STUDIES ON CLONING AND EXPRESSION OF PROTEIN-L ISOASPARTATE-O-METHYLTRANSFERASE GENE OF *CARICA PAPAYA*

काशी हिन्दू विश्वविद्यालय BANARAS HINDU UNIVERSITY

THESIS
SUBMITTED FOR THE DEGREE OF

Masters of Science
in
Plant Biotechnology

Submitted by

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I have great pleasure in forwarding the thesis entitled **"Cloning and expression of ProteinL-isoaspartate-O-methyltransferase gene of *Carica papaya*"** submitted by **Ms. AKANKSHA GUPTA**, **Roll No. 20430PLB001**, in partial fulfillment of the requirements for the degree of **Master of Science** in **Plant Biotechnology**, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi and placing on record that he has completed the requisite residential requirements as contained in the statue of the University.

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Thanking you

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(Course Coordinator)

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Studies on" Cloning and expression of Protein-L-isoaspartate-O-methyltransferase gene of *Carica papaya*"

by

AKANKSHA GUPTA

Thesis submitted in partial fulfilment of the requirements for the degree of

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LIST OF ABBREVIATIONS

Asn	Asparagine
Asp	Aspartyl
AdoMet	S-adenosyl- L- methionine
bp	Base pair
DNA	Deoxyribonucleic acid
EtBr	Ethidium bromide
EDTA	Ethylene -di- aminetetraacetic acid
et al	(et albeit) and elsewhere
fig	Figure
IsoAsp	L-Isoaspartyl
IPTG	Iso propyl-D-thiogalactopyranoside
mM	Millimole
ng	Nanogram
PAGE	Polyacrylamide gel electrophoresis
PMTs	Protein methyltransferase
PIMT	Protein -L- iso- aspartyl-O- methyltransferase
PCMT	Protein carboxyl methyltransferase
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SAM	S- adenosylmethionine
SDS	Sodium dodecyl sulfate
TAE	Tris acetic acid EDTA
Tm	Melting temperature
UV	Ultraviolet
µg	Microgram
µl	Microliter

CONTENT

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Misfolded, unfolded, and abnormal proteins are formed as a result of errors in post-translational modification of proteins and the abortion of chaperone machinery. Proteins are constantly damaged by a variety of extrinsic and intrinsic factors. Sugars, aldehydes, reactive nitrogen and oxygen species, as well as oxidative modification of their lateral chain of amino acids, can harm them. Many proteins' conformational stability and activity are affected as a result of this. To prevent the accumulation of abnormal proteins, the majority of damaged proteins are degraded by the proteasome system or repaired by protein repair enzymes like PIMT.

Methylation of proteins is a complex and multifaceted process **(Paik *et al*., 1971).** Protein Methyltransferases (PMTs) are the enzymes that catalyze the methylation of proteins **(Clarke *et al*.,1993).** S-adenosylmethionine (SAM) is used as a methyl group donor by protein methyltransferase enzymes **(Clarke *et al*.,2013).** Polypeptides are sensitive to a range of spontaneous, structural modifications that can cause structural and biological activity to be impacted. By regulating histone tail modification, PMTs play a functional role in cell response in stressful situations and protein repair **(Visick *et al*.,1998).** Deamidation and dehydration of Asn and Asp of amino acid are examples of protein damage. Under oxidative stress, the conversion of Asparginyl (Asn) and Aspartyl (Asp) residues to L-Isoaspartyl (IsoAsp) residues as a major compound occurs via the formation of the L-Succinimide intermediate **(Mishra PKK *et al*., 2019, Mahawar M. *et al*., 2019).** The nucleophilic attack of the adjacent amino acid nitrogen on the side chain carbonyl stimulates the alteration of Asp and Asp residues, resulting in the formation of a cyclic ring between the side chain and the main chain, resulting in the formation of a succinimide intermediate **(Clarke *et al*.,1992).**

IsoAsp can affects the protein in many physiological conditions. Succinimides intermediates are unstable products that are hydrolyzed into a mixture of aspartyl and isoaspartyl residues in a nonenzymatic manner. Isoaspartyls make up about two-third of the new products, and with the addition of an extra carbon to the main chain, they have a higher risk of interrupting structure of the protein and thus it's bioactivity **(Clarke *et al*.,1987).**

PIMT is a highly conserved protein repair enzyme found in a wide variety of organisms including bacteria, plants, and vertebrates such as mammals (**O'Connor *et al*., 2006**). The efficacy of PIMT in a variety of organisms, as well as its protein repair functions, have been thoroughly investigated. Plants acquire higher levels of isoAsp in proteins in response to heat and oxidative stress, which has been proven to have a deleterious impact on their growth, development, and survivability.

There are only a few protein repair enzymes known. One of them, Protein Iso-Aspartate Methyltransferases (PIMT), also known as Protein Carboxyl Methyltransferases (PCMT), is responsible for converting Iso-Aspartate (abnormal amino acid residues) to Aspartate (normal amino acid residues). PCMT is an enzyme found in a wide range of living organisms **(Mishra PKK *et al*., 2019, Mahawar M. *et al*., 2019).**

Transfer of methyl group by PIMT and subsequent methylation leads to reformation of succinimide intermediate. The methyl group from S-adenosyl-L-methionine (SAM) to the alpha carboxyl of the Iso-Aspartyl group is transferred and further hydrolysis converts it to (normal amino acid) aspartyls residues **(Kindrachukt *et al*., 2003).**

Overexpression of protein-repair enzymes, in particular, has been shown to extend lifespan, supporting the theory that senescence is caused by a decline in cellular repair processes **(Knight *et al*., 2000).** The ability of these proteins to increase longevity is thought to be due to an increased ability to deal with damaged or misfolded proteins, which limit the organism's lifespan. The expression level of the PIMT enzyme increases in highly stressful situations. It was also shown that PIMT activity is crucial for plant growth, development, and survival under heat and oxidative stress conditions. In addition, it was discovered that under heat and oxidative stress conditions, catalase and superoxide dismutase acquire iso-Asp residues, resulting in a considerable reduction in their catalytic activity.

The ability of these proteins to increase longevity is thought to be due to a higher ability to deal with damaged or misfolded proteins, which limit the organism's lifespan. PIMT genes have been identified and characterized from a variety of species. Also, PIMT activity being found from a variety of sources in plants **(Thapar *et al*., 2001, Mudgett *et al*., 1997, Shen-Miller *et al*., 1995).**

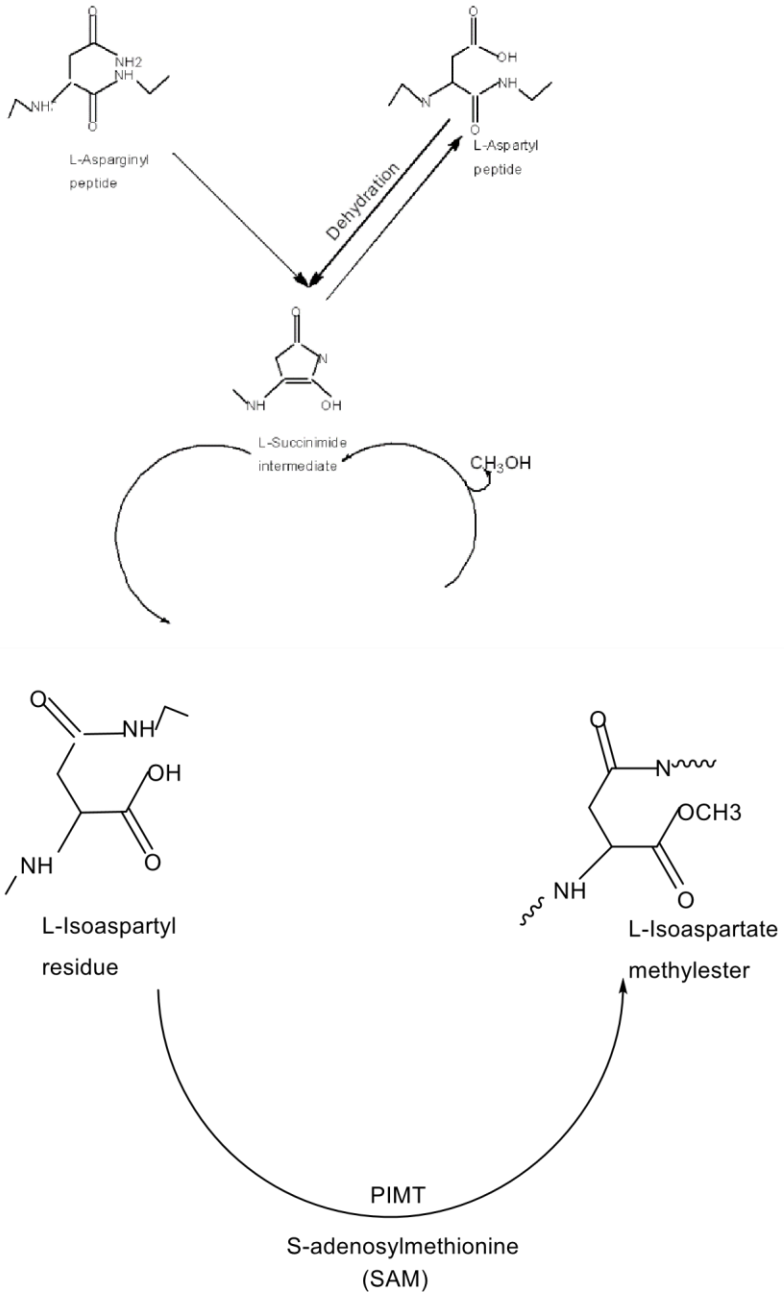


Fig. 1.1 Mechanism of PIMT enzyme in stress conditions: PIMT restores the iso-asp to asp residues through a succinimide intermediate**.**

We were particularly interested in cloning and expression of this enzyme from Papaya (*Carica papaya*). The expression of rPIMT of *Carica papaya* in *Escherichia coli* is investigated in this work.

REVIEW OF LITERATURE

Throughout their lives, plants are subjected to a range of stressful circumstances that might disrupt their regular growth and development. Salinity stress is one of the most severe environmental pressures. Stresses have a negative impact on a plant's overall development and survival **(Isayenkov and Maathuis, 2019).** Plants, particularly seeds, suffer from spontaneous protein breakdown, just like other species, as a result of ageing or stressful conditions. Proteins lose their biological function as a result of excessive stress, and they undergo numerous covalent modifications that cause kinks in their structure, disrupting their biological activity. Protein mutations can also change how proteins work. For example, oxidative damage to a protein's active site might result in the loss of enzymatic activity **(Stadtman *et al*., 1990).** Proteins that are misfolded, unfolded, or aberrant accumulate in cells, resulting in cell death. To prevent abnormal protein accumulation, the majority of proteins are destroyed by the ubiquitin proteosome system or repaired by protein repair enzymes. Protein-l-iso-aspartate-o-methyltransferases (PIMT) are one of the few protein repair enzymes identified.

