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

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

Submitted by:
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July, 2014

DECLARATION

I, hereby declare that the work which is being presented in the thesis entitled, **"Class I patatin genes from Indian potato (*Solanum tuberosum* L.) cultivars: isolation and partial characterization"** in the partial fulfillment of the requirement for the award of degree of Master of Science in Biotechnology, Thapar University, Patiala, is an authentic record of my own research work carried out under the guidance and supervision of **Dr. N. Das**, Professor, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this dissertation has not been submitted to any other university or institute for award of any other degree.

Date: July 18, 2014

Place: Patiala

Manjeet Kaur

(Manjeet Kaur)

CERTIFICATE

This is to certify that the dissertation entitled **"Class I patatin genes from Indian potato (*Solanum tuberosum* L.) cultivars: isolation and partial characterization"** submitted by **Manjeet Kaur** (Roll No. 301201010) in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, to Thapar University (Deemed University)is a record of student's own work carried out by her under our guidance and supervision. 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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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Abbreviations	**Name**
Bp	Base-pair
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyltransferase
CaMV 35S	Cauliflower mosaic virus 35S
cDNA	Complementary DNA
dNTP	2′-deoxynucleoside-5′-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
GUS	Glucouronidase
IgE	Immunoglobulin E
IPTG	Isopropyl-β-D-Thiogalactopyranoside
Kb	Kilo base
kDa	Kilo-daltons
LA	Luria Agar
LB	Luria Broth
M	Molar
mM	Milli-molar
PCR	Polymerase chain reaction
Pmoles	Picomoles
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
UTR	Untranslated region
X-GAL	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

Abstract

The potato (*Solanum tuberosum* L.) is highly nutritious non-grain starchy food crop belonging to *Solanaceae* family. It is a short day and C3 plant grown in cool season in temperate regions. Usually the potato cultivars are tetraploid species. The tubers are the sink organs for the storage of starch and other abundant storage proteins. Among the storage proteins of tubers the major soluble glycoprotein is a ~40 kDa protein known as 'Patatin' which is encoded by a large multigene family. The sequence variation in the upstream of 5′-flanking regions of patatin genes leads to the division of patatin genes in two classes: Class I and Class II. A 22-bp insertion is present only in the 5′-UTR of class II patatin genes. Class I patatin genes are expressed at elevated levels mainly in the tubers, whereas class II patatin genes are expressed in roots and tubers but at a very lower level as compared with class I patatin genes. The present study mainly deals on molecular cloning and partial characterization of 5′-flanking regions of class I patatin genes of Indian potato cultivars namely Kufri Jyoti (KJ) and Kufri Chipsona-2 (CS-2). A specific set of forward and reverse primer as designed in our laboratory based on the class I patatin gene sequence available in the data base (Accession no. X87216) were used in order to amplify the 5′-flanking regions of class I patatin genes. The sizes of the PCR-amplified products were: ~1.5 kb and ~2.0 kb specific to KJ; ~1.0 kb specific to CS-2. The PCR amplified products clearly suggested significant sequence variations in the upstream of 5′-flanking regions of patatin genes of different potato cultivars. All these DNA bands were cloned into the *Sma*I site of a plasmid vector pUC19. For partial characterization, restriction analyses were done to check the presence of the cloned inserts, and PCR was carried out to check the specificity and intactness of the inserts. Based on the sizes of the PCR-amplified products, the following conclusions could be made. The ~1.5 kb DNA band specific to the cv. KJ, in particular, possibly represents a new patatin gene not reported earlier. However, the other DNA bands may show some degree of variations. In order to identify tuber specificity, classification, microheterogeneity and strength of the promoters, sequencing and functional characterization are required.

Chapter 1

INTRODUCTION

The potato (*Solanum tuberosum* L.) is a non-grain food crop belonging to family *Solanaceae*. Potato originated in the highlands of the Peruvian Andes-mountains in South America on the border between Bolivia and Peru, 8,000 years ago. It is the indigenous species of Andes. Potato reached Europe from Andes through Spanish explorers around 1570. During 16th century AD the potato was subsequently brought to territories and ports throughout the world by European sailors. Soon it became an important food staple and field crop. There are now over a thousand different varieties of potatoes. Potatoes have become an integral part of much of the world's cuisine. It is the world's third-largest food crop, following rice and wheat. Now, China is the world's largest potato-producing country, and nearly a third of the world's potatoes are harvested in China and India. Potatoes are more nutritious, faster growing, need less land and water and can thrive in worse growing conditions than any other major crop. They are a good source of vitamin C, potassium, carbohydrates, protein, calcium and phosphorus.

Potatoes are a short day and C3 plants grown in cool season and in temperate regions. They have a low light saturation point. Proficient root development in potato cultivars occurs at soil temperatures 15° to 20°C. Potatoes are grown on wide range of soil from sandy to clay loams. Soil for potato cultivation should have low salinity and good drainage properties for root aeration. (http://www.yara.us/agriculture/crops).

1.1 Morphological features of potato plant

Potato is a tetraploid between diploid species i.e., *S. stentotomum* and *S.sparsipilum* with subsequent chromosome doubling (Ramanna and Hermsen, 1979).The genus *Solanum* consists of approximately 2000 members, from this only about 160 wild and seven cultivated species are able to form tubers. Potato plant has fibrous root system. Roots are pale in color, fleshy, thick and elongated referred as rhizomes. It has dark green, broad and compound leaves with oval leaflets. They bear white, pink, blue, or purple flowers with yellow stamens. Generally, tubers of varieties with white flowers have white skin, while those of varieties with colored flowers have pinkish skins. After flowering, some varieties of potato plants produce small green fruits, each fruit containing up to 300 seeds. Potato fruit contains an alkaloid solanine which is toxic in nature, so not suitable for consumption. Potato stems could be of three kinds including leafy stems, stolons and tubers. Potatoes are the specialized storage tissues which develop by modification of underground stem. Potato plant produces multiple tubers. Tubers bear small nodes with buds ‘eyes’ which sprout in warm conditions. These are the sink organs where starch is synthesized from sucrose and stored in amyloplasts.

