Class I patatin genes from Indian potato *(Solanum tuberosum L.)* cultivars: molecular cloning and partial characterization

A Dissertation
Submitted in partial fulfillment of the requirement
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Masters of Science in Biotechnology

Under the guidance of
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Certificate

This is to certify that the thesis entitled **"Class I patatin genes from Indian potato *(Solanum tuberosum L.)* cultivars: molecular cloning and partial characterization"** submitted by Anchal Sharma (Roll no.301101003) in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology, to Thapar University (Deemed University), Patiala, is a record of student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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I

Declaration

I hereby declare that the work which is being presented in this thesis **"Class I patatin genes from Indian potato *(Solanum tuberosum L.)* cultivars: molecular cloning and partial characterization"** submitted by the undersigned in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology, 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, Department of Biotechnology and Environmental Sciences, 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.

Date: July 15, 2013

Place: Patiala **(Anchal Sharma)**

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Place: Patiala Anchal Sharma

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List of Abbreviations

Abbreviations	Name
Amp	Ampicillin
Bp	Base pair
CaMV 35S	Cauliflower mosaic virus 35S
dNTP	2′-deoxynucleoside-5′-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
GUS	Glucuronidase
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
Kb	Kilo base
LA	Luria agar
LB	Luria broth
M	Molar
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
pI	Isoelectric point
Pmoles	Picomoles
SDS	Sodium dodecyl sulphate
STET	Sucrose Tris EDTA Triton X100
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
TSS	Transcription start site
UTR	Untranslated transcribed region
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

Abstract

Promoter is the region of DNA that initiates and regulates transcription of particular gene. Strong promoter permits a high rate of transcription. So to drive the expression of any gene of interest either in a constitutive or cell-type specific manner a strong promoter is required. In potato tubers, patatin is a major soluble and storage protein. Based on sequence features, patatin gene is further classified into two classes: Class I and Class II.

The present study is focused on molecular cloning and characterization of patatin gene promoters from Indian potato cultivars namely Kufri Chipsona-1 (CS-1) and Kufri Jyoti (KJ). Primers were designed based on the class I patatin gene sequence available in GenBank database. Two different forward primers and one reverse primer were used to amplify promoter region of CS-1 and KJ. The PCR amplified DNA products were cloned in pUC19 vector. The partial characterization was done through PCR and restriction digestion. Putative recombinant clones are likely to be class I specific and can be further characterized using sequencing approach. Further characterization is required in order to estimate promoter's efficiency to derive a foreign gene expression and to have knowledge of gene regulation.

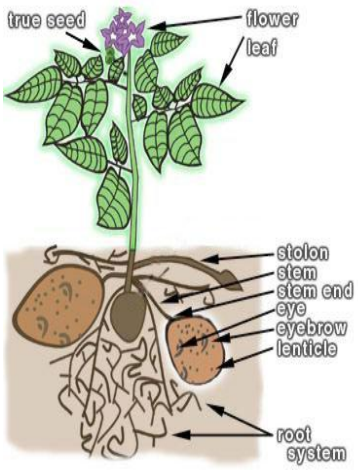
Chapter 1

Introduction

Potato (*Solanum tuberosum* L.) is a tuberous, starchy crop belongs to *Solanaceae* family, an economically important family that includes tomato, pepper, aubergine (eggplant), petunia and tobacco. Since 5000 BCE it has become a staple crop in many countries. Potato was first domesticated in the region of modern-day southern Peru and extreme north western Bolivia. Potatoes were first introduced outside Andes region and then it was shipped to Europe where it remains as an essential crop. From Europe it was introduced to North America and it expands rapidly over the world. China is the world's largest potato-producing country. Today, potato ranks third in terms of total global production after wheat and rice with a worldwide production of 330 million tons in 2009 (http://faostat.fao.org/). It is a cool season crop grown in temperate region (Ewing, 1981). Because of its importance as food, potatoes have been investigated as an agricultural crop. It is a short day plant and a C3 plant with a low light saturation point (Demagante and Zaag, 1988). It is a herbaceous, dicotyledonous plant with alternate stolons underground and alternate leaves on the stem above ground. Root growth is usually at a depth 20-25 cm but in rich soils roots of some varieties of potato may reach upto 90-100 cm. Different species (including *Solanum tuberosum* L., *S. ajanhuiri, S. curtilobum, S. caucha, S. goniocalyx, S. phureja, S. juzepczukii, and S. stenotomum*) are recognized as potato species (Struik and Wiersema, 1999) and over 230 wild potato species are known (Harris, 1992). It is a part of the diet of billion consumers in developing countries by providing roughly half of the world's annual production (Ghislain et al, 1999). This starchy crop is rich in protein, iron, magnesium, potassium and vitamin B and C, fat and fibre. It is also important crop in terms of dry matter production (2.2 t ha^{-1}), energy (216 $MJha^{-1}day^{-1}$) and nutrition (Beukema and Vander Zaag, 1990). India ranks 4^{th} in area and 3^{rd} in the world in production of potato after China and Russian Federation. Potato is produced in an area of 14 lakh ha with a production of 250 lakh tonnes and productivity of 17.86 tonnes per ha.

1.1 Characteristic features and morphology of potato

Potato is a nightshade plant cultivated as an annual and is susceptible to frost and freezing. *Solanum tuberosum* is a hybrid between a diploid species i.e. *S. stentotomum* and *S.sparsipilum* with subsequent chromosome doubling (Ramanna and Hermsen, 1979). It is a tetraploid species. Potatoes are specialized storage tissues which develop by modification of somatic structure plant. The genus *Solanum* has about 2000 members and including tuber bearing species.



Potato plant has compound leaves, bearing white and purplish flowers. It could have three kinds of stems including sprouts (leafy stems), stolons and tubers (Beukema and Zaag, 1979; Struik and Wiersema, 1999). Tuber is swollen underground stem used in commercial propagation. The tubers bear lateral buds (eyes) which grow into a new plant under favourable conditions. Potato tubers are sink organs where starch is synthesized from the sucrose (transported from the source leaves). Potato plant produces a fibrous root system arising from the underground portion of stem. The skin color of potato tuber can be brownish white to deep blue depending upon the cultivar.

1.2 Potato tubers

Tubers are the modified stem enlarged to function as a storage tissue. Tubers expand radially by a process of cell expansion and limited cell division (Cutter, 1982). The bulk cells i.e. cortical and pith cells which are found in mature tubers are modified for the purpose of starch and storage protein accumulation. When tuber expands periderm beneath the epidermis get sloughed off. Tuberization initiates with an enlargement of existing pith cells in the sub-apical regions of the stolon, followed by rapid cell division in parenchyma cells, particularly those associated with the primedulla and inner cortex (Li, 1985). During this growing and filling stage, the tuber is highly metabolically active (Ewing et al, 1992) and two major biochemical changes occur, accumulation of starch and formation of storage proteins (Appeldoorn et al, 1997; Li, 1985).

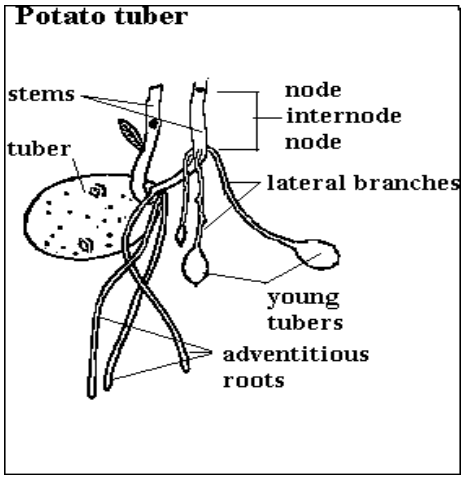
Stolon initiation ⟶ elongation ⟶ subapical swelling ⟶ induction of specific protein

Developmental processes during tuberization

There are two main biological roles of tubers:

- They can store carbon and nitrogen in metabolized form.
- They can act as propagules, which are able to sprout and give rise to new plants. For this process, they need sufficient nutrients to support the requirements of the plant. This property is not present in all type of tubers.

So potato tubers serve as a sink organ where assimilates are accumulated during all stages starting from tuber initiation and formation. Afterwards tuber goes through a filling and a storage phase. So during tuber life cycle, potato undergoes several developmental phases (Ewing and Struik, 1992). Depending on the genotype, dormancy phase lasts for 18-33 weeks (Cutter, 1978).