2.1 Protein-l- iso-aspartate-o-methyltransferases

Aspartyl (Asp) and asparaginyl (Asn) residues in proteins spontaneously convert to isoaspartate (iso-Asp) due to normal post-translational modification, depending on the half-life of the protein **(Güttler *et al*., 2013).** However, it has been demonstrated that under specific conditions, their rate of production accelerates. Iso-Asp formation causes protein structure to be distorted, resulting in protein unfolding and aggregation. As a result, iso-Asp formation has been linked to protein dysfunction **(Dimitrijevic *et al*., 2014, Kern *et al*., 2005)** As a result, cellular survival suffers.

In bacteria, the protein-l-isoaspartyl -o-methyl transferase (PIMT) methylates the -carboxyl group on iso-Asp residues by utilizing the methyl group of S-adenosyl-L-methionine (AdoMet), resulting in methyl esters. PIMT partially restores protein function(s) by mending iso-Asp to Asp, which improves cellular survival under stress conditions **(Dimitrijevic *et al*., 2014).**

2.2 Repair mediated by PIMT mechanism

Succinimide is formed when asp/asn residues in proteins are changed spontaneously or under stress. When succinimide is hydrolyzed, it produces isoAsp and Asp in a 3:1 ratio (**Vigneswara *et al*., 2006)**. Using the protein repair enzyme PIMT, transfer of the methyl group from S- adenosyl methionine to iso- Aspartate residues to generate iso- Aspartyl methyl esters. Iso-Aspartyl methyl esters are highly unstable and quickly hydrolyze to create succinimide intermediate. With several cycles of such reactions, the aspartyl residues are recovered, and proteins resume their function **(Dimitrijevic *et al*., 2014, DeVry *et al*., 1999).**

2.3 The impact of deleting the PIMT gene on the long-term survival of a wide range of creatures

E. coli PIMT gene knockout strain **(Li and Clarke., 1992, Visick *et al*., 1998, Hicks *et al*., 2005).** was hypersensitivity to oxidative, thermal, and other stresses. The PIMT overexpressing *E. coli* cells, on the other hand, demonstrated increased tolerance to oxidative and thermal stressors **(Verma *et al*., 2010, Kindrachuk *et al*., 2003).**

The methyltransferase independent activities of PIMT were found to be responsible for the increased survival capacity of PIMT overexpressing cells under extremes of temperature. **(Kindrachuk *et al*., 2003).**

In the binding region of PIMT for AdoMet, a structure crystallographic research in *E. coli* revealed the presence of two highly conserved Glu81 (E81) and Glu104 (E104) residues **(Kindrachuk *et al*., 2003).**

In the same study, glutamine residues in PIMT of *E. coli* cells (E81A and E104A mutants) were *in-situ* mutated to alanine to investigate the particular effects of PIMT on cellular viability. When exposed to temperature stress, overexpressed wild and inactive PIMT (E81A) resulted in elevated but equivalent survival rates, with the E104A inactive mutant showing the best cellular survival. The findings proposed a different role for E104A inactive mutant since it lacked methyltransferase activity. Other than its usual duty of enzymatic repair, PIMT plays an important role in cellular survival. Furthermore, when the E104A inactive mutant was subjected to

temperature stress, a western blot analysis revealed that the DnaK chaperone protein was overexpressed. As a result, the study emphasis the importance of PIMT's non-methyltransferase role in improving cellular survival via heat shock protein induction. As a result, this research suggests that PIMT plays a role in E. coli survival that is both methyl-transferase dependent and independent **(Kindrachuk *et al*., 2003).**

2.4 Aspects of PIMT's structure and catalysis

Three subdomains make up the PIMT protein **(Skinner *et al*., 2000).** The core subdomain is preserved and resembles other SAM-dependent methyl transferases (except in topological relationships). It has the ideal form to allow the methyltransferase process to take place in a suitable environment **(Kagan *et al*., 1994).** Serine/threonine rich β strands line the active site of this enzyme. These hydrophilic amino acids promote hydrogen bonding, which enhances enzyme interaction with iso-Asp residues and increases the enzyme's affinity for its substrate. The binding/active site of PIMT from *Thermotoga maritima* was carefully investigated, and it was discovered that this enzyme only has one isoAsp binding site (Skinner et al., 2000). PIMT in Homo sapiens, on the other hand, has four isoAsp residue binding sites **(Lowenson *et al*., 1992, Lowenson *et al*., 1991**). According to structural analyses, PIMT has three hidden charged residues that are important for catalytic reactions and SAM binding (**Ryttersgaard *et al*., 2002, Dutta *et al*., 2012).** Traditional catalytic reactions are distinguished by two features of PIMT activity. It's been proposed that, unlike traditional enzymes, it works without the formation of any covalent intermediates. Despite the fact that SAM is required as a methyl donor, no PIMT cofactors, such as metal ions, have yet to be discovered. The iso-Asp carboxylate's oxygen atom is a powerful nucleophile, capable of catalyzing substrate conversion via transferase **(Skinner *et al*., 2000).**

2.6 Carica papaya's Distribution and Economic significance

Papaya is a tropical American fruit that is native to southern Mexico and Central America. Papaya is also native to southern Florida, having been brought by Calusa forefathers no later than

300 CE. In the 16th century, Spaniards brought papaya to the Old World. Papaya farming has now spread to Hawaii, Central Africa, India, and Australia, making it practically pantropical. Papaya populations in the wild are mostly restricted to naturally disturbed tropical forest. Following large hurricanes, papaya is abundant on Everglades hammocks, although it is normally uncommon. Papaya flourishes and reproduces swiftly in canopy gaps in the rain forests of southern Mexico, but it dies off in mature closed-canopy forests **(Chávez-Pesqueira *et al*., 2017, M. F *et al*., 2011)**

Plants are a rich source of antioxidants. *Carica papaya* L., a flowering and dicotyledonous plant categorized as violales order, Caricaceae family, *Carica* L. genus, and papaya species, is an example of an antioxidant-rich plant **(Aradhya *et al*., 2004).**

Carica papaya L. has a single hollow pale greenish to brownish stem with scars that bears large leaves and round fruits. Furthermore, this plant is grown in nations near the equator, such as Malaysia, Brazil, South America, Australia, and Indonesia. Based on its geographical range, the *Carica papaya* L. plant is known by several distinct names, including kepaya, paw paw, and tapaya. In fact, each component of the plant, including the fruit, roots, leaves, and seeds, is praised for its therapeutic properties. As a result, it's been employed as a conventional therapy regimen for a variety of illnesses **(Kong *et al*., 2021).**

Some of the plant's medical effects are due to its antioxidative qualities, which protect cells from oxidative stress. Papain is the most frequently used proteolytic enzyme derived from the *Carica papaya* L. It has been used to aid in the tenderization and digesting of meat. It's worth noting that papain has shown a lot of promise as a drug. It has been proposed that it may have drug-like characteristics for atherosclerosis and other disorders involving monocyte-platelet aggregate (MPA)-regulated inflammation. The advantages of *Carica papaya* extracts and chemical ingredients have been evaluated in relevant and noteworthy research. The goal of this study is to compile and evaluate research findings related to the *Carica papaya*'s antioxidant qualities and its use as a medicinal, cosmeceutical, and nutraceutical product **(Kong *et al*., 2021).**

2.5 Effect of stress on early growth of *Carica papaya*

Heat stress is defined as a temperature rise that exceeds a threshold level for a long enough length of time to cause irreparable harm to plant growth and development. It is a complicated function of temperature intensity (in degrees), duration, and rate of growth. Excessive radiation and high temperatures are two of the most limiting variables affecting plant development and agricultural productivity in tropical areas. Pre- and post-harvest damages can include scorching of leaves and twigs, sunburns on leaves, branches, and stems, leaf senescence and abscission, shoot and root development suppression, fruit discoloration and damage, and yield reduction **(Guilioni *et al*., 1997, Ismail *et al*., 1999, Vollenweider *et al*., 2005).** To maximize production and quality, it is vital to understand how papaya interacts with environmental elements such light, wind, temperature, relative humidity, soil water, and soil physical and biological properties.

Oxidative stress occurs when the equilibrium between antioxidants and prooxidants is disrupted, affecting redox signaling and resulting in cell and tissue damage. Studies have assessed the antioxidant properties of *Carica papaya* fruit extracts at the cellular level, in keeping with literature demonstrating the pluripotent actions of tropical fruits.

Additional proof of papaya fruit's antioxidant effects came from studies employing cellular models of oxidative stress. Extracts of seed from ripe and unripe fruit dramatically lowered oxidative stress levels in human pre-adipocytes (SW872) and hepatocellular cancer cells (HepG2) subjected to hydrogen peroxide at a concentration of 2 mg dry weight/mL (H_2O_2). Its cytoprotective effects against oxidative-inflammation were demonstrated by the maintenance of mitochondrial viability, reduction of intracellular reactive oxygen species levels, and mediation of pro-inflammatory cytokine secretory levels (Tumor necrosis factor-, interleukin-6, monocyte chemoattractant protein-1) **(Somanah *et al*., 2017).**

2.7 Cloning and expression of gene

A cloning vector is a short bit of DNA that may be kept in an organism for a long time and into which a foreign DNA fragment can be introduced for cloning. DNA from a virus, a cell from a higher creature, or a bacterium's plasmid can all be used as cloning vectors. The inclusion of

restriction sites, for example, allows for the easy insertion of a DNA fragment into the vector or the removal of a DNA fragment from the vector. A restriction enzyme can be used to cut the vector and foreign DNA, resulting in DNA fragments with blunt ends or overhangs known as sticky ends, which can then be joined together by molecular ligation. After cloning a DNA fragment into a cloning vector, it can be subcloned into a more specific vector. Cloning vectors come in a variety of shapes and sizes, but genetically modified plasmids are the most frequent. Plasmids, bacteriophages (such as phage), cosmids, and bacterial artificial chromosomes (BACs) are all used as cloning vectors in *E. coli.*

However, some DNA pieces, such as very large DNA fragments, cannot be kept stable in *E. coli* and must be transferred to another cell, such as yeast. Yeast artificial chromosomes (YACs) are one type of cloning vector. Every cloning vector has characteristics that make it easy to insert or delete a gene. It's possible that this is a multiple cloning site (MCS) or poly linker, which has many restriction sites. The restriction sites in the MCS are first cleaved by restriction enzymes, and then a PCR-amplified target gene, which has also been digested with the same enzymes, is ligated into the vectors with DNA ligase. The target DNA sequence can be inserted into the vector in a precise orientation. If required, the restriction sites can be employed to subclone the vector into another vector **(Gorman *et al.*, 2015).** One such example is pPROEXHTa (Fig. 2.1).