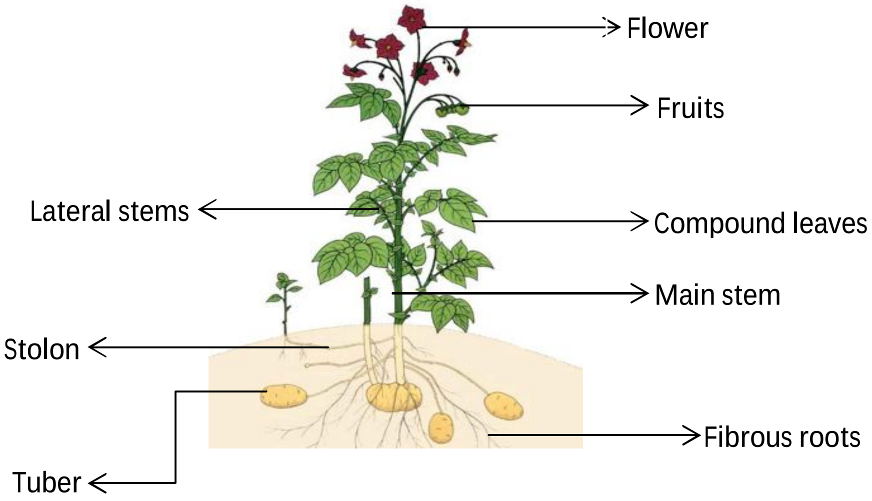


Fig. 1 Schematic view of Potato Plant

1.2 Biology of potato tubers

Potato tubers are swollen, fleshy, underground stems that serve as sink organs for the storage of starch synthesized from sucrose, and other abundant proteins. Tuberization is the result of activity of several interdependent processes i.e., stolon initiation and elongation, sub apical swelling, cell division, and the induction of specific proteins. It is a complex developmental process, influenced by various factors such as photoperiod, temperature and endogenous levels of phytohormones (Aksenova et al. 2012). This process is favored in short days (long nights) having low light saturation point. Tuberization initiates at low temperatures. Tubers expand radially by the process of cell expansion and limited cell division (Cutter 1982). 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. Beneath the epidermis, a periderm is formed which gets sloughed off as the tuber expands. Tuberization initiates in stolon by enlargement of existing pith cells in its sub apical region followed by rapid cell division in parenchyma cells particularly associated with primedulla and inner cortex (Li. 1985). Tubers are metabolically highly active during this growing and filling stage (Ewing et al. 1992). The process of tuberization is accompanied with the expression of a large number of both constitutive and developmentally regulated genes which are involved in various housekeeping functions, starch biosynthesis and accumulation of different soluble proteins. For stolon differentiation darkness is an important factor required whereas sucrose and cytokinins are involved in tuber initiation on stolons (Paiva et al. 1983). Growth regulators also affect tuberization. Gibberellic acid (GA_3) inhibits tuberization whereas ethylene stimulates the process (Stallknecht and Farnsworth, 1982). 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 ranges of 1-3mM.

Biological roles of tubers are:

- They can store carbon and nitrogen in metabolized form.
- They can also act as propagules which are able to give rise to new plants by sprouting, but this property is not present in all type of tubers.

Nutrient profile of potato tubers:

Potato is a highly nutritious, digestible and whole some food. About 70-80% of potato tuber is water content and rest is dry matter. The major reserve materials present are complex carbohydrates, starch grains and proteins.

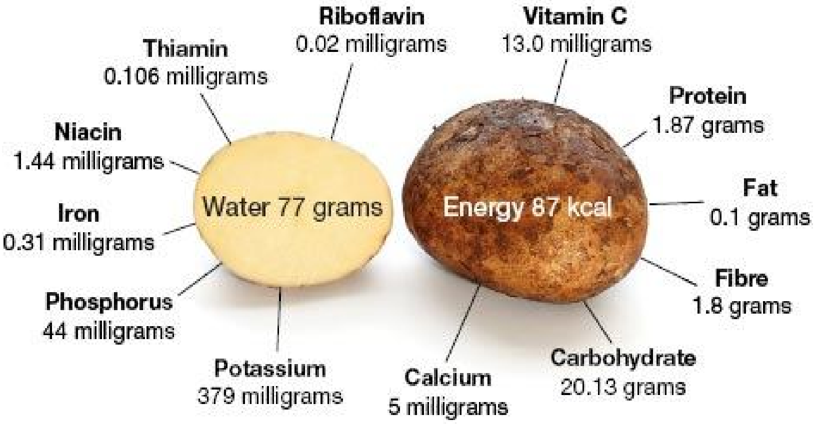


Fig. 2 Nutrient composition of Potato tubers

Two major biochemical attributes that differentiate tubers from other somatic tissues of potato are:

- Accumulation of large amount of starch.
- Accumulation of a set of relatively abundant proteins also called as storage proteins.

Amongst the storage proteins of tubers, major storage protein is a 40 kDa glycoprotein called ‘Patatin’. Other proteins are type II proteinases inhibitors, Bowman-Birk proteinase inhibitors and Kunitz trypsin inhibitors (Stiekema et al. 1988). Proteinase inhibitors provide defense against pathogens and other predators (Gatehouse and Boulter, 1983).

Out of 50% of tuber protein present in potato, 40% of the total soluble protein is contributed by ‘patatin’ (Prat et al. 1990). Patatin is present in high amounts in potato tubers so, it functions as a storage protein. Storage proteins, in general, can be defined as proteins whose major role is to act as source of nitrogen, sulphur and carbon. They may enable the plant to survive under 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.

1.3 Patatin-the major soluble protein in the tubers

Patatin is one of the major soluble glycoprotein in potato tubers encoded by multigene family. Patatin protein family represents the primary storage protein in potato tubers. These proteins also exhibit enzymatic function as lipid acyl hydrolases and have both plant defense and antioxidant activities (Strickland et al. 1995; Sharma et al. 2004). There are approximately 10-18 copies of patatin gene in each monoploid (12 chromosomes) potato genome Twell D and Ooms G (1988). All the patatin gene, map to a single locus on chromosome 8 (Ganal et al. 1991; Wenzler et al. 1989 a). Most of the patatin proteins are immunologically and biochemically indistinguishable (Mignery et al. 1984). The protein coding regions of patatin genes are highly homologous with one another, in most cases with more than 90% nucleotide sequence identity. Patatin is mainly localized in vacuoles, which is consistent with its hydrolytic activity. It is synthesized with an N- terminal signal peptide, which allows the polypeptide to enter in the lumen of endoplasmic reticulum. During the transport to endoplasmic reticulum, the signal sequence is cleaved off and the protein processes further to become N-glycosylated. The molecular mass of patatin monomer ranges between 39 and 43 kDa.

Patatin is also expressed in roots but at a 100-fold lower level then in tubers (Pikaard et al. 1987). Tuberization is always accompanied by patatin expression but in some instances patatin expresses in non-tuberous tissues. Patatin is expressed in petioles and stems of potato plant induced for tuberization upon removal of tubers and stolons. Under high levels of sucrose patatin accumulates in leaves of potato plant.

The promoter and protein coding regions of patatin genes up to position -87 remains homologous but upstream of this region the promoters diverge. This allows the patatin genes to divide in two classes: Class I and Class II. Class II patatin genes are characterized by the presence of a 22 bp sequence in the 5′-untranslated region which is absent in Class I patatin gene. Class I and Class II patatin genes have different expression patterns: class I patatin genes are mainly expressed in tubers whereas class II patatin genes are expressed in both tubers and roots but at a much lower level then class I patatin genes (Pikaard et al. 1987).