The process of tuberization can be delayed in the presence of high nitrogen levels (supplied in the form of ammonium or nitrate ions) between the range of 1-3mM. Tuberization can be ceased in high temperatures in both short and long photoperiods. Gibberellic acid (GA) is an inhibitory factor for tuber growth. Ethylene seems to stimulate tuberization and is antagonistic to the inhibitory effects of gibberellic acid (Stallknecht and Farnsworth, 1982).

1.3 Major constituents in the potato tubers

The main reasons for the increasing popularity of the potato in third-world countries are the high nutritional value of the tubers along with the simple vegetative mode of propagation. The potato is a wholesome food with all the important and necessary dietary constituents. Potato contains important mineral elements such as: iron 0.01%, sulphur 0.15%, magnesium about 0.1%, calcium 0.05%, potassium, boron, copper, silicon, manganese, iodine and fluorine (Salaman, 1985). Potato tubers contain 77.5% water and 22.5% solids such as: protein 2.0%, carbohydrates (with 0.6% crude fibre), 19.4%, fat 0.1% and ash 1.0% (Talburt and Smith, 1967). Different nitrogeneous compounds such as free amino acids, storage proteins and nitrate are also present in the potato tuber (Ahmad, 1977).

The accumulation of large amounts of starch and the accumulation of a set of relatively abundant proteins (which are apparently like 'storage proteins') refer to two major biochemical attributes that differentiate tubers from other somatic tissues of potato. **Patatin** is the major glycoprotein that accumulates in tubers. Some inhibitor proteins are also found to accumulate in tubers for example type II proteinase inhibitor (chymotrypsin and trypsin/chymotrypsin), a Bowman-Birk proteinase inhibitor and Kunitz inhibitor (Stiekema et al, 1988). It has been postulated that inhibitor proteins provide defence against pathogens and other predators.

Out of 50% of tuber protein present in potato, 40% of the total soluble protein is represented as 'patatin'. The high amount of patatin in potato tubers argues for its function as a storage protein. Storage proteins, in general, can be defined as proteins whose major role is to act as stores of nitrogen, sulphur and carbon. They may enable the plant to survive periods of adverse conditions, and may provide nutrients to support the growth of new plants as seedlings (from seeds) or shoots (from tubers). They act as sink for nitrogen (and probably also sulphur), accumulating in greater amounts under conditions of excess nutrient supply. As a storage protein, patatin is mainly localized in the plant cell vacuoles.

1.4 Patatin-major soluble protein in the tuber

The major protein that is found in potato tubers was a globulin which was earlier named as 'tuberin' (Osborne and Campbell, 1986). Racusen and Foote (1980) reported that a glycoprotein of Mr about 45000 Da named it as Patatin. Patatin gene is encoded by a multigene family composed of approximately 64-72 copies in the tetraploid cultivar. The molecular mass of patatin monomer ranges between 39 and 43 kDa. It has been reported that all patatin genes are clustered on long arm of chromosome 8 (Wenzler et al, 1989 a).

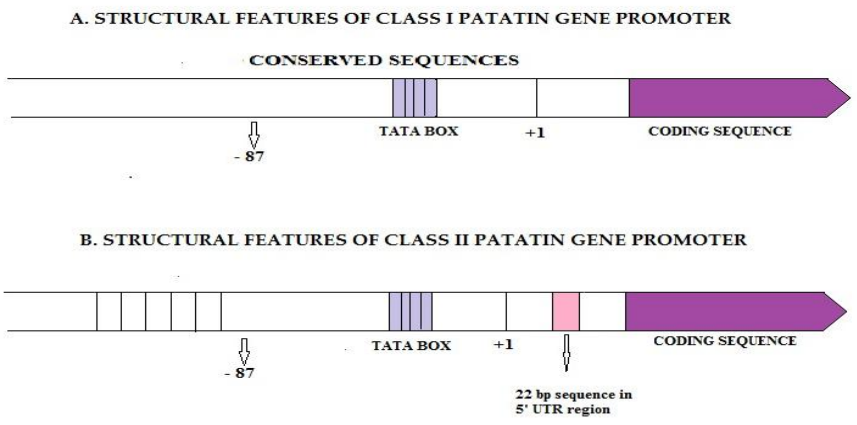


Fig. 1 Schematic view showing Patatin gene promoters

The patatin gene is further subdivided into two classes: Class I and Class II. The presence of 22 bp insertion sequence in the 5´ UTR region in Class II differentiates it from Class I patatin gene (Pikaard et al, 1987). Class I patatin gene is predominantly expressed in tubers whereas Class II patatin gene expressed in certain cell types of tuber and roots and with 50 to 100 lower level than Class I patatin gene (Pikaard et al, 1987; Mignery et al, 1988). Patatin is not only a storage protein (unlike all storage protein) but also it has enzymatic activities of nonspecific lipid acyl hydrolase (LAH), (Racusen et al, 1986; Shewry 2003), phospholipase A2 (Senda et al, 1996), β 1, 3-glucanase (Tonón et al, 2001) and β 1, 2-xylosidase (Peyer et al, 2004).

The biochemical and other properties of patatin proteins are depend on the genotype of cultivar. However, the real physiological role of patatin in potato tubers has not yet been completely established (Bárta and Curn, 2004; Pots et al, 1999; Shewry 2003). Various enzymatic activities mentioned above, high level of essential amino acid index with value of about 86.1% (Bártová and Barta 2009), its characteristics such as solubility (Kärenlampi and White 2009), high foaming activity (Ralet and Gueguen 2000), antioxidative potential (Liu et al, 2003) make patatin an interesting factor to study and it can be a good protein source in food and biotechnological applications.

Chapter 2

Review of literature

2.1 Biochemical attributes of patatin

Patatin is a 40 KDa glycoprotein showed extensive heterogeneity with forms differing in electrophoretic mobility at pH 8.6 and in mobility on SDS-PAGE (Park et al, 1983). Patatin is encoded by a multigene family with 10-15 members per haploid genome. All patatin genes are clustered on long arm of chromosome 8 (Ganal et al, 1991). About 33% of the patatin residues have been estimated to adopt α-helical and 46% a β-stranded structure (Pots et al, 1998). There was a linear relationship between the amount of patatin, expressed as a percentage of total soluble protein and the logarithm of tuber weight from 0.3 to 300g (Paive et al, 1983). It was demonstrated that patatin expressed in leaves of transgenic tobacco was glycosylated on two sites (asparagine 60 and asparagine 90) with typical small complex glycans comprising xylose, fructose, mannose and N-acetyl glucosamine in a ratio of 1:1:3:2, which is the same as the ratio of these sugars present in patatin isolated from potato tubers (Sonnewald et al, 1989b). This glycosylation has no influence on proteolytic stability or enzymatic properties.

Patatin exhibits enzyme activity: Galliard (1971) purified an enzyme from potato tubers that catalysed the deacylation of a range of lipid substrates (mono and diacylphosholipids, galactosyl diglycerides, mono and diglyceride). Subsequent studies demonstrated that this acyl hydrolase activity was due to patatin (Racusen, 1984), and that it also exhibits an esterase activity over PNP laurate, PNC acetate, α- napthyl laurate, β-napthyl acetate and phenyl acetate substrates (Racusen, 1986). The specificity of the acyl hydrolase has since been studied in more detail (Andrews et al, 1988; Anderson et al, 2002) particularly its activity as a phospholipase on phospholipid and lysophospholipid substrate (Senda et al, 1996; Hirschberg et al, 2001). The esterase activity has also been confirmed by its expression in transgenic tobacoo plants (Rosahl et al, 1987).