The vector carries a selectable marker that enables the selection of positively transformed cells. Antibiotic resistance is frequently used as a marker, with the beta-lactamase gene, for example, conferring resistance to the penicillin group of beta-lactam antibiotics, such as ampicillin. Some vectors include two selectable markers, such as the plasmid pACYC177, which contains both the ampicillin and kanamycin resistance genes (***Nicola et al., 2003***).

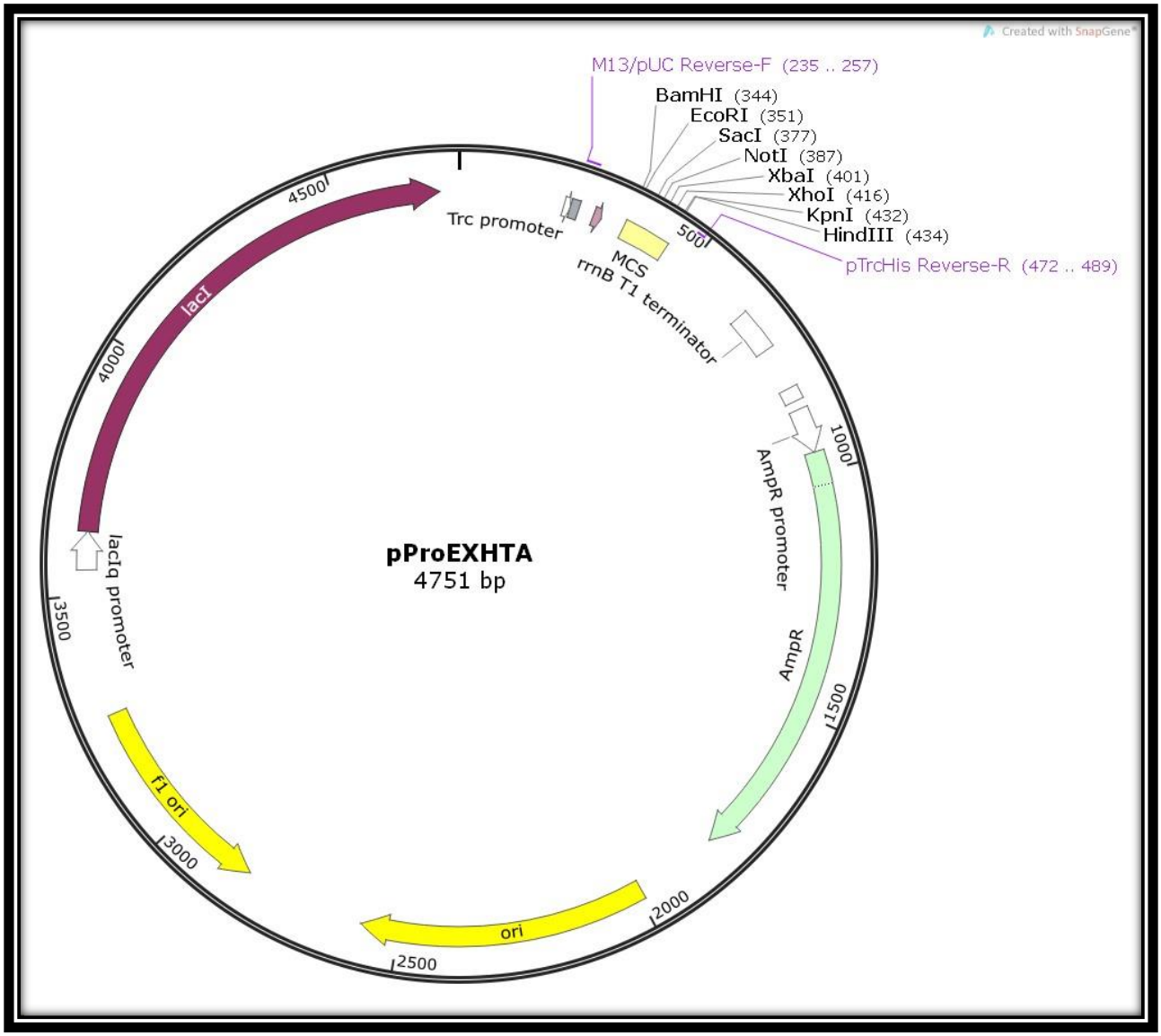


Fig.2.1 Vector map of pProEXHTa).

- **Plasmid Type-** Bacterial Expression
- **Promoter** – Trc
- **Size** – 4751bp
- **Tag1-** (His)6, N terminal
- **Bacterial resistance**- ampicillin

2.8 Competent cells

Competent cells are bacterial cells that are capable of up taking foreign DNA from the environment through a process known as transformation. In Streptococcus pneumoniae, Griffith was the first to report it. E. coli cells are more likely to ingest the DNA if the cell wall has been altered. Salt and temperature shock treatment are widely used to make cells competent. Rapidly developing cells are frequently more readily made competent than those at slower development phases. After the transformation step, the cells may keep the gained genetic information. The procedure is mostly used to introduce recombinant plasmid DNA into bacterial cells that are capable of doing so. This method does not need the use of a donor cell, but rather DNA from the environment. Competent cells have modified cell walls that allow DNA to pass through more easily. To become competent cells, certain cells needed to be treated to chemical or electrical treatments. The conventional way for preparing such cells is to treat them with calcium ions.

Material and Methods

The current study was conducted in the Unit of Teaching of Veterinary Clinical Complex, Faculty of Veterinary and Animal Sciences, R.G.S.C, B.H.U, with the goal of **"Cloning and expression of Protein-L-isoaspartate-O-methyltransferase gene of *Carica papaya*"** This chapter eventually contains detailed descriptions of all the materials, equipment, and methodologies acquired during the investigation.

3.1. Materials and reagents

List of important equipments and reagent are given below-

3.1.1. Glassware and plasticware:

During the experiment, beakers, funnels, measuring cylinder, and other glass apparatus were utilized. They were procured either from Glassco (India) or Schott Durans (Germany). Plasticwares and Micro Pipettes (10 µl, 200 µl, 1000 µl) were from various companies such as Abdos (India) and Thermo Fischer (USA). Micropipettes Tips, Petri plates, 1.5 ml and 2 ml centrifuge tubes (Abdos and Tarsons), PCR tubes (Abdos) were procured.

3.1.2. Equipments:

Gel electrophoresis (GeNei), PCR (Bio- Rad) T100 – Thermal Cycler, Microcentrifuge (Remi), UV- Transilluminator (Laby, India) Incubator, Thermal Shaker (Logitech DNA), Nano- Drop Spectrophotometer (Thermo Fischer), Autoclave (Equitron), Vortex (Tanco, India), Laminar Hood (Biogen Scientific, Meerut U.P, India), Magnetic stirrer (Glassco, India), were used during the experiments.

3.1.3. Chemicals:

Agarose low EEO (SRL, India), 10X TAE Buffer PH-8.3, Methanol, Glacial acetic acid, Bromophenol Blue, CBB.R-250, Ethidium Bromide (SRL cat-93079), Isopropyl-β-D- Thiogalactopyranoside (IPTG), Ampicillin (SRL-61314), Luria Bertani Agar Miller (SRL-47436), Luria Bertani Broth, Miller (SRL-29817), TEMED (SRL), Nickel Chloride (SRL), 10X Tris Glycine- SDS Buffer (SRL-57806), 10X

Phosphate Buffered Saline (PBS)(SRL-78529), 2.5 Tris -SDS Buffer PH-8.8 (Himedia-ML039), Tris SDS Buffer PH-6.8 (Himedia-ML040), Acrylamide/ Bis- acrylamide (29:1) 30% (Himedia-ML037), 10X Quick cut green Buffer (TaKaRa), Q. cut EcoRI (TaKaRa-1611), Q. cut HindIII (TaKaRa-1615), Oligo$(dt)_{18}$ Primer (Thermo Scientific RefS0132), 5X Reaction Buffer (Thermo Scientific Ref-K1612), Revert Aid Reverse Transcriptase (Thermo Scientific Ref- K1612), RiboLock RNase Inhibitor (Thermo scientific), G_2 Green Master Mix (G_0 Taq Ref- M782A), Low Range OG 10 Colored Protein Ladder (TaKaRa Cat-#3471), T4DNA Ligase 10X Buffer (Promega Ref- 126B), T4 DNA Ligase Ref- M180A, pfu DNA Polymerase (Promega Ref- M774A), pfu 10X Buffer (Promega Ref- M776A), 10mM dNTP Mix (Thermo Scientific).

3.1.4. Plant material:

The hybrid variety of *Carica papaya* L seed Papaya Honey Gold was purchased from an authorized seed store, Mishra Beej Bhandar Pvt. Ltd Mirzapur UP.

Fig.3.1. Seeds of *Carica papaya*

3.1.5. Soil selection and Seed Sowing

Because soil plays an important role in delivering nutrients for proper plant growth, the quality of soil is a critical factor in plant development. For our experiment, soil was collected from the R.G.S.C campus farm at B.H.U. For better results, the soil was fertilized with manure, and the

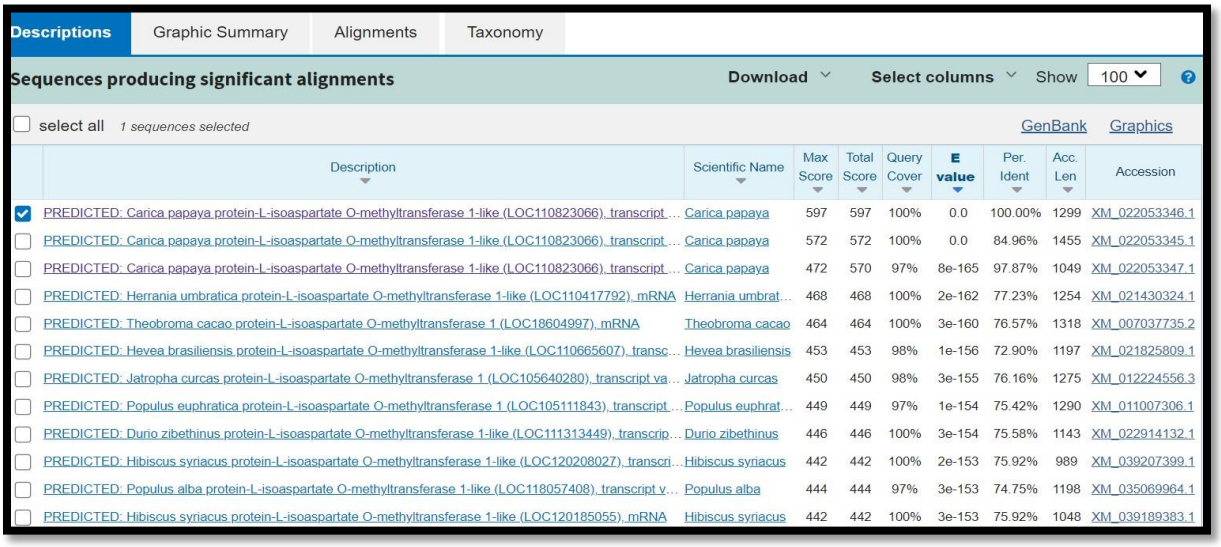
seeds were treated with fungicide for 30 minutes before being sown in ground containing 4 to 5 seeds each, and kept for 15 days in a normal humid climate.

