Strategies for Making Ti-plasmid based Genetic Constructs using Patatin Gene Promoters for Functional Characterization

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

Under the guidance of
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CERTIFICATE

This is to certify that the thesis entitled **"Strategies for Making *Ti*-plasmid based Genetic Constructs using Patatin Gene Promoters for Functional Characterization"** submitted by Shipra Singh (Roll no.301001023) in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology, to Thapar University (Deemed University), Patiala, is a record of student's own work carried out by her under our supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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DECLARATION

I hereby declare that the work which is being presented in this thesis **"Strategies for Making*Ti*-plasmid based GeneticConstructs using Patatin Gene Promotersfor Functional Characterization"** submitted by the undersigned in partial fulfillment of the requirement for the award of Degree of Master of Sciences in Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. N. Das,** AssociateProfessor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India. The matter embodied in this thesishas not been submitted in part or full to any other university or institute for the award of any degree.

Date: July 14, 2012

Place: Patiala**(Shipra Singh)**

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Place: Patiala Shipra Singh

Table of Contents

List of Abbreviations

Abbreviations	Name
Amp	Ampicillin
bp	Base pair
CaMV 35S	Cauliflower mosaic virus 35S
DNA	Deoxyribonucleic acid
dNTP	2′-deoxynucleoside-5′-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
GUS	Glucuronidase
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
kb	Kilo base
kJ	Kilo joule
L	Liter
LA	Luria agar
LB	Luria broth
M	Molar
mg	Milligram
mg g^{-1}	Milligram per gram
mg L^{-1}	Milligram per liter
mg min^{-1}	Milligram per minute
mg mL^{-1}	Milligram per milliliter
µg	Microgram
µg mL^{-1}	Microgram per milliliter
min	Minute
mL	Millilitre
mM	Millimolar
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
pI	Isoelectric point
pmoles	Picomoles
RNA	Ribonucleic acid
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
STET	Sucrose Tris EDTA Triton X100

TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
TSS	Transcription start site
UTR	Untranslated region
V	Volt
v/v	Volume per volume
w/v	Weight per volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

ABSTRACT

Potato (*Solanumtuberosum*L.) is one of the prominent crops capable of nourishing the world's population. Food and Agriculture Organisation of the United Nations has acknowledged potato as the food for the future in order to fight global poverty and hunger.Tuberization in potato is a complex developmental process influenced by various metabolic, environmental and developmental signals. Tuberization in potato is accompanied by the formation of starch and expression of relatively abundant proteins. Patatins, in potato are major storage proteins encoded by a multigene family comprising of 10-18 copies per haploid genome. The patatin gene family in potato is distinguished in to two classes: Class-I and Class-II based on their sequence morphology and expression patterns. Many events underlying tuberization aspects in potato remains obscure both at the biochemical and molecular level. The importance of tuber-expressed promoters has drawn a lot of attention in terms of both basic and applied research. Keeping in view of the above, in the present study efforts were made to isolate and characterize different members of Class-I and Class-II patatin gene family. The background work carried out in our laboratory yielded a number of Class-I and Class-II patatin clones with partial coding region designated as PTV03, PTV01, PK03 and PTV04 respectively as revealed through partial sequencing. For functional characterization studies of these clones, only the promoter regions were amplified and cloned in to pUC19 plasmid vector and well characterized through restriction analyses and PCR. One of the Class I patatin clone PK03 was subsequently cloned in to the *Agrobacterium* based *Ti*-plasmid vector (pBI121) and well characterized through PCR. The resulting recombinant *Ti*-plasmids will be used to transform potato systems for functional characterization of the patatin promoters. Moreover, further efforts are required in making binary genetic constructs using the remaining patatin promoters.

Chapter 1
Introduction

1.1. Potato crop

Potato (*Solanumtuberosum* L.) is a member of the *Solanaceae*, an economically important family that includes tomato, pepper, aubergine (eggplant), petunia and tobacco. The Potato is indigenous to the Peruvian and Bolivian Andes mountains in South America, where it was discovered by Spanish explorers in 1532 (Brown, 1993). Thepotato was shipped to Europe and introduced in the Spain in 1573. From Europe it was introduced to North America and rest of the world (Brown, 1993; Zuckerman, 1998). It is the major tuber crop that is grown in temperate regions. It is a cool season crop (Ewing, 1981) that requires an optimal average temperature between 20-25°C. It is a short day plant and a C3 plant with a low light saturation point (Demagante and Zaag, 1988). It is known as a herbaceous, succulent, dicotyledonous plant with alternate stolons underground and alternate leaves on the stem above ground; stems are about 30-100 cm tall. A potato plant can have three kinds of stems including sprouts (leafy stems), stolons and tubers (Beukema and Zaag, 1979;Struik and Wiersema, 1999).It is the third most important food crop after wheat and rice, with a worldwide production of 330 million tons in 2009 (http://faostat.fao.org/).Potato provides roughly half of the world's annual production of all root and tuber based foods, making it the leading non-cereal crop. It is a part of the diet of half a billion consumers in the developing countries (Ghislain et al., 1999).It is also important crop in terms of dry matter production (2.2 t ha^{-1}), energy (216 $MJha^{-1}day^{-1}$) and nutrition (Beukema and Vander Zaag, 1990). India ranks 4^{th} in area and 3^{rd}in the world in production of potato after China and Russian Federation. Potato is produced in an area of 14 lakh ha with a production of 250 lakh tonnes and productivity of 17.86 tonnes per ha.

Different species (including *Solanumtuberosum*L., *S. ajanhuiri, S. curtilobum, S. caucha, S. goniocalyx, S. phureja, S. juzepczukii, and S. stenotomum*) are recognized as potato species (Struik andWiersema, 1999) and over 230 wild potato species are known (Harris, 1992).

Besides the great economic relevance, research on potatoes has many advantages: they are easily transformable and therefore amenable to genetic manipulation, and they can be rapidly propagated both in tissue culture and through cuttings.

1.2. Tuber Biology

Tubers are underground, fleshy stems with eyes and they are suitable for ware, food processing, seed, animal feed and non-industrial use (Beukema and Vander Zaag 1979; Struikand Wiersema, 1999). Potato plants undergo several phases of development during the tuber life cycle (Ewing and Struik, 1992). They start with tuber initiation and formation, after which the tuber goes through a filling and a storage phase. During all these stages the potato tuber serves as a sink organ where assimilates are accumulated. The dormancy phase follows and last for 18-33 weeks (Cutter, 1978), depending on the genotype. The sprouting of the tuber marks the beginning of the phase where the tuber becomes a source organ to form the new growing plant.

Tuberisation is a complex develomantal process involving the differentiation of underground stolons (a modified stem) in to a specialised storage organ, the tuber (Moorby, 1978). The process of tuberisation comprises inhibition of the longitudinal growth in the tip of the stolon followed by the initiation and growth of the tuber (Cutter, 1978). Potato tubers develop initially from an enlargement of existing pith cells in the sub-apical regions of the stolon, followed rapidly by cell divisions in most parenchyma cells, particularly those associated with the primedulla and inner cortex (Li, 1985). These divisions are thought to cease early in tuber development, once the tuber has reached 30-40 g of fresh weight (Peterson et al., 1985). The final tuber size is determined by further increase in cell volume of parenchyma tissue (Taylor et al., 1992 a, b). During this growing and filling stage, the tuber is highly metabolically active (Ewing et al., 1992) and two major biochemical changes occur, accumulation of starch and formation of storage proteins (Appeldoorn et al., 1997; Li, 1985). There are many environmental factors that affect tuber formation, but nitrogen levels, temperature and light have the greatest effect (Jackson, 1999).

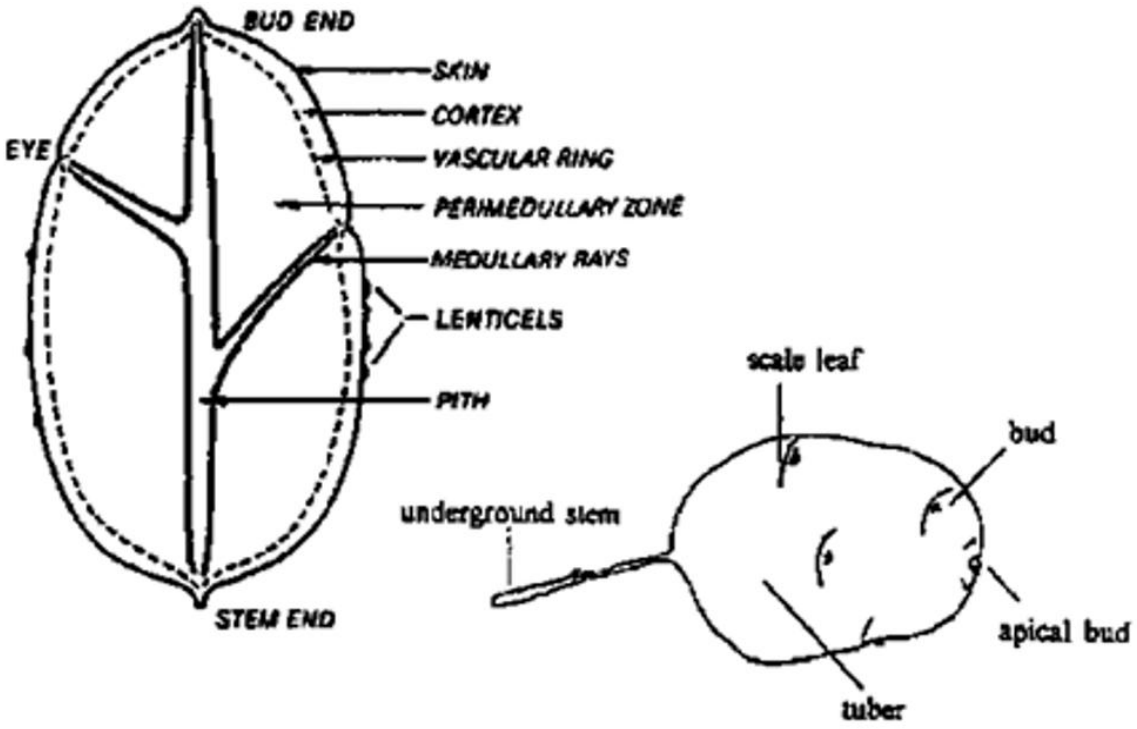


Diagram of a longitudinal section of a potato tuber (Rastovski et al., 1981)