Role of patatin in plant defence mechanism: Hydrolytic activity has also been described recently for patatin as an acidic β 1,3-glucanase (Tonón et al, 2001). β 1,3-glucanases are thought to contribute to plant defence to fungal pathogens by digesting β-1,3-glycans in hyphal cell walls and often form part of the pathogenesis-related (PR) protein response (Shewry and Luca, 1997; Van Loon and Van Strien, 1999). This may imply that patatin plays a role in the defence of potato tubers. A role of patatin in defence against pest and pathogens is also indicated by two other observations. First, the inclusion of patatin in artificial diets resulted in the inhibition of growth of larvae of corn rootworm, Diabrotica spp. (Stickland et al, 1995). Comparison of the enzymatic and inhibitory properties of patatin fractions from different cultivars showed that galactolipase activity was correlated with growth inhibition,

but not phospholipase or acyl hydrolase activity. It was concluded that patatin may provide defence against the insect pest by effects on lipid metabolism. A patatin like protein with galactolipase activity was also induced by draught stress in leaves of Cowpea (*Vigna unguiulata*) (Matos et al, 2000, 2001), indicating that patatin may play a wide role in stress response.

Allergic response: Potato may elicit allergic responses in humans either when consumed as food or by skin contact with raw potatoes. Seppala et al, (1999) showed that patatin bound to IgE (a class of immunoglobulins specific for allergenic response) from children with a positive skin-prick test to raw potato, and also showed that the purified patatins gave positive skin- prick tests in allergenic children. This was subsequently confirmed by more detailed studies including skin exposure test and oral challenge (Majamoa et al, 2001), and patatin has been given the allergen designation Sol t 1. Heat treatment of potato results in decreased allergenicity, which appears to result from aggregation with other potato proteins rather than denaturation of patatin itself (Koppelman et al, 2002). Patatin also showed sequence similarity to allergen protein of latex i.e. Hev b 7 protein (43 KDa) (Kostyal et al, 1998; Sowka et al, 1998; Breiteneder et al, 1999).

2.2 Isolation, expression patterns and functional characterization of patatin genes

Several cDNA and genomic clones of patatin have been isolated and a number of nucleotide sequences have been determined. Most patatin isoforms have nearly identical amino acid sequences and are immunologically identical within a given cultivar as well as among cultivars (Park et al, 1983). The protein coding regions and the promoter regions up to position -87 of all genes analyzed so far are homologous, whereas upstream from this point the promoters diverge, allowing the genes to be divided into Class I and Class II category. The conserved region contains the CAAT and TATA homologies as well as a homology to the core enhancer sequence.

Class I and Class II patatin genes: The mature Class I and Class II patatins comprise of about 360 amino acid residues but are synthesized with N- terminal signal sequences of 23 residues (Mignery et al, 1984). This is consistent with their transport via the endomembrane systems leading to deposition in vacuoles (Sonnewald et al, 1989a). Immunochemical studies demonstrated that patatin is located in vacuoles in tubers and in leaves induced for its expression (Sonnewald et al, 1989), an observation which is consistent with N- glycosylation taking place in the endoplasmic reticulum and golgi apparatus (Kermode and Bewley, 1999). Preliminary comparisons by N- terminal sequencing and Ouchterlony double diffusion using polyclonal antiserum to total soluble proteins indicated that the component proteins of patatin are closely related (Park et al, 1983) and this was confirmed by the analysis of cloned cDNAs

and genes (Mignery et al, 1984, 1988). This showed existence of two classes of mRNA and genes, with the former sharing about 98% sequence identity. The encoded proteins, showed some minor differences in sequence, particularly in the N- terminal region, which was in agreement with the heterogeneity observed previously in directly determined N- terminal sequences (Park et al, 1983), but also differed in the presence (Class II) or absence (Class I) of a 22 bp sequence within the 5´untranslated regions. There is a functional division between the two classes: Class I genes are expressed predominantly in tuber, while Class II genes are expressed at 50 to 100 fold lower levels than Class I transcripts in roots and tubers (Pikaard et al, 1987; Mignery et al, 1988). Comparison between the sequences of various patatin clones revealed a high degree of homology of both Class I and Class II genes in the region of the first exon and in part of the upstream sequence. Unlike Class I patatin genes, Class II genes are characterized by the presence of 22 bp sequence in the 5' UTR region.

Class I Patatin promoters: Patatin also accumulates in leaves and stems of potatoes grown under field conditions (Racusen et al, 1983). The leaves incubated with high concentrations of sucrose induced expression of Class I patatin gene (Paiva et al, 1983; Rosahl et al, 1986; Jefferson et al, 1990). Removal of tubers and auxiliary buds can result in the accumulation of patatin, other tuber proteins and starch in stems and petioles, without any swelling or tuber formation (Paiva et al, 1983). To check the expression of Class I patatin gene, gene fusion have been made between the 5' flanking sequences of different Class I patatin genes using GUS reporter gene (β-glucuronidase) and genes directed the expression of GUS (β-glucuronidase) to high levels in tubers and at low levels in leaves, stems and roots of greenhouse grown plants (Jefferson et al, 1990; Rocha-Sosa et al, 1989; Wenzler et al, 1989b). Blundy et al, 1990 reported that the expression of Class I patatin gene fusions in transgenic potato varies with both gene and cultivar. The promoters of Class I genes, PS20 and PS3/27, were transcriptionally fused to β-glucuronidase and transformed into the potato cultivars Desiree and Maris Bard. Examination of the expression levels in large populations of microtubers indicated that the PS20 promoter produced β-glucuronidase activities 5-fold lower in Desiree than Maris Bard whereas the PS3/27 promoter showed similar levels in both cultivars. Furthermore, the relative expression levels from the two promoters were reversed in the two cultivars.

Class II Patatin promoters: Patatin gene studies were carried out by fusing it with the CAT (Chloramphenicol acyltransferase) reporter gene (Twell and Ooms, 1988). It expressed low levels of CAT in tubers and roots. Fusions of Class II promoters to the gene encoding GUS that did not include 22 bp sequences in the 5' leader appeared to be regulated normally, with

low levels of expression in the root and tuber. This observation suggested that the 22 bp sequence was not significant in determining the patterns of expression of the Class II genes.

Structural and functional characterization of patatin genes: A further series of experiments have been conducted using different lengths of 5' patatin gene regions fused to GUS, with the aim of defining the *cis*-acting regions necessary for the observed patterns of expression (Jefferson et al, 1990) as well as to test the presence of 'tuber-specific' elements and 'sucrose-specific elements' as separate entities on the same promoter. The minimum promoter length that gave sucrose responsiveness is extended 360 bp towards 5' of the transcriptional start site. Transgenic potato plantlets grown *in vitro* on high levels of sucrose (used to induce tuberization) showed higher levels of GUS activity than when grown on low sucrose. It was also demonstrated that patatin transcription, as measured by GUS activity, could be induced by high concentrations of sucrose both in the light and in the dark in isolated node cuttings (Jefferson et al, 1990). Detailed studies of the 5´ upstream sequences of a patatin gene have been reported by Holdsworth et al (1992) and Grierson et al (1994), aimed at identifying specific sequences and trans acting factors that determine developmental regulation and sucrose inducibility. This had led to the identification of a new type of DNA binding protein, called storekeeper (STK) which is thought to regulate patatin gene expression (Zourelidou et al, 2002; Kapoor et al, 1975; Liedl et al, 1987). Consequently a number of studies have been carried out on the structure and properties of patatin, particularly on its stability and thermal aggregation in relation to the production of functional proteins on an industrial scale.

Naumkina et al, (2006) studied the comparison of the activity of Class I patatin promoter (B33 promoter) fused with the reporter gene during heterological expression of B33::GUS in Arabidopsis (*Arabidopsis thaliana* L.) and homological expression of the same DNA construct in potato. Promoter activity was estimated from quantification of β-glucuronidase (GUS) activity. It was shown that, during heterological expression in arabidopsis seedlings, B33 promoter manifested a tissue-specificity and inducibility, although in a different manner than during homological expression in potato. In non induced arabidopsis seedlings, B33 promoter was most active in the roots, whereas, after induction with sucrose treatment, it became most active in cotyledons. 10 mM sucrose was sufficient for a manifold activation of B33 promoter in intact seedlings. The degree of B33 promoter induction by sucrose in arabidopsis seedlings was strictly organ-specific and increased in the following sequence: root < hypocotyl < cotyledons. 150-200 mM sucrose enhanced B33 promoter activity in cotyledons by 200 to 300 times i.e. much stronger than in potato organs. Glucose and fructose were less efficient than sucrose. Phytohormones affecting tuber formation in potato

(gibberellins, auxins, and cytokinins) did not affect significantly B33 promoter activity in arabidopsis.