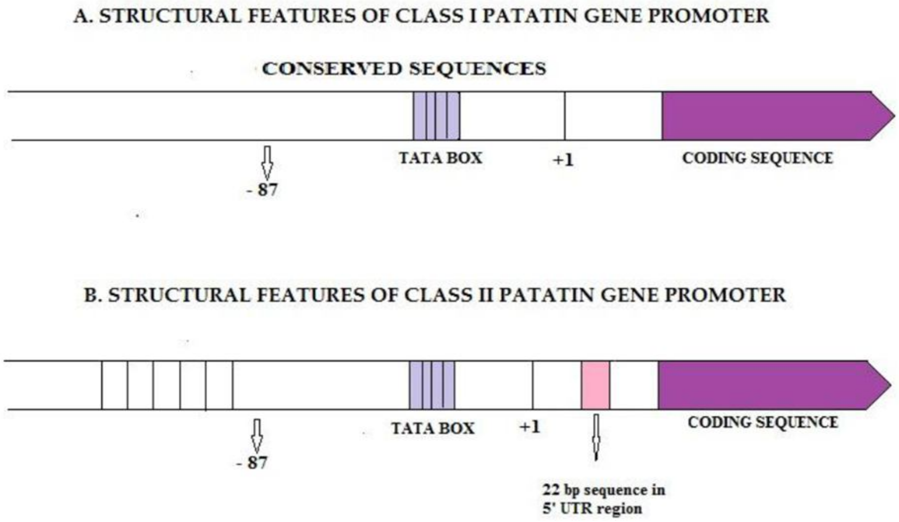


Fig. 3 Schematic view of Patatin gene promoters

Many studies have been done to know about organization, sequence and expression of patatin genes, but very less is known about the functional properties of patatin. Physiological role of patatin is still unknown (Shewry, 2003; Barta and Curn, 2004).

Chapter 2

REVIEW OF LITERATURE

The current section mainly deals with existing knowledge on patatin, particularly with the regulation of Class I patatin genes at biochemical, molecular and genetic level as described in the literature. By performing SDS-PAGE, molecular mass of patatin was estimated to be ~ 40 kDa (Racusen et al. 1984). Patatin protein shows extensive heterogeneity with forms differing in electrophoretic mobility at pH 8.6 on SDS-PAGE (Park et al. 1983). Patatin gene is encoded by a multigene family composed of approximately 64-72 copies in the tetraploid potato cultivar. About 33% of the patatin residues have been estimated to adopt α-helical and 46% β-stranded structure (Pots et al. 1998). All the Patatin genes are immunologically identical with in a cultivar as well as among the cultivars (Park et al. 1983). Patatin genes are further subdivided into two classes: Class I and Class II (Pikaard et al. 1987). Class I genes differs from class II genes by the presence of a 22 bp insertional sequence in 5′-UTRs of class II gene. The mature class I and class II patatin comprises 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 system leading to deposition in vacuoles (Sonnewald et al. 1989). Class I and class II genes have differential expression patterns. Class I genes are expressed predominately in tubers whereas class II are expressed at 50-100 lower level in roots than class I transcripts. By EM-immunocytochemistry it was shown that patatin is localized mainly in vacuoles of potato tubers (Sonnewald et al. 1989). The patatin protein is N-glycosylated but glycosylation has no influence on proteolytic stability and enzymatic properties.

2.1 Classification, molecular and functional characterization of patatin gene

Patatin is a group of major soluble glycoproteins present in potato tubers. They are encoded by multigene family with an estimated copy number of 10 to 18 per haploid genome, depending on cultivar (Twell and Ooms, 1988). Several cDNA and genomic clones were isolated and complete nucleotide sequence of both the promoter and the coding regions were determined for some of them (Bevan et al. 1986; Mignery et al. 1988; Rosahl et al.1986). Based on the results it was estimated that the sequences up to position -87 upstream from the transcription start point of the all patatin genes analyzed, are homologous suggesting commonality in the core promoter activities. But further upstream of position -87 the promoters diverge. This divergence leads to the division of patatin genes in two sub classes: class I and class II. The conserved regions contain the CAAT and TATA homologies as well as a homology to the core enhancer sequence.

2.1.1 Isolation of class I patatin gene and its expression pattern: Patatin cDNA pcT58 probe was used to screen the genomic library established in λ-phage (Rosahl et al.1986) and it yielded 13 hybridized clones. Further these clones were screened for class I patatin gene by southern blot analysis and two class I patatin genes were identified namely B24 and B33. Comparison with other genomic clones revealed a high degree of homology between class I and class II genes in the region of first exon and in some part of upstream sequence. Homology to class II genes ends after position -86 whereas the homology to other class I genes extends up to 1.7 kb (Bevan et al. 1986). 208 Nucleotides long direct repeat was seen in B33, which was itself composed of sub repeats. These types of repeats were also seen in class I genes analyzed so far.

To check the expression pattern of class I patatin genes, a chimeric gene was constructed containing the 5′-flanking region of class I patatin gene B33 fused with coding region of beta-glucuronidase gene and transferred into potato. Expression level was highest in tubers followed by stems and roots and lowest in leaves (Rocha-Sosa et al. 1989). By performing immunocytochemical assays, it was analyzed that the glucuronidase activity of the chimeric patatin gene is restricted to parenchymatic cells only. Histochemical assays revealed that no expression was seen in periderm as this tissue is composed of dead cells. A variety of environmental, metabolic and developmental factors are involved in the control of patatin gene expression.

Further studies of class I patatin gene promoter revealed that a highly conserved 100-bp region containing two repeat domains, the A-box and the B-box is important for the regulation of class I patatin gene expression (Grierson et al. 1994). It was observed that nuclear proteins bind to A-box and B-box and these proteins were speculated to be transcription factors. The protein that binds to B-box has been identified named ‘Storekeeper’ and it is thought to be regulator of patatin gene expression (Zourelidou et al. 2002).

2.1.2 Sucrose inducibility of class I patatin gene promoter: Class I gene promoter drive predominant expression in the tubers, but they are not strictly tuber specific; since the expression of chimeric gene (patatin–GUS construct) could be highly induced in cultured stem and leaf explants by increasing the concentration of sucrose. Expression patterns of class I patatin chimeric-GUS gene in leaves of the transgenic potato plants kept in MS medium containing high (7%) and low (2%) levels of sucrose were studied by histochemical and flurometric GUS assay. It was revealed that high sucrose concentration had a prominent effect in terms of expression of the chimeric gene in leaves (Rocha-Sosa et al. 1989). The expression was seen in both spongy and palisade mesophyll cells and in epidermal cells but is absent in vascular tissue and rarely detectable in hair cells and guard cells.

2.1.3 Identification of cis-acting elements in 5′-flanking regions of class I patatin gene promoter: A series of experiments had been conducted with the aim to defining the *cis*-acting elements (i.e. enhancers/silencers) necessary for the differential expression in different cell types/tissues. The experiments were also designed to test whether the 'tuber specific' elements and 'sucrose responsive' elements were separate identities on the same promoter (Jefferson et al. 1990). It was revealed that a minimal promoter extending 360 bp, 5′ of the transcriptional start site gave sucrose responsiveness as measured in *in vitro* experiments by growth of nodal explants on high and low sucrose concentrations and tuber specific expression measured form glass house-grown plants. Promoter extending to 600 bp of the transcriptional start site showed higher levels of expression in organs other than tubers and in tissues grown on lower level of sucrose. Addition of longer promoter regions, extending up to 3500 bp, 5′end of the transcription start site led to higher level of tuber specific expression and lower expression in leaves and other organs. The most proximal region of patatin promoter i.e. 360 bp region extending from 5′ of the transcription start site was fused with a heterologous truncated CaMV 35S promoter and high degree expression was seen in tubers (Benfey et al. 1989). The data obtained from these experiments showed that two pairs of repeats found at – 154 to – 224 bp and at – 471 bp to – 579 bp act as *cis*-elements that confer tuber specific expression (Kuhlemeier et al. 1987).