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

1.3. Biochemical aspects of tubers

According to Talburt and Smith (1967) white potato tubers contain 77.5% water and 22.5% solids such as: protein2.0%, carbohydrates (with 0.6% crude fibre) 19.4%, fat 0.1% and ash 1.0%. Potato contains important mineral elements such as: iron 0.01%, sulphur 0.15%, magnesium about0.1%, calcium 0.05%, potassium, boron, copper, silicon, manganese, iodine

and fluorine which are needed for health (Salaman, 1985). Three forms of sugar (sucrose, fructose and glucose) and also different nitrogeneous compounds (including free amino acids, storage proteins and nitrate) are present in the potato tuber (Ahmad, 1977).A single potato tuber (of medium size) has about half the daily adult requirement of vitamin C whereas some other staples like rice and wheat have none (CIP website at http:// www.cipotato.org).The potato is a wholesome food with all the extremely important and necessary dietary constituents, which are needed for health and growth (Li, 1985; Burton, 1989). Compared to other roots and tubers and also many cereals, potato tubers have a high ratio protein to carbohydrates with a high nutritional value of the protein (Shekhawat et al., 1994).

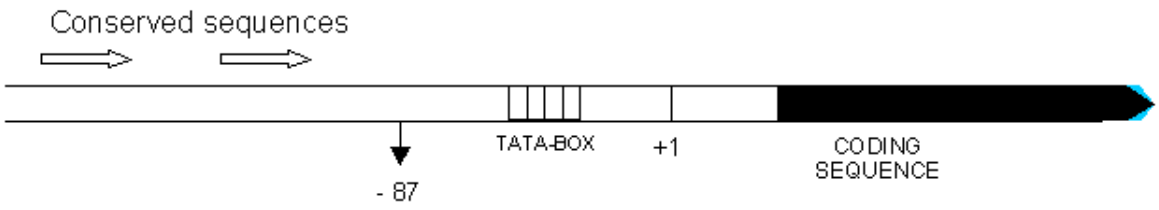
There are two differences in the biochemistry of tubers and other somatic tissues of the potato: the accumulation of large amounts of starch and the accumulation of a set of relatively abundant proteins that may be akin to 'storage proteins'. The major protein amongst those which accumulate in tubers is a glycoprotein called 'patatin', with the other proteins being a type II proteinase inhibitor (chymotrypsin and trypsin/chymotrypsin), a Bowman-Birk proteinase inhibitor and Kunitz proteinase inhibitor. They have been characterised mostly on the basis of sequence comparisons (Stiekemaet al., 1988). Together with patatin, these species account for 50% of tuber proteins but up to 40% of the total soluble protein of potato tubers is represented by 'patatin'. The high amount of patatin in potato tubers argues for its function as a storage protein. Storage proteins, in general, can be defined as proteins whose major role is to act as stores of nitrogen, sulphur and carbon. They may enable the plant to survive periods of adverse conditions, and may provide nutrients to support the growth of new plants as seedlings (from seeds) or shoots (from tubers). They act as sink for nitrogen (and probably also sulphur), accumulating in greater amounts under conditions of excess nutrient supply. As a storage protein, patatin is mainly localized in the plant cell vacuoles.

1.4.Patatins: Major soluble proteins of potato tubers

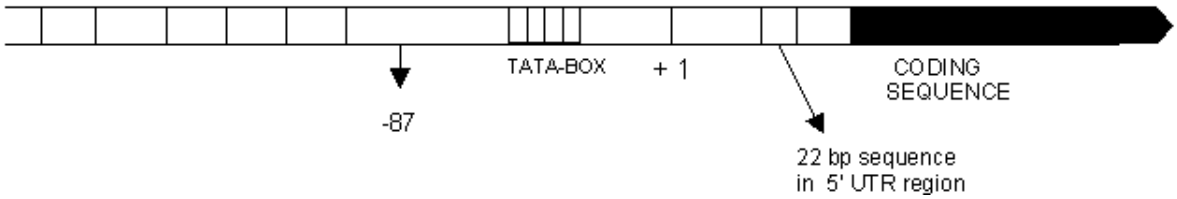
Osborne and Campbell (1986) reported that the major protein in potato tubers was a globulin which they termed 'tuberin'. More recently, Racusen and Foote (1980) reported that a glycoprotein of Mr about 45000 Da accounted for about 20% of the total soluble protein in potato and proposed the alternative name ' patatin', based on 'Patata' which is the original American Indian-derived Spanish word for potato. The name patatin has since become widely accepted. The major protein of potato tubers, called patatin, is a family of immunologically

identical isoforms of glycoproteins, comprising up to 40% of the total protein in tubers. In potato, the patatins are encoded by a multigene gene family with 10-15 members per haploid genome. These genes can be divided in to 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(Pikaardet 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 (Pikaardet al., 1987; Migneryet 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:



The molecular mass of patatin monomer ranges between 39 and 43 kDa. It was determined that one to threeglycosylations (by sugar antenna with molecular mass of about 1.1-1.2 kDa) have the main effect on the mentioned molecular mass range (Sonnewald et al., 1989; Pots, 1999). Patatin appears to serve as a storage protein, but unlike most other plant storage proteins, it has also surprising enzymatic activities of nonspecific lipid acyl hydrolase (LAH), (Racusen et al., 1986; Shewry, 2003), phospholipase A2 (Senda et al., 1996), β 1,3-glucanase (Tonón et al., 2001) and β 1,2-xylosidase (Peyer et al., 2004). These findings have supported the concept that patatin is

not only a storage protein but could also be a part of the potato defense mechanism (de Souza Cândido et al., 2011).

However, the real physiological role of patatin in potato tubers has not yet been completely established (Bárta and Curn, 2004; Pots et al., 1999; Shewry, 2003). The mentioned enzymatic activities of patatin and, furthermore, its characteristics such as solubility (Kärenlampi and White, 2009), high foaming activity (Ralet and Gueguen, 2000), antioxidative potential (Liu et al., 2003) and high level of essential amino acid index with value of about 86.1% (Bártová and Barta, 2009) make patatin an interesting protein source for use infood and biotechnological applications.Variability of protein content in potato tubers, patatin relative abundance in total protein, and also biochemical and other properties of patatin proteins are influenced particularly by genotype (cultivar).

1.5. Relevance of the present study

Patatins present in potato tubers and other parts of the potato plant is encoded by a multigene family consisting of large number of genes. Only a few members of Class-I and Class-II family are studied both at the biochemical and molecular level. However, many questions are yet to be addressed with respect to the regulation of expression of the individual members of the patatin gene family. Till date, there is no report available on patatins with respect to the corresponding promoters, their relative strengths along with the biochemical aspects from our own Indian potato cultivars. Further,unlike constitutive expression of genes, tissue-specific expression is the result of several molecular interactions. Patatin gene promoters 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.

Therefore in order to have molecular insights on patatin genes, the aim of the present study is to carry out molecular cloning and characterization of patatin gene promoters from Indian potato cultivars. The present work would also help in doing a comparative analysis of Class-I and Class-II patatin gene promoters in terms of their efficiency and regulation.

Chapter 2

Literature Review

2.1. Characteristics of patatin

Patatin is encoded by a gene family with 10-15 members per haploid genome. Genetic mapping of the patatin genes indicates that all genes map to a single locus on chromosome 8 (Ganal et al., 1991). Park et al. (1983) estimated the molecular mass of patatin to be about 40,000 Da and showed extensive heterogeneity with forms differing in electrophoretic mobility at pH 8.6 and in mobility on SDS-PAGE. Paive et al.(1983) demonstrated that there was a linear relationship between the amount of patatin, expressed as a percentage of total soluble protein, and the logarithm of tuber weight from 0.3 to 300 g, with patatin forming 40% of the total soluble protein in tubers above 200 g. About 33% of the patatin residues have been estimated to adopt α-helical and 46% a β-stranded structure (Pots et al., 1998).