Studies on functional analysis of a Class I patatin gene SK24-1 in microtuber formation of transgenic potatoes revealed that expression of SK24-1, cDNA clone in *Escherichia coli* possessed lipid acyl hydrolase (LAH) activity. Transformed potato plants were obtained via *Agrobacterium*-mediated transformation using the chimeric constructs containing the sense and antisense cDNA under the control cauliflower mosaic virus 35S (CaMV 35S) promoter. In some sense transformed plants, both sense patatin RNA and LAH activity were increased and further resulted in a significant increase of percentage of plantlets that formed microtubers and numbers of microtubers per plantlet *in vitro*. All antisense plants displayed a reduction in LAH activity. Moreover, expression of antisense cDNA in some antisense transformed plants led to a significant decrease in the number of microtubers formed. These results suggest that SK24-1 was involved in regulating microtuber formation (Huaijun et al, 2007).

Differential expression of patatin genes: In order to understand the molecular mechanism underlying the complex control of patatin expression as well as the differential expression of the two classes of patatin genes several members of the patatin gene family representing Class I and Class II have been isolated. The class-specific expression of patatin genes was investigated by analyzing four new patatin genes. A Class I patatin gene from cv. Berolina as well as a Class I and two Class II patatin genes from the monohaploid cultivar AM 80/5793 were isolated and partially sequenced. Sequence comparison indicates rearrangements as the major source for the generation of diversity between the different members of the classes. The expression of single genes was studied in potato plants transformed with chimeric genes where the putative patatin promoters were fused to the GUS reporter gene. A detailed histochemical analysis reveals that both Class I genes are expressed in the starch-containing cells of potato tubers and in sucrose-induced leaves. The Class II gene pgT12 shows the same expression pattern in root tips and in the vascular tissue of tubers, whereas no activity was detectable for pgT4. Thus the expression pattern of both classes of genes seems to be stable at least within or even between different cultivars (Liu XY et al, 1991).

Histochemical analysis has shown that patatin transcription occurs in most cell types of the potato tuber. No expression was observed in the periderm, as this tissue is composed of dead cells. In leaves and shoots that were grown on high concentrations of sucrose, patatin transcription was observed in several different cell types, such as in mesophyll cells, epidermal cells and several cell types associated with the vascular system. The distribution of patatin transcription in various tissues can be understood in terms of distribution of sucrose in those tissues. High levels of GUS activity associated with the internal phloem in patatin-GUS

transformants reflect the predominant use of these conducting elements for sucrose transport. A further series of experiments have been conducted that have used different lengths of 5' patatin gene regions fused to GUS, with the aim of defining the *cis*-acting regions necessary for the observed patterns of expression (Jefferson et al, 1990). The addition of the longer promoter regions, extending to 3500 bp 5' of the transcriptional start site, led to greater degrees of tuber-specific expression, both by increasing the amount of transcription in the tuber and by decreasing it in leaves and other organs. In the *in vitro* experiments, a similar trend in GUS expression was observed: i.e. addition of longer promoters led to increased levels of GUS activity on high sucrose medium and lower levels on medium containing low levels of sucrose. These observations suggest that sucrose responsiveness and tuber specificity may be functionally equivalent in activating the patatin promoter. As the highest levels of GUS activity are found in tubers rather than other organs grown on sucrose there may be further quantitative elements that confer higher levels of expression in tuber. The patterns of transcription observed in potato plants could be due to mechanisms conferring tuber specificity or they could reflect the concentrations of sucrose found in different tissues. To distinguish between these possibilities, a detailed examination was made of the function of the region of a Class I patatin promoter previously implicated in conferring tissue-specific and sucrose inducible expression. Internal deletions of this region revealed the presence of highly conserved 100 bp region comprising two conserved sequence elements, the A-box and the B box. The B repeat region acted as a positive activator of transcription in the tuber and was also responsible for a degree of sucrose inducibility. The distal region of A repeat repressed transcription in leaf and tuber tissue, while the proximal region of the A repeat was able to confer sucrose responsiveness. Each of these regions specifically bound nuclear proteins, which may be putative transcription factors involved in conferring these responses. The region found to confer sucrose inducible expression was conserved among some other genes that are also regulated by exogenous sucrose (Grierson et al, 1994).

Relevance of the present study

Patatin is encoded by a mutigene family consisting of large number of genes. Only few members of both Class I and Class II has been identified at molecular level. However many more members are yet to be identified with respect to the regulation of expression of the individual members of the patatin gene family from our own Indian potato cultivar. Potato plants suffer from a variety of viral, bacterial, nematode and fungal diseases, which have serious consequences in terms of tuber yield and consumer acceptance. The application of cross protection strategies, using promoters that are abundantly expressed in tubers can be used to combat with viral and fungal diseases. However, till date there are few report available on patatin with respect to its corresponding promoters and its relative strength. Patatin promoters are used in molecular farming for the production of novel proteins and produce very large amount of protein per acre. So by fusion with a patatin promoter, cheap production of proteins could be achieved. Patatin promoter control the specific gene expression in a tissue so the transgene driven by these type of promoters will only be expressed in some specific tissue without affecting rest of the plant. There are several DNA sequences present in promoter region such as *cis*-acting elements which are recognized by proteins called transcription factors. These proteins activate or suppress the expression of gene by binding with *cis*-acting elements. So by modifying expression of a particular promoter region, a desired result could be achieved. Hence isolation and characterization of different patatin gene promoters are very important area of research today. Since tubers specific promoters could be used as molecular tools to the molecular biologist with regard to both basic and applied research. Different India potato cultivar with their rich genetic resources could be used for isolation and characterization of different patatin genes of large multigene family. Keeping this in view, this study is quiet relevant.

Background work in our laboratory

Earlier we focused on the molecular cloning of the 5' flanking regions of patatin genes from different potato cultivars namely Kufri Chipsona-1(CS-1), Kufri Chipsona-2 (CS-2), Kufri Chandramukhi (KCM) and Kufri Jyoti (KJ). A total of five partial Class I patatin genes were isolated from different potato cultivars and submitted to the NCBI GenBank database under the Accession numbers JX124227, cv. Kufri Jyoti; JX124228, cv. Kufri Chipsona-1; JX124229, cv. Kufri Chipsona-1; JX124230, cv. Kufri Chandramukhi; and JX124231, cv. Kufri Chandramukhi. In order to amplify the 5' flanking regions of the patatin genes from these cultivars, a series of combination of different forward primers and reverse primer were designed based on the Class I and Class II patatin gene sequences available in the database. Therefore in order to have more molecular insights on patatin genes, the aim of the present

study is to carry out molecular cloning and characterization of patatin gene promoters from Indian potato cultivars. The present work would also help in doing a comparative analysis of Class I and Class II patatin gene promoters in terms of their efficiency and regulation

Objectives

The following objectives are framed based on the literature survey and background work in our laboratory

- Isolation of genomic DNA from Indian potato cultivars
- PCR amplification of partial patatin genes having 5´-flanking regions with the help of Class I gene-specific primers
- Molecular cloning and partial characterization of the patatin genes

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Procurement of potato germplasm and other materials:

The germplasm of various potato cultivars such as Kufri Chipsona-1, Kufri Jyoti were procured from Central Potato Research Institute (CPRI), Shimla. Various enzymes used were purchased from Bangalore Genei Pvt.Ltd, Bangalore and Amersham Biosciences Ltd. Hongkong. The required chemicals were bought from Sisco Research Laboratories Pvt. Ltd, and Himedia Pvt. Ltd, Mumbai. Primers used were from Bangalore Genei Pvt.Ltd, Bangalore.

3.1.2 Strains and Plasmids

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

- pUC19 : GenBank accession no. X02514 (Yanish- Perron et al, 1985)
  The above bacterial strain was routinely maintained in the laboratory. *E.coli* DH5α strain was maintained on Luria agar medium, whereas, those transformed with pUC19 plasmid were maintained on Luria agar-Ampicillin medium. pUC19 is a commonly used plasmid cloning vector in *E.coli* (Yanisch-Perron et al, 1985). Its size is of 2686 bp, has high copy number plasmid. It carries a 54 bp multiple cloning site that contains unique sites of restriction endonucleases.

