MOLECULAR CLONING STUDIES ON PATATIN GENE PROMOTERS FROM POTATO CULTIVARS

A

Thesis submitted
In partial fulfilment for the award of the
Degree of Master of Science in Biotechnology

BY

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CERTIFICATE

This is to certify that the thesis entitled, **“MOLECULAR CLONING STUDIES ON PATATIN GENE PROMOTERS FROM POTATO CULTIVARS”** submitted by Saloni Bansal in partial fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology, to Thapar Institute of Engineering and Technology (Deemed University), Patiala is a record of student’s own work carried out by her under my supervision and guidance. The 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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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled, "**MOLECULAR CLONING STUDIES ON PATATIN GENE PROMOTERS FROM POTATO CULTIVARS**" in partial fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala, is an authentic record of my own work during the period of six months from January 2005 to June 2005, under the guidance of **Dr. N. Das**, Assistant Professor, Thapar Institute of Engineering and Technology, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

Date :   **(Saloni Bansal)**
Place : Patiala

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The whole contribution for my achievements goes to **My parents, Brother, Sister,** and **Friends** who always stood by me during thick and thin, and providing me the moral support and resources to finish my work.

(SALONI BANSAL)

ABSTRACT

Promoters are regarded as molecular biological tools crucial for regulation of gene expression. Tissue-specific or cell type-specific promoters, operate in a particular tissue or cell type and also at certain developmental stages of a plant. These promoters could become very important in terms of driving expression of any gene of interest. Patatin, a major potato tuber protein is encoded by a multigene family. The present study is mainly focussed on molecular cloning and characterization of the 5’ flanking regions of class-I patatin genes from different potato cultivars.

Here, four potato cultivars were chosen namely Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Chandramukhi (KCM) and Kufri Jyoti (KJ). In order to amplify the 5’ flanking regions of the patatin gene from these cultivars, two different forward primers and one reverse primer was designed based on the class-I patatin gene sequence available in the database (Accession no. X87216). The reverse primer here was used as common. A number of DNA bands got amplified from different cutivars using two sets of primer pairs and then analyzed by agarose gel electrophoresis. Cultivar-wise variation was also noted. The cultivar KJ showed marked variation in terms of the size of the amplified products. The PCR amplified products as obtained using two sets of primers were examined carefully which suggests that the sequences proximal to transcription start site of the gene are relatively conserved and divergence occurs in the farther upstream regions. Only a few DNA bands were chosen for cloning and partial characterization which were as follows: two amplified DNA products (~ 2.2 kb & ~ 2.0 kb) from CS-1 and one (~ 1.4 kb) from KCM corresponding to the first set of primer; similarly one amplified DNA product (~ 0.8 kb) from CS-1 and another (~ 2.0 kb) from KJ DNA corresponding to the second set of primer pair. The selected PCR amplified products were cloned into the *Sma*I site of pUC19 vector. The putative transformants were selected on the basis of α-complementation (blue/white) for isolation of plasmids. Restriction analyses were carried out to check the presence of cloned inserts. PCR approach was adopted to check first the intactness of the inserts and secondly to see gene specificity. Taken together the results indicate that the cloned inserts correspond to the 5’ flanking region of class-I patatin gene in potato. Further sequencing and functional characterization are required to identify tuber-specific and efficient class-I patatin gene promoters.

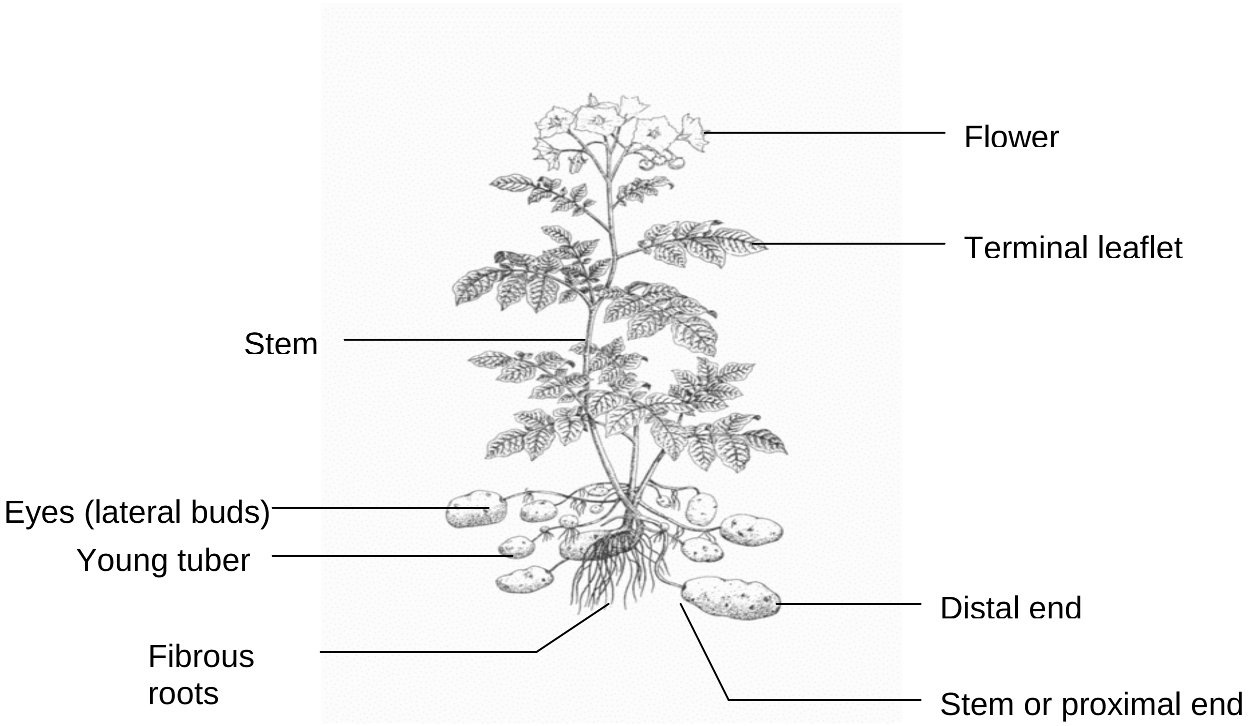
1. INTRODUCTION

The potato (*Solanum tuberosum* L.) is a vegetable crop of major economic importance worldwide. It is the fourth most cultivated food crop after wheat, rice and maize. As such, potato growers produce about 325 million tonnes of potato annually. The potato is a short duration crop that produces a large amount of calories in a short period of time. Potato belongs to Solanaceae family, which encompasses about 90 genera and 2800 species. The genus *Solanum* has about 2000 members and includes all tuber bearing species, as well as *Solanum tuberosum*, the species to which all common potato cultivars belong. The most common cultivated species, *tuberosum*, is a tetraploid that is thought to have arisen as a hybrid between the diploid species *S. stentotomum* and the diploid weed S. *sparsipilum*, with subsequent chromosome doubling (Ramanna and Hermsen, 1979).