Fig.3.2. Grown *Carica papaya* plant

3.1.6. Primer Designing

The *Carica papaya* PIMT protein sequence was obtained from **Uniport**, and the coding sequence (CDS) of the PIMT gene was determined using **tBLASTn** (blast.ncbi.nlm.nih.gov). For PCR amplification of the target DNA sequence, the primers play a very important role. The primers were designed manually and checked by Primer BLAST (www.ncbi.nlm.nih.gov › tools › primerblast). Oligo Analyzer (eurofinsgenomics.eu › en › ecom) tool for binding characteristics.

We wanted restriction sites of EcoRI and HindIII in our desired sequence for clone formation, so included restriction sites for sequences of EcoRI and HindIII in primers (both forward and reverse primer) and few extra nucleotide sequences to provide support for restriction endonuclease.



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — Select columns — Show 100

select all 1 sequences selected — GenBank — Graphics

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	PREDICTED: Carica papaya protein-L-isoaspartate O-methyltransferase 1-like (LOC110823066), transcript…	Carica papaya	597	597	100%	0.0	100.00%	1299	XM_022053346.1
☐	PREDICTED: Carica papaya protein-L-isoaspartate O-methyltransferase 1-like (LOC110823066), transcript…	Carica papaya	572	572	100%	0.0	84.96%	1455	XM_022053345.1
☐	PREDICTED: Carica papaya protein-L-isoaspartate O-methyltransferase 1-like (LOC110823066), transcript…	Carica papaya	472	570	97%	8e-165	97.87%	1049	XM_022053347.1
☐	PREDICTED: Herrania umbratica protein-L-isoaspartate O-methyltransferase 1-like (LOC110417792), mRNA	Herrania umbrat…	468	468	100%	2e-162	77.23%	1254	XM_021430324.1
☐	PREDICTED: Theobroma cacao protein-L-isoaspartate O-methyltransferase 1 (LOC18604997), mRNA	Theobroma cacao	464	464	100%	3e-160	76.57%	1318	XM_007037735.2
☐	PREDICTED: Hevea brasiliensis protein-L-isoaspartate O-methyltransferase 1-like (LOC110665607), transc…	Hevea brasiliensis	453	453	98%	1e-156	72.90%	1197	XM_021825809.1
☐	PREDICTED: Jatropha curcas protein-L-isoaspartate O-methyltransferase 1 (LOC105640280), transcript va…	Jatropha curcas	450	450	98%	3e-155	76.16%	1275	XM_012224556.3
☐	PREDICTED: Populus euphratica protein-L-isoaspartate O-methyltransferase 1 (LOC105111843), transcript …	Populus euphrat…	449	449	97%	1e-154	75.42%	1290	XM_011007306.1
☐	PREDICTED: Durio zibethinus protein-L-isoaspartate O-methyltransferase 1-like (LOC111313449), transcrip…	Durio zibethinus	446	446	100%	3e-154	75.58%	1143	XM_022914132.1
☐	PREDICTED: Hibiscus syriacus protein-L-isoaspartate O-methyltransferase 1-like (LOC120208027), transcri…	Hibiscus syriacus	442	442	100%	2e-153	75.92%	989	XM_039207399.1
☐	PREDICTED: Populus alba protein-L-isoaspartate O-methyltransferase 1-like (LOC118057408), transcript v…	Populus alba	444	444	97%	3e-153	74.75%	1198	XM_035069964.1
☐	PREDICTED: Hibiscus syriacus protein-L-isoaspartate O-methyltransferase 1-like (LOC120185055), mRNA	Hibiscus syriacus	442	442	100%	3e-153	75.92%	1048	XM_039189383.1

Fig.3.3. tBLASTn Results of PIMT amplicon of *Carica papaya*.

Carica papaya PIMT gene primers were used to amplify the gene. The GC content of the primer was used to calculate the optimum annealing temperature.

$$T_A = [2\times(A+T) + 4\times(G+C)] - 4$$

$$T_m$$

The Tm temperature of the primer was 62 °C and 61 °C for forward and reverse primer, respectively, and the annealing temperature was 56 °C. The above designed oligo primer for PIMT gene of *Carica papaya* were custom synthesized by from **Eurofins Genomics India Pvt. Ltd.**

Table3.1 Sequence of the primers used to amplify the *Carica papaya* PIMT gene.

Primer	Sequence 5' → 3'	Length	Tm in °C	GC content
Forward Primer	ATGAATTCATGCGAGCACTATCGCCATTA	29	63.85	41.38
Reverse Primer	ATAAGCTTTCAATAGCCGCGCAATTGAG	28	63.66	42.86

3.2. Experimental methods:

3.2.1. Extraction of Total RNA of Plant:

RNA isolation kit (Cat no-MB603 Hi pura plant and fungal RNA Miniprep purification kit Himedia, India) was used to extract the Total RNA. In the presence of liquid nitrogen, fresh leaves (100 mg) were ground to a fine paste using a mortar and pestle (the equipment were dry and properly clean before RNA isolation). Added 1ml of RNAxpress reagent to the ground plant material right away and thoroughly mixed it in. To allow complete separation of the nucleotide complex, transferred the mixture to a 2 ml capped tube and incubated for 5 minutes at room temperature. To separate the phases, added 200 µl of chloroform to the sample and shook for 15 seconds, then set aside for 10 minutes at room temperature before centrifuging the mixture at 13000 rpm for 15 minutes at 4 °C. Three layers of the mixture are formed. The lower organic phase (which contains protein), the interphase (which contains cell debris and DNA), and the upper aqueous phase (containing RNA) were all present. Later, transferred the aqueous phase containing the RNA to a new tube and added 1 ml of binding solution without disturbing the other two layers. Pipetted the entire solution into a 15 ml tube after thoroughly mixing it. 775 µl ethanol, was gently pipetted into the above solution in a 2 ml uncapped collection tube. Loaded the lysate into a Hielute miniprep spin column. The flowthrough was discarded after centrifuging for 1 min at 10,000 rpm. The spin columns were filled with 700 µl of prewash solution (RW1), centrifuged for 1 min at 10,000 rpm, and discarded the flowthrough. Added 500 µl of dilute wash solution to the spin column for final washing, centrifuged for 2 min at 10,000 rpm, then empty spun for 1 min at 14,000 rpm. Transferred the spin column to a new 2 ml centrifuge tube, add 50 µl of elution solution directly onto the spin column, and centrifuged at 10,000 rpm for 1 min. The eluted RNA was stored at -20 °C until it was needed.

3.2.2. Agarose gel electrophoresis

Agarose gel (1 %) was prepared in tris acetic acid EDTA (TAE) buffer to illustrate the integrity of RNA extracted from plants. TAE buffer composition is discussed in Annexure I.

The agarose suspension (0.3 g of agarose in 30 ml of 1X buffer) was boiled in microwave for 2 minutes. Allowed to cool for 5 minutes, added 5 µl of ethidium bromide (10 mg/ml) then slowly stirred. EtBr is an intercalating agent that binds to DNA or RNA to allow it to visualize under UV light. The agarose gel was poured into the gel casting tray and left to set. The comb was removed

without disturbing the well once the gel had solidified, and the agarose gel was placed in the buffer tank submerged in 1X TAE buffer. Place each sample in its own well: The samples were loaded as given below:

- Lane 1: 5 µl 1 kb base pair ladder
- Lane 2: 10 µl of RNA of *Carica papaya*
- Lane 3: 10 µl of RNA of *Carica papaya*

The gel was visualized using a UV transilluminator after being run for 45 minutes at 50 volts.

3.2.3. First strand cDNA synthesis

For cDNA synthesis kit was used (Thermo Scientific Revert Aid first strand cDNA synthesis kit, cat no: K1621). The RNA that has been extracted served as a direct template. In a sterile, nucleasefree tube, added the following reagents.

Table 3.2. List of reagents for first strand cDNA synthesis.

Sl. No.	Components	Volume	Volume
1.	**Template RNA**	11µl	10µl
2.	**Primer (oligo dT)**	1 µl	1 µl
3.	**Hexamer Primer**		1 µl
4.	**Total volume**	12 µl	12 µl
5.	**5X reaction buffer**	4 µl	4 µl
6.	**Ribolock RNase inhibitor**	1 µl	1 µl
7.	**10mM dNTP mix**	2 µl	2 µl
8.	**Revert aid RT**	1 µl	1 µl
	Total volume	20 µl	20 µl

• Gently mixed, then centrifuged for a few seconds.

- Incubated for 60 minutes at 42 ºC with oligo(dt) followed by 5 minutes at 70 °C to terminate the reaction.

3.2.4. Amplification of PIMT gene of *Carica papaya L.:*

Amplification of cDNA was done using PCR. PCR master mix and other reaction components, as listed in Annexure II, are used to make the PCR mixture.

After thawing, PCR reagents were gently vortexed and centrifuged. Placed the following reagents in a thin-walled PCR tube on ice. The reaction has a total volume of 20 µl, and the reagents were added in the following order

- ✓ PCR master mix
- ✓ NFW (nuclease free water)
- ✓ Template
- ✓ Primer (forward and reverse both)

PCR steps:

Table 3.3. Conditions used for polymerase chain reaction.

Sl. No.	PCR Steps	Temperature	Time
1.	**Initial Denaturation**	95 ºC	5 min
2.	**Denaturation**	94 ºC	45 sec
3.	**Annealing**	54 ºC to 64 ºC	45 sec
4.	**Extension**	72 ºC	1 min
5.	**Final Extension**	72 ºC	10 min
	No of cycles	35 cycles	

Table3.4. Temperature gradient used for PCR amplification

Sl. No.	Temperature gradient
1.	64.0 ºC
2.	63.2 ºC
3.	62.0 ºC
4.	60.1 ºC
5.	57.8 ºC
6.	55.9 ºC
7.	54.7 ºC
8.	54.0 ºC

3.2.4.1. Gel electrophoresis of PCR products

This is done on a 2% agarose gel. At 100 volts, the gel was run for 30 minutes. The gel was then examined under a UV transilluminator.