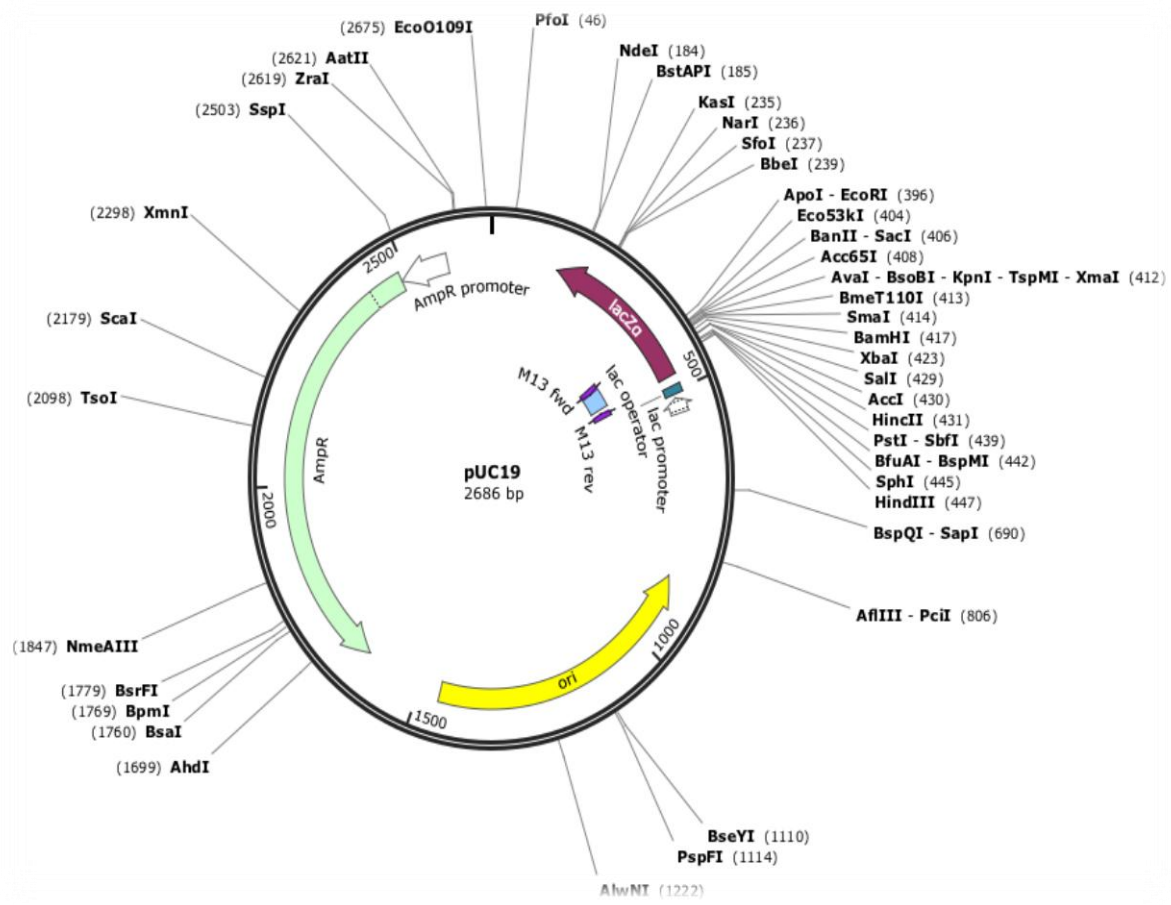


Fig. 2 Schematic view of pUC19

3.1.3 Media used

Luria Bertani Medium:

Yeast extract - 0.5 % (w/v)

Tryptone - 1.0 % (w/v)

NaCl - 1.0 % (w/v)

Agar - 1.5 % (w/v)

For preparing LA - Ampicillin medium, ampicillin was added to the LA medium at the working concentration of 50 mg/ml autoclaving.

3.1.4 Buffers used

Gel Loading Buffer (5X)

Sucrose - 35 % (w/v)

EDTA - 50 mM (pH 8.0)

Tris – 25 mM

Bromophenol blue - 0.2 % (w/v)

STET buffer

Sucrose - 8 % (w/v)

Triton X 100 - 0.5 % (w/v)

EDTA - 50 mM (pH8.0)

Tris HCl - 10 mM (pH8.0)

TBE Buffer (5X)

Tris Base - 54 g/L

Boric acid - 28 g/L

EDTA - 3.8 g/L

The pH of the buffer was set at 8.0

TE Buffer (1X)

Tris HCl - 10 mM (pH 8.0)

EDTA - 1 mM (pH 8.0)

3.1.5 Enzymes used

Restriction enzymes:

Various restriction enzymes such as *Eco*RI, *Hind*lll and *Sma*I were used in this study. Restriction digestion was carried out in buffer supplied by manufacturer. Depending on specific enzyme, reaction was carried out at appropriate temperature and BSA added as required.

Other enzymes

Ribonuclease A

Stock solution - 10 mg/ml

Working solution - 10-15 mg/ml

DNAse free Ribonuclease A was prepared in a buffer containing 10 mM Tris (pH 8.0) and 15 mM NaCl. To prepare DNAse free RNAse, the solution was boiled for 10 minutes, followed by the slow cooling, after which it was dispensed into aliquots and then stored at - 20 °C for subsequent use.

Lysozyme

Stock solution - 10 mg/ml

Working solution - 300-400 mg/ml

Freshly prepared lysozyme was used in regular work.

T4 DNA ligase

Stock conc. - 400 U/ ml

Working conc. - 40 U/ ml

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

Klenow fragment of DNA polymerase I

Stock conc. - 5 U/ ml

Working concentration - 2 U/ 50 ml of the reaction volume.

3.1.6 Other chemicals

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

Stock conc. - 20 mg/ml

Working conc. - 20 mg/ml

It was prepared by dissolving the required amount in N, N- dimethyl formamide.

IPTG (Isopropyl thio β- D- galactoside)

Stock conc. - 100 mg/ml

Working conc. - 100 mg/ml

It was prepared in fresh and sterile water.

3.2 Methods

3.2.1 Isolation of genomic DNA from potato

Genomic DNA isolation was carried out following the procedure as described by Kumari V. et al, (2012). 2.0- 3.0 g of plant material was washed with sterile water and pulverized to fine powder using mortar and pestle in liquid nitrogen. It was 0.3 then transferred quickly to a conical flask containing 15 ml extraction buffer [50 mM Tris-HCl pH 8.0, 50 mM ethylene diaminetetraacetic acid (EDTA) pH 8.0, 250 mM NaCl and 15% (w/v) sucrose] which was maintained at 65°C. Proper mixing was done and it was incubated at 65°C for 20 min with intermittent gentle shaking. Then 5 ml of 5.0 M potassium acetate solution (pH 5.5) was added, mixed vigorously and incubated further on ice for 20 min and then centrifuged at 4000 *g* and 4°C for 25 to 30 min. The supernatant was filtered through two layers of fine cloth and 0.70 volume of isopropanol was added, mixed gently and incubated at -20°C for 2 to 3 h followed by centrifugation at 10000 *g* and 4°C for 15 min. The crude DNA pellet was washed with ice cold 70% ethanol, then 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). The DNA was then precipitated using 0.1 volume of 3.0 M sodium acetate (pH 5.5) and 2.0 volume of dehydrated ethanol, which was finally dissolved in 200 to 250 µl of TE buffer and stored at -20°C. The DNA was finally dissolved in 50 to 60 µl of TE buffer. In the case of suspended macromolecules in DNA solution, small spin was required to remove the impurities; the clarified supernatant was then transferred to sterile microfuge tube and stored at -20°C for further use.