1.1 About potato plant

Overall morphological features:

Solanum tuberosum L. is a herbaceous perennial (can grow upto 20 - 40 inches high) cultivated as an annual, and is susceptible to frost and freezing. It is a cool season crop that requires optimal average temperature between 50° to 65°F. The ends of its underground stems or stolons enlarge greatly to form a few to more than 20 tubers of variable shape and size. There are two principal storage organs in higher plants – the seed and tubers. Tubers are fundamentally different from seeds in their anatomy and development. These are the somatic storage tissues, which develop by the modification of the somatic structures of the plant. Potato plant produces a fibrous root system arising from the underground portion of the stem. The compound leaves manufacture the starch that is stored in potato's underground tubers. The plant bears white or purplish flowers. The skin of the potato tuber varies in color from brownish white to deep purple. The commercially significant portion of the plant is the tuber, which is a swollen underground stem. The tubers bear lateral buds (eyes) that, when the conditions are right, grow into new plants. Thus, tubers are used in commercial propagation since the true seed are heterozygous and highly variable, used primarily in crop improvement (The morphological features of potato plant are shown in the next page).



Schematic view of a potato plant

Characteristic features of potato tubers:

Morphologically, potato tubers are modified stems, which expand radially by a process of cell expansion and limited cell division (Cutter, 1982). Under field conditions, induction of this differentiation process is controlled by a number of environmental conditions such as photoperiod, temperature and nitrogen supply. In addition, genetic factors also play a critical role. Tuber formation is the most critical physiological function involved in potato production. It is the result of the activity of several interdependent processes like: stolon initiation and elongation, subapical swelling, cell division and the induction of specific proteins. Plant tubers share one or two biological roles. The first is to store carbon and usually also nitrogen in a form that can be mobilized when required. The second property that is shared by most, but not all, tubers is that they act as propagules, which are able to sprout and give rise to new plants. In this case, they need to contain a sufficiently wide range of nutrients to support the requirements of the plant until it is capable of independent growth.

Biochemically, there are two obvious differences between tubers and other somatic tissues of the potato: first, the presence of large amounts of starch (around 20% of fresh tuber weight) and secondly, the presence of a set of relatively abundant proteins (2% of fresh tuber weight) that may be akin to storage proteins in a loose sense. The cortical and pith cells, which are the bulk of the cells in mature tuber, are modified for the purposes of starch and storage protein accumulation. Potato tubers also contain many vitamins such as vitamin C, riboflavin, thiamine and niacin. Among many minerals found in potatoes are calcium, potassium, phosphorus, and magnesium. Like most somatic tissues, the tuber has indeterminate growth and may have no complex temporal or spatial patterns of gene expression beyond those involved in periderm differentiation and starch and storage protein biosynthesis.

1.1 Patatin: the major protein constituent of potato tubers

The morphological process of tuberization is accompanied by a variety of biochemical changes. Protein composition changes dramatically during stolon-tuber transition resulting in the formation of a much simplified protein complement consisting of only a few highly abundant proteins. Up to 40 % of the total soluble protein of potato tubers is represented by a family of immunologically identical glycoproteins with a molecular wt of ~ 40 kD which have been given the name ‘patatin’ (Racusen and Foote, 1980; Park, 1983). The protein is encoded by a multigene family of 50 -70 genes, which have been divided into classes I and II on the basis of sequence homology. The class-I genes encode the majority of tuber patatins.

As a storage protein, patatin is mainly localized in the plant cell vacuoles. Seed storage proteins are polymorphic mixtures of components. In the case of patatin, it is also possible to recognize two distinct groups of proteins and/or genes showing differential regulation. The content of patatin gradually increases as tuberization proceeds, reaching maximum levels when the tubers are ready to harvest. Thereafter, patatin levels decrease as the tubers age in storage and start to sprout (Hannapel, 1990, 1991). The tissue specificity of patatin, its presence in high amounts in potato tubers, and its developmental regulation strongly suggests that patatin plays a major role like storage protein. Unlike most other storage proteins, patatin displays lipid acyl hydrolase and acyl transferase activities (Racusen, 1984, 1986; Rosahl *et al*., 1987; Andrews *et al*., 1988). In addition to tubers, patatin is also expressed in roots, albeit at a 100-fold lower level (Pikaard *et al*., 1987). It also accumulates in leaves and stems of potatoes grown under field conditions (Racusen, 1983). Thus, the expression of patatin is not essentially tissue specific like typical seed storage proteins.

Potato tubers contain several low molecular weight proteinase inhibitors that inhibit the activity of trypsin, chymotrypsin and other proteases, thus decreasing the digestibility and the biological value of the ingested protein. Ones that have been characterized, mostly on the basis of sequence comparisons to other proteinase inhibitors, include Bowman-Birk, Kunitz, chymotrypsin and trypsin/ chymotrypsin inhibitors (Steikema *et. al*., 1988). The latter are best characterized in potato and represent type-II proteinase inhibitors. Together with patatin, these species account for 50% of tuber proteins. Proteinase inhibitors also accumulate during seed development, and it has been postulated that they provide defense against pathogens and other predators. Like patatin, these proteins can accumulate in stems and leaves during normal plant growth.