3.2.5. cDNA Amplification by Pfu DNA polymerase

Pfu DNA polymerase was used for error free amplification of cDNA (Cat no- M774A). The proofreading activity of the Pfu DNA polymerase ensures that the amplified DNA is error-free. The reagents are added in the following order to the two reactions, each of which has a total volume of 25 µl:

Table 3.5. Reagents used for cDNA amplification by Pfu DNA polymerase.

Sl. No.	Reaction Mixture	Volume
1.	Pfu DNA poly 10X buffer	5µl
2.	dNTP Mix 10 mm	1µl
3.	Forward primer	1µl
4.	Reverse primer	1µl
5.	DNA template	2µl
6.	Pfu DNA polymerase	1µl
7.	NFW	39µl
Total volume		50µl

PCR steps:

Table 3.6. Conditions used for PCR with Pfu DNA polymerase:

Sl. No.	PCR Steps	Temperature	Time
1.	Initial Denaturation	95ºC	2min
2.	Denaturation	94ºC	45 sec
3.	Annealing	54ºC to 64ºC	45sec
4.	Extension	72ºC	2min
5.	Final Extension	72ºC	10min
No of cycles		35 cycles	

3.2.5.1. Gel electrophoresis of PCR products

This is done on a 2% agarose gel. At 100 volts, the gel was run for 30 minutes. The gel was then examined with a UV transilluminator.

3.2.6. Isolation of plasmid

The plasmid isolation kit (Nucleo Spin Plasmid REF-740588.50) is used to isolate plasmid. The plasmid culture (pProEXHTa) was provided by my guide Dr. Prasanta Kumar K. Mishra Assistant Professor Department Unit of teaching veterinary clinical complex, FVAS, B.H.U. Grown the plasmid culture overnight in LB broth with antibiotics (Ampicillin). In Annexure III, the ingredients of LB broth and antibiotics are listed. Isolated the plasmid from the overnight culture on the next day morning used 1.5 mL of a saturated *E. coli* LB culture in each of three tubes, and centrifuge for 2 minutes at 10,000 rpm. Removed maximum liquid and discarded the supernatant. By adding 250 µl Buffer A1 to a sample tube, lysed the cells, then vortexed the pellet to resuspend that completely. Incubated at room temperature for 5 minutes after adding 250 µl Buffer A2 and mixing it 6 -times by inverting the tube. Added 300 µl of Buffer A3 and mixed it by inverting the tube 6-8 times until the sample turned colorless, then centrifuged for 5 min at 14,000 rpm at room temperature to separate the two layers. Took 700 µl from the transparent layer. In a collection tube, put the nucleo-spin plasmid column. Placed 700 µl of the supernatant to the plasmid spin column and centrifuged for 1 minute at 11,000 rpm, discarding the flowthrough and placing the plasmid spin column back into the collection tube. Added 500 µl of Buffer AW and centrifuged for 1 minute at 11,000 rpm for additional washing. Added 600 µl of Buffer A4 and centrifuged at 11,000 rpm for 1 minute. Removed the flowthrough and replaced the nucleospin column to the collection tube. Empty spun for 2 minutes at 11,000 rpm to dry the silica membrane, then discarded the collection tube. In a 1.5 ml centrifuge tube, placed the nucleospin column and 35 µl of Elution Buffer. Incubated for 1 minute at room temperature before centrifuging for 1 minute at 11,000 rpm.

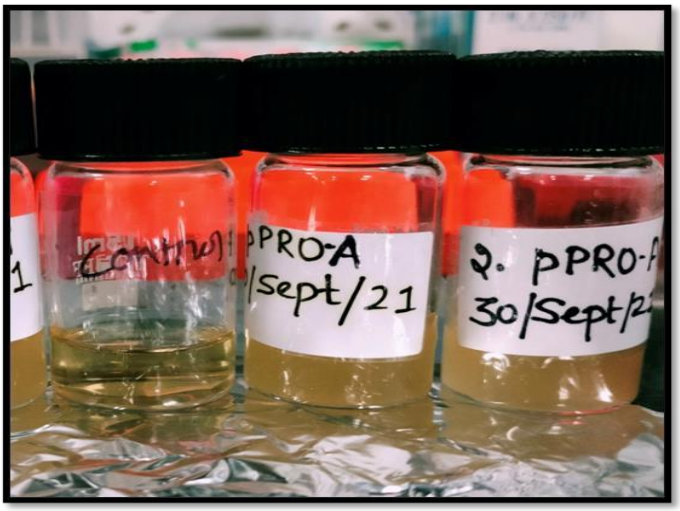


Fig.3.4 DH5-α culture harboring plasmid (pProEXHTa)

3.2.6.1. Gel electrophoresis of isolated plasmid

This was done on a 2% agarose gel. At 100 volts, the gel was run for 30 minutes. The gel was then examined with a UV transilluminator.

3.2.7. Restriction endonuclease digestion of PCR product and plasmid (pProEXHTa)

To ligate the PCR product and the vector, we used the restriction enzymes (Takara quick cut) EcoRI and HindIII to double digest the PCR product and the plasmid (pProEXHTa). The reagents were added to the four reactions in the following order, two for the PCR product and two for the Vector, each of which has a total volume of 50 µl:

EcoR1 (G|AATTC HindIII (A|AGCTT

CTTAA|G) TTCGA|A)

Table3.7. Reaction mixture for restriction endonuclease digestion of PCR product and plasmid (pProEXHTa)

Sl. No	Components	Volume
1.	**PCR product / vector(pProEXHTa)**	10 µl
2.	**NFW (nuclease free water)**	33 µl
3.	**10X Buffer Green**	5 µl
4.	**EcoRI**	1 µl
5.	**HindIII**	1 µl
Total Volume		50 µl

- Spun for 1 minute after adding all of the reagents.
- Incubated in water at 37 °C overnight.
- After the incubation period, the enzyme was denatured by heat shock at 80 °C for 10 minutes in a water bath.

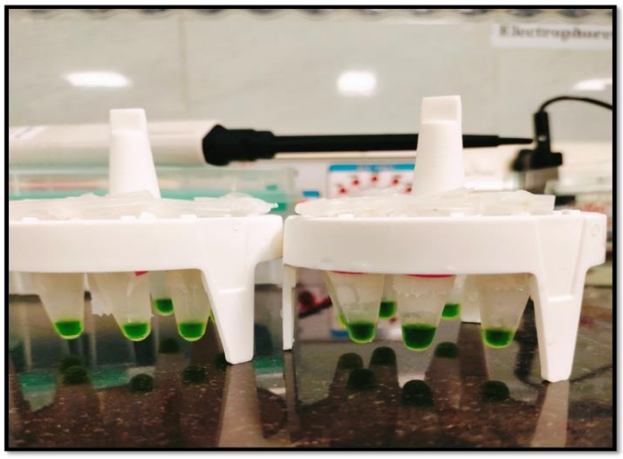


Fig. 3.5. Restriction endonuclease digestion of PCR product and pProEXHTa

3.2.8. Extraction of digested PCR product and plasmid (pProEXHTa) from agarose gel

The **Sure Extract Gel Extraction Kit (Cat no. NP36105**) was used to extract DNA from gels. The protocol that came with the kit was followed exactly. Using a sterile scalpel, carefully removed the digested gel fragment from the agarose gel. Added 200 µl of Buffer SET to 100 mg of agarose gel. After adding the buffer, incubated the sample for 5 minutes in a water bath at 50 °C, vortexed the gel every 2 to 3 minutes until the gel slice was completely dissolved, then incubated it for another 5 minutes at 50 °C in the water bath. Placed the sample on the Sure extract spin column in the collection tube and centrifuged for 2 minutes at 13,000 rpm, discarding the flowthrough and returning the spin column to the collection tube. Added 700 µL of Buffer SET3 and centrifuged at 13,000 rpm for 1 minute, then discarded the flowthrough. To dry the membrane of the column, spun down it for 2 minutes at 13,000 rpm, discarded the collection tube, and placed the spin column in a 1.5 ml fresh centrifuge tube. Allowed 35 µl of SEB to settle in the sure extract spin column for 1 minute at room temperature before centrifuging for 1 minute at 12,000 rpm.

- The double digested PCR product excised weighed 330 mg.
- The excised double digested plasmid (pProEXHTa) had a weight of 383 mg.

3.2.9. Nanodrop reading of double digested PCR product and plasmid

Nanodrop readings were taken using a nanodrop spectrophotometer. The principle of the nanodrop spectrophotometer is based entirely on the Beer-Lambert Law, which states that absorbance is proportional to concentration. A drop of sample (1 to 2 µl) is placed on a small pedestal in the nanodrop spectrophotometer. Lowered the arms to make contact with the liquid and then pulled up a little, aspirated the liquid. The sample nanodrop reading is:

- PCR digested product- **6 ng/µl**.
- Plasmid (pProEXHTa) digested product- **2.5 ng/µl**

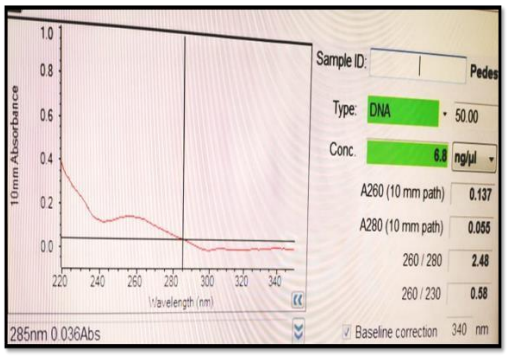


Fig.3.6.1 Nano-drop reading of PCR product

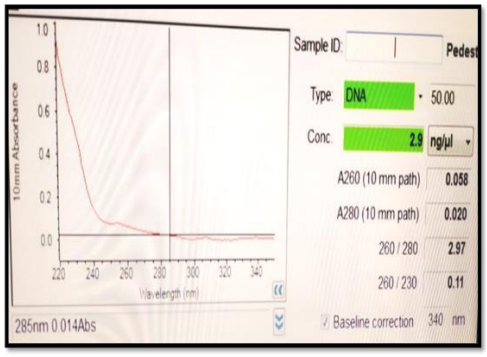


Fig.3.6.2. Nano-drop reading of plasmid (pProEXHTA)

3.2.10. Ligation

For ligation, the T4 DNA Ligase enzyme (Promega) was used.