Sonnewald et al.(1989 b) demonstrated that patatin expressed in leaves of transgenic tobacco was glycosylated on two sites (asparagine 60 and asparagine 90), with typical small complex glycans comprising xylose, fructose, mannose and N-acetyl glucosamine in a ratio of 1:1:3:2, which is the same as the ratio of these sugars present in patatin isolated from potato tubers. The mature Class-I and Class-II patatins comprise about 360 amino acid residues but are synthesized with N- terminal signal sequences of 23 residues (Mignery et al., 1984).This is consistent with their transport via the endomembrane systems leading to deposition in vacuoles (Sonnewald et al., 1989 a). Immunochemical studies demonstrated that patatin is located in vacuoles in tubers and in leaves induced for its expression (Sonnewald et al., 1989), an observation which is consistent with N- glycosylation taking place in the endoplasmic reticulum and golgi apparatus (Kermode and Bewley, 1999)

Several cDNA and genomic clones of patatin have been isolated and a number of nucleotide sequences have been determined. The protein coding regions and the promoter regions up to position -87 of all genes analyzed so far are very homologous, whereas upstream from this point the promoters diverge, allowing the genes to be divided into two classes. This conserved region contains the CAAT and TATA homologies as well as a homology to the core enhancer sequence.

Within a class, there are additional large regions of homology, but differences exist that allow the patatin genes to be further divided into several discrete subclasses. Preliminary comparisons by N- terminal sequencing and Ouchterlony double diffusion using polyclonal antiserum to total soluble proteins indicated that the component proteins of patatin are closely related (Park et al; 1983) and this was confirmed by the analysis of cloned cDNAs and genes (Mignery et al., 1984, 1988). This showed the existence of two classes of mRNA and genes, with the former sharing about 98% sequence identity. The encoded proteins, showed some minor differences in sequence, particularly in the N- terminal region, which was in agreement with the heterogeneity observed previously in directly determined N- terminal sequences (Park et al., 1983), but also differed in the presence (Class-II) or absence (Class-I) of a 22 bp sequence with in the 5′untranslated regions.

Pots et al. (1999 b) showed that patatin comprised 10 peaks by reversed-phase high pressure liquid chromatography that could be separated in to four pools by ion-exchange chromatography. These pools represented 62% (A), 26% (B), 5%(C) and 7%(D) of the total fraction, and each comprised isoforms and masses of about 40400 and 41600 Da, which were considered to be due to differences in glycosylation. No differences in the properties or conformational stability of the pools were observed. In other studies, the same group has investigated the effects of pH and temperature on the stability and aggregation of whole patatin fractions (Pots et al., 1998 a, b, 1999 a, c).

2.2. Biological activity of patatins

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

in the activity of the products of Class-I and Class-II genes the former being idendical to those of the form present in potato tubers (Höfgen and Willmitzer, 1990).

A further type of hydrolytic activity has also been described recently for patatin as, an acidic β 1,3-glucanase (Tonón et al., 2001). β 1,3-glucanases are thought to contribute to plant defence to fungal pathogens by digesting β-1,3-glycans in hyphal cell walls and often form part of the pathogenesis-related (PR) protein response (Shewry and Luca, 1997; van Loon and van Strien, 1999). This may imply that patatin plays a role in the defence of potato tubers.

A role of patatin in defence against pest and pathgens is also indicated by two other observations. First, the inclusion of patatin in artificial diets resulted in the inhibition of growth of larvae of corn rootworm, Diabrotica spp. (Stickland et al., 1995). Treatmant of patatin with di-isopropyl flucophosphate inhibited its phospholipase, galactolipase and acylhydrolase activities, and also eliminated its negative effect on larval growth. Comparison of the enzymatic and inhibitory properties of patatin fractions from different cultivars showed that galactolipase activity was correlated with growth inhibition, but not phospholipase or acyl hydrolase activity. Inhibitory activity was also reduced by provision of cholesterol in diet. It was concluded that patatin may provide defence against the insect pest by effects on lipid metabolism. A patatin like protein with galactolipase activity is also induced by draught stress in leaves of Cowpea (*Vignaunguiulata*) (Matos et al., 2000, 2001), indicating that patatin may play a wide role in stress response.

The second indication that patatin play a role in plant defense comes from the studies of tobacco leaves infected with tobacco mosaic virus (Dhondt et al., 2000). Three genes encoding patatin-like proteins were rapidly induced on infection, with one of the protein exhibiting phosholipase A_2(PLA_2) activity. The increase in PLA_2 occurred before the accumulation of fatty acid- derived defence signals (12-oxophytodienoic acid and jasmonic acid), and it is suggested that PLA_2 initiates the synthesis of these by releasing fatty acid substrates from membrane lipids.

An intriguing report, whose significance is still not understood, is that the gene responsible for the STURDY mutant of Arabidopsis encodes a patatin-like protein (Huang et al., 2001). Thisactivation tagged mutant is characterised by a stiff inflorescence stem, thick leaves, short siliques, large seeds, round flowers and delayed growth.

Allergenicity of patatin:Potato may elicit allergenic responses in humans and children, either when consumed as food or by skin contact with raw potatoes. Seppälä et al. (1999) showed that patatin bound toIgE (a class of immunoglobulins specific for allergenic response) from children with a positive skin-prick test to raw potato, and also showed that the purified patatins gave positive skin- prick tests in allergenic children. This was subsequently confirmed by more detailed studies including skin exposure test and oral challenge (Majamoa et al., 2001), and patatin has been given the allergen designation Sol t 1.Heat treatment of potato results in decreased allergenicity, which appears to result from aggregation with other potato proteins rather than denaturation of patatin itself (Koppelman et al., 2002)

Earlier work had shown that a major allergen of latex called Hev b 7, was a 43000 Da protein with sequence homology to patatin (Kostyal et al., 1998; Sowka et al., 1998; Breiteneder et al., 1999) and it came as no surprise that patients with allergy to natural rubber also show in vitro reactivity of IgE to patatin (Seppälä et al., 2009). Similarly,a related allergenic protein is also present in tomato (Reche et al., 2001).

2.3. Regulation of Patatin synthesis

The patatin present in tuber is almost solely encoded by Class-I transcripts with Class-II transcripts being about 50-100 times less abundant (Mignery et al., 1988). However,Pikaard etal.(1987) showed that roots also contain small amounts of an immunologically distinct form of patatin which appears to be encoded by Class-II transcripts.Tubers are usually formed from underground stolons but can also form above ground from auxiliary buds as a result of injury, disease or removal of stolons and tubers. These tubers accumulate patatin to similar levels to those in tubers, i.e. approx. 40-45% of total soluble proteins. Removal of tubers and auxiliary buds can result in the accumulation of patatin, 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 and accumulation of patatin are also induced in leaves incubated with high concentrations of sucrose (Paivaet al., 1983; Rosahl et al., 1986; Rocha-Sosa et al., 1989; Jefferson et al., 1990), but patatin gene expression in tubers is inhibited by wounding (Logemann et al., 1988) and in whole plants and induced stem cuttings by treatment with gibberellic acid (Hannapel et al., 1985).

Northern blots show that tuber and root patatin mRNAs are of similar size and immunoprecipitation of *in vitro* translation products also shows the patatin precursors of same molecular weight in roots and tubers. This suggests that post-translational processing may differ in both roots and tubers (Pikaardet al., 1987). 5′ S1 nuclease and primer extension mapping suggests that the class of patatin transcripts expressed in roots (Class-II transcripts) to be a subset of patatin transcripts expressed in tubers (Classes I and II). Although Class-II transcripts of both tubers and roots appear qualitatively the same by primer extension, S1 mapping and hybridization stringency experiments but there is no direct evidence to suggest that the identical Class-II patatin genes are expressed in both organs. All these data demonstrate that expression of the patatin multigene family is differentially regulated in tubers and roots.

The transcription of patatin was observed in several different cell types, such as in mesophyll cells, epidermal cells and several cell types associated with the vascular system when leaves and shoots were grown on high concentrations of sucrose. Such a 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,* thatinduction is specific for sucrose (Jefferson et al., 1990; Wenzleret 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. The uneven distribution of GUS activity found in young tubers may be related to the fact that sucrose is unloaded from the phloem bundles in the medullary regions of the tuber, and this localized high concentration gives rise to the preferential accumulation of GUS activity in these areas. All the above studies on the expression of patatin-GUS genes used large regions of the promoter of three different Class-I patatin genes.

Chromatin immunoprecipitation method show that there was dramatic increase of patatin gene expression during the transition from stolons to tubers coincides with an increase of histone H4 lysine acetylation. 3′ rapid amplification of cDNA ends were used to profile expression of different patatin genes during tuber development. The profiling results revealed differential expression patterns of specific patatin gene groups throughout six different stages of tuber development. One group of patatin gene transcripts, designated patatin gene group A was found to be the most abundant group during all stages of tuber development. Other patatin gene groups,

with a 48 bp insertion in the 3′untranslated region, are not expressed in stolons but display a gradual increase in expression level following the onset of tuberization. These results demonstrate that the patatin genes exhibit alterations in chromatin state and differential transcriptional regulation during the developmental transition from stolons into tubers, in which there is an increased demand for protein storage (Stuper et al., 2005).

2.4. Functional Characterization of Patatin gene promoters

A further series of experiments have been conducted using different lengths of 5′patatin gene regions fused to GUS, with the aim of defining the *cis*-acting regions necessary for the observed patterns of expression (Jefferson et al., 1990) as well as to test the presence of 'tuber-specific' elements and 'sucrose-specific elements' as separate entities on the same promoter. The minimum promoter length that gave sucrose responsiveness, as measured in *in vitro* experiments by growth of nodal explants on high and low sucrose concentrations and tuber-specific expression, as measured in samples taken from glasshouse-grown plants, extended 360 bp 5′ of the transcriptional start site.