1.3 Application of genetic engineering in the potato tubers

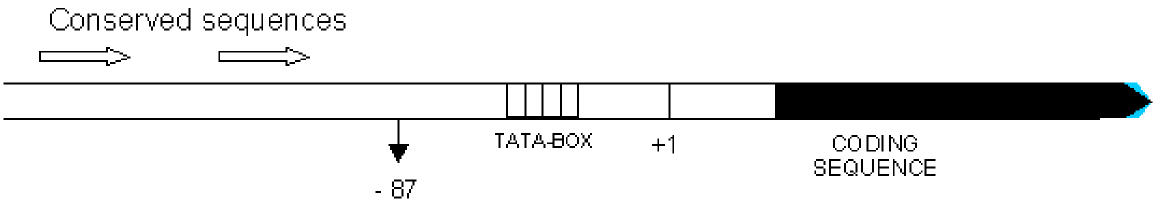
Unlike constitutive expression of genes, tissue-specific expression is the result of several molecular interactions. There are promoters that control gene expression in a tissue-specific (more precisely in a cell type-specific) manner and according to the developmental stage of the plant. The transgenes driven by these type of promoters will only be expressed in tissues where the transgene product is desired, leaving the rest of the tissues in the plant unmodified by transgene expression. Tissue-specific promoters may be induced by endogenous or exogenous factors, so they can be classified as inducible promoters as well. The promoter region consists of DNA sequences called *cis*-acting elements, which are recognized by proteins called transcription factors. These proteins activate or suppress the transcription of a gene by binding to the *cis*-control elements. 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, is an obvious way of combating several plant viruses. Tubers account for a very large amount of protein produced per acre, and if this could be harnessed by using fusions with a tuber-specific class-I patatin gene promoter, cheap production of proteins could be achieved. In this regard it is important to study the upstream regulatory sequences of the patatin gene from different potato cultivars.

2. LITERATURE REVIEW

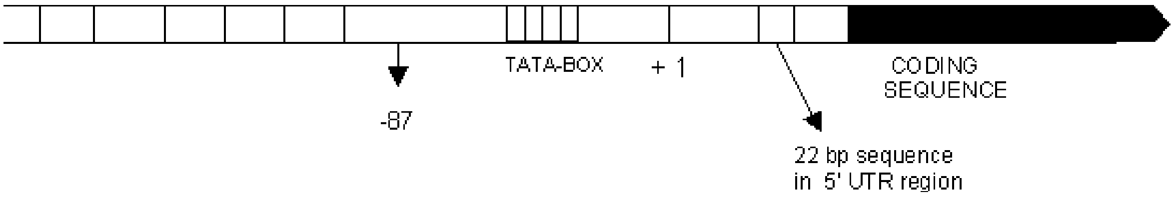
Patatin has an estimated molecular mass on SDS/PAGE of 43 kD (Racusen *et al.*, 1984) accounts for about 40 % of the total soluble protein in potato. It is estimated that about 33% of the patatin residues adopts a α-helical and 46% a β-stranded structure (Pots *et al*., 1998). The enzyme is N-glycosylated on two sites (asparagine 60 and asparagine 90), with typical small complex glycans (Sonnewald *et al*., 1989b). This glycosylation has no influence on proteolytic stability or enzymatic properties. 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). All patatin genes isolated express a protein that has a 23 amino acid N-terminal extension having all the attributes of a signal sequence that directs proteins to the endomembrane system (Sonnewald *et al*., 1989a).

In potato, the patatins are encoded by a gene family with 10-15 members per haploid genome. These genes can be divided into two classes, class-I and class-II, based on the presence or absence of a 22 bp insertion in the 5' UTR. Class-II patatin genes are easily characterized by the presence of a 22 bp sequence in the untranslated leader which is absent in class-I patatin genes (Pikaard *et al*., 1987). 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). The relative proportion of the two classes varies among cultivars.

A. STRUCTURAL FEATURES OF CLASS-I PATATIN GENE PROMOTER:



B. STRUCTURAL FEATURES OF CLASS-II PATATIN GENE PROMOTER:



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.

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). The expression of class-I patatin genes can also be induced in leaves incubated with high concentrations of sucrose (Paiva *et al*., 1983; Rosahl *et al*., 1986; Jefferson *et al*., 1990). Patatin also accumulates in leaves and stems of potatoes grown under field conditions (Racusen *et al*., 1983). Gene fusions have been made between the 5’ flanking sequences of different class-I patatin genes using GUS reporter gene (β-glucuronidase), and the expression of these has been studied in transgenic potato and tobacco plants. The 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).

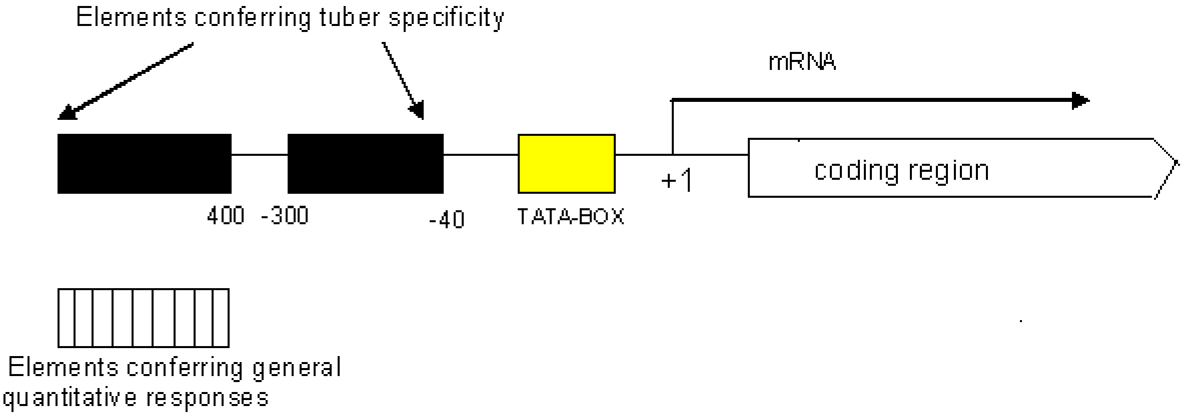
Histochemical analysis has shown that patatin transcription occurs in most cell types of the potato tuber. No expression was observed in the periderm, as this tissue is composed of dead cells. In leaves and shoots that were grown on high concentrations of sucrose, patatin transcription was observed in 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. It has been demonstrated *in vitro* that induction is specific for sucrose (Jefferson *et al*., 1990; Wenzler *et al*., 1989b) at an optimal concentration of 300-500 mM, while other sugars such as glucose and fructose are not effective. 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).

It was found that patatin promoter-GUS fusion genes are regulated by sucrose in tobacco (Wenzler *et al*. 1989a), a plant that has no endogenous patatin genes or lipid acyl hydrolase activity and no propensity to form tubers. The levels of expression were about 10 times lower than were observed for the same gene fusion in potato, and the difference in GUS activity after growth on low and high concentrations of sucrose was not as marked as in potato. This indicated that a similar signal transduction pathway involving sucrose concentration as an effector might be present in tobacco.