According to the formula: ng of vector × kb size of insert × Molar ratio of insert

kb size of vector Vector

For 10 µl of vector = 25 ng of vector ×0.896 kb of insert × 3

4.77 kb 1

= 14.088 ng

Table 3.8. Ligation Mixture:

Sl. No	Components	Volume
1.	Vector (pProEXHTA)	10 µl
2.	PCR Product	3 µl
3.	Ligation Buffer	2 µl
4.	T4 DNA Ligase enzyme	1 µl
5.	NFW	4 µl
	Total Volume	20 µl

- After an overnight incubation at 15 °C (in chilled water), the tube was transferred to 4 °C for 4 hours.

3.2.11. Transformation of ligated product into DH5 alpha competent cells:

DH5 alpha Competent Cells from **Himedia (cat no. MBT111A)** were used. In DH5 alpha cells, place 10 µl of PCR-ligated product (100 µl). Heat shock at 42 °C in a water bath for 1 minute after 30 minutes on the ice. After that, it was placed in the ice for two minutes. To the competent cells, I added 900 µl of SOC medium. Incubated for 1 hour at 37 °C and 225 rpm. 5 minutes of centrifugation at 4000 rpm. Supernatant was removed. Using an L-shaped spreader, it was then poured onto Ampicillin plates. Incubated at 37 °C for an overnight period.

Fig.3.7. Chemically competent DH5-α cells

3.2.12. Colony selection of PIMT clone

In ten LB Tubes (5 ml), add 5 µl of ampicillin (100 mg/mL). By inoculation loop, selected single isolated colonies from Amp petri plates and incubated for 3-4 hours at 37 °C and 225 rpm in an incubator.

3.2.13. Colony PCR

After 4 hours of incubation, growth appeared in the LB tube. Transferred 1 mL of culture to each of the two 1.5 mL tubes, centrifuged for 2 minutes at 10,000 rpm to remove the supernatant, then add 50 µl of NFW to lyse the cells in each tube and placed it in a water bath at 100 °C for 10 minutes. This lysate can then be used as a PCR template. PCR master mix composition and other reaction components, as listed in Annexure II.

PCR steps:

Table 3.9. Colony PCR conditions:

Sl. No	PCR Steps	Temperature	Time
1.	**Initial Denaturation**	95 ºC	5 min
2.	**Denaturation**	94 ºC	45 sec
3.	**Annealing**	54 ºC to 64 ºC	45 sec
4.	**Extension**	72 ºC	1 min
5.	**Final Extension**	72 ºC	10 min
No of cycles		30 cycles	

3.2.14. Transformation of clone into BL21 competent cells

The plasmids were isolated from PCR positive DH5-alpha cells. The construct with rtPIMT was further transformed to BL21 Competent cells **(MBT143 HiPurA)** to study the protein expression of the PIMT protein in E. coli. For transformation to BL21 cells the protocol described under 3.2.11 was followed without any modifications.

3.2.15. Confirmation of clone by double digestion

Insert release by double digestion recombinant clone(pProEXHTa+PIMT) by restriction enzyme EcoRI and HindIII and electrophoresis on a 2% agarose gel confirmed the clone (pProEXHTa with insert PIMT).

3.2.16. Induction

IPTG at different concentration (Isopropyl-β-D-1-thiogalactopyranoside) was added to the mid log phase culture culture to study protein expression. IPTG, or isopropyl-D-1-thiogalactopyranoside, is a chemical reagent that mimics allolactose and induces gene expression by binding to a repressor in the lac operon. When lactose enters cells, it gets converted into allolactose. It acts as an inducer, causing genes in the lac operon to start transcription. The culture containing PIMT construct was grown overnight (pProEXHTa with PIMT insert). The culture was split to in five different LB tubes, subcultured for 3 hours till the $O.D._{600}$ reached 0.6. One was uninduced, while the other four LB tubes were induced with IPTG at four different concentrations: 0.25 mM, 0.5 mM, 1 mM, and 2 mM, respectively. From the uninduced and other four, collected 1 mL of culture in a 1.5 mL tube after 1 hour. The cultures were centrifuged and made into pellet by centrifugation. The supernatant was discarded from all tubes and pellets were stored at -20 °C till further use. For expression kinetic study, we added IPTG at a specific concentration (1 mM) to one LB tubes keeping one tube uninduced. For four hours, the cultures were induced. At one hour interval (till 4 hours), took 1 mL from the induced tube and transferred to a 1.5 mL tube. For 2 minutes, spun at 10,000 rpm. Collected pellets, discarded supernatant, and stored at -20 °C immediately.

Calculation:

Preparation of 100 mM IPTG

- IPTG 119 mg in 5 ml double distilled water
- Different concentration of IPTG was calculated by using the following formula:

$$C_1V_1=C_2V_2$$

C_1= 100 mM, V_1=? V_2= 5 ml

C_2=different concentration of IPTG

1. For 0.25 mM IPTG concentration we added 12.5 µl of stock solution.
2. For 0.5 mM IPTG concentration we added 25 µl of stock solution.
3. For 1 mM IPTG concentration we added 50 µl of stock solution.
4. For 2 mM IPTG concentration we added 100 µl of stock solution.

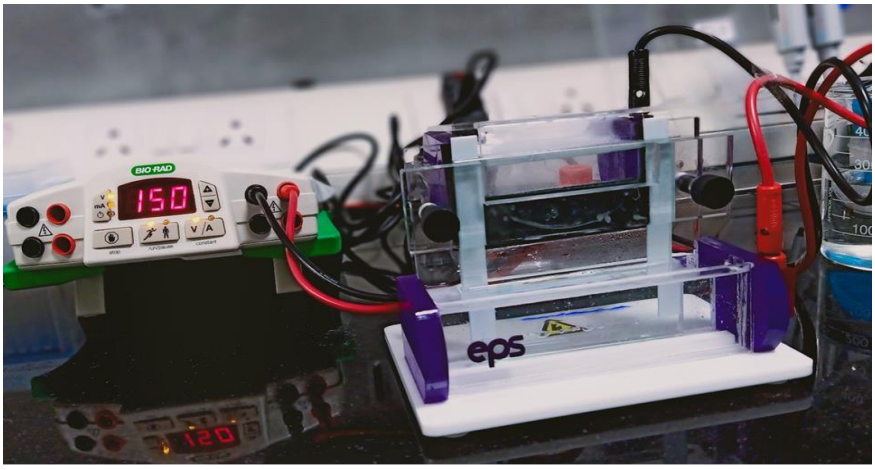
3.2.16.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDSPAGE) analysis of PIMT protein.

The SDS- PAGE is used for protein analysis. We prepared a 12 % of SDS gel. Gel composition is mentioned in annexure IV.

For loading of the sample to the gel, we used the following chemicals:

- ✓ Added 100 µl PBS (Phosphate buffered saline) to a pellet and thoroughly mixed it.
- ✓ Using β ME (β-mercaptoethanol), added 25 µl of sample buffer.

- ✓ Placed in a hot water bath for 5 minutes before loading it into the well.
- ✓ Till the sample crossed the stacking phase, the gel was run at 50 volts, then increased the voltage to 150 volts.
- ✓ Performed gel staining with CBB-G-250 and destaining with aqueous methanol + acetic acid solution.



SDS -PAGE gel electrophoresis

Fig.3.8. SDS-PAGE gel electrophoresis apparatus

- ✓ We looked protein expression from two perspectives. The first was done at the same time but with a different concentration of IPTG, and the second was done at a different time but with the same concentration of IPTG.

Table 3.10.1 Loading sequence of samples in the first SDS gel

Protein Marker(M)	**Uninduced (1hr)**	**Gap**	**Induced 0.25 mM (1hr)**	**Induced 0.5 mM (1hr)**	**Induced 1 mM (1hr)**	**Induced 2 mM (1hr)**
5 µl	30 µl		30 µl	30 µl	30 µl	30 µl

Table 3.10.2 Loading sequence of samples in the second SDS gel

Protein Marker(M)	**Uninduced (0hr)**	**Gap**	**Induced 1 mM (1hr)**	**Induced 1 mM (2hr)**	**Induced 1 mM (3hr)**	**Induced 1 mM (4hr)**
5 µl	30 µl		30 µl	30 µl	30 µl	30 µl

RESULTS AND DISCUSSION

"Cloning and protein expression of the PIMT gene of *Carica papaya*" is the title of the current study. The findings of the research are discussed in this chapter.

1.1. Extraction of total RNA from leaves of *C. papaya*:

After gel electrophoresis (fig.4.1), multiple bands were seen in a smearing pattern. As per the banding pattern and known molecular weight the bands have been designated in the fig 4.1. This represents the total RNA pool of the papaya leaves.

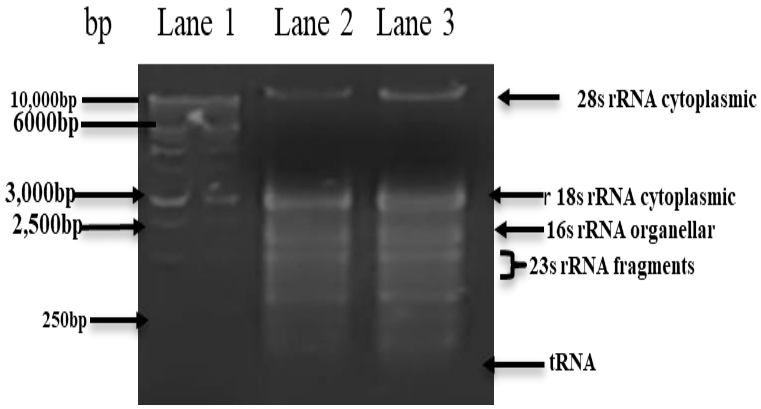


Fig. 4.1. Fig. 4.1. Total RNA isolated from *Carica papaya* leaves. Lane 1: 1 Kb ladder: Lanes 2 and 3: total RNA of *Carica papaya.*

1.2. Amplification of PIMT gene of *Carica papaya L.:*

The PCR amplified PCR product of PIMT ***Carica. papaya*** using PIMT gene-specific primers was detected just below the 900 bp region (Fig.4.2) (Amplicon size: papaya -867 bp + additional sequence of primers: 29 bp, **total size of 896 bp.**

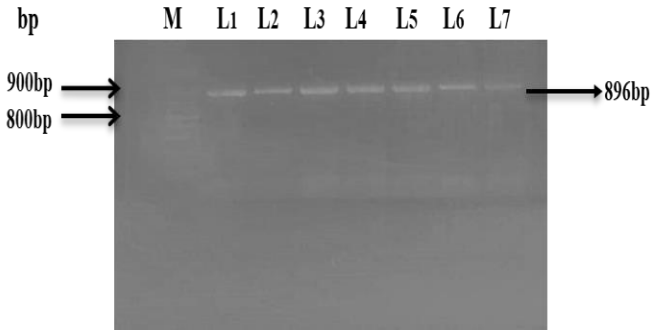


Fig:4.2. Agarose gel electrophoresis of PCR amplicon: The above gel is a 2 % agarose gel where lane M: 100 bp ladder, L1 to L7: amplicons formed at different Ta. The arrow indicates PCR product.