Studies of Class-II promoters using gene fusions to the CAT (Chloramphenicol acyltransferase) reporter gene have also been carried out (Twell and Ooms, 1988). 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-Topferet al., 1989). Expression of this chimaeric gene was also found in callus and suspension cultures of potato.

Detailed studies of the 5′upstream sequences of a patatin gene have been reported by Holdsworth et al. (1992) and Grierson et al. (1994), aimed at identifying specific sequences and trans acting factors that determine developmental regulation and sucrose inducibility. This had led to the

identification of a new type of DNA binding protein, called storekeeper (STK) which is thought to regulate patatin gene expression (Zourelidou et al., 2002; Kapoor et al., 1975; Liedl et al., 1987).Consequently a no. of studies has been carried out on the structure and properties of patain, particularly on its stability and thermal aggregation in relation to the production of functional proteins on an industrial scale.

Blundy et al., 1990 reported that the expression of Class-I patatin gene fusions in transgenic potato varies with both gene and cultivar. The promoters of two Class-I genes, PS20 and PS3/27, were transcriptionally fused to β-glucuronidase and transformedinto the potato cultivars Desiree and Maris Bard. Examination of the expression levels in large populations of microtubers indicated that the PS20 promoter produced β-glucuronidase activities 5-fold lower in Desiree than MarisBard whereas the PS3/27 promoter showed similar levels in both cultivars. Furthermore, the relative expression levels from the two promoters were reversed in the two cultivars.

Naumkina et al., 2006 studied the comparison of the activity of Class-I patatin promoter (B33 promoter) fused with the reporter gene during heterological expression in B33::GUS transgenic arabidopsis (*Arabidopsis thaliana*L.) plants and homological expression of the same DNA construct in potato. Promoter activity was estimated from quantification ofβ-glucuronidase (GUS) activity. It was shown that, during heterological expression in arabidopsis seedlings, B33 promoter manifested a tissue-specificity and inducibility, although in a different manner than during homological expression in potato. In noninducedarabidopsis seedlings, B33 promoter was most active in the roots, whereas, after induction with sucrose treatment, it became most active in cotyledons. 10 mM sucrose was sufficient for a manifold activation of B33 promoter in intact seedlings. The degree of B33 promoter induction by sucrose in arabidopsis seedlings was strictly organ-specific and increased in the following sequence: root < hypocotyl < cotyledons. 150-200 mM sucrose enhanced B33 promoter activity in cotyledons by 200 to 300 times i.e., much stronger than in potato organs. Glucose and fructose were less efficient than sucrose. Phytohormones affecting tuber formation in potato (gibberellins, auxins, and cytokinins) did not affect significantly B33 promoter activity in arabidopsis.

Studies on functional analysis of a Class-I patatin gene SK24-1 in microtuber formation of transgenic potatoes revealed that expression of SK24-1, cDNA clone in *Escherichia*

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

In order to understand the molecular mechanism underlying the complex control of patatin expression as well as the differential expression of the two classes of patatin genes several members of the patatin gene family representing Class-I and Class-II have been isolated. The class-specific expression of patatin genes was investigated by analyzing four new patatin genes. A Class-I patatin gene from cv. Berolina as well as a Class-I and two Class-II patatin genes from the monohaploid cultivar AM 80/5793 were isolated and partially sequenced. Sequence comparison indicates rearrangements as the major source for the generation of diversity between the different members of the classes. The expression of single genes was studied in potato plants transformed with chimeric genes where the putative patatin promoters were fused to the GUS reporter gene. A detailed histochemical analysis reveals that both Class-I genes are expressed in the starch-containing cells of potato tubers and in sucrose-induced leaves. The Class-II gene pgT12 shows the same expression pattern in root tips and in the vascular tissue of tubers, whereas no activity was detectable for pgT4. Thus the expression pattern of both classes of genes seems to be stable at least within or even between different cultivars (Liu XY et al., 2003).

Objectives

Based on the literature survey and background work in our laboratory, the following objectives are framed:

- Designing of oligonucleotide primers specific to Class-I and Class-II patatin gene promoters
- Molecular cloning and characterization of only the promoter region of patatin genes from Indian potato cultivars
- Molecular approaches for making *Ti*-plasmid based Patatin promoter-GUS genetic constructs

Chapter 3

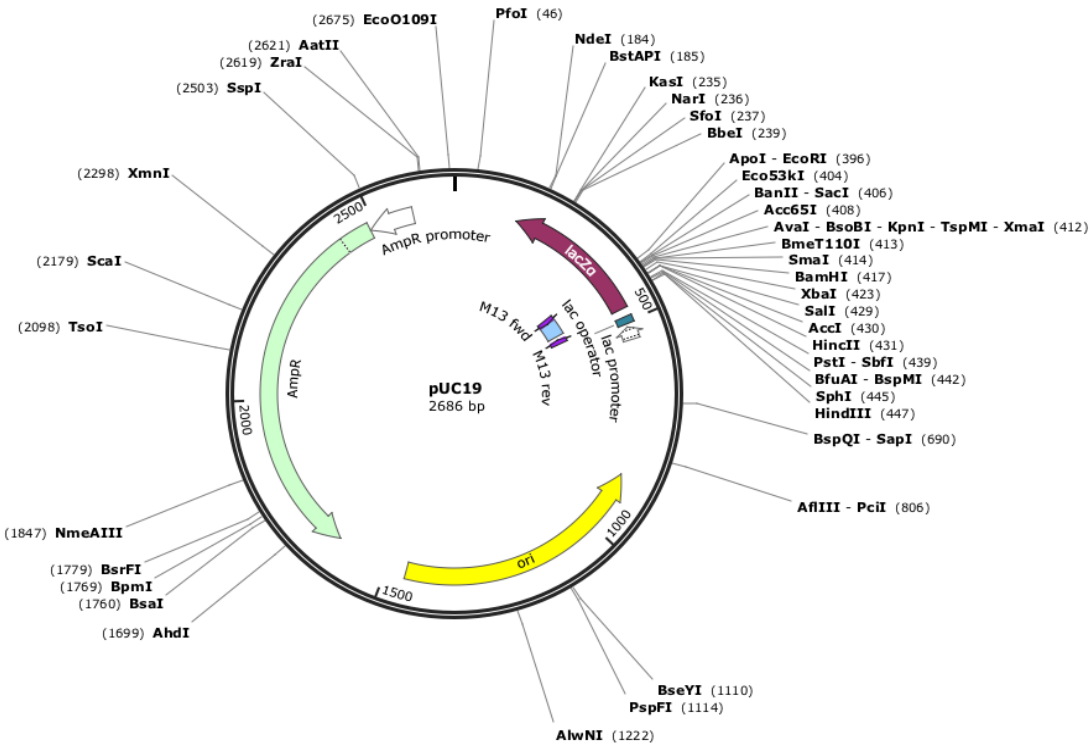
Materials and Methods

3.1. Materials

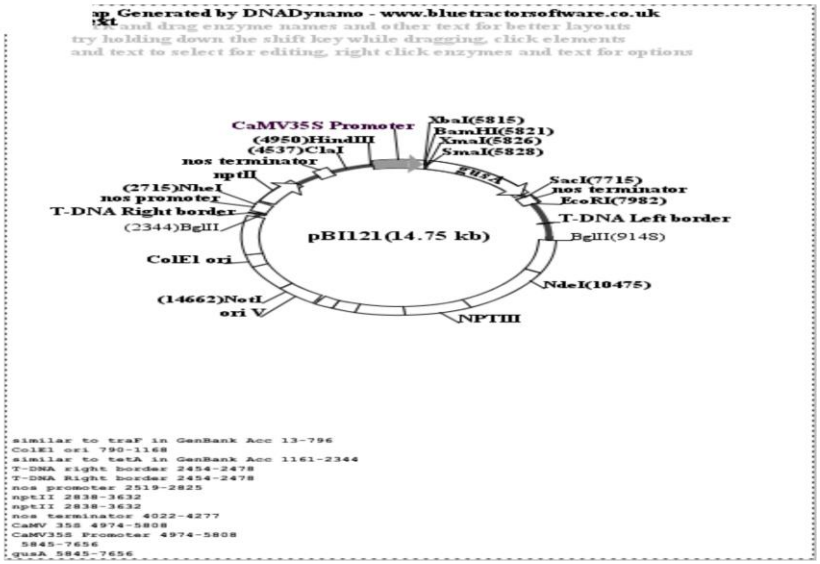
3.1.1. Bacterial strains and plasmids

***E. coli* DH5α:** supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 *rel*A1 *E.coli* DH5α strain was maintained on Luria agar mediumwhereas those transformed with pUC19 plasmid were maintained on Luria agar medium containing 50 µg mL^{-1} of ampicillin.

pUC19 Vector: pUC19 (GenBank Accession No: X02514) is a commonly used plasmid cloning vector in *E.coli* (Yanisch-Perron et al. 1985). Its size is 2686 bp. It is a high copy number plasmid. It carries a 54 bp multiple cloning site that contains unique sites for a number of different hexanucleotidespecific restriction endonucleases.



pBI121 Vector: pBI121 (GenBank Accession No: AF485783) is binary vector most widely used in plant transformation system. Its size is ~14.75 kb.