Studies of class-II promoters using gene fusions to the CAT (chloramphenicol acyltransferase) reporter gene have also been done (Twell and Ooms, 1987). Class-II promoters expressed low levels of CAT in tubers, and similar low levels in 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. Expression profile of a member of class-II patatin subfamily has been studied in potato and tobacco using β-glucuronidase as reporter gene. Histochemical analysis revealed high expression in a few defined cells in potato tubers and in a specific layer of both potato and tobacco root tips. In contrast to the developmentally and metabolically regulated class-I patatin gene, this gene was not inducible by elevated levels of sucrose (Koster-Topfer *et al.*, 1989). A comparative analysis of the 5' upstream sequences of B33 patatin gene (class-I patatin gene) reveals a high degree of homology to both class-I and class-II genes up to position -87, whereas upstream from this upto at least - 1.7 kb, sequences are homologous only to class-I genes (Bevan *et al*., 1986; Mignery *et al.*, 1988).

Detailed studies of the 5' upstream sequences of a patatin gene have been reported, aimed at identifying specific sequences and *trans*-acting factors that determine the developmental regulation and sucrose-inducibility. This has 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).



PUTATIVE FUNCTIONAL DOMAINS OF CLASS-I PATATIN

In 1970, Galliard reported the partial purification of an acyl hydrolase that was active on a range of lipid substrates. Subsequent studies demonstrated that acyl hydrolase activity was due to patatin (Racusen *et al.*, 1984). In addition, it also acts as an esterase. The lipid acyl hydrolase activity of patatin could be important for the rapid degradation of cell membranes and thus rapid degradation of certain metabolites. Thus, patatin might be implicated in the defense of potato tubers.

A study on patatin, the main storage protein in potatoes, reports induction of allergic reactions in children (Seppala *et al*., 1999). This was subsequently confirmed by more detailed studies including skin exposure tests and oral challenge, and patatin has been given the allergen designation Sol t 1. Potatoes are a major source of starch for food and industrial uses, with the tuber proteins forming a by-product. The proteins are usually recovered in an aggregated denatured state, which limits their use to low value feed for livestock. However, native potato proteins have promising functional properties (e.g. formation and stabilization of emulsions and foams) as well as good nutritional quality. Also, patatin provides an interesting opportunity for application of its catalytic power as a mono-acyl esterase or ester synthase at low water activities (Macrae *et al*., 1998).

AIM OF THE PRESENT STUDY

The present study aims at the cloning and characterization of 5’ flanking regions of class-I patatin genes from the Indian potato cultivars namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chandramukhi and Kufri Jyoti. These varieties are suitable to our own agro-climatic conditions. There is no such report available yet in the literature. Studying on class-I patatin gene promoters at molecular level is important because of its tuber specificity. Apart from this, such promoters could be very helpful to drive expression of any gene of interest in these cultivars. Various potato cultivars will be considered in this study. This will provide us scope to compare different patatin class-I gene promoters in terms of their regulation. For this purpose PCR-based approach is going to be adopted.

3. MATERIALS

3.1 PROCUREMENT OF POTATO GERMPLASM AND OTHER MATERIALS:

- The germplasm of various potato cultivars such as Kufri Chandramukhi, Kufri Chipsona-1, Kufri Chipsona-2, 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 were brought from Bangalore Genei Pvt.Ltd., Bangalore.

3.2 STRAINS AND PLASMIDS:

- *E.coli* DH5α: *sup*E44 Δ*lac*U 169 (φ80 *lac*ZΔM15) *hsd*R17 *rec*A*end*A1 *gyr* A 96 *thi*-1 *rel*A1
- pUC 19 (2686): 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, where as, those transformed with pUC19 plasmid were maintained on Luria agar-Ampicillin medium.

3.3 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 μg/ml autoclaving.

3.4 BUFFERS USED

Gel Loading Buffer (5X):

Sucrose - 35 % (w/v)
EDTA - 50 mM (pH 8.0)
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)

3.5 ENZYMES USED

3.5.1 RESTRICTION ENZYMES:

Various hexacutter restriction enzymes such as *Eco*RI, *Bam*HI, *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.

3.5.2 OTHER ENZYMES

Ribonuclease A

Stock solution - 10 mg/ml

Working solution - 10 - 15 μg/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 μg/ml

Freshly prepared lysozyme was used in regular work.

T4 DNA ligase

Stock conc. - 400 U/ μl

Working conc. - 40 U/ μl

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

Klenow fragment of DNA polymerase I

Stock conc. - 5 U/ μl

Working conc. - 2 U/ 50 μl of the reaction volume.

OTHERS

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

Stock conc. - 20 mg/ml

Working conc. - 20 μg/ml

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

IPTG (Isopropyl thio ß- D- galactoside)

Stock conc. - 100 mg/ml

Working conc. - 100 μg/ml

It was prepared in fresh and sterile water.

4. METHODS

4.1 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 encodes the 23 amino acid transit pepide of the patatin gene. The latter 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: (i) 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 5° C, so that the GC content and length must be chosen accordingly. (6) The annealing temperature should be about 5° C lower than the melting temperature. (7) Sequenœs with long runs of a single nucleotide should be avoided. (8) 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 & PT-F610 belong 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.

4.2 POLYMERASE CHAIN REACTION:

The total plant DNA was used as a template and polymerase chain reaction was carried out using the primer pairs as mentioned in the above section. The composition of typical 50 µl PCR reaction was as follows:

PCR Buffer	used at a concentration of 1X
Template DNA	- 0.1 to 1 µg
Primer 1	- 10 pmoles
Primer 2	- 10 pmoles
dNTPs	- 50 µM
Taq DNA polymerase	- 3 U/ µl

During PCR, the temperature cycling parameters were as given below:

Initiation denaturation at 94ºC, 1 min 30 sec

Denaturation	- 94ºC, 1 min
Annealing	- 50ºC, 2min
Polymerization	- 72ºC, 3 min

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

4.3 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 to which ethidium bromide dye was added (0.5 μg/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.

4.4 KLENOW TREATMENT:

Klenow fragment is a proteolytic product of *E.coli* DNA polymerase I, which retains polymerization and 3' to 5 ' exonuclease activity. Klenow treatment was carried out as a polishing step of the PCR amplified products because, *Taq* DNA polymerase has a tendency to add extra 'A' residue at 3' ends. Apart from this, some 3' recessed termini also occur in PCR amplified products. 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.