2.1.4 Studies on class II patatin genes: Class II patatin genes are characterized by the presence of 22 bp insertion sequence in the 5′-untranslated region. Class II promoters were fused to CAT (chloramphenicol acetyltransferase) (Twell D and Ooms G 1987). Fusion of class II promoter that does not include 22 bp sequences with GUS gene expressed normally with low level of expression in roots and tubers. From this observation it was analyzed that 22 bp sequence does not have a significant role in determining the expression patterns of class II genes.

2.2 Biochemical attributes of patatin protein

Patatin exhibits enzymatic activity. Patatin has a lipid acyl hydrolase activity and can cleave a variety of free fatty acids from glycolipids and phospholipids (Racusen, 1984). It has specificity for acyl chains of lengths 10-16 (Vancanneyt et al. 1989). Acyl hydrolase activity increases when tubers get disrupted. Patatin also shows esterase activity and it was confirmed by expression of patatin in transgenic tobacco plants (Rosahl et al. 1987). Lipid acyl hydrolase activity was characterized by cloning patatin cDNA clone pGM01 in baculo virus vector (Andrews et al. 1988). Galliard in 1970 reported that patatin also exhibits acyl transferase activity along with acyl hydrolase activity. Addition of methanol to the lipid acyl hydrolase reaction mixture permitted the formation of methyl esters with 30% (v/v) methanol, leading to a maximum of 75% methyl ester conversion of the original substrate. The tuber lipid acyl hydrolase is normally sequestered in lysosomes (Wardale and Galliard, 1975), thus biologically inactive, but upon mechanical damage or pathogen invasion the enzyme would be released. Patatin provides substrates for production of toxic fatty acid derivatives and waxes that could inhibit the invasion of pathogens. Patatin is also involved in phytoalexin production, since it would release arachidonic acid, which is a known potent elicitor of phytoalexins (Bostock et al. 1981).

Apart from enzymatic functions, patatin exhibits plant defense and antioxidant activities (Strickland et al. 1995; Liu et al. 2003). Patatin exhibits hydrolytic activity as an acidic β 1,3- glucanase (Tonon et al. 2001). β 1,3-glucanases contribute to plant defense by digesting β-1,3-glycans in hyphal cell walls of fungal pathogens 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 defense of potato tubers. Inclusion of patatin in artificial diets resulted in inhibition of growth of larvae of corn rootworm, *Diabrotica spp*. (Strickland et al. 1995). This indicates that patatin plays a role in plant defense against pests and pathogens.

Patatin also elicit allergic responses in humans and children either when consumed or by skin contact with raw potatoes. Immunoblotting results show strong IgE (a class of immunoglobulins specific for allergic responses) binding to a 43-kDa protein, identified as patatin, the main storage protein of potato tubers. Astwood et al. (2000) identified the IgE-binding epitopes of patatin and within them, substitutions in amino acid residues that appear to mitigate IgE binding to these peptides. 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). A major allergen of latex, called Hev b 7 of molecular wt. 43KDa had a sequence homology to patatin (Kostyal et al. 1999; Briteneder et al. 1999).

Relevance of the present study

Patatin genes are encoded by a large multigene family. The presence of many patatin genes in the potato genome indicates that different members of this multigene family are likely to have varying 5′-flanking sequences. 5′-flanking regions of different patatin genes have diverse *cis*-regulatory sequence motifs i.e. enhancers/silencers and characteristic sequence features and so they respond to different environmental, metabolic and developmental signals. Patatin promoters are used in molecular farming for the production of novel proteins in large amount. So by gene-fusion with a patatin promoter, cheap production of proteins could be achieved. However, only few members of class I and class II patatin genes have been identified at molecular level till date. It is noteworthy that many more functional patatin genes are yet to be isolated and characterized. Isolation and characterization of functional patatin genes help in understanding expression patterns, regulation and over all biological role of the individual members of multigene family. Therefore, molecular cloning studies would help in isolating efficient tuber specific promoters from potato; particularly from the Indian potato cultivars since their vast genetic resources still remain hugely untapped. Moreover, sequence features of the patatin promoters could be analyzed for identification of the *cis*-regulatory elements essential for high level tuber-specific expression. Present work will be useful in getting efficient promoters for targeted gene expression, and also in the areas of crop improvement and ‘molecular farming’.

Background work in our laboratory

Earlier, in our laboratory the focus was on cloning and characterization of 5′-flanking regions of class I patatin genes from the Indian potato cultivars namely Kufri Chipsona-1(CS-1), Kufri Chipsona-2 (CS-2), Kufri Chandramukhi (KCM) and Kufri Jyoti (KJ). The isolation of Class I patatin genes, and functional characterization of their promoters had been actively persuaded in our lab. A total of five partial Class I patatin genes were isolated from different potato cultivars and submitted to the NCBI GenBank database under the Acc. No’s JX124227, cv. Kufri Jyoti; JX124228, cv. Kufri Chipsona-1; JX124229, cv. Kufri Chipsona-1; JX124230, cv. Kufri Chandramukhi; and JX124231, cv. Kufri Chandramukhi; respectively. A series of combinations of forward and reverse primers were designed based on class I and class II patatin gene sequences available in data base to amplify the 5′-flanking regions of these Indian potato cultivars. Aim of the present study is to do molecular cloning studies for partial characterization of the patatin genes of these Indian potato cultivars.

Objectives

- Isolation of genomic DNA from the Indian potato cultivars
- Amplification of partial class I patatin genes having 5′-flanking regions by PCR approach using gene-specific primers
- Molecular cloning and partial characterization of the amplified DNA products

Chapter 3

MATERIALS AND METHODS

3.1 Plants and other materials

3.1.1 Potato germplasm procurement

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

3.1.2 Other materials

Various enzymes used were purchased from Bangalore Genei Pvt.Ltd., Bangalore and Amersham Biosciences Ltd. Hong Kong. The chemicals required were bought from Sisco Research Laboratories Pvt. Ltd, and Himedia Pvt. Ltd, Mumbai. Primers used were synthesized by Bangalore Genei Pvt.Ltd, Bangalore. The gel extraction Qiagen kit was purchased from Genetix.

3.1.3 Strains and plasmids

E.coli DH5α: supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1

The strain *E.coli* DH5α was routinely maintained on Luria agar medium whereas the transformant E.coli DH5α having pUC19 plasmid vector was maintained on Luria agar-Ampicillin (50µg mL$^{-1)}$ medium. pUC19: GenBank Accession no. X02514 (Yanisch-Perron et al. 1985). pUC19 is a commonly used cloning vector .It’s size is 2686 bp having 54-bp multiple cloning site in which there are unique sites of different restriction endonucleases. It is a high copy number plasmid.