3.2.2 Designing of primers

The following oligonucleotide primers were designed based on the available genome sequence corresponding to Class I patatin gene in GenBank database (Acc. No. X87216). The 1770 bp nucleotide sequence of Class I patatin gene is comprised of the following structural features : The TATA box starts at the base 1338 and the transcription start site is located at the base 1361 corresponding to the first exon. So the above gene sequence provides an extended Class I patatin gene promoter. The translational start site of the gene is present at the base 1407. The nucleotide bases from 1407 to 1475 encode the 23 amino acid transit pepide of the patatin gene. The later refers to vacuolar targeting signal. To ensure that there are minimum chances of non-specific amplification, some important factors kept in consideration while designing of primers were: (1) PCR primers should be 10-24 nucleotides in length. (2) The GC content should be 40% - 60%. (3) The primer should not be self-

complementary or complementary to any other primer in the reaction mixture, to prevent primer-dimer and hairpin formation. (4) Melting temperatures of primer pairs should not differ by more than 5º C, so that the GC content and length must be chosen accordingly. (5) The annealing temperature should be about 5°C lower than the melting temperature. (6) Sequences with long runs of a single nucleotide should be avoided. (7) Primers with significant secondary structure are avoided. The features of three primers (each 20 mer) specific for Class I patatin genes are briefly discussed here: Two forward primers were designed from different regions of upstream sequence patatin gene. This could facilitate in studying different lengths of patatin promoter and to see the divergence in the upstream sequences of various patatin isoforms. PT-F074 is a forward primer that corresponds to the bases 74-93 of the genome sequence having G+C content 40 %. The sequence of PT- F074 is

5´ – TAA TTG ACC GGA GAC TAT AC – 3´

PT-F610 is other forward primer that corresponds to the bases 601-629 of the genome sequence having G+C content 35 %. The sequence of PT- F610 is:

5´—TTC TTA TCA ATT CTG ACG TG –3´

As the transcription start site is located at 1338 base, both the forward primers: PT- F074 and PT-F610 belongs exclusively to the extended promoter region of the gene. PT- R1478 is a reverse primer that complementary to the bases 1478-1459 of the genome sequence having G+C content 45 %. The sequence of PT-R1478 is:

5´ – CGT AGC ACA TGT TGA ACT AG –3´

The gene sequence corresponding to the reverse primer lies in the region encoding the transit peptide of the patatin gene i.e. 23 amino acid peptide coding region. The purpose was to see whether the sequences are conserved in the coding region in patatin gene family members.

3.2.3 Polymerase Chain Reaction

PCR is used to amplify a specific region of a DNA strand (the DNA target) using forward and reverse primer. It is an iterative process. It consists 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. In this genomic DNA were taken as template using different primer combinations as following:

PTF074 and PTR1478 - for cv. Kufri Chipsona 1 (CS-1)

PTF610 and PTR 1478- for cv. Kufri Jyoti (KJ)

Total reaction volume: 50µl

Template DNA	3µl
10X Buffer	5 µL
Forward Primer	10 pmoles
Reverse Primer	10 pmoles
dNTP's	25 mM
Sterile D.D. Water	To make up the volume 50 µL
Taq DNA polymerase	3 U/µL

The thermal cycling parameters were as given below:

Step	Temperature	Time
Denaturation	94ºC	1 min
Annealing	55ºC	2 min
Polymerization	72ºC	3 min

The reaction was carried out for 30 cycles with final extension at 72ºC, 5 min.

3.2.4 Agarose gel electrophoresis

PCR samples were analysed using Agarose gel electrophoresis using standard methods (Sambrook-A laboratory manual). 0.7% agarose gel was made in 0.5X TBE buffer to which ethidium bromide dye was added (0.5 mg/ml) and casted in a gel tray. The DNA samples were loaded after mixing well with the gel loading buffer and electrophoresis was carried out at 2 - 5 V/cm till the tracking dye covered two-third of the gel length. Finally, the DNA bands were visualized under UV light.

3.2.5 Klenow treatment

DNA polymerase I, Large (Klenow) Fragment is a proteolytic product of *E. coli* DNA polymerase I which retains polymerization and 3'→ 5' exonuclease activity, but has lost 5'→ 3' exonuclease activity. *Taq* DNA polymerase has a tendency to add extra 'A' residue at 3' ends. It is a polishing step removes extra 'A' residue from 3' ends making PCR product truly blunt ended. Here Klenow treatment was carried out for 30 min at room temperature in presence of dNTPs mixture. The Klenow treated PCR products were then purified, precipitated and finally dissolved in TE Buffer.

3.2.6 Gel elution of DNA bands

Qiagen kit was used to purify desired band of DNA for further cloning. For purification of DNA bands QIAEX II agarose gel extraction protocol was followed. For this, 0.8% agarose in 1X TAE buffer was used to run DNA samples along with a control lane in which sample

was also loaded. The gel was run for sufficient time and then, the control lane was excised with the help of a clean scalpel and visualized on a UV-transilluminator. The position of DNA band of interest was marked and then, corresponding band was excised from samples (without UV exposure). The gel slice was weighed in 1.5 ml of microfuge tube. Following this, three volumes of Buffer QX1 was added to one volume of gel as per the manufacturer's instructions. QIAEX II solution was resuspended by vortexing for 30 seconds and 20 µL of it was added to each sample. The samples were incubated at 50°C for 10 min with intermittent vortexing after every 2 min in order to solubilize the agarose. This was done to keep QIAEX II in suspension. The samples were centrifuged for 30 seconds at high speed and the supernatant was carefully removed with a pipette. The pellet was washed with 500 µL of Buffer QX1. For this, the pellet was first resuspended by vortexing and then the sample was centrifuged for 30 seconds followed by removal of supernatant. In the same manner, the pellet was washed with 500 µL of PE Buffer twice, supplied with the kit. 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 for 30seconds. The supernatant containing the purified DNxA was carefully transferred into a clean microfuge tube. The above two steps were repeated to increase the yield. Lastly, the eluates were combined and 2.5 µL was loaded on 0.8 % agarose gel in order to check the yield.

3.2.7 Processing of vector

3.2.7.1 Restriction digestion reaction

Restriction enzyme is used to digest double stranded DNA at specific site within or adjacent to the recognition sequences. The Restriction digestion reaction was set up according to manufacturer's instructions and was usually carried out in a reaction volume of 15 µL for 2½ hours at optimum temperatures, depending upon the restriction enzyme.

pUC 19 DNA	1.0 µg
Restriction enzyme	1µl (2 units)
Buffer	Used at a concentration of 1X (as supplied by the manufacturer)
Sterile distilled water	To make up desired volume

3.2.8 Ligation reaction

A ligation reaction was set up in order to ligate the insert into vector (pUC19), using the enzyme T4 DNA ligase. It catalyzes the formation of phosphodiester bond between the

juxtaposed 5'-phosphate and 3'-OH termini in the duplex DNA. It can join blunt as well as the cohesive end termini. The main components of a ligation reaction are as follows:

Linearized vector	0.5 µg
Insert	1.0 µg
T4 DNA ligase Buffer	Used at a concentration of 1X
T4 DNA Ligase enzyme	1 µl (~30 units)

The reaction volume was made up to 15 µL and the reaction mixture was incubated at 18°C for 6-7 hours. Ligation reaction was carried out in two sets for two different inserts respectively. Each ligation mix was individually used to transform competent *E.coli* DH5α.

3.2.9 Genetic transformation of *E. coli* DH5α with recombinant plasmid

E.coli DH5α was transformed with various DNA samples using the standard $CaCl_2$ method (Mandel and Higa, 1970).

Preparation of competent cells: 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 in 10 mL of ice-cold 50 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 50 mM $CaCl_2$ and kept in ice for 2½ hours.

Transformation: 100 µL of the competent cell suspension was dispensed in sterile microfuge tubes and kept at 0°C. 6-7µL 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 to all the tubes, followed by the addition of 1ml 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. Each of the obtained white transformant was further purified by streaking it to single colony.

3.2.10 Isolation of plasmid DNA in small scale

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 37ºC/120 rpm for overnight. Cells were harvested from 1.5 ml overnight grown culture in microfuge tubes and centrifuge it at 8000 rpm for 10 min. 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 37ºC 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, 1/10th volume of 3M sodium acetate (CH3COONa) and equal volume of isopropanol was added and incubated at 4ºC for 25 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.

Chapter 4

Results and Discussion

Patatin is a multigene family comprising of several members respond to different metabolic and developmental signals. The present work focused on the class I patatin genes which are predominantly expressed in tubers. In this study, the promoter regions of Class I patatin genes were amplified from potato cultivars namely Kufri Chipsona 1 (CS-1) and Kufri Jyoti (KJ). From the above potato cultivars, PCR amplified DNA products were analyzed and then cloned in pUC19. Finally, inserts were partially characterized by restriction digestion and PCR analysis. The results as obtained by step-wise experiments are given in the following sections.

