyay S (2005) A Basic Helix-Loop-Helix Transcription Factor in Arabidopsis, MYC2, Acts as a Repressor of Blue Light–Mediated Photomorphogenic Growth. Plant Cell 17:1953–1966

Yanagisawa S, Schmidt RJ (1999) Diversity and similarity among recognition sequences of Dof transcription factors. Plant J 17:209–214

Yanisch-Perron C, Viera J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp8 and pUC19 vectors. Gene 33:103–119

Yu WC, Joyce PJ, Cameron DC, McCown BH (2000) Sucrose utilization during potato microtuber growth on bioreactors. Plant Cell Rep 19:407−13

Zeeman SC, Kossmann J, Smith AM (2010) Starch: Its Metabolism, Evolution, and Biotechnological Modification in Plants. Annu Rev Plant Biol 61:209–234

Zhang ZJ, Li HZ, Zhou WJ, Takeuchi Y, Yoneyama K (2006a) Effect of 5-aminolevulinic acid on development and salt tolerance of potato (*Solanum tuberosum* L.) microtubers *in vitro*. Plant Growth Regul 49:27–34

Zhang ZJ, Zhou WJ, Li HZ (2005) The role of GA, IAA and BAP in the regulation of *in vitro* shoot growth and microtuberization in potato. Acta Physiol Plant 27:363–369

Zhang ZJ, Zhou WJ, Li HZ, Zhang GQ, Subrahmaniyan K, Yu JQ (2006b) Effect of jasmonic acid on *in vitro* explant growth and microtuberization in potato. Biol Plant 50:453–456

Zhu Q, Song B, Zhang C, Ou Y, Xie C, Liu J (2008) Construction and functional characteristics of tuber-specific and cold-inducible chimeric promoters in potato. Plant Cell Rep 27:47–55

Zourelidou M, de Torres-Zabala M, Smith C, Bevan MW (2002) Storekeeper defines a new class of plant-specific DNA binding proteins and is a putative regulator of patatin expression. Plant J 30: 489–497

Zrenner R, Schuler K, Sonnewald U (1996) Soluble acid invertase determines the hexose-to-sucrose ratio in cold-stored potato tubers. Planta 198:246–252

Status of Publications in the peer-reviewed journals

1. **Raghavendra Aminedi**, Niranjan Das (2013) Microtuberization in the potato (*Solanum tuberosum* L.) cultivars: Effects of varying concentrations of the plant growth regulators. Potato Research, Springer (Provisionally Accepted)

2. **Raghavendra Aminedi**, Gunveen Wadhwa, Niranjan Das, Bonamali Pal (2012) Shape dependent bactericidal activity of TiO2 for the killing of Gram-negative bacteria *Agrobacterium tumefaciens* under UV Torch Irradiation. Environmental Science and Pollution Research 20 (9):6521-6530

3. Vijay Kumari, Anshu Bansal, **Raghavendra Aminedi**, Dhakshi Taneja, Niranjan Das (2012) Simplified extraction of good quality genomic DNA from a variety of plant materials. African Journal of Biotechnology 11 (24): 6420-6427

4. Bonamali Pal, Isha Singh, Kunal Angrish, **Raghavendra Aminedi**, Niranjan Das (2012) Rapid photokilling of Gram-negative *Escherichia coli* bacteria by platinum dispersed titania nanocomposite films. Materials Chemistry and Physics 136: 21-27

5. **Raghavendra Aminedi**, Niranjan Das (2013) Class I patatin genes from potato (*Solanum tuberosum* L.) cultivars: molecular cloning, sequence analyses and assessment of the promoter activities under field and *in vitro* conditions (Under Review)

Full Papers published in the Conference Proceedings

1. **Raghavendra A**, Bansal S, Balain V, Das N (2011) Molecular strategies for isolation and functional characterization of patatin gene promoters from Indian potato cultivars. AChemE2011, Thapar University, Patiala, 130-136.
2. Kumari V, Bansal A, **Raghavendra A**,Taneja D, Das N (2011) Isolation and quality checking of DNA from different plant materials by employing simple methods. AChemE 2011, Thapar University, Patiala, 89-96.

Abstracts published in the Conference Proceedings

1. **Raghavendra A**, Bansal S, Das N (2009) Molecular approaches for identification and characterization of tuber-expressed promoters from Indian potato cultivars. National Symposium on Biotech 2009: Present and Future Perspectives, Punjabi University, Patiala.
2. Das N, Aggarwal A, Kumar R, **Raghavendra A** (2010) Molecular approaches for isolation and characterization of seed-specific gene promoters from different oilseed cultivars of *Brassica.* National Conference on Emerging Trends in Biopharmaceuticals: Relevance to Human Health & 4th Annual Convention of Association of Biotechnology and Pharmacy. Thapar university, Patiala.
3. Das N, **Raghavendra A**, Balain V (2011) Molecular cloning and characterization of patatin gene promoters from the Indian potato cultivars. 98th Indian Science Congress. SRM University, Chennai.
4. **Raghavendra A**, Khandal H, Das N (2011) Effect of sucrose and chlorocholine chloride on *in vitro* production of microtubers in the potato cultivars. 98th Indian Science Congress. SRM University, Chennai.
5. Das N, Kapoor R, Gupta M, **Raghavendra A** (2011) Effect of varying concentrations of cytokinin, N^6-benzyladenine on microtuberization in the potato cultivars. 98th Indian Science Congress. SRM University, Chennai.