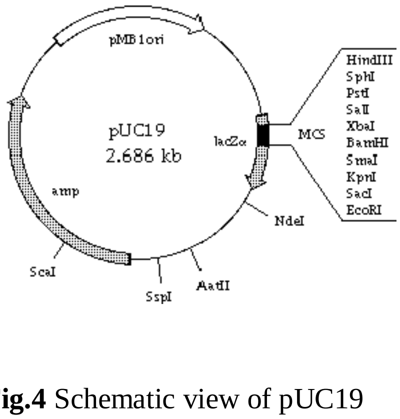


Fig.4 Schematic view of pUC19

3.1.4 Media

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 50μg mL^{-1} .Media was used after autoclaving it.

3.1.5 Buffers

Gel loading buffer (5X)

Sucrose - 35 % (w/v)

EDTA - 50 mM (pH 8.0)

Tris – 25 mM

Bromophenol blue - 0.2 % (w/v)

Solutions used for plasmid isolation by alkali lysis method

Solution I - 50 mM glucose, 10 mM EDTA, 25 mM Tris.HCl pH 8.0

Solution II - 0.2 M NaOH, 1.0% SDS (needs to be freshly prepared)

Solution III - 3.0 M with respect to potassium and 5.0 M with respect to acetate

TBE (5X) buffer

Tris Base - 54 g L^{-1}

Boric acid - 28 g L^{-1}

EDTA - 3.8 g L^{-1}

The pH of the buffer was set at 8.0

TE buffer

Tris.HCl - 10 mM (pH 8.0)

EDTA - 1 mM (pH 8.0)

3.1.6 Enzymes used in molecular techniques

Restriction enzymes

Restriction enzymes such as *Eco*RI, *Hind*III, *Bam*HI 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 was added as required (for e.g. BSA is required during *Bam*HI restriction digestion reaction).

Other enzymes

Ribonuclease A

Stock solution - 10 mg mL^{-1}

Working solution - 10-15 mg mL^{-1}

DNAse free Ribonuclease A was prepared in a buffer containing 10 mM Tris (pH 8.0) and 15 mM NaCl. The solution was boiled for 10 minutes, followed by the slow cooling by placing at room temperature for 2 hours, and then stored at - 20 °C for subsequent use.

Lysozyme

Stock solution - 10 mg mL^{-1}

Working solution - 300-400 mg mL^{-1}

Lysozyme was freshly prepared during regular work.

T4 DNA Ligase

Stock conc. - 400 U mL^{-1}

Working conc. - 40 U mL^{-1}

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

Klenow fragment of DNA polymerase I

Stock conc. - 5U mL^{-1}

Working concentration - 2U 50 mL^{-1} of the reaction volume.

3.1.7 Chemicals/Biochemicals

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

Stock conc. - 20 mg mL^{-1}

Working conc. - 20 μg mL^{-1}

X-gal was prepared by dissolved in N, N- dimethyl formamide.

IPTG (Isopropyl thio ß- D- galactoside)

Stock conc. - 100 mg mL^{-1}

Working conc. - 100 μg mL^{-1}

It was prepared in fresh and sterile (autoclaved) water.

3.2 Methods

3.2.1 Genomic DNA isolation from potato cultivars

Genomic DNA was isolated from the potato cultivars by the protocol described by Kumari et al. (2012). 2.0 to 3.0 g of plant material was taken and was washed with sterile water and grounded to fine powder in the presence of liquid nitrogen using mortar and pestle. The fine powder was then transferred to conical flask containing 15 mL extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA (pH 8.0), 250 mM NaCl and 15% (w/v) sucrose) maintained at 65°C. Contents were mixed properly and incubated at 65°C for 20 min with intermittent gentle shaking. Then 5 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 g at 4°C for 25 min. The supernatant was filtered through a fine muslin cloth and 0.70 volume of isopropanol was added, mixed gently and incubated at -20°C for 4-5 hrs. DNA was extracted by centrifugation at 10000 g at 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). Further purification of DNA was done by treatment with DNase –free RNase followed by solvent extraction step twice using mixture of phenol: chloroform: isoamyl alcohol (25:24:1). DNA was then precipitated using 0.1 volume of 3.0 M sodium acetate (pH 5.5) and 2.0 volumes of ethanol and the DNA was finally recovered by dissolving in 200-250 μL of TE buffer and stored at -20°C. For miniscale isolation i.e. 0.1 to 0.2 g plant material, same protocol was scaled down accordingly. The quality and quantity of DNA was initially checked spectrophotometrically by measuring the A260/A280 ratio and 260 nm respectively.

3.2.2 Designing of Class I patatin gene specific 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). Class I patatin gene sequence of 1770 bps is comprised of the following structural features: The TATA box starts at the base 1338 and the transcriptional start site is located at the base 1361 corresponding to first exon. So, the above gene sequence provides an extended class I patatin gene promoter. The translational start site is present at the base 1407. The 23 amino acid transit peptide of patatin gene is encoded by nucleotides bases from 1407 to 1475. This peptide refers to vacuolar targeting signal.

➢ To ensure minimum chances of non-specific amplification, important factors kept in consideration while designing the primers were:

- PCR primers should be 10-24 nucleotides long.
- GC content should be 40-60%.
- The primer should not be self-complementary or complementary to any other primer to form primer-dimer or hair pin.
- Melting temperatures of primer pairs should not differ by more than 5º C, so the GC content and length must be chosen accordingly.
- The annealing temperature should be about 5°C lower than the melting temperature.
- Long run sequences of a single nucleotide should be avoided.
- Primers with significant structures 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 of patatin gene. This could facilitate in studying different lengths of patatin promoter and to check the divergence in the upstream sequences of patatin isoforms.

PT-F074 is a forward primer that corresponds to bases 74-93 of the genome sequence having 40% G + C content.

Sequence of PT-F074: 5′-TAATTGACCGGAGACTATAC -3′

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

Sequence of PT-F610: 5′-TTCTTATCAATTCTGACGTG -3′

As the transcription start site is located at 1338 base, both forward primers belong exclusively to extended promoter region of the gene.

PT-R1478 is a reverse primer that is complementary to the bases 1478-1459 of the genome sequence having 45% G + C content.

Sequence of PT-R1478: 5′-CGTAGCACATGTTGAACTAG -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)

PCR was used to amplify a specific DNA sequence in a simple, rapid and automated manner using forward and reverse primer. PCR is repeated cycling of three steps: heat denaturation of template DNA (94°C); annealing of primers to the complementary sequences in template DNA (55°C); extension of annealed primers by a thermo stable DNA polymerase (72°C).

In PCR, genomic DNA preparations from the following two potato cultivars were used:

- Kufri Chipsona-2 (CS-2)
- Kufri Jyoti (KJ)

Primer pair used to carry out PCR was: PT-F610 and PT-R1478

Composition of PCR reaction:

Total reaction volume: 50 µL	
Template DNA	3 µL
Buffer 10X	5 µL
Forward primer	10 pmoles
Reverse primer	10 pmoles
dNTP’s	25mM
Sterile deionized water	volume made up to 50 µL
Taq DNA polymerase	3U/µL

The thermal cycling parameters were as given below:

Step	*Temperature*	*Time*
Denaturation	94°C	1 min
Annealing	55°C	2 min
Polymerization	72°C	3 min

The reaction was carried out for 30 cycles with final extension at 72°C for 5 minutes.