4.5 ELECTRO–ELUTION OF DNA BANDS:

Run 0.8% agarose gel in 1X TAE buffer in which required amount of ethidium bromide (0.5 µg/ml) was added. Electrophoresis was carried out at 70 volts (for approx. 1:30 hrs) using 1 - 2 V/cm voltage gradient. After resolution of bands, agarose slice containing the desired bands was excised with the help of a sterile blade, transferred to a prepared dialysis bag and submerged with 0.6 - 0.7 ml 1X TAE buffer. The eluted DNA sample was transferred in clean microfuge tube and solvent extraction was done with phenol twice followed by solvent extraction with mixture of phenol and chloroform (1:1) once. Centrifugation was done at 8,000 rpm for 10 min. Transferred the aqueous layer in clean microfuge tube and added 1/10th volume of 3 M sodium acetate (CH_3COONa), followed by the addition of 2.5 X volumes of ethanol. Mixed well and kept at - 20°C. DNA was precipitated and washed with 70% ethanol, air-dried and finally dissolved in 13-15 μl of TE buffer. Stored at - 20 °C for subsequent use.

4.6 SETTING UP RESTRICTION DIGESTION REACTION:

Restriction endonucleases are the enzymes that recognize short DNA sequences and cleave the double stranded DNA at specific sites within or adjacent to the recognition sequences. The main components of a digestion reaction are-

DNA - 0.5 - 1µg
Restriction enzyme - 1 - 10 units (as supplied by the manufacturer)
Buffer - used at a concentration of 1X (as supplied by the manufacturer)
BSA - used at a concentration of 1X (as supplied by the manufacturer)
Sterile distilled water - to make up the desired volume

Usually restriction digestion was carried out in a reaction volume of 15 µl for 2 - 3 hrs at temperatures depending upon restriction enzymes used.

4.7 SETTING UP 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 ~ 0.3 µg
Insert - 0.3 to 0.6 µg
T4 DNA Ligase enzyme - 1 - 10 units (as supplied by the manufacturer)

T4 DNA Ligase buffer containing PEG 8000 was used at a concentration of 1X. The reaction volume was made up to 15 µl and the reaction was set up 21°C for 3 hours.

4.8 TRANSFORMATION OF *E.coli* DH5α STRAIN:

E.coli was transformed with the ligation mix using the standard $CaCl_2$ method. To prepare the competent cells, a single bacterial colony was inoculated in 25 ml of LB (Luria Broth) and incubated at 37°C overnight. The overnight grown culture was reinoculated into 25 ml fresh LB and allowed to grow up to 0.4 O.D at 590 nm. The culture was then cooled in ice and the cell pellet was recovered by centrifugation at 6000 rpm for 6 min. The pellet was resuspended well in 15 ml of ice-cold 0.1M $CaCl_2$, and recovered again by centrifugation. The pellet was then resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$ for at least two hrs at 0°C for the development of ‘competence’. 100 µl of the competent cell suspension was dispensed into sterile microfuge tubes at 0°C. 2 - 3 µl of the ligation mixture was added to the cell suspension, mixed well and then kept at 0°C for 30 minutes. Heat shock was given to all the tubes at 42°C for 2 minutes, followed by the addition of 1 ml LB and further incubation at 37°C for 1 hr. 100 µl of the above transformed cells were plated on LA - ampicillin medium containing X-gal and IPTG. The plates were then incubated at 37°C for 16 - 18 hrs. The transformants were selected based on α-complementation (blue/white selection).

4.9 SMALL SCALE ISOLATION OF PLASMID DNA FROM BACTERIAL TRANSFORMANTS:

Plasmids were isolated by rapid boiling method. Bacterial transformant colonies were inoculated aseptically in 4.5 ml LB containing ampicillin in test tubes. The culture was incubated overnight at 37°C for 120 rpm. Cells were harvested from 1.5 ml overnight grown culture in microfuge tubes. The dried pellet was loosened by vortexing, followed by resuspension in 800µl of STET buffer. 20 µl of lysozyme was added to the bacterial suspension and mixed well. Each microfuge tube containing cell suspension was then kept in boiling water bath for 1.5 minutes. After cooling it down to room temperature, high speed centrifugation (12,000 rpm) was carried out for 15 minutes. Pellet formed was discarded and 2.0 µl of RNase solution was added to the supernatant to remove the contaminating RNA. After incubation at 37°C for 45 minutes, equal volume of phenol: chloroform was added, mixed for 5-7 minutes and centrifugation was performed at 10,000 rpm for 10 minutes. To the upper aqueous layer, 1/10th volume of 3 M sodium acetate (CH_3COONa) and equal volume of isopropanol was added for precipitation. Solution was incubated at 4°C for 45 minutes. Then it was centrifuged at 10,000 rpm for 10 minutes. The DNA pellet was washed with 70% ethanol to ensure the removal of excess salts and other impurities. Finally, DNA pellet was air dried at room temperature and dissolved in 20-30 µl of TE buffer.

5. RESULTS AND DISCUSSION

Different members of patatin multigene family in potato respond to different metabolic and developmental signals. The present thesis work focused on the patatin genes which are predominantly expressed in the tuber, thus showing tissue-specific expression. In this study, the promoter regions of class-I patatin genes were amplified from potato cultivars namely Kufri Chipsona 1 (CS-1), Kufri Chipsona 2 (CS-2), Kufri Chandramukhi (KCM) and Kufri Jyoti (KJ). These cultivars are suitable to our agro-climatic conditions. The PCR-amplified DNA products as derived from the above potato cultivars were analysed and then cloned in plasmid vector pUC19. Finally the inserts were partially characterized. The results as obtained step-wise are given in the following sections:

5.1 POLYMERASE CHAIN REACTION :

The total DNA from different potato cultivars were used as template in PCR. Potato total DNA was isolated from micropropagated plantlets (as shown in Fig. 1) using a simple and efficient procedure. Potassium acetate was introduced in a particular step in order to remove carbohydrates and other bulk impurities.