4.1 Isolation of potato genomic DNA

Isolation of total genomic DNA was done by following procedure as described in 3.2.1 from different Indian cultivars namely Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Jyoti (KJ), Ashoka (As). These cultivars are agronomically important. The quality of genomic DNA was checked through agarose gel electrophoresis (Fig. 3A) followed by restriction digestion with *Sau*3A1 enzyme (Fig. 3B).

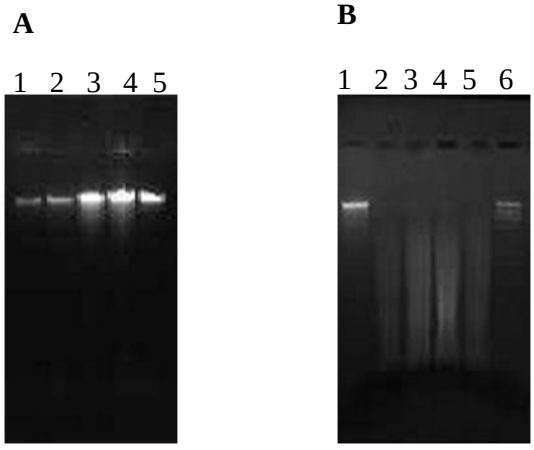


Fig. 3 Agarose Gel Electrophoresis showing Genomic DNA and digestion of genomic DNA with *Sau3A1*

A Lane 1- λ DNA; Lane 2- Kufri Chipsona-1(CS-1); Lane 3- Kufri Chipsona-2 (CS-2); Lane 4- Kufri Jyoti (KJ); Lane 5- Ashoka (As); **B** Lane 1- Genomic DNA (contro) l; Lane 2- Kufri Chipsona-1 (CS-1) digested with *Sau3A1*; Lane 3- Kufri Chipsona-2 (CS-2) digested with *Sau3A1*; Lane 4- Kufri Jyoti (KJ) digested with *Sau3A1*; Lane 5- Ashoka(As) digested with *Sau3A1*; Lane 6- 500 bp ladder

4.2 Isolation of partial patatin genes consisting promoter regions

From above four cultivars, two cultivars namely Kufri Chipsona-1 and Kufri Jyoti were selected in order to amplify Class-I partial patatin genes consisting mostly the 5' flanking

regions. First PCR was carried out with primer pair, PT-F074 (forward primer) and PT-R1478 (reverse primer) using genomic DNA of Kufri Chipsona-1 as template. Two DNA amplified products i.e. ~2.0 kb and ~2.5 kb were obtained as shown in Fig.4A. Second PCR was carried out with genomic DNA of Kufri Jyoti (as template) and primer pair, PT-F610 (forward) and PT-R1478 (reverse). ~1.8 kb and ~2.0 kb DNA fragments were obtained as shown in Fig. 4B. PCR conditions are mentioned in section 3.2.3. PCR amplified DNA band of ~2.5 kb from Kufri Chipsona-1 and ~2.0 kb DNA band from Kufri Jyoti were used for cloning and characterization.

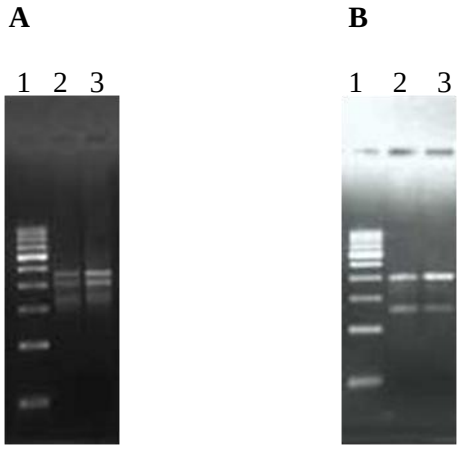


Fig. 4 PCR amplified products using patatin specific primers

A Lane 1- 500 bp ladder; Lanes 2 & 3 coresspond to CS-1

B Lane 1- 500 bp ladder; Lanes 2 & 3 coresspond to KJ

4.3 Molecular cloning and characterization

Preparation of plasmid vector: The plasmid vector pUC19 was isolated from the E. *coli* DH5α strain by boiling preparation method. Further, it was digested with restriction enzyme *Sma*I (a blunt end cutter) as blunt-ended PCR amplified DNA products were required to be cloned into the *Sma*I site of the plasmid vector as shown in Fig. 5.

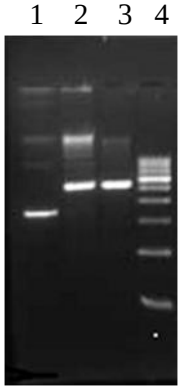


Fig. 5 Restriction analysis of pUC19 with *Sma*I enzyme

Lane 1- Control pUC19; Lanes 2& 3 pUC19 digested with *Sma*I Lane 4- 500 bp Ladder

4.4 Gel elution of DNA bands

4.4.1 Recovery of DNA bands using Qiagen kit

PCR products i.e. ~2.5 kb DNA (CS-1) and ~2.0 kb (KJ) were treated with Klenow polymerase. For ligation, the termini of the target DNA fragments and the vector should be compatible. Generally, the PCR-amplified products are not truly blunt ended. Therefore, these DNA products were polished with the Klenow fragment of *E. coli* DNA polymerase I (as described in the section 3.2.5). Further DNA bands were purified by Qiagen kit (following QIAEX II agarose gel extraction protocol). Fig. 6 A & B show eluted DNA fragments.

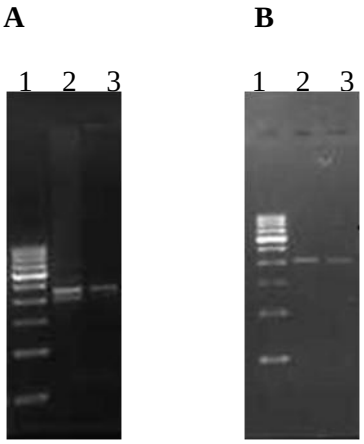


Fig. 6 Agarose Gel Electrophoresis showing eluted DNA bands

A Lane 1- 500 bp ladder; Lane 2- CS-1 specific PCR products and Lane 3 refers to the eluted band

B Lane 1- 500 bp ladder; Lane 2- KJ specific PCR product and Lane 3 refers to the eluted band

4.5 Putative recombinant clones of CS-1 and KJ

In this study, two set of blunt-end ligation reactions were carried out separately using purified PCR amplified DNA products and linearized pUC19, and incubated overnight at 16ºC. Then ligation mixtures were used for transformation of *E. coli* DH5α strain. A number of white transformant colonies were obtained on ampicillin plates containing X-gal and IPTG corresponding to each set of ligation mix. The putative white colonies were further purified to single colonies. Plasmid DNA was isolated from white colonies obtained and further analyzed to check the presence of inserts. Further putative clones were analyzed in agarose gel on the basis of mobility. In Fig. 7 putative clones of CS-1 and KJ show lesser mobility as compared to control pUC19. Hence, these clones are likely to have desired gene. Further, characterization of clones was carried out using restriction digestion and PCR. Kufri Chipsona-1 specific putative clones were designated as C1-13, C1-15 and C1-17; likewise, Kufri Jyoti putative clones designated as K1-3 and K1-8.

4.6 Characterization of the putative clones

4.6.1 Restriction analysis of the recombinant plasmids: Restriction analysis of the above recombinant plasmids was carried out using the restriction enzymes namely *Eco*RI and *Hind*III. Restriction analyses of the CS-1 derived clones are shown in Fig. 7 A and B. Restriction analysis indicated that C1-13 was not digested with either of the enzymes where as clone C1-15 and C1-17 were clearly digested by both the enzymes.