1.3. cDNA amplification by Pfu DNA polymerase

The total RNA isolated was used for synthesis of cDNA by RT-PCR. The cDNA formed was used for amplification of PIMT gene by Taq polymerase and Pfu DNA polymerase. *Carica. papaya* PIMT gene-specific primers were used for amplification of the targeted region. A band was detected just below the 900 bp region in both (Fig.4.3)**.**

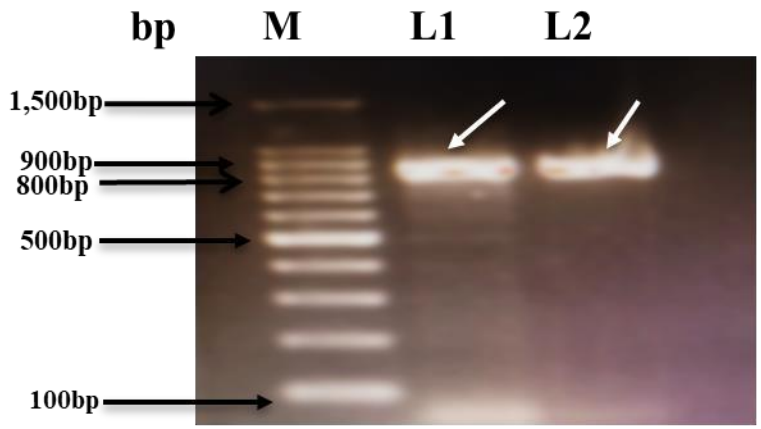


Fig:4.3 Agarose gel electrophoresis of PCR products. M: 100 bp ladder, L1: PCR product of Taq DNA polymerase, L2: PCR amplicon by Pfu DNA polymerase.

1.4. Isolation of plasmid

A 1 % agarose gel was used to visualize the isolated plasmid. the plasmid showed characteristics two bands indicating the intactness of the plasmid while isolation.

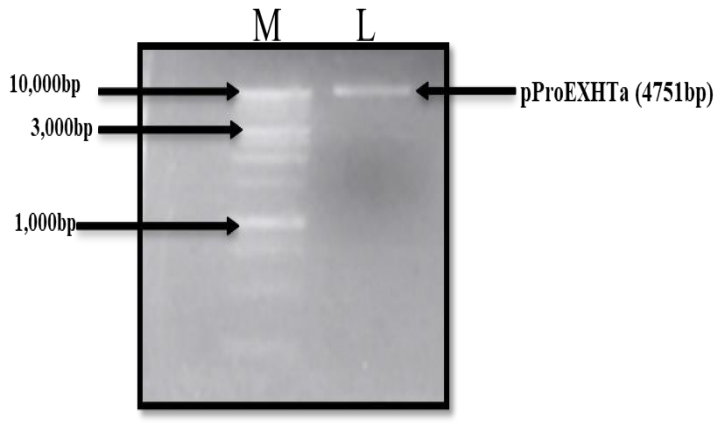


Fig.4.4. Agarose gel electrophoresis of pProEXHTa: M: 1 Kb ladder, L: Plasmid (pProEXHTa).

1.5. Transformation of ligated product into DH5 - α competent cells:

The gel purified PIMT and pProEXHTa was ligated after RE digestion. Transformation into DH5 alpha cells and seeding those transformed cell onto Ampicillin agar plates produced few white transparent recombinant colonies (Fig:4.5).

Fig: 4.5. *Carica papaya* PIMT pProEXHTa–DH5-α, grown on LB ampicillin agar plates: The arrow marks indicate the bacterial colonies (DH5- α) transformed with ligated products.

1.6. Colony PCR

Colony PCR of transformed colonies (having recombinant plasmid pProEXHTa) with PIMT gene specific primers for confirmation of insert was done. The PCR amplicon showed a band of 896 bp.

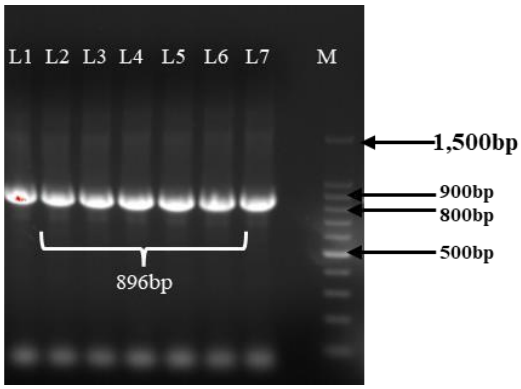


Fig:4.6. Visualization of colony PCR product on a 2 % agarose gel: L1 to L7: Colony PCR amplicon of size 896 bp, Lane M: molecular weight marker.

1.7. Transformation of clone into BL21 competent cells

To investigate protein expression, the rPIMT-pProEXHTa isolated from DH5 alpha cells were transformed into BL21 competent cells, which were then seeded onto ampicillin agar plates. They were incubated overnight at 37 ºC resulting in a few white transparent recombinant colonies (Fig:4.7)

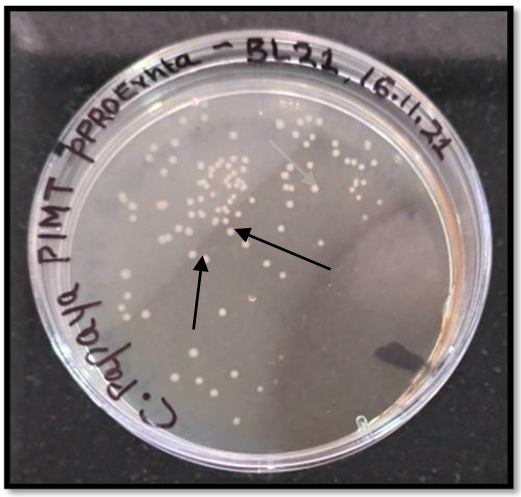
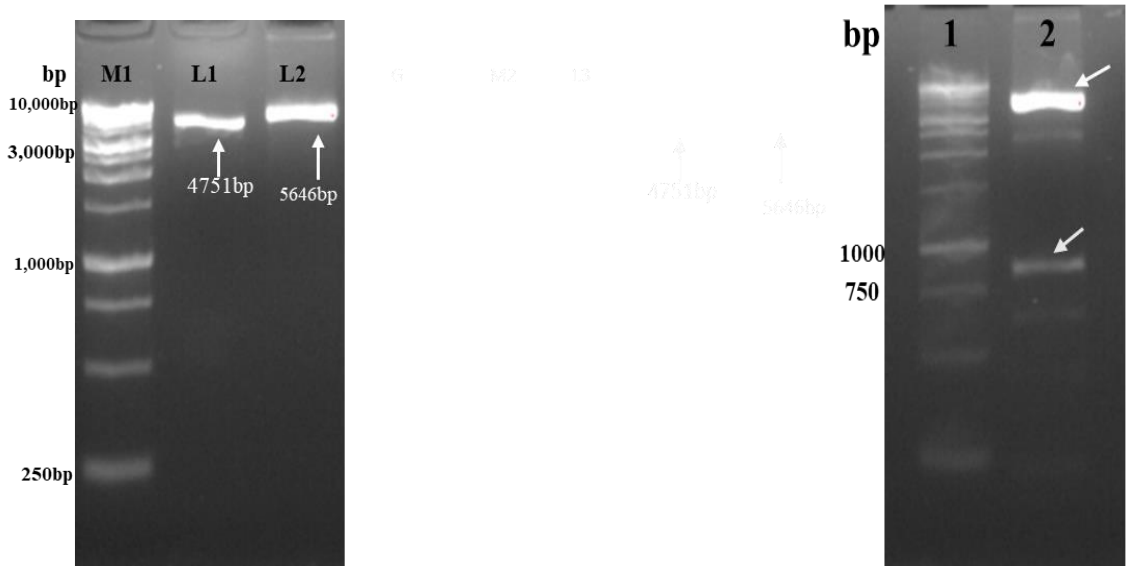


Fig: 4.7. *Carica. papaya* PIMT-pProEXHTa – BL21 competent cells, grown on LB ampicillin agar plates: Arrow marks indicate transformed BL21 colonies.

1.8. Confirmation of clone by double digestion

The Recombinant plasmid isolated was (pProEXHTa+PIMT) confirmed by double digestion with restriction enzyme EcoRI and HindIII and insert release of the desired size i.e. 896 bp (fig.4.8).



(a) **(b)**

Fig:4.8 Confirmation of Papaya PIMT clone with plasmid pProEXHTa by (a) Gel retardation: M1: 1 kb ladder, L1: single digested plasmid (pProEXHTA) without any insert, L2: single digested recombinant Plasmid (pProEXHTA with PIMT insert) (b) Insert release.: 1: 1 kb ladder, 2: The arrow marks in figure (b) represents digested pProEXHTa and released.