3.2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using standard methods (Sambrook- a laboratory manual). 0.7% agarose gel was made in 0.5X TBE buffer and ethidium bromide dye (0.5μg mL^{-1}) was added to it. Gel was then casted in the casting tray. The DNA samples were loaded in the wells after solidification of gel. Electrophoresis was carried out in 0.5X TBE (running buffer) at 2 – 5 Volt per 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

Klenow fragment is produced by the proteolytic cleavage of *E. coli* DNA polymerase I by protease subtilisin. It retains the 5′-3′ polymerase and 3′-5′ exonuclease activity. Processing of PCR products is done by klenow treatment. This treatment makes the PCR products truly blunt ended by removing an extra ‘A’ residue from 3′ ends. This is also called the polishing step.

Reaction mixture for Klenow treatment:

Total reaction volume: 40 μL	
PCR amplified products	22 μL
Klenow enzyme buffer	2 μL
dNTPs	0.9 μL
Klenow enzyme	1 μL
Sterile deionized water	volume made up to 40 μL

Klenow treatment was carried out at 28°Cfor 40min and the reaction was stopped by incubating at 65°C water bath for 5-7 min. The klenow treated PCR products were then purified, precipitated and finally dissolved in TE buffer.

3.2.6 Elution of DNA bands from agarose gel

Qiagen kit was used to elute the DNA bands of Kufri–Chipsona-2 and Kyfri Jyoti for cloning purposes. QIAEX II agarose gel extraction protocol was followed for elution of DNA bands. DNA samples were run in 0.8% agarose in 1X TAE buffer. In one lane, DNA sample was loaded as control. This control lane was excised with a clean scalpel and visualized under UV-trans illuminator to mark the position of DNA bands. The corresponding DNA bands were excised from the gel (without UV exposure) by matching the position of bands in the control lane. The gel slices containing the DNA bands were weighed in 1.5 mL microfuge tubes. As per the manufacturer's instructions, three volumes of QX I were added to one volume of gel. Following this, 20 µL of QIAEX II was added and mixed properly by vortexing for 30 seconds. The tubes were then incubated at 50°C in water bath for 10 minutes with intermittent vortexing after every 2 minutes for 30 sec in order to solubilize the agarose. This was done to keep QIAEX II in suspension. The samples were then centrifuged at 13,000 rpm for 30 sec and supernatant was removed very carefully with the help of a pipette. The pellet was washed with 500 uL of buffer QX I by vortexing (for resuspending the pellet) and then centrifuging at high speed for 30 sec followed by removal of supernatant. In the same manner, pellet was washed two times with 500 µL of PE buffer. Pellet was properly air dried in the laminar air flow until it appeared in a white powdery form. 7 µL of DEPC treated water and 7 µL of TE buffer was added to the dried pellet. Pellet was resuspended in TE buffer by vortexing for 15-20 sec. It was incubated at 50°C water bath for 2 minutes. Then centrifugation was done at 13,000 rpm for 30 sec. The supernatant containing the purified DNA was carefully transferred to a fresh sterile microfuge tube. The last two steps were again performed to increase the yield of DNA. 2.5 µL of eluted DNA was loaded in 0.8% agarose gel to check the yield.

3.2.7 Preparation of linearized vector

Restriction digestion reaction: Restriction enzymes are the enzymes that cut or digest the DNA with in or adjacent to the specific recognition nucleotide sequences known as restriction sites. Restriction digestion is usually carried out in 15 µL volume for 2½ hours at optimum temperatures, depending on the nature of restriction enzymes used.

pUC 19	1 µL
Restriction enzyme	1 µL (2 units)
Buffer	Used at a concentration of 1X (as supplied by manufacturer)
Sterile deionized water	To make up the final volume

Phenol-chloroform extraction of linearized plasmid vector was done to remove the impurities such as restriction enzymes left behind after restriction digestion reaction which can inhibit the ligation reaction.

3.2.8 Ligation reaction

Ligation reaction was set up to ligate the insert into plasmid vector pUC 19. The enzyme T4 DNA ligase was used in order to carry out ligation. The ends of vector DNA and the insert are covalently ligated by the formation of phosphodiester bonds between the 3′-hydroxyl group of one DNA terminus and 5′-phosphoryl of another. T4 DNA ligase has the ability to join both blunt end and cohesive end termini.

Ligation reaction is composed of following components:

Total reaction volume: 15 µL	
Linearized vector	3 µL
Insert	5 µL
T4 DNA ligase buffer (blunt ended)	3 µL (used at a concentration of 1X)
T4 DNA ligase	1 µL

The reaction mixture was kept at 18°C for overnight. Ligation reaction was carried out in three different sets for 3 different inserts, two inserts were of KJ and third was of CS-2. Each ligation mix was used separately to transform competent E.coli DH5α.

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

E.coli DH5α was transformed with different recombinant plasmids containing desired DNA inserts from cultivars Kufri Jyoti and Kufri Chipsona -2 using the standard $CaCl_2$ method (Mandel and Higa 1970).

Preparation of competent cells: A single colony of *E.coli* DH5α from a freshly grown plate was inoculated in 25mL of Luria broth and incubated at 37°C for overnight with vigorous shaking (200-250 cycles per minute in a rotary shaker). 200µL of the above grown culture was further inoculated in 20 mL of fresh Luria broth in aseptic conditions and incubated at 37°C for 2-3 hours in a rotary shaker to obtain an O.D. around 0.4 to 0.5 at 590 nm. The culture was kept on ice to slow down the metabolism of cells for 5 minutes. Culture was pelletized by centrifugation at 8000rpm for 10 min. Cell pellet was resuspended in 10mL of ice cold 50mM $CaCl_2$ and kept in ice bath for 5-10 min. The cells were recovered by centrifugation at 8000 rpm for 10 minutes at 4°C. Pellet was then resuspended in 1mL of pre-chilled 50mM $CaCl_2$ and kept on ice for 2½ hours.

Transformation: 100µL of competent cells was dispensed in pre-chilled sterile microfuge tubes. 7µL of ligation mix was added to each microfuge tube containing competent cells suspension. It was mixed well and kept at 4°C for 30 minutes. Heat shock was given to all the microfuge tubes at 42°C for 2 min followed by addition of 1mL Luria broth and incubation at 37°C for 1½ hour. The culture was then centrifuged at 7000 rpm for 7 min. 600-800µL of supernatant was discarded to concentrate the cells. The pellet was resuspended with the help of a pipette. 100µL of the above transformed cell suspension was plated on Luria-agar (LA) plates containing ampicillin (50 µg mL^{-1}) with X-Gal and IPTG. The plates were incubated for overnight at 37°C. The transformants were selected by blue-white screening. Each pure white colony containing the recombinant pUC I9 was picked up with a sterile tooth pick and patches were made on LA plates containing ampicillin with X-Gal and IPTG.