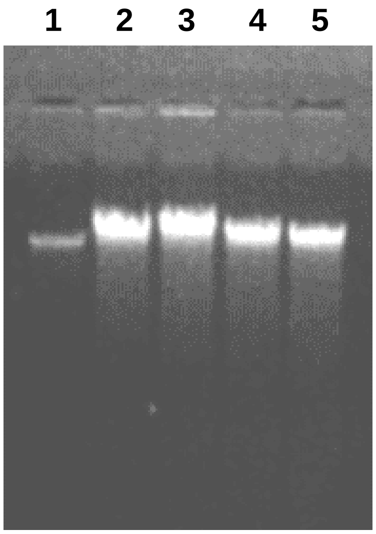


Fig. 1. Total DNA from different potato cultivars

Lane 1, Bacteriophage λ DNA; Lane 2, Total DNA of Kufri Chipsona-1; Lane 3, Total DNA of Kufri Chipsona-2; Lane 4, Total DNA of Kufri Chandramukhi; Lane 5, Total DNA of Kufri Jyoti

In order to amplify the 5’ flanking region of class-I patatin gene from the above potato cultivars following sets of primers were used: PT-F074 & PT-R1478 (first set of primer pair): PT-F610 & PT-R1478 (second set of primer pair). Here PT-R1478 served as a common reverse primer. The temperature cycling parameters as employed during PCR are given in section 4.2. The PCR amplified products were separated on 0.8% agarose gel containing ethidium bromide. The DNA bands were clearly visible and distinct which indicated that the primer combinations worked well for the class-I patatin gene. Fig. 2 shows the PCR amplified DNA products obtained using first set of primer pair. In case of CS-1 the size of the most prominent DNA band was ~ 2.2 kb. Along with this fragment, a DNA band of ~ 2.0 kb was also obtained. The same pattern was also found in other cultivars namely KCM and CS-2 although less prominent in the latter suggesting that the corresponding regions in the genome are more or less conserved in these cultivars. A distinctive feature is that a prominent DNA band of approx. 1.4 kb was obtained only in the cases of KCM and CS-2 potato cultivars. This size was consistent as per designing of the primers. The potato cultivar KJ showed a marked difference in this regard as the size of the amplified DNA band was approx. 2.7 kb as found only in this case reflecting considerable divergence.

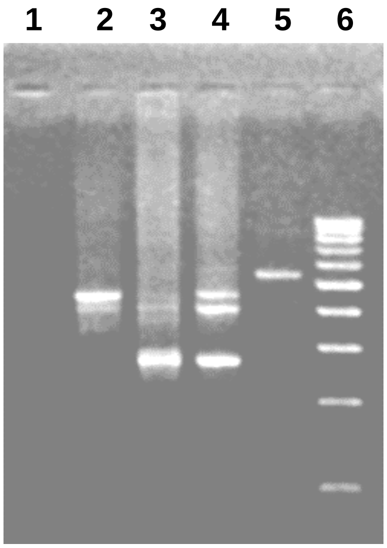


Fig. 2. PCR amplified DNA products using first set of primer pair (PT-F074 and PT-R1478)

Lane 1, Control PCR without template; Lane 2, CS-1 total DNA as template; Lane 3, CS-2 total DNA as template; Lane 4, KCM total DNA as template; Lane 5, KJ total DNA as template; Lane 6, 500 bp DNA ladder

The PCR amplified DNA products as obtained using the second set of primer pair i.e., PT-F610 & PT-R1478 are shown in Fig. 3. From all the four potato cultivars, an easily noticeable DNA band of size ~ 2.0 kb is obtained. In addition, another prominent DNA band of approx. 0.8 kb was obtained in all the cultivars except KJ variety. This size of DNA also matches with the corresponding class-I patatin gene sequence in the database. This data clearly suggests that TATA box proximal regions of the patatin gene promoters are relatively conserved in the potato cultivars although the variation was noted in the KJ variety that needs separate attention.

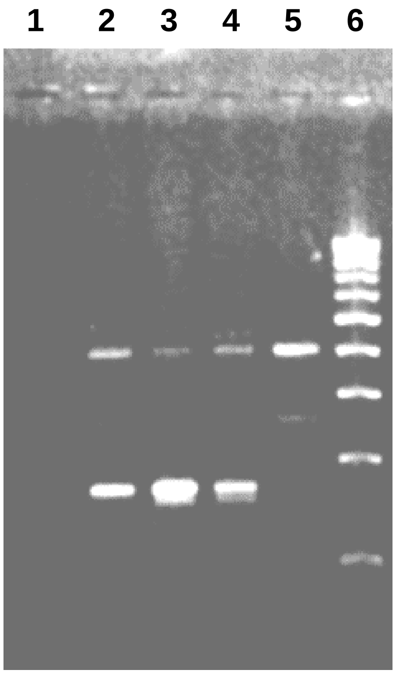


Fig. 3. PCR amplified DNA products using second set of primer pair (PT-F610 & PT-R1478)

Lane 1, Control PCR without template. Lane 2, CS-1 total DNA as template; Lane 3, CS-2 total DNA as template; Lane 4, KCM total DNA as template; Lane 5, KJ total DNA as template; Lane 6, 500 bp DNA ladder

The Fig. 4. provides us a scope to compare and analyze the amplified DNA bands corresponding to the first and second set of primer pairs in each potato cultivar. The preliminary data suggests that the distal class-I patatin promoter regions are quite divergent in the cases of CS-1 and KJ.

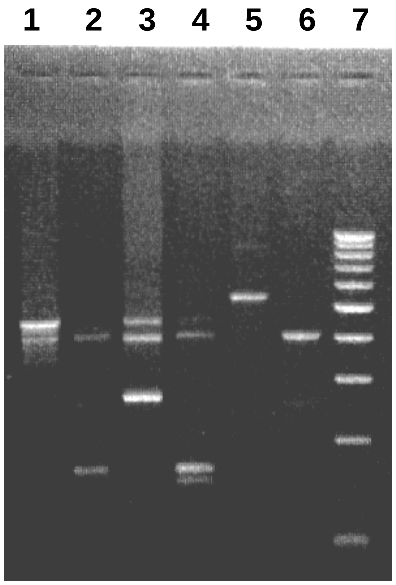


Fig. 4. Comparison of the PCR amplified DNA products corresponding to the first and second set of primer pairs

Lanes 1 & 2, CS-1 total DNA as template using first and second set of primers respectively; similarly, Lanes 3 & 4 correspond to KCM total DNA as template whereas Lanes 5 & 6 correspond to KJ total DNA as template; Lane 7, 500 bp DNA ladder

Only a few PCR-amplified products were chosen for further molecular cloning and characterization studies as given below cultivar-wise.