3.1.2. Media used

Luria-Bertani (LB) Medium

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

3.1.3. 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	8.0% (w/v)
Triton X-100	0.5% (w/v)
EDTA	50 mM (pH= 8.0)
Tris-HCl	10mM (pH= 8.0)

TBE Buffer (5X):

Tris Base	$54gL^{-1}$
Boric Acid	$28gL^{-1}$
EDTA	$3.8gL^{-1}$((pH= 8.0)

TAE Buffer (5X):

Tris-HCl	24.2 gL^{-1}
Glacial acetic acid	5.7 ml L^{-1}
0.5 M EDTA	10 mlL^{-1} (pH= 8.0)

TE Buffer (1X):

Tris-HCl	10mM (pH= 8.0)
EDTA	1.0 mM (pH= 8.0)

3.1.4. Enzymes used

Restriction Enzymes

Various hexacutter restriction enzymes such as *Eco*R1, *Bam*H1, *Hind*III and *Sma*1were used in the present study. Restriction digestion was carried out in buffer supplied by the manufacturer. Depending upon specific enzyme, reaction was carried out at appropriate temperature.

Description of some restriction enzymes used in this study:

Restriction enzymes	Restriction site	Reaction temperature
*Eco*R1	GAATTC	37˚C
*Bam*H1	GGATCC	37˚C
*Hind*III	AAGCTT	37˚C
*Ecl*136II	GAGCTC	37˚C
*Sac*1	GAGCTC	37˚C
*Sma*1	CCCGGG	28˚C

Other Enzymes

Description of Different Enzymes:

Various enzymes	Stock conc.	Working conc.	Other relevant details
Lysozyme	10 mg mL^{-1}	300-400 μg mL^{-1}	Sterile water was used for stock preparation.
Ribonuclease A	10 mg mL^{-1}	10-20 μg mL^{-1}	Dissolved in 10 mMTris-HCl (pH 8.0) and 15 mMNaCl buffer and kept in boiling water bath for 10 min followed by slow cooling to room temperature and stored at -20 ºC for subsequent use
T4 DNA Ligase	400UμL^{-1}	~ 40 U μL^{-1}	The enzyme was diluted using dilution buffer as provided by the manufacturer.
Klenow Fragment of *E.coli* DNA polymerase I	5 U μL^{-1}	2 U per 50 µL of reaction volume	Klenow enzyme treatment was carried in the buffer as supplied by the manufacturer
Taq DNA Polymerase	3 U μL^{-1}	3 U μL^{-1}	

3.1.5. Other Chemicals

Chemicals/Biochemicals	Stock conc.(mg mL^{-1})	Working conc.(μg mL^{-1})	Solvent used
Ethidium bromide	5	0.5-1.0	Sterile water

X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside)	20	20	N, N-dimethyl formamide
IPTG (Isopropyl β-D-thiogalactopyranoside)	100	100	Sterile water
Ampicillin	50	50	Sterile water
Kanamycin	50	50	Sterile water

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

3.2. Methods

3.2.1. Background work in our laboratory

Earlier we focused on the molecular cloning studies of the 5′ flanking regions of patatin genes from different potato cultivars.For this, four potato cultivars were chosen namely Kufri Chipsona-1(CS-1), Kufri Chipsona-2 (CS-2), KufriChandramukhi (KCM) and KufriJyoti(KJ).In order to amplify the 5′ flanking regions of the patatin genes from these cultivars, a series of combination of different forward primers and reverse primer were designed based on the Class I and Class-II patatin gene sequences available in the database.

3.2.2. Polymerase chain reaction

PCR is iterative process, consisting of three cycling parameters, heat denaturation of DNA template, annealing of oligonucleotide primers to single stranded DNA templates, and extension of the annealed primers by a thermostable DNA polymerase. In this study, the recombinant pUC19 clones PTV-04, PTV-03, PTV-01 and PK03 were used as templates and PCR was carried out using the respective oligonucleotide primers. The combinations were as follows:

a) PT2-F025 & PT1-RV (For PTV-04); b) PT2-F067 & PT1-RV (For PTV-03)
c) PT2-F067 & PT1-RV (For PTV-01); d) PTF-610 & PT1-RV (For PK03)

PCR was carried out in 50 µL reaction volume and various components were used as follows:

Template DNA	0.1 to 1.0 µg
10X Buffer	5 µL
Forward Primer	10 pmoles
Reverse Primer	10 pmoles
dNTP’s	25 µM
Sterile D.D. Water	To make up the volume 50 µL
Taq DNA polymerase	3 U/µL

The thermal cycling parameters were set as follows:

Initial Denaturation(Pre-PCR)	94°C	1 min
Denaturation	94°C	1 min
Annealing	55°C	2 min
Polymerization	72°C	1 min
Final Extesion(Post-PCR)	72°C	7min

The reaction was carried out for 30 cycles.

3.2.3. Setting up a restriction digestion reaction

Restriction endonucleases are enzymes that recognize short DNA sequences and cleave double stranded DNA at specific sites within or adjacent to the recognition sequences. Restriction digestion reaction was set up according to manufacturer's instructions and was usually carried out in a reaction volume of 15 µL for 2½ hours at optimum temperatures, depending upon the restriction enzyme.

3.2.4. Agarose gel electrophoresis

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

3.2.5. Cloning in to pUC19 plasmid vector

Processing of insert for ligation:Generally, the PCR amplified DNA products are not truly blunt-ended as the enzyme *Taq* DNA polymerase has a tendency to add an extra 'A' residue at the 3' end of both the strands along with 3' recessed termini. As a polishing step here, Klenowtreatment served two purposes: firstly, removal of the extra 'A' residue at the 3' ends; secondly, filling up the recessed 3' termini in the amplified DNA products. For this purpose, 20µL of amplified DNA products i.e. the inserts were dissolved in minimum volume of water. In the same tube, a 50 µL of reaction volume was set up by adding required amount of 10X Klenow enzyme buffer, dNTP-mix and finally 1-2 Uof Klenow enzyme. The reaction was carried out at 28°C for 40 min and then terminated by incubating at 65°C for 5-7 min. Then truly blunt ended PCR products were digested with *Bam*H1 so as to obtain inserts blunt ended at 3′ end and

cohesive ended at 5′ end. The processed inserts were purified using QIAEX II agarose gel extraction protocol.

Processing of plasmid vector for ligation:For cloning of amplified PCR product, plasmid pUC19, a high-copy plasmid vector was used.2-3 µgof pUC19 DNA was first digested completely with hexacutter*Sma*1 that produced blunt-ended termini followed by restriction digestion with *Bam*H1. The completion of the digestion was further checked through agarose gel electrophoresis using a proper control. The processed pUC19 vector having one side blunt ended as a result of *Sma*1 digestion and other side cohesive ended due to *Bam*H1 digestion was purified using QIAEX II agarose gel extraction protocol.

3.2.6.Recovery of DNA bands using Qiagen kit

QIAEX II agarose gel extraction protocol was followed for purification of DNA bands. For this, DNA samples were run in two lanes on 0.8 % agarose in 1X TAE buffer along with a control lane in which sample was also loaded. The gel was run for sufficient time and then, the control lane was excised with the help of a clean scalpel and visualized on a UV-transilluminator. The position of DNA bands of interest was marked and then, corresponding bands were excised from sample lanes without UV exposure. The gel slices were weighed separately in 1.5 mL microfuge tube. Following this, three volumes of Buffer QX1 was added to one volume of gel as per the manufacturer's instructions. QIAEX II solution was resuspended by vortexing for 30 seconds and 20 µL of it was added to each sample. In order to solubilize the agarose, the samples were incubated at 50°C for 10 min with intermittent vortexing after every 2 min. This was done to keep QIAEX II in suspension. The samples were centrifuged for 30 seconds at high speed and the supernatant was carefully removed with a pipette. The pellet was washed with 500 µL of Buffer QX1. For this, the pellet was first resuspended by vortexing and then the sample was centrifuged for 30 seconds followed by removal of supernatant. In the same manner, the pellet was washed with 500 µL of PE Buffer twice, supplied with the kit. The pellet was air dried until it appeared powdery white. 7 µL of sterile water and 7 µL of TE buffer were added to the pellet and resuspended by vortexing. It was incubated at 50°C for 10 min and then centrifuged for 30 seconds. The supernatant containing the purified DNA was carefully transferred into a clean microfuge tube. The above two steps were repeated to increase the yield. Lastly, the eluates were combined and 2.5 µL was loaded on 0.8 % agarose gel in order to check the yield.

3.2.7. Ligation reaction

A ligation reaction was set up in order to ligate the insert into vector, 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.To improve the efficiency of ligation, the reaction was carried out at 21°C and PEG 8000 (Poly Ethylene Glycol) was included in the ligation buffer.

The components of a ligation reaction are as follows:

Vector DNA	>0.3 to 0.5 µg
Insert DNA	0.3 to 0.5µg
T4 DNA Ligase Buffer	Used at a concentration of 1X
T4 DNA Ligase Enzyme	1-10 U

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

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

*E.coli*DH5αwas transformed with various DNA samples using the standard $CaCl_2$method(Mandel and Higa, 1970).