3.2.10 Plasmid isolation

Alkali lysis method: At mini scale plasmid isolation was carried out by alkali lysis method as described by Brinboim and Doly (1979). Transformed *E.coli* colony was inoculated in 25 ml of Luria broth containing ampicillin (50µg mL^{-1}). The culture was inoculated at 37°C for overnight with vigorous shaking. 1.5mL of the above grown culture was poured in microfuge tubes and centrifuged at 8000 rpm for 5min. supernatant was decanted and pellet was resuspended in 200µL of ice cold Solution I with vigorous vortexing followed by addition of 30µL of lysozyme (10mg mL^{-1}) with proper mixing. Then 400µL of freshly prepared solution II was added and mixed gently. The tubes were then stored on ice for some time. Then 300µL of ice cold Solution III was added and mixed by gentle inversion of tubes till curdy white precipitate was formed. The tubes were stored on ice for 15 to 20 min. the tubes were centrifuged at 12,000 rpm for 10 min at 4°C. Supernatant was carefully transferred to fresh microfuge tubes. DNA was precipitated with equal volume of isopropanol and was kept at 4°C for 30 min. Then centrifugation was done at 10,000 rpm for 10 min at 4°C. Supernatant was decanted and pellet was air dried and dissolved in 30µL of TE buffer. Further purification of crude plasmid was done by adding 370 µL of sterile water to crude plasmid dissolved in TE buffer, followed by addition of 4µL DNase free RNase to remove RNA contamination. The tubes were then incubated at 37°C for 30 – 45 min. Extraction of DNA was done with equal volume of phenol and chloroform. Then centrifugation was done at 8000 rpm for 10 min and upper aqueous layer was transferred to fresh microfuge tube and 1/10th volume of 3M sodium acetate was added and mixed well for 5 min. Double volume of dehydrated alcohol was added and mixed properly. It was kept at -20°C for overnight. Centrifugation was done at high speed for 10 min and supernatant was decanted. Pellet was washed with 70% ethanol and air dried and then dissolved in 30µL TE buffer.

Chapter 4

RESULTS AND DISCUSSION

Patatin is encoded by a multigene family and different members of this multigene family respond to different metabolic and developmental signals. The present project work focused on molecular cloning of the 5′-flanking regions of class I patatin gene promoters from Indian potato cultivars Kufri Jyoti and Kufri Chipsona-2 in plasmid vector pUC19 and partial characterization by restriction digestion and PCR analysis.

The results obtained by step-wise experiments are given in the following sections:

4.1 Isolation of potato genomic DNA

Total genomic DNA was isolated from agronomically important Indian potato cultivars namely Kufri Jyoti and Kufri Chipsona-2 (see section 3.2.1 of 'Materials & Methods'). The quality of genomic DNA was checked by agarose gel electrophoresis as shown in Fig.5.

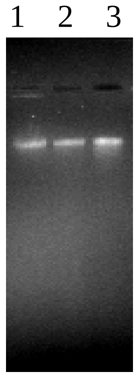


Fig. 5 Genomic DNA isolation from potato cultivars, Kufri Jyoti and Kufri Chipsona-2

Lane 1- λ DNA; Lane 2- Kufri Jyoti (KJ) genomic DNA; Lane 3- Kufri Chipsona-2 (CS-2) genomic DNA

4.2 Isolation of partial class I patatin genes consisting promoter regions

The 5′-flanking regions of class I patatin genes of the potato cultivars Kufri Jyoti and Kufri Chipsona-2 were amplified using primer pair PT-F610 (forward primer) and PT-R1478 (reverse primer). Two DNA fragments of Kufri Jyoti were amplified of sizes ~ 1.5 kb (named as K I) and ~ 2.0 kb (named as K II); one DNA fragment of Kufri Chipsona-2 was amplified of size ~ 1.0 kb (named as C I) as shown in Fig. 6. All these PCR amplified DNA fragments from Kufri Jyoti and Kufri Chipsona-2 were used for cloning and partial characterization.

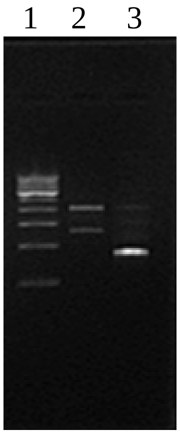


Fig. 6 PCR amplified products using gene specific primers

Lane 1- 500 bp ladder; Lane 2- PCR amplified bands of KJ; Lane 3- PCR amplified band of CS-2

4.3 Molecular cloning studies

4.3.1 Preparation of plasmid vector: The plasmid vector pUC I9 was isolated from *E.coli* DH5α (pUC19) strain by alkali lysis method. The intactness of the isolated plasmid DNA vector was checked through restriction digestion with different restriction enzymes such as, *Eco*RI, *Hind*III and *Sma*I (shown in Fig. 7). For cloning purpose, the plasmid DNA vector was linearized by restriction digestion with *Sma*I (blunt ended hexacutter). The PCR amplified products from KJ and CS-2 were cloned in *Sma*I site of the plasmid vector pUC19.

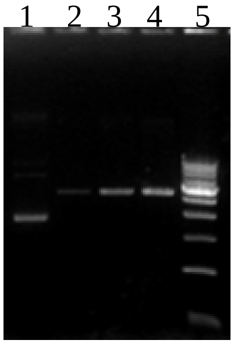


Fig. 7 Restriction analysis of pUC19 with *Eco*RI, *Hind*III and *Sma*I enzymes

Lane 1- control pUC19; Lane 2- pUC19 digested with *Eco*RI; Lane 3- pUC19 digested with *Hin*dIII; Lane 4- pUC19 digested with *Sma*I; Lane 5- 500 bp ladder

4.3.2 Elution of DNA bands

Recovery of DNA bands using Qiagen kit: PCR products of KJ i.e. ~ 1.5 kb and ~ 2.0 kb and of CS-2 i.e. ~1.0 kb DNA fragments were treated with Klenow enzyme. For ligation, the termini of vector and insert should be compatible to each other. Generally, the PCR amplified DNA products are not truly blunt ended. Therefore, these DNA fragments were polished with Klenow fragment of *E.coli* DNA polymerase I. Further DNA bands were purified by Qiagen kit (following QIAEX II agarose gel extraction protocol). Fig. 8 A & B shows eluted DNA fragments.

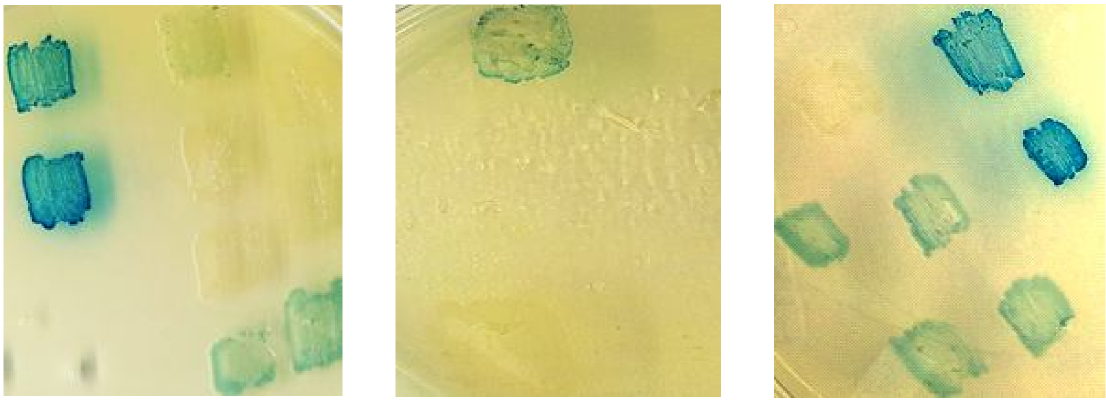
Fig. 8 Agarose gel electrophoresis showing eluted DNA bands of KJ and CS-2.