CS-1 : ~ 2.2 kb & ~ 2.0 kb (using first primer set), ~ 0.8 kb (using second primer set)
KCM : ~ 1.4 kb (using first primer set)
KJ : ~ 2.0 kb (using second primer set)

5.2 MOLECULAR CLONING & CHARACTERIZATION

<u>Klenow treatment of PCR amplified DNA products</u>: 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 as the enzyme *Taq* DNA polymerase has a tendency to add an extra 'A' residue at the 3' end of both the strands. Moreover, the PCR amplified DNA may have 3' recessed termini also. Therefore, these DNA products were polished with the Klenow fragment of *E. coli* DNA polymerase I (as described in the section 4.4) in presence of dNTPs. The Klenow-treated DNA bands were then recovered by electroelution as described in section 4.5.

<u>Linearization of plasmid vector</u>: The plasmid vector pUC19 was isolated from the E*. coli* DH5α strain by boiling preparation (as described in section 4.9). 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 said site of the plasmid vector.

In this study, five different blunt-end ligation reactions were carried out separately using PCR amplified DNA products as mentioned above and linearized pUC19 for around 3 hr at 21^0C.

<u>Transformation and selection of transformants:</u> The above 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 a number of white colonies and further analyzed to check the presence of inserts. The different recombinant plasmids were designated as follows:

The inserts of the following recombinant plasmids <u>pSN-PK01</u> (~ 2.2 kb); <u>pSN-PK02</u> (~ 2.0 kb) and <u>pSN-PK03</u> (~ 0.8 kb) correspond to the potato cultivar CS-1. Similarly, the inserts of the recombinant plasmids <u>pSN-PM01</u> (~ 1.4 kb) & <u>pSN-PN01</u> (~ 2.0 kb) correspond to the potato cultivars KCM and KJ respectively.

RESTRICTION ANALYSIS OF THE RECOMBINANT PLASMIDS:

Restriction analysis of the above recombinant plasmids were carried out using the restriction enzymes namely *Eco*RI, *Bam*HI and *Hind*III. *Eco*RI & *Bam*HI digestion pattern are shown in Fig. 5 & Fig. 6 respectively. It is apparent that there is no internal *Eco*RI and *Bam*HI site in the cloned genomic inserts.

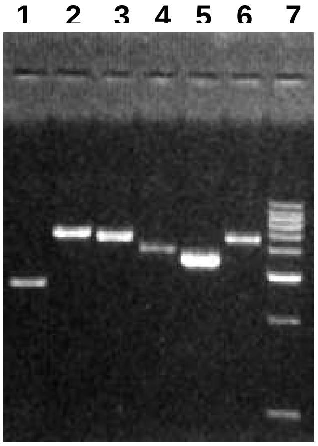


Fig. 5. Restriction digestion of recombinant plasmids with *Eco*RI

Lane 1, pUC19 with *Eco*RI; Lane 2, pSN-PK01 with *Eco*RI; Lane 3, pSN-PK02 with *Eco*RI; Lane 4, pSN-PM01 with *Eco*RI; Lane 5, pSN-PK03 with *Eco*RI; Lane 6, pSN-PN01 with *Eco*RI; Lane 7, 1.0 kb DNA ladder

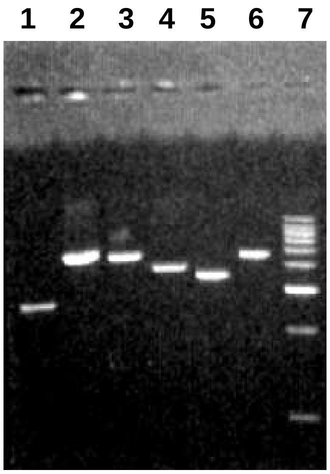


Fig. 6. Restriction digestion of recombinant plasmids with *Bam*HI

Lane 1, pUC19 with *Bam*HI; Lane 2, pSN-PK01 with *Bam*HI; Lane 3, pSN-PK02 with *Bam*HI; Lane 4, pSN-PM01 with *Bam*HI; Lane 5, pSN-PK03 with *Bam*HI; Lane 6, pSN-PN01 with *Bam* HI; Lane 7, 1.0 kb DNA ladder

The restriction analysis of the recombinant clones with *Hind*III is shown in Fig.7. It is quite apparent that majority of the inserts lack internal *Hind*III site. Only in the case of pSN-PK02, a DNA fragment of size approx. 1.8 kb was released by *Hind*III digestion. The genomic inserts in the clones pSN-PK01 and pSN-PK02 showed different restriction pattern although derived from the same cultivar CS-1 using the first set of primer pair. It is likely that the above inserts may correspond to different members of the class-I patatin multigene family.

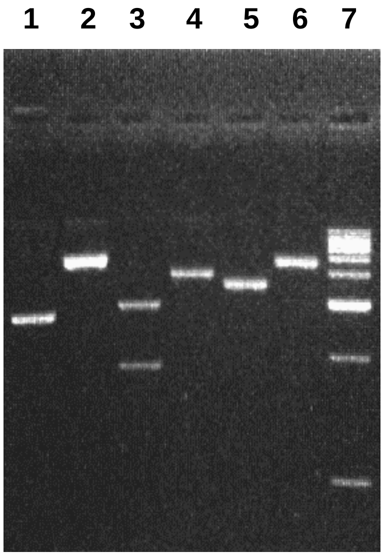


Fig. 7. Restriction digestion of recombinant plasmids with *Hind*III

Lane 1, pUC19 with *Hind*III; Lane 2, pSN-PK01 with *Hind*III; Lane 3, pSN-PK02 with *Hind*III; Lane 4, pSN-PM01 with *Hind*III; Lane 5, pSN-PK03 with *Hind*III; Lane 6, pSN-PN01 with *Hind*III; Lane 7, 1.0 kb DNA ladder

PARTIAL CHARACTERIZATION OF THE RECOMBINANTS:

The recombinant plasmids were used as templates separately to carry out PCR using the same primer pairs as used earlier where total potato DNA used as template. The first set of primer pair i.e., PT-F074 & PT-R1478 worked well for the clones namely pSN-PK01, pSN-PK02 & pSN-PM01 thus confirming the intactness of the cloned genomic inserts (as shown in Fig. 8) as compared with the amplified DNA bands obtained directly using total potato DNA as template. In case of pSN-PM01, one of its variant clone showed consistent result in this regard (Lane 6 of Fig. 8) which was pursued later for further characterization. The above variation might result from some modification during cloning procedure.