Preparation of competent cells:A single bacterial colony was inoculated in 25 mL of Luria broth and incubated at 125 rpm at 37°C for overnight. A small aliquot of overnight grown culture was used to re-inoculate 25 mL of fresh Luria broth and then incubated at 37°C with shaking to obtain an O.D. around 0.4-0.6 at 590 nm. The culture was kept on ice to arrest the cell growth. Cell pellet was recovered by centrifuging the cells at 6500 rpm for 10 minutes. The pellet was resuspended properly in 10 mL of ice-cold 100 mM $CaCl_2$. The cells were recovered by centrifugation at 6500 rpm for 10 minutes. The pellet was resuspended in 1.0 mL of ice-cold 100 mM $CaCl_2$ and kept in ice for 2½hours.

Transformation of plasmid vector: 100 µL of the competent cell suspension was dispensed in sterile microfuge tubes and kept at 0°C. 6-7 µL of ligation mix was added to each tube containing competent cell suspension, mixed well and kept at 4°C for 30 minutes. Heat shock was performed at 42°C for 2 minutes to all the tubes, followed by the addition of 1 mL LB and

incubation at 37°C for 1½ hour. Centrifugation was carried out at 6500 rpm for 6-7 min and 600-800 µL of supernatant was discarded to concentrate the cells. The pellet was resuspended and 100 µL of the above transformed cell suspension was plated on LA medium containing ampicillin (50 µg mL^{-1}) with X-Gal and IPTG. The plates were incubated at 37°C for 16-18 hours. The transformants were further analyzed on the basis of blue/white colour selection in the case of pUC19 vector.Each of the obtained white transformant was further purified by streaking it to single colony.

3.2.9. Miniprep isolation of plasmid DNA

Boiling method:Plasmid isolation in mini scale was carried out by boiling prep method as described by Holmes and Quigley (1981). In this process bacterial transformant colonies were inoculated aseptically in 4.5 mL LB containing ampicillin in test tubes. The culture was incubated at 37°C/120 rpm for overnight. Cells were harvested from 1.5 mL overnight grown culture in microfuge tubes. The pellet was loosened by vortexing, followed by resuspension in 800 µL of STET buffer. 30 µL of lysozyme was added to the bacterial suspension and mixed well. Each microfuge tube containing cell suspension was kept in boiling water bath for 1.5 min. After cooling down to room temperature high speed centrifugation (12,000 rpm) was carried out for 15 min. After removing the pellet, 2.0 µL of RNase solution was added to the supernatant to remove the contaminating RNA. After incubation at 37°C for 45 min equal volume of phenol: chloroform was added, mixed for 5-7 min and centrifugation was performed at 10,000 rpm for 10 min. To the upper aqueous layer, $1/10^{th}$ volume of 3M sodium acetate (CH_3COONa) and equal volume of isopropanol was added and incubated at 4°C for 45 min for the precipitation of plasmid DNA. Then the tubes were centrifuged at 12,000 rpm for 15 min. The DNA pellet was washed with chilled 70% ethanol to ensure the removal of excess salts and other impurities. Finally, DNA pellet was air dried at room temperature and dissolved in 20-30 µL of TE buffer.

3.2.10. Cloning in *Agrobacterium*-mediated *Ti*-plasmid based vector pBI121 vector

Processing of inserts for ligation:To carry out cloning in *Ti*-plasmid based vectors, the obtained recombinant pUC19 clones corresponding to all the inserts were subjected to double digestion with *Bam*H1 and *Ecl*136II as a result of which the released inserts were blunt ended on one side and cohesive ended on other side. The processed inserts were purified according to QIAEX II agarose gel extraction protocol.

Processing of pBI121 vector for ligation:To make the ends of the vector compatible with the ends of inserts, pBI121 vector was first subjected to restriction digestion with *Hind*IIIfollowed by Klenow enzyme treatment in a reaction volume of 25µl using the buffer of the restriction enzyme (*Hind*III), dNTP′s at a concentration of 1mM and Klenow enzyme (3U/µL) at a temperature of 30˚C for 20 minutes. Further, the vector was digested with *Bam*H1so as to obtain pBI121 vector blunt ended on one side and cohesive ended on the other side. The processed vector was purified according to QIAEX II agarose gel extraction protocol.

3.2.11. Genetic transformation of *E. coli* DH5α with recombinant *Ti*-plasmid vectors

*E.coli*DH5αwas transformed with various DNA samples using the standard $CaCl_2$method(Mandel and Higa, 1970). The transformed cell suspension was plated on LA medium containing kanamycin (50 µg mL^{-1}). The plates were incubated at 37°C for 16-18 hours. Each of the obtained white transformant was further purified by streaking it to single colony.

3.2.12. Characterization of the putative recombinant pBI121 clones

Plasmid DNA isolated from white transformant colonies was further analyzed for screening of recombinant pBI121 clones. The recombinant clones were characterized through appropriate restriction enzymes and PCR. Also, the recombinant clones were checked through PCR using the corresponding primer pairs, gene specific forward primer and GUS (reporter gene in pBI121 vector) specific reverse primer.

Chapter 4

Results and Discussion

4.1. Background research work on patatin gene promoters in our laboratory

The 5′ flanking sequences of both the Class-I and Class-II patatin genes as generated in our laboratory were used as the template to further amplify only the promoter region of the corresponding clones inorder to carry out functional characterization.The recombinant pUC19 clones PTV03, PTV01 and PK03 corresponds to Class-I patatin genes and PTV04 corresponds to Class-II patatin genes. The recombinant clones PTV04, PTV03, PTV01 and PK03 were analyzed through agarose gel electrophoresis (Fig.1A & 1B).

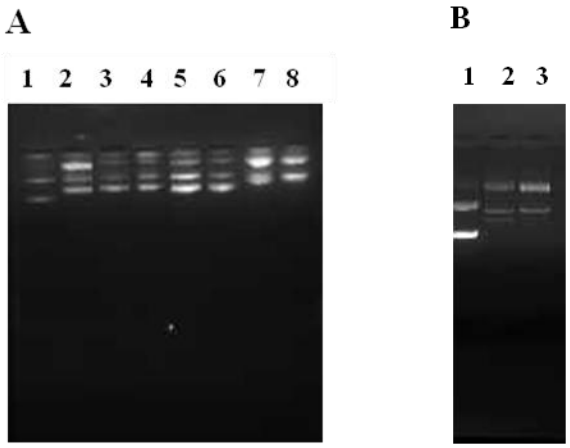


Fig. 1.Agarose gel electrophoresis of recombinant pUC 19 clones (PTV04, PTV03, PTV01 and PK03)

A) Lane 1, control pUC19 DNA; Lanes 2 to 4, recombinant clone PTV04; Lanes 5 and 6, recombinant clone PTV03; Lanes 7 and 8, recombinant clone PTV01; B) Lane 1, control pUC19 DNA; Lanes 2 and 3, recombinant clone PK03 through restriction digestion with *Eco*R1 and *Bam*H1 respectively.

The recombinant clones were linearized indicating the absence of internal site of the respective enzymes in the insert molecules. The sizes of the recombinant clones were analyzed by comparing with the known molecular weight DNA marker through agarose gel electrophoresis. The size of the recombinant clones PTV04, PTV03 and PTV01digested with *Eco*R1and *Bam*H1 were found to be approximately 3.1, 3.1 and 3.6 kb respectively (Fig. 2A & 2B). Similarly, the size of the recombinant clone PK03 was found to be approximately 3.4 kb (Fig. 3A & 3B).

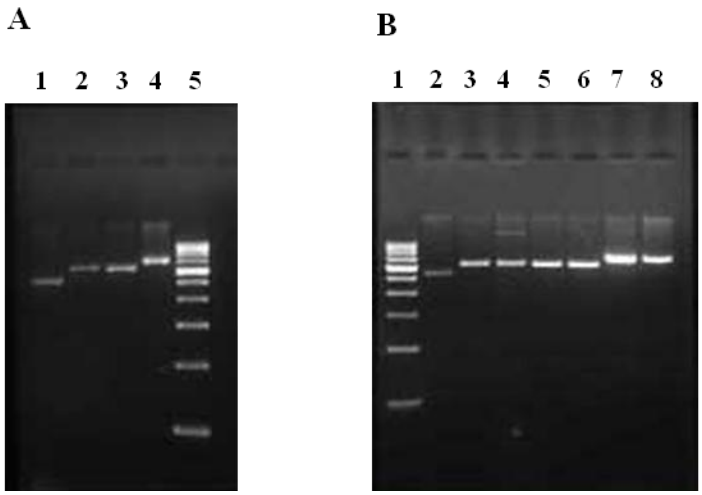


Fig. 2. Restriction analysis of recombinant pUC19 clones (PTV04, PTV03 and PTV01) with *Eco*R1and *Bam*H1

A) Lane 1, pUC19 linearized with *Eco*R1; Lane 2, PTV04 digested with *Eco*R1; Lane 3, PTV03 digested with *Eco*R1; Lane 4, PTV01 digested with *Eco*R1; Lane 5, 0.5 kb DNA ladder; B) Lane 1, 0.5 kb DNA ladder; Lane 2, pUC19 linearized with *Bam*H1; Lane 3and 4, PTV04 digested with *Bam*H1; Lane 5 and 6, PTV03 digested with *Bam*H1; Lane 7 and 8, PTV01 digested with *Bam*H1