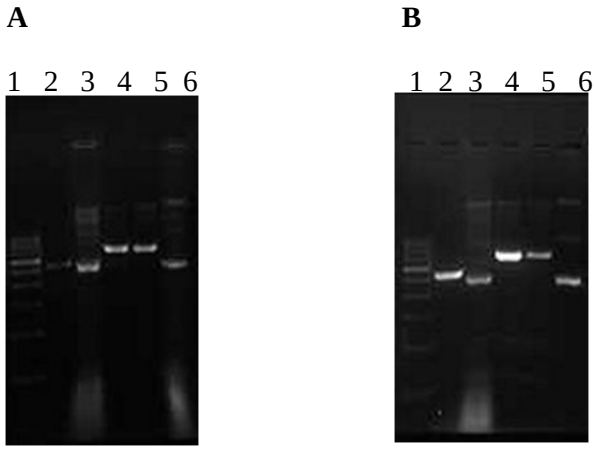


Fig. 7 Restriction analyses of CS-1 derived clones with *Eco*R1 and *Hind*III

A Lane 1, 500 bp ladder; Lane 2, pUC19 digested with *Eco*R1; Lane 3, C1- 13 digested with *Eco*R1; Lane 4, C1-15 digested with *Eco*R1; Lane 5, C1- 17 digested with *Eco*R1; Lane 6, undigested C 1-13 as control

B Lane 1, 500 bp ladder; Lane 2, pUC19 digested with *Hind*III; Lane 3, C1- 13 digested with *Hind*III ; Lane 4, C1-15 digested with *Hind*III ; Lane 5, C1- 17 digested with *Hind*III; Lane 6- undigested C 1-13 as control

Likewise, restriction analyses of the KJ derived clones are shown in Fig. 8 A and B. Restriction analysis indicated that both the clones i.e. K1-3 and K1-8 where digested with both the enzymes. Interestingly, K1-3 and K1-8 where found to differ with respect to the digestion pattern with *Eco*R1. Moreover, in case of *Hind*III digestion the mobility of the linearized bands were found to vary indicating difference in their sizes.

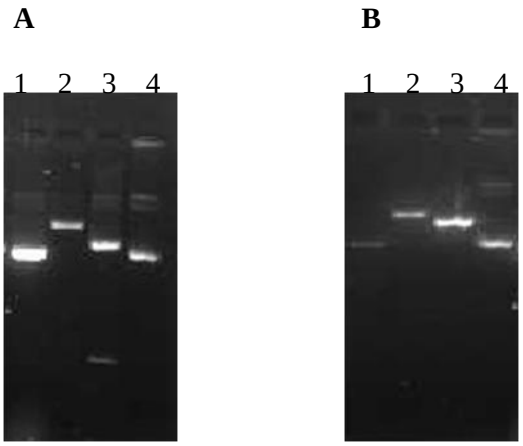


Fig. 8 Restriction analyses of KJ derived clones with *Eco*R1 and *Hind*III

A Lane 1, pUC19 digested with *Eco*R1; Lane 2, K1-3 digested with *Eco*R1; Lane 3, K1-8 digested with *Eco*R1; Lane 4, undigested K1-3 as control

B Lane 1, pUC19 digested with *Hind*III; Lane 2, K1- 3 digested with *Hind*III ; Lane 3, K1-8 digested with *Hind*III ; Lane 4, undigested K1- 3 as control

4.6.2 PCR characterization of the putative clones

Apart from restriction analyses, the putative clones were used as templates to carry out PCR using the respective gene specific primers. As mentioned earlier, for CS-1 derived clones, PT-F074 and PT-R1478 primers were used in the PCR. As shown in Fig. 9 A, the size of the amplified band was similar for the clones C1-13, C1-15, C1-17 i.e. ~2.5 kb as expected. However, extent of PCR amplification was found to vary between the clones then as evident from the relative intensities of the amplified band. All these clones need to be firther characterized by sequencing. It is apparent that the clone C1-15 could be more promising. Likewise, for the KJ derived clones, PT-F610 and PT-R1478 primers were used in the PCR. As shown in Fig. 9 B, the size of the amplified DNA band for the clone K1-3 was ~2.0 kb as expected; whereas there was no amplification for the clone K1-8 under similar conditions suggesting that the latter could be a spurious clone. PCR data clearly suggested that C1-15 and K1-3 were quiet promising and very likely to contain potato genomic DNA fragments encoding Class I patatin genes as proposed in the objectives of the study. However all these clones need to be further characterized by sequencing.

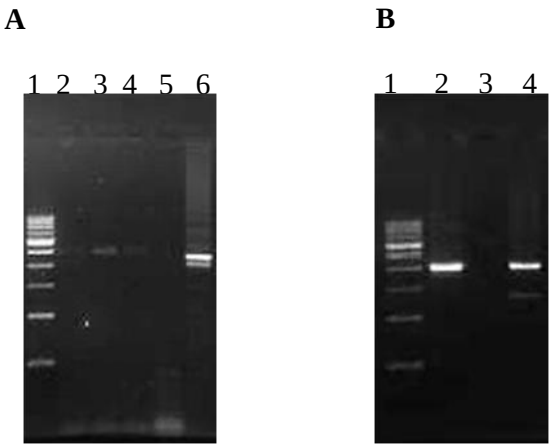


Fig. 9 Characteriztion of the putative clones by PCR approach. **A** correspondes to the primer pair PT-F074 and PT-R1478 was used. **B** correspondes to the primer pair PT-F610 and PT-R1478.

A Lane 1, 500bp ladder; Lanes 2, 3 & 4 correspond to templates C1-13, C1-15& C1-17; Lane 6, CS-1 genomic DNA derived original PCR as control **B** Lane 1, 500 bp ladder; Lane 2 & 3 correspond to templates K1-3 & K1-8, respectively Lane 4, KJ genomic DNA derived original PCR as control

(NOTE: Lane 5 of Fig. 9 A may be ignored here since this lane was used for other purpose.)

Concluding Remarks

Patatin (Class I and Class II) is encoded by a relatively large multigene family. It is known that patatin genes mostly vary in their 5´flanking regions. The study of different members of the gene family and identification of promoter region is very important with regard to both basic and applied aspects of research. As compared to its relatively large family size in potato, only a few members have been studied at molecular level till date. Many more functional patatin genes belonging to either of the classes are yet to be isolated and further characterized from potato cultivars. Such efforts are required to understand the spatio-temporal nature of expression and transcriptional regulation of the Class I patatin genes.

In this study, efforts were made for isolation of partial Class I patatin genes from the potato cultivar namely Kufri Chipsona-1 and Kufri Jyoti through PCR approach using gene specific primers. A few promising putative clones were obtained as characterized by PCR. All these clones need to be further characterized by sequencing. Earlier in our laboratory, a few Cass I patatin gene promoters were isolated and characterized from some of the Indian potato cultivars. Our preliminary observations and critical comparison with the previous ones clearly suggest that the putative clones as obtained in study are likely to encode distinct patatin genes which are hitherto unknown. So in this context this study is quiet relevant and useful in the area of plant molecular biology/ biotechnology.

Summary

The thesis work could be summarized as follows:

- Isolated total genomic DNA from four potato cultivars namely CS-1, CS-2, KJ, and As and their quality checked through agarose gel electrophoresis. The DNA samples were used as template in PCR.
- A total of three oligonuleotides (20-mer each) namely PT-F074 & PT-F610 (forward primers) along with PT-R1478 (reverse primer) were designed based on the Class I patatin gene sequence available in GenBank database (Accession no. X87216). Two forward primers were designed exclusively from the Class I patatin promoter region whereas, the reverse primer PT-R1478 was designed from the transit peptide coding region in this region. PT-R1478 was used as common reverse primer in both combinations.
- Polymerase chain reactions (PCR) were carried out using DNA templates with the first set of primer pair (PT-F074 and PT-R1478) for CS-1, and the second set of primer pair was used (PT-F610 and PT-R1478) for KJ. The amplified DNA products were analyzed by agarose gel electrophoresis.
- Klenow reaction was carried to make the amplicons blunt-ended. Cloning was done in the *Sma*I site of pUC19 vector. For transformation *E.coli* DH5α strain was used. The transformants were selected based on the α-complementation (blue/white colony selection) followed by isolation of plasmid DNA samples.
- In order to check presence of DNA inserts restriction analyses was carried by using various enzyme and PCR was also carried out using same set of primers to check the intactness of the cloned inserts. Preliminary characterization suggest that the clone inserts are likely to correspond Class I partial patatin genes having mostly the 5´ flanking regions.

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