1.9. Pair wise sequence Alignment of retrieve Sequence and Clone of *Carica Papaya*

The *Carica papaya* L Var- Papaya Honey Gold PIMT gene clone is 100 % identical to the retrieved sequence of the *Carica papaya* PIMT gene from NCBI. It indicates a conserved sequence of pimt across different varieties of the *C. papaya*.

```
# Program: needle
# Rundate: Fri 6 May 2022 08:01:50 #
Commandline: needle
#    -auto
#    -stdout
#    -asequence emboss_needle-I20220506-080822-0087-39617108-p2m.asequence
#    -bsequence emboss_needle-I20220506-080822-0087-39617108-p2m.bsequence
#    -datafile EDNAFULL
#    -gapopen 10.0
```

```
#    -gapextend 0.5 #
-endopen 10.0
#    -endextend 0.5
#    -aformat3 pair
#    -snucleotide1
#    -snucleotide2
# Align_format: pair
# Report_file: stdout
########################################
#=======================================
#
# Aligned_sequences: 2
# 1: EMBOSS_001
# 2: EMBOSS_001
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 867
# Identity:     867/867 (100.0%)
# Similarity:   867/867 (100.0%)
# Gaps:           0/867 ( 0.0%)
# Score: 4335.0
#
#
#=======================================

EMBOSS_001         1 ATGCGAGCACTATCGCCATTAATTGCGTATGGTTGCCGCTCTTGTTCGCC     50
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001         1 ATGCGAGCACTATCGCCATTAATTGCGTATGGTTGCCGCTCTTGTTCGCC     50

EMBOSS_001        51 CCTTCTAAAGCGATTTCTATCCTGTAACCATCGTCGCCTACCTCTCTTCT    100
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001        51 CCTTCTAAAGCGATTTCTATCCTGTAACCATCGTCGCCTACCTCTCTTCT    100

EMBOSS_001       101 ACACTCTTTCTTCTTTTCCTCACAATCACAATCTCTCTCATCTCCTTCTC    150
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       101 ACACTCTTTCTTCTTTTCCTCACAATCACAATCTCTCTCATCTCCTTCTC    150

EMBOSS_001       151 ACGGGTAACTCTCTCTTCTTCTGCATGGAGCGACTGTGGAGTGGCAGTGG    200
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       151 ACGGGTAACTCTCTCTTCTTCTGCATGGAGCGACTGTGGAGTGGCAGTGG    200

EMBOSS_001       201 CATTAACCAGAATAAAGCAATGGTTGAGAATTTGCAGCATTATGGAGTAA    250
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       201 CATTAACCAGAATAAAGCAATGGTTGAGAATTTGCAGCATTATGGAGTAA    250

EMBOSS_001       251 TTAGTTCAAAGAAAGTTGCTGAAGTGATGGAGACTGTAGATAGAGCTTTG    300
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       251 TTAGTTCAAAGAAAGTTGCTGAAGTGATGGAGACTGTAGATAGAGCTTTG    300

EMBOSS_001       301 TTTGTGCCAGATGGAACACCGCCTTATGTTGATAGCCCAATGTCAATTGG    350
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       301 TTTGTGCCAGATGGAACACCGCCTTATGTTGATAGCCCAATGTCAATTGG    350

EMBOSS_001       351 TTACAATGCAACAATTTCTGCTCCTCATATGCATGCAACATGTCTTCAGC    400
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       351 TTACAATGCAACAATTTCTGCTCCTCATATGCATGCAACATGTCTTCAGC    400

EMBOSS_001       401 TGCTGGAGAAGAATTTGCAGCCTGGGATGCATGCTTTAGATGTTGGCTCA    450
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       401 TGCTGGAGAAGAATTTGCAGCCTGGGATGCATGCTTTAGATGTTGGCTCA    450
```

```
EMBOSS_001       451 GGAACTGGATATTTGACAGCCTGTTTTGCGGTGATGGTGGGAAAAGAAGG    500
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       451 GGAACTGGATATTTGACAGCCTGTTTTGCGGTGATGGTGGGAAAAGAAGG    500

EMBOSS_001       501 CTGTGCTGTTGGTGTTGAACATATTCCTGAATTAGTTGCTTCTTCAATAA    550
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       501 CTGTGCTGTTGGTGTTGAACATATTCCTGAATTAGTTGCTTCTTCAATAA    550

EMBOSS_001       551 AAAATATTGAGAAGAGTGCAGCAGCTCCATTGTTGGAACAAGGTTCCCTT    600
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       551 AAAATATTGAGAAGAGTGCAGCAGCTCCATTGTTGGAACAAGGTTCCCTT    600

EMBOSS_001       601 TCCATTCATGTCGGAGATGGAAGACTAGGGTGGCAAGAGCTTGCTCCTTA    650
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       601 TCCATTCATGTCGGAGATGGAAGACTAGGGTGGCAAGAGCTTGCTCCTTA    650

EMBOSS_001       651 TGATGCCATACATGTGGGGGCGGCAGCTCCAGATATTCCTCAGGCTCTCG    700
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       651 TGATGCCATACATGTGGGGGCGGCAGCTCCAGATATTCCTCAGGCTCTCG    700

EMBOSS_001       701 TTGACCAATTGAAGCCTGGAGGCAGAATGGTAATACCAGTGGGAAATATT    750
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       701 TTGACCAATTGAAGCCTGGAGGCAGAATGGTAATACCAGTGGGAAATATT    750

EMBOSS_001       751 TTCCAAGATCTGAAGGTGGTAGACAAAAACTTGGATGGCTCTATCAGTAT    800
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       751 TTCCAAGATCTGAAGGTGGTAGACAAAAACTTGGATGGCTCTATCAGTAT    800

EMBOSS_001       801 TCACAATGAAACATCTGTGCGATATGTTCCTTTGACCAGCCGTGATTCTC    850
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_001       801 TCACAATGAAACATCTGTGCGATATGTTCCTTTGACCAGCCGTGATTCTC    850

EMBOSS_001       851 AATTGCGCGGCTATTGA    867
                     |||||||||||||||||
EMBOSS_001       851 AATTGCGCGGCTATTGA    867


#---------------------------------------
#---------------------------------------
```

Fig. 4.9: Pair-wise sequence alignment of retrieved sequence and clone of *Carica papaya*

1.10. SDS- PAGE analysis of PIMT protein

Upon induction with different concentration of IPTG for 1 hour, it was observed that a band corresponding to the molecular weight of 35 k Da was present in the induction lanes (Fig.4.10.1) and absent in the uninduced lane. The intensity of the band was maximum at 1 mM IPTG and remained stable in higher concentration. So, it was decided to use IPTG at 1 mM concentration and to study its expression with respect to time. Considering the fact that loading was equal in all

lanes, a gradual increase in protein expression was seen with respect to time. The expression was maximum at 4 hours (Fig.4.10.2).

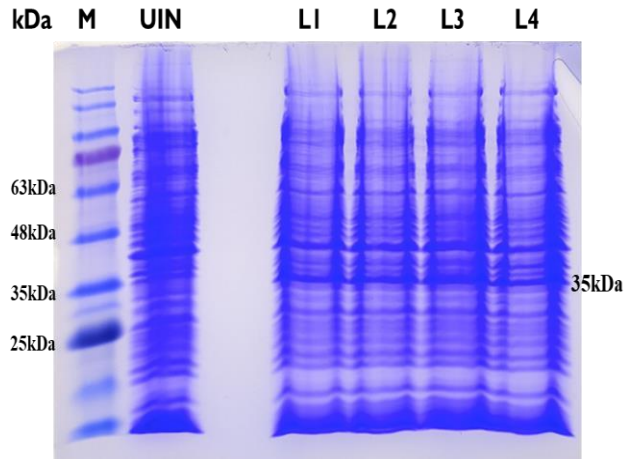


Fig: 4.10.1. SDS-PAGE gel image of PIMT expression after 1 hour at different concentration of IPTG: M-marker, UNI-uninduced at 1hr, L1-L4 represents induction after 1 hour at IPTG concentration of 0.25, 0.5, 1, 2 mM.

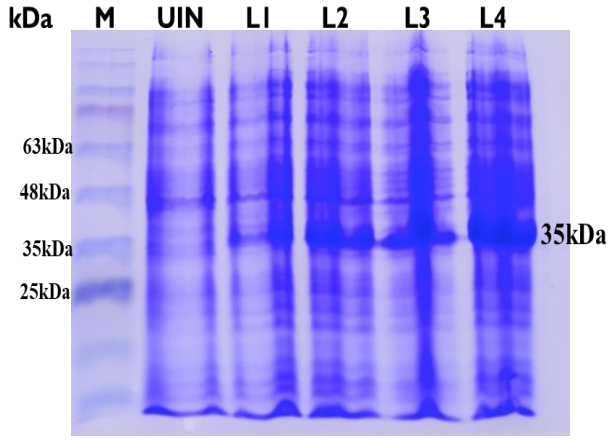


Fig: 4.10.2. SDS-PAGE gel image of PIMT expression kinetics: M-Marker, UIN-uninduced at 0 hours, L1-induced at 1 hours, L2-induced at 2 hours, L3-induced at 3 hours L4induced at 4 hours

SUMMARY AND CONCLUSION

Protein -L- IsoAspartyl-O-Methyltransferase (PIMT) has been widely studied across different phyla and classes of eukaryotes and prokaryotes. As a protein repairing enzyme it plays significant role in the protein function by repair of abnormal iso-Asp residues. There are only few reports of plant PIMTs and less is known about their characteristics. In the present study we have tried to check the expression of PIMT of *C. papaya* in a heterologous expression system of *E. coli*. Plant leaves were used for isolation of total RNA followed by synthesis of cDNA. Using PIMT specific primers, the gene coding for PIMT of *C. papaya* was amplified. The amplified gene was used as insert to a prokaryotic expression system pProEXHTa. The construct was confirmed by various methods like restriction endo nuclease digestion, PCR amplification etc. After confirmation the positive clone was further propagated for induction with IPTG. The effect of different concentration of IPTG was studied on expression pattern and it was observed that maximum expression obtained at the concentration of 1 mM of IPTG. Further, time dependent expression of PIMT was studied and maximum expression was seen after 4 hours of induction with 1mM of IPTG.

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ANNEXURE-I

Agarose gel electrophoresis

1. Agarose gel

Chemical	Gel (in %)	Weight of agarose	TAE solution
Agarose	0.8 %	0.24 g	30 ml
	1 %	0.3 g	
	1.5 %	0.45 g	
	2 %	0.6 g	

2. Preparation of 1X TAE from 10X TAE.

1 litre of 1X TAE 100ml of 10X TAE dissolved in 900ml of distilled water.

ANNEXURE-II

1. PCR components

Components	Final volume
Template DNA	2 µl
Master Mix Mix (2X)	10 µl
Forward Primer	1 µl
Reverse Primer	1 µl
NFW	6 µl
Total Volume	20 µl

ANNEXURE-III

Media preparation:

A. Luria Bertani Broth in 1L.

Reagent	Amount
Yeast extract	5 g
Tryptone	10 g
Sodium chloride	10 g
Double distilled water	1000 ml

Autoclave at 121ºC for 20 min.

B. Luria Bertani Agar in 1L.

Reagent	Amount
Yeast extract	5 g
Tryptone	10 g
Sodium chloride	10 g
Agar	15 g
Double distilled water	1000 ml

Autoclave at 121ºC for 20 min.

ANNEXURE-IV

SDS- PAGE gel composition

1. Resolving gel – p^H 8.8

Reagent	10%
30% Acrylamide	1.65 ml
2.5 X Tris SDS Buffer	2 ml
Distilled water	1.35 ml
Total volume	5 ml
APS	50 µl
TEMED	20 µl

Place it in the casting plates and wait for it to solidify.

2. Staking gel- p^H 6.8

Reagent	10%
30% Acrylamide	835 µl
5 X Tris SDS Buffer	1 ml
Distilled water	3.165 ml
Total volume	5 ml
APS	50 µl
TEMED	20 µl

Place it in the casting plates and wait for it to solidify.