A Lane 1- 500 bp ladder; Lane 2- KJ specific PCR products; Lane 3- ~ 2.0 kb eluted band of KJ(K II); Lane 4- ~ 1.5kb eluted band of KJ (K I)

B Lane 1- 500 bp ladder; Lane 2- CS-2 specific PCR product; Lane 3- ~1.0 kb eluted band of CS-2 (C I)

4.3.3 Putative recombinant clones of KJ and CS-2

In this study, three sets of blunt end ligation reactions were carried out separately using purified eluted DNA fragments of KJ and CS-2 and linearized vector pUC19, and incubated overnight at 18°C. These ligation mixtures containing recombinant pUC19 (r-pUC19) were used to transform *E.coli* DH5α. Transformation was carried out by using the standard $CaCl_2$ method (see section 3.2.9 of Materials & Methods). A number of white transformed colonies were obtained on LA- ampicillin plates containing X-Gal and IPTG corresponding to each ligation mix. The putative white colonies were further purified to single colonies shown in Fig. 9 A, B & C.



A B C

Fig. 9 A Putative white colonies containing r-pUC19 corresponding to insertK I of KJ

Fig. 9 B Putative white colonies containing r-pUC19 corresponding to insert K II of KJ

Fig. 9 C Putative white colonies containing r-pUC19 corresponding to insert C I of CS-2

Plasmid DNA was isolated from the white colonies by alkali lysis method (see section 3.2.10 of Materials & methods) and further analyzed to check whether the plasmid contain desired inserts or not. Putative clones were analyzed in agarose gel on the basis of mobility of bands. Kufri Jyoti specific putative clones of K I fragment i.e. ~ 1.5 kb size were designated as K I-1, K I-2 and K I-3; and of K II fragment i.e. ~ 2.0 kb size was designated as K II-1. Kufri Chipsona-2 specific putative clone of size ~ 1.0 kb was designated as C I. Fig. 10 shows the putative clones of KJ and CS-2 having lesser mobility then control pUC19. Hence, these clones are likely to have desired inserts, except K I-3, as its size is comparable to control pUC19. So, it may be a spurious clone.

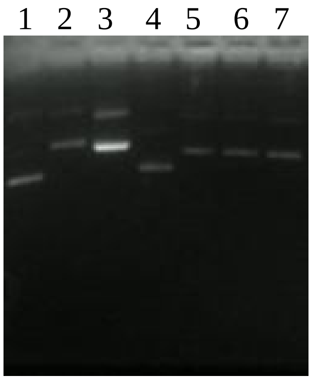


Fig. 10 Comparison of putative clones of KJ and CS-2 with control pUC19

Lane 1- control pUC19; Lane 2- K I-1; Lane 3- K I-2; Lane 4- K I-3; Lane 5- K II-1; Lane 6- C I (1); Lane 7- C I (2).

4.4 Partial characterization of putative clones

Restriction digestion of recombinant plasmids: Restriction digestion of the recombinant plasmids carrying desired inserts was performed using restriction enzymes namely *Eco*RI and *Bam*HI. By restriction digestion it was analyzed that all the putative clones of KJ and CS-2 were digested with both the enzymes and it was apparent that there was no internal site for these restriction enzymes in genomic cloned inserts of KJ and CS-2 as shown in Fig. 11 A & B

A

B

Fig. 11 A Restriction digestion of putative clones of KJ and CS-2 with *Eco*RI

Fig. 11 B Restriction digestion of putative clones of KJ and CS-2 with *Bam*HI

A Lane 1- 500 bp ladder; Lane 2- control pUC 19 digested with *Eco*RI; Lane 3- K I-1 digested with *Eco*RI; Lane 4- K I-2 digested with *Eco*RI; Lane 5- K I-3 digested with *Eco*RI; Lane 6- K II-1 digested with *Eco*R1; Lane 7- C I(1) digested with *Eco*RI; Lane 8- C I(2) digested with *Eco*RI

B Lane 1- 500 bp ladder; Lane 2- control pUC 19 digested with *Bam*HI; Lane 3- K I-1 digested with *Bam*HI; Lane 4- K I-2 digested with *Bam*HI; Lane 5- K I-3 digested with *Bam*H1; Lane 6- K II-1 digested with *Bam*H1; Lane 7- C I(1) digested with *Bam*HI; Lane 8- C I(2) digested with *Bam*HI

4.5 PCR characterization of putative clones

Apart from restriction analysis, the recombinant plasmids containing the inserts were used as templates to carry out PCR using the gene-specific primers i.e. PT-F610 (forward primer) and PT-R1478 (reverse primer) both for KJ and CS-2. As shown in Fig. 12 only the K I-2 i.e. the 1.5 kb size DNA insert was amplified in PCR. During PCR amplification and subsequent processing of the amplified DNA fragments by molecular techniques, possibly there could be damage at the DNA ends, very likely due to nuclease actions. Sometimes the ends of the amplified DNA could be incomplete. Probably this may explain why PCR amplification did not occur while the clones K II-1 and C I were used as templates. However, this needs to be further addressed.

Fig. 12 PCR based characterization of putative clone of KJ

Lane 1-500 bp ladder; Lane 2- Mother PCR KJ; Lane 3- PCR amplified K I-2 (~ 1.5 kb insert of KJ)

Concluding remarks

Patatin is encoded by a relatively large multigene family. Both Class I and Class II patatin genes differ mostly in their 5′-flanking regions. Therefore, the study of promoter regions of different patatin genes of the multigene family is important with regard to basic and applied aspects of research. Molecular cloning studies would help in isolating functional patatin genes from different potato cultivars, since there vast genetic resources still remain untapped till date.

In this study efforts were made for the isolation of partial class I patatin genes from the potato cultivars namely Kufri Jyoti and Kufri Chipsona-2 through PCR approach using gene specific primers. ~1.0 kb DNA fragment of CS-2 amplified by PCR using specific primer pair was consistent with other potato cultivars as observed in our laboratory earlier. However, further sequence analysis of this CS-2 derived DNA fragment may reveal some degree of microheterogeneity which could influence the overall functionality of its promoter region. The cv. KJ specific ~2.0 kb amplified DNA product appeared to be consistent with the earlier cloning studies in our laboratory (data not shown). Interestingly, ~1.5 kb PCR amplified-DNA product as obtained from the cv. KJ appeared to encode a distinct member of the patatin multigene family not documented earlier. Therefore, this ~1.5 kb DNA fragments needs to be thoroughly characterized by sequencing. Thorough sequence characterization along with functional characterization of the individual 5′-flanking regions as isolated in this study would reveal their tissue specificity and overall functionality.

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