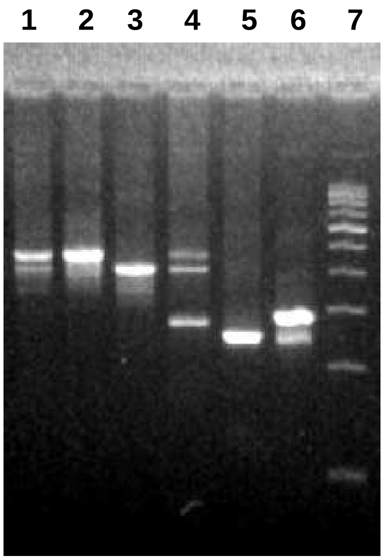


Fig. 8. PCR based characterization of cloned inserts
(Corresponding to first set of primer pair: PT-F074 & PT-R1478)

Lane 1, CS-1 total DNA as template; Lane 2, pSN-PK01 as template; Lane 3, pSN-PK02 as template; Lane 4, KCM total DNA as template; Lane 5, variant of pSN-PM01as template; Lane 6, pSN-PM01 as template; Lane 7, 500 bp DNA ladder

Similarly, the second set of primer pair PT-F610 & PT-R1478 was employed for the recombinants pSN-PK03 & pSN-PN01 (as shown in Fig. 9). The result was consistent as expected, again showing the intactness of the cloned inserts.

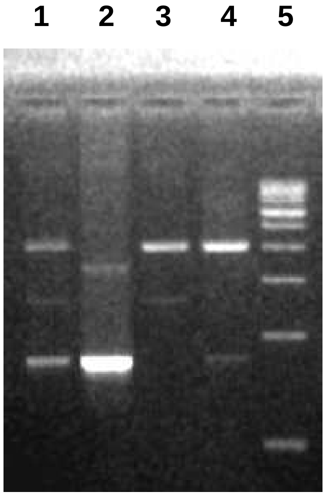


Fig. 9. PCR based characterization of cloned inserts

(Corresponding to second set of primer pair: PT-F610 & PT-R1478)

Lane 1, CS-1 total DNA as template; Lane 2, pSN-PK03 as template; Lane 3, KJ total DNA as template; Lane 4, pSN-PN01 as template; Lane 6, 500 bp DNA ladder

To the end of this study, the following recombinant clones pSN-PK01, pSN-PK02 and pSN-PM01 were further characterized by PCR using nested set of primers i.e. PT-F610 & PT-R1478. In other words, the primer PT-F610 served as an internal primer. The size of the amplified DNA bands appeared to be same i.e. ~ 0.8 kb in all the cases (Fig. 10). This result strongly suggests that TATA box proximal regions are conserved in class-I patatin gene family. Microheterogeneity if any could be revealed further through sequencing only. However, distal promoter regions appeared to be divergent that could be related with tissue specificity, more precisely tuber specificity. For this purpose, functional characterization needs to be carried out.

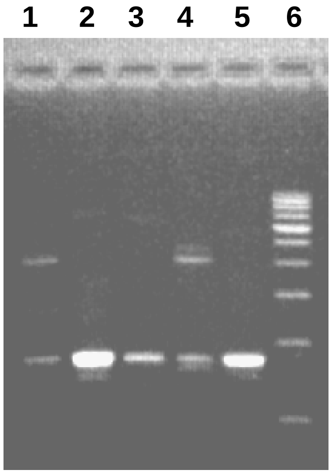


Fig. 10. Characterization of cloned inserts by nested PCR

(Nested set of primer pair, PT-F610 & PT-R1478)

Lane 1, CS-1 total DNA as template; Lane 2, pSN-PK01 as template; Lane 3, pSN-PK02 as template; Lane 4, KCM total DNA as template ; Lane 5, pSN-PM01 as template; Lane 6, 500 bp DNA ladder

In this molecular cloning studies, total DNA from a number of potato cultivars were used as template and PCR was carried out to amplify the 5' flanking regions using class-I patatin gene-specific primers. Results clearly indicate that patatin is encoded by a gene family. It is of quite interest to note that one specific set of primer was able to amplify more than one DNA product from most of the cultivars except Kufri Jyoti. The apparent variation as noted in KJ variety needs to be thoroughly understood. Here we focussed only on a few amplified DNA bands for cloning and characterization studies. Partial characterization indicates that the cloned inserts correspond to the 5' flanking regions of class-I patatin gene. Further sequencing and functional characterization of the cloned genomic inserts would help to identify the *cis*-regulatory elements that could be responsible for conferring tuber specificity. The remaining amplified DNA products also require further equal attention. The purpose is to understand first the molecular mechanism underlying the complex control of patatin expression and secondly to recognize efficient tuber-specific promoters.

SUMMARY

The work components of this thesis work could be summarized as follows:

- Isolated total DNA from four potato cultivars namely CS-1, CS-2, KCM, and KJ and their quality checked through agarose gel electrophoresis. The DNA samples were used as template in PCR.
- Three oligonucleotide primers: 20-mer each, namely PT-F074, PT-F610 (forward primers) and PT-R1478 (reverse primer) were designed based on the class-I patatin gene sequence available in GenBank database (Accession no. X87216). Out of these, primer PT-R1478 served as common reverse primer in two different primer combinations used. 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 as this region is likely to be conserved in various members of patatin multigene family.
- Polymerase chain reaction (PCR) was carried out using DNA templates as mentioned above with the first set of primer pair (PT-F074, & PT-R1478) and the second set of primer pair (PT-F610 & PT-R1478). The amplified DNA products were analyzed by agarose gel electrophoresis. Cultivar-wise variation was noted.
- Corresponding to the first set of primer pair, distinct DNA bands of size ~ 2.1 kb & ~ 2.0 kb (from CS-1) and ~ 1.4 kb (from KCM) were chosen for molecular cloning studies. Similarly, with respect to the second set of primer pair, DNA products of size: ~ 0.8 kb (from CS-1) and ~ 2.0 kb (from KJ) were also chosen.
- The above amplicons were polished by Klenow enzyme of *E. coli.* DNA polymerase I prior to cloning into 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.
- Restriction analyses were carried out by using various enzymes in order to check the presence of DNA inserts as well as their orientation. Furthermore, the recombinant clones were used as template separately to carry out PCR using same set of primers to check intactness of the cloned inserts. PCR was also carried out using nested primers for their characterization. The data suggests that the above cloned inserts correspond to the 5' flanking region of class-I patatin gene of potato.

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