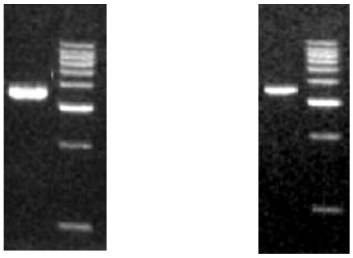


Fig. 3. Restriction analysis of recombinant pUC19 clone (PK03) with*Eco*R1and *Bam*H1A) Lane 1, PK03 linearized with *Eco*R1; Lane 2, 1.0 kb DNA ladder; B) Lane 1, PK03 linearized with *Bam*H1; Lane 2, 1.0 kb DNA ladder

4.2. Designing of oligonucleotide primers

In order to study the 5′flanking regions of both the Class-I and Class-II patatin genes in potato, the following oligonucleotide primers were designed based on the available patatin gene sequences in the GenBank database and were designated as follows:

PT2-F025, the first forward primer, was designed from the 5′ flanking region of the Class-II patatin gene (Accession No. X07030). This gene sequence is 474 bp long and only 320 bp correspond to the promoter region. It was chosen because it shared more sequence similarity with the other members of patatin gene family. The primer corresponds to the bases 25-44 of the above gene and has the following sequence:

5' – TGT TAT ATC AGT AAT CAA TC – 3'

PT2-F067, the other forward primer, was designed from the upstream promoter region of another Class-II patatin gene (Accession no. X60397). The promoter region of this gene is approx. 1.5 kb in length, therefore it provides an extended 5′flanking region for cloning. It was done in order to account for any divergence observed in the far upstream regions from the transcription start site. The primer corresponds to the bases 67-86 and has the following sequence:

5' – ATT ATA GTG CTG AGT CTA GA – 3'

PT-F610, the other forward primer, was designed from the promoter region of Class-I patatin gene (Accession no. X87216). The promoter region of this gene is approx. 1.7 kb in length. The primer corresponds to the bases 610-629 and has the following sequence:

5'—TTC TTA TCA ATT CTG ACG TG –3'

PT1-RV,the reverse primer, was designed covering the transcription start site region of the patatin gene. The reverse primer was designed based on the Class-I patatin gene sequence (Accession No. X87216) as it was expected to be conserved among different classes of patatin isoforms. The above primer is complementary to the bases 1351-1371 of the patatin gene and has the following sequence:

5' – GC GGA TCC GTG CTT TGA GCA TAT AAC AAG-3'

4.3. PCR amplification of the promoter region from the source recombinant pUC19 clones

In order to specifically amplify the promoter region from the recombinant clones, PCR was carried out by using PTV04, PTV03, PTV01 and PK03 as template with the respective primer pairs:

a) PT2-F025 & PT1-RV (PTV-04); b) PT2-F067 & PT1-RV (PTV-03);
c) PT2-F067 & PT1-RV (PTV-01); d) PT-F610 & PT1-RV (PK03)

The PCR cycling parameters adopted for all the recombinant clones are as follows: 1 cycle at 94°C for 1 minute (Pre-PCR), 30 cycles of (94°C for 1 minute, 55°C for 2 minutes and 72°C for

2 minutes) and the last cycle at 72°C for 7 minutes (Post-PCR). The PCR products were resolved through agarose gel electrophoresis. The sizes of the amplified products were determined by comparing with the known molecular weight DNA marker. The size of the PCR products amplified from the recombinant clones PTV04, PTV03 and PTV01 were found to be approximately 0.4 kb, 0.4 kb and 0.9 kb respectively as shown in Figure 4A. Similarly, the size of the PCR product amplified from the recombinant clone PK03 was found to be approximately 0.7 kb as shown in Figure 4B. From the figure, it is evident that the amplified products of PTV04 and PTV03 are of approx. 0.4 kb. Although they have the similar size, but the sequence may differ and the heterogeneity could be explored through sequencing. In the case of the PCR product amplified from PTV01, the size is approx. 0.9 kb and the difference in the size indicates that it may represent a different member of the gene family.

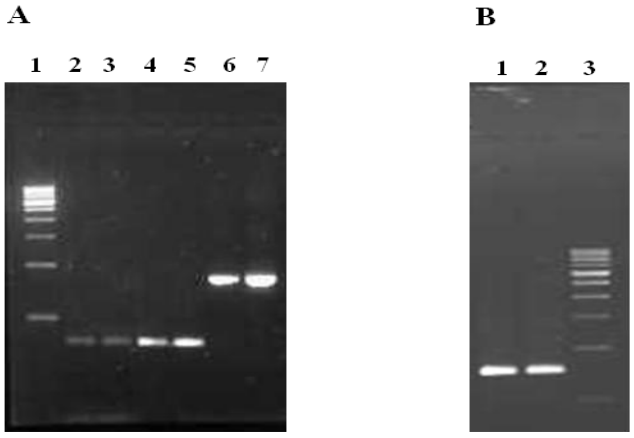


Fig. 4. PCR amplified products of recombinant pUC19 clones (PTV04, PTV03, PTV01 and PK03)

A) Lane 1, 0.5 kb DNA ladder; Lane 2 and 3, PCR amplified product (~0.4 kb) using PTV04 as template; Lane 4 and 5, PCR amplified product (~0.4 kb) using PTV03 as template; Lane 6 and 7, PCR amplified product (~0.9 kb) using PTV01 as template B) Lanes 1 and 2, PCR product (~0.7 kb) using PK03 as template; Lane 3, 0.5 kb DNA ladder

4.4. Molecular Cloning of patatin gene promoters

***Processing of PCR amplified DNA products*:** 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 5′ recessed termini also. Therefore, these DNA products were polished with the Klenow

fragment of *E. coli* DNA polymerase I (as described in the section3.2.5) in presence of dNTPs. These PCR products were then subjected to restriction digestion with *Bam*H1 as the 3′ end of the PCR products contain *Bam*H1 site. The processed DNA bands were then recovered by using QIAGEN gel extraction kit as described in section 3.2.6.

Linearization of plasmid vector: The plasmid vector pUC19 was isolated from the *E. coli* DH5α strain by boiling preparation (as described in section 3.2.9). Further, it was digested with restriction enzymes *Sma*I (a blunt end cutter) and *Bam*H1 (hexacutter) so as to generate vector with compatible ends to ligate PCR amplified DNA products truly blunt ended on one side and *Bam*H1 site on the other end.

In this study, four different ligation reactions were carried out separately using PCR amplified DNA products as mentioned above and linearized pUC19 for overnight at 16°C.

Transformation and selection of transformants: 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 inserts of the following recombinant plasmids corresponding to PTV04, PTV03, PTV01 and PK03 were designated as pPT2-PTV04(~0.4 kb); pPT1-PTV03 (~ 0.4 kb), pPT1-PTV01 (~ 0.9 kb) and pPT1-PK03 (0.7 kb).

4.5. Characterization of putative recombinant pUC19 clones

Restriction analysis of the recombinant plasmids:Restriction analysis of the above recombinant plasmids were carried out using the restriction enzymes namely *Bam*H1 and *Sac*1.Putative recombinant pUC19 clones screened on the basis of α complementation were characterized by double digestion with *Bam*H1and *Sac*1 restriction enzymes. As expected, inserts of the size approx. 0.4 kb, 0.4 kb and 0.9 kb were released from recombinant pUC19 clones pPT2-PTV04(~ 0.4 kb); pPT1-PTV03 (~ 0.4 kb) and pPT1-PTV01 (~ 0.9 kb) respectivelyas shown in Figure 5A. Similarly, insert of the size approx. 0.7 kb was released from the recombinant pUC19 clone pPT1-PK03 (~0.7 kb) as shown in Figure 5B.

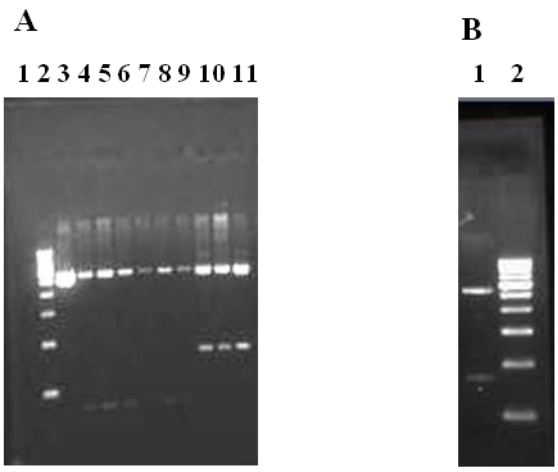


Fig. 5. Restriction analysis of putative recombinant pUC19 clones with *Bam*H1 and *Sac*1

A) Lane 1, 0.5 kb DNA ladder; Lane 2, control pUC19 digested with *Bam*H1 and *Sac*1, Lanes 3, 4 and 5, pPT2-PTV04 clones digested with *Bam*H1 and *Sac*1; Lanes 6, 7 and 8, pPT1-PTV03 clones digested with *Bam*H1 and *Sac*1; Lanes 9, 10 and 11, pPT1-PTV01 clones digested with *Bam*H1 and *Sac*1; B) Lane 1, pPT1-PK03 digested with *Bam*H1 and *Sac*1; Lane 2, 0.5 kb DNA ladder

PCR characterization of putative recombinant clones: The recombinant plasmids were used as templates separately to carry out PCR using their respective primer pairs. By using the primer pairs PT2-F025 & PT1-RV, PT2-F067 & PT1-RVa band of ~0.4 kb, ~0.4 kb, ~0.9 were amplified from the putative recombinant pUC19 clones pPT2-PTV04, pPT1-PTV03, pPT1-PTV01 respectively as shown in Figure 6A. Similarly, a band of ~0.7 kb was amplified from the putative recombinant clone pPT1-PK03 by using the primer pair PT-F610 & PT1-RV as shown in Figure 6B.

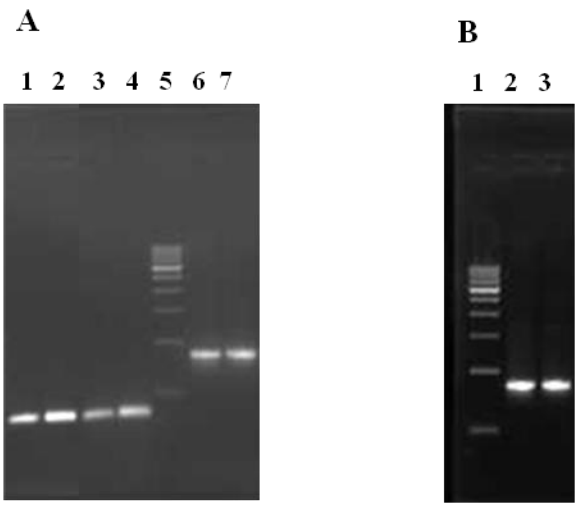


Fig.6.PCR characterization of putative recombinant pUC19 clones

A) Lanes 1 and 2, PCR amplified product (~0.4 kb) using pPT2-PTV04 as template; Lanes 3 and 4, PCR amplified product (~0.4 kb) using pPT1-PTV03 as template; Lane 5, 0.5 kb DNA ladder; Lanes 6 and 7, PCR amplified product (~0.9 kb) using pPT1-PTV01 as template; B) Lane 1, 0.5 kb DNA ladder; Lanes 2 and 3, PCR amplified product (~0.7 kb) using pPT1-PK03 as template

4.6.Construction of *Ti* – plasmid based genetic constructs for*Agrobacterium*transformation

For construction of *Ti*-based genetic constructs, one of the above promoter sequences, pPT1-PK03 was cloned into a binary vector pBI121 by replacing its CaMV35S promoter located upstream of the reporter gene GUS. For proper directional cloning in to pBI121, the inserts from the corresponding pUC19 clones were subjected to double digestion with *Ecl*136II and *Bam*H1. The resulting recombinant clone of pBI121 was designated as pBI-PK03. The cloned promoter was well characterized through PCR.The orientation of the promoter was also checked by amplifying the region of GUS gene along with the promoter specific primer. The characterization of promoter sequence through PCR is shown in Figure 7.

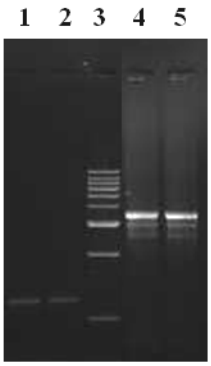


Fig. 7. PCR characterization of recombinant pBI121 clone (pBI-PK03)
Lanes 1 and 2, pBI-PK03 insert amplified from recombinant pBI121 clone; Lane 3, 0.5 kb DNA ladder; Lanes 4 and 5, pBI-PK03-GUS fragment amplified from recombinant pBI121 clone

4.7. Sequence analysis of the recombinant clones: We availed the DNA sequencing services of Bangalore GeNei for sequencing of the clones PTV04-1, PTV01-1 and PTV03-3 and PK03 using M13 forward primer. BLAST search was used to know the sequence similarity. It was observed that the sequence of PTV04 clone was around 90% homologous to the available Class-II patatin gene sequences. Thereby it was confirmed that the PTV04 clone belongs to Class-II patatin gene family. It is interesting to note that there was heterogeneity amongst the sequences as there were still around 8-9% gaps between the homologous sequences. This indicates the novelty of the sequence generated which is important for the purpose of functional characterization. Sequences of PTV01 and PTV03 and PK03 clones were also analyzed for sequence similarities. It was observed that they were homologous to promoter regions of class I patatin gene sequences. This suggests that there is considerable similarity among the 5'-flanking regions of both Class-I and Class-II patatin genes.

All these data collectively suggest that PTV04 clones isolated in this study are specific to Class-II patatin gene promoters and represent only a part of the gene, containing its regulatory region. In-depth understanding at molecular level is only possible through further functional characterization.

CONCLUSIONS

Patatins, in potato are encoded by a multigene family comprising of more than 10-12 copies per haploid genome. Based on the structural features and expression patterns, patatins in potato are divided in to two classes: Class-I and Class-II. It is believed that the expression of the different members of the gene family is influenced by various developmental, metabolic and environmental signals. The 5′-flanking regions of the patatin genes are highly diverse and it is of interest to study the individual members of the gene family for the identification of novel *cis*-acting elements that play a key role in gene regulation. The importance of tuber specific promoters has drawn a lot of attention in terms of basic and applied research. Keeping this in view, earlier we have isolated a number of Class-I and Class-II promoters containing partial coding region and the cloned inserts were characterized through PCR and the sequencing data revealed that they belong to the different members of the gene family. In the present study, efforts were made to make Ti-plasmid based genetic constructs to study the functional characterization of the patatin promoters through transgenic approaches. For functional characterization only the promoter regions were amplified from the source patatin clones of Class-I promoters (PTV-03, PTV-01, PK03); Class-II promoter (PTV-04) and cloned in the pUC19 plasmid vector. The resulting pUC19 clones of Class-I and Class-II patatin promoters were designated as pPT1-PTV-03, pPT1-PTV-01, pPT1-PK03 and pPT2-PTV-04 respectively. Among these clones, a Class-I patatin clone pPT1-PK03 was subjected to double digestion and the released insert was cloned in to the *Agrobacterium* based Ti-plasmid vector pBI121 by replacing the CaMV 35S promoter region to generate Class-I patatin promoter-GUS fusion (pBI-PK03). The resulting recombinant clone pBI-PK03 was well characterized and the orientation of the insert was checked through PCR. Further, functional characterization of the Class-I patatin promoter can be carried out by *Agrobacterium*-mediated genetic transformation of the potato system with pBI-PK03, a binary genetic construct generated in the present study.

Chapter 5

SUMMARY

The present study is focused on the Molecular Cloning and Functional Characterization of Class-I and Class-II patatin gene promoters. The background work carried out in our laboratory resulted in identifying a few promoter sequences corresponding to both the classes of the gene family. At present, further attempts were made to carry out the molecular cloning of these promoters in *Ti*-plasmid based vectors followed by transformation to potato plants to study the functional characterization of the promoter sequences.Various experimental steps as adopted in the study are briefly discussed below:

- The putative recombinant clones containing the partial coding region of the Class-I and Class-II patatin gene family used in the present study are PTV03, PTV01, PK03 and PTV04 respectively.
- Three oligonucleotide forward primers were designed (PT2-F025, PT2-F067 and PTF-610, each a 20-mer) based on the Class-II and Class-I patatin gene sequences available in GenBank database. The forward primer, PT2-F025 was designed from the upstream promoter region of Class-II patatin gene (Accession No. X07030). Another forward primer, PT-F610 was designed based on the Class-I patatin gene sequence available in GenBank database (Accession no. X87216).The reverse primer, PT1-RV was designed based on the gene sequence X87216 from the region covering transcription start site and the region seems to be conserved among the different members of the gene family.
- The source putative recombinant clones were well characterized through restriction enzymes *Eco*R1 and *Bam*H1 and PCR by using their respective primer pairs.
- In order to specifically amplify the promoter region from the recombinant clones, PCR was carried out by using PTV04, PTV03, PTV01 and PK03 as template with the respective primer pairs. a) PT2-F025 & PT1-RV (PTV-04); b) PT2-F067 & PT1- RV (PTV-03); c) PT2-F067 & PT1-RV (PTV-01); d) PT-F610 & PT1-RV (PK03).

- Polymerase Chain Reaction (PCR) was carried out using the source recombinant pUC19 clones as the template DNA.
- The amplified PCR products were polished with Klenow enzyme, digested with *Bam*HIand cloned in to the *Sma*1 and *Bam*H1 site of pUC19 vector.
- The resulting recombinant pUC19 clones corresponding to PTV04, PTV03, PTV01 and PK03 were designated as pPT2-PTV04, pPT1-PTV03, pPT1-PTV01 and pPT1-PK03 respectively.
- The above recombinant clones were further characterized through restriction analysis by *Bam*H1 and *Sac*1 followed by PCR using their respective primer pairs.
- The recombinant clone pPT1-PK03 was further subjected to double digestion with BamH1 and Ecl136II and the insert was cloned in to pBI121 by replacing the CaMV 35S promoter to generate pPT1-PK03-GUS fusion.
- The resulting pPT1-PK03-GUS fusion was well characterized through PCR by using its respective primer pair and the orientation of the insert was checked through promoter specific forward primer and GUS specific reverse primer.
- Further attempts are being made to carry out molecular cloning studies of pPT2-PTV04, pPT1-PTV03, pPT1-PTV01 in *Agrobacterium* mediated *Ti*-plasmid based vector followed by functional characterization in transgenic potato lines.

Chapter 6

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