Cloning and characterisation of *myo-inositol phosphate synthase gene* coding for phytates in *Dolichos lablab* L.

by

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(2017-11-006)

THESIS
Submitted in partial fulfilment of the requirement
for the degree of

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DECLARATION

I, hereby declare that the thesis entitled **"Cloning and characterisation of** *myo-inositol phosphate synthase* **gene coding for phytates in** *Dolichos lablab* **L."** is a bonafide record of research work done by me during the course of research and that this thesis has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled 'Cloning and characterisation of *myo-inositol phosphate synthase* gene coding for phytates in *Dolichos lablab* L.' is a record of research work done independently by Sreedevi Jagal Kishore (2017-11-006) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the Advisory Committee of Ms. Sreedevi Jagal Kishore, a candidate for the degree of **Master of Science in Agriculture** with major field in Plant Biotechnology, agree that the thesis entitled **"Cloning and characterisation of *myo-inositol phosphate synthase* gene coding for phytates in *Dolichos lablab* L."** may be submitted by **Sreedevi Jagal Kishore** in partial fulfillment of the requirement for the degree.

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ABBREVATIONS

ABA	Abscisic acid
BLAST	Basic local alignment search tool
BLASTn	Nucleotide basic local alignment search tool
BLASTp	Protein basic local alignment search tool
CAPS	Cleaved amplified polymorphic DNA
DEPC	Diethyl pyrocarbonate
DHNA	Dihydroneopterin aldolase
DICER	RNase III endonuclease
E.coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra Acetic acid
G	Gram
IMP	Inositol monophosphate phosphatase
Ins	Inositol/ *myo* inositol
$InsP_1$	Inositol monophosphate
$InsP_3$	Inositol triphosphate
$InsP_5$	Inositol pentakisphosphate

InsP$_5$-K	Inositol pentakisphosphate kinase
InsP$_6$	Inositol hexakisphosphate
InsP$_6$-K	Inositol hexakisphosphate kinase
InsP$_7$	Diphosphoinositol pentakisphosphate
InsPs	Inositol phosphates
IPK1	Inositol pentakisphosphate 2- kinase
IPK2	Inositol trisphosphate 3/6- kinase
IPKs	Inositol phosphate kinases
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITPK	Inositol tris/tetra phosphate 5/6- kinase
LT	Low-temperature
lpa	Low phytic acid
MAFFT	Multiple alignment using fast Fourier transform
MEGA	Molecular evolutionary genetics analysis

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Introduction

1. INTRODUCTION

Hyacinth bean or dolichos bean (*Dolichos lablab* L.) is one of the most ancient and extensively cultivated crops in tropical regions of Asia, Africa and America. This crop has multipurpose uses as pulse, vegetable and fodder. It is mainly cultivated for green pods, and various vegetarian dishes can be prepared out of its dry seeds also. This vine is native of tropical regions of Africa. It is an excellent nitrogen fixer and is also grown as a cover crop for livestock fodder purpose. In India, lablab is mostly confined to peninsular region and is extensively cultivated in Karnataka, some districts of Tamil Nadu, Kerala, Andhra Pradesh and Maharashtra. Karnataka accounts for about 90 per cent of both area and production of *Dolichos* in India with a cultivated area of 0.085 million hectares and production of 0.030 million tonnes (Laxmi *et al.*, 2016). It is considered as a potential crop for sustainable agriculture in dry land ecosystems assuring food and security to small and marginal farmers (Raghu *et al.*, 2018).

Food legumes are good sources of proteins and hence utilised to reduce the problem of protein deficiency in several parts of the world. In spite of the presence of many anti-nutritional factors such as trypsin inhibitors, phytins, saponins, legumes are considered to be the major source of protein in countries having short supplies of animal protein. The major storage form of phosphorus (P) in mature cereal and legume seeds is phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate, $InSP_6$) which strongly chelates to metallic cations such as potassium, magnesium, calcium, iron and zinc to form a mixed salt called phytate (Lott, 1984).

Dolichos bean has a fairly high content of phytic acid and its concentration among the cultivars varies from 1000 to 1350 mg/ 100g (Deka and Sarkar, 1990). Monogastric animals including humans, lack the enzyme phytase and hence, the undigested phytate complexes pass through intestinal tract. Thus, reducing the phytate level in legumes can increase their nutritional value.

The gene *myo-inositol phosphate synthase* (*MIPS*) is understood to play the major role in phytate synthesis in leguminous crops (Loewus and Murthy, 2000). The *MIPS* gene helps in inositol metabolism and catalyses the conversion of glucose-6-phosphate to myo-inositol phosphate, the first product of inositol biosynthesis. At the same time, this gene has diverse biological roles and participate in several cellular processes like signal transduction, stress response, cell wall biogenesis, growth regulation, osmo-tolerance, IAA metabolism, membrane trafficking and in phytic acid synthesis (Loewus & Murthy, 2000). In 2018, Shanmugham has reported that the reduction of phytic acid to more than 50 per cent will affect its yield or germination ability. Thus, it is impossible for the plant to germinate and to grow if this gene is missing or if this gene is not expressed normally.

In the last decade, a great concern has emerged to develop food crops with low phytates (Holm *et al.*, 2002). Even with that, the essential physiological roles of phytates in the plant system raise many concerns while breeding to this end. Multiple phosphate synthase genes are understood to contribute to the phytate levels in plants (Wongkaew, 2010). Thus, knocking down of one or few major genes may generate lablab accessions having sufficient amount of phytates only to sustain the essential plant physiological functions. However, a precise understanding on the minimum possible levels of phytates in each crop remains a challenge. RNA interference is a novel approach to silence the genes in plant system and this strategy is currently used extensively to generate plants with improved consumption qualities (Perera *et al.*, 2018). To silence any gene through RNAi strategy, precise mRNA sequence information is mandatory. Even though *Dolichos* is a major pulse crop, the mRNA sequence information for *MIPS* gene is not available. Thus, this study was taken up with the objective to clone, sequence and annotate *myo-inositol phosphate synthase* gene from the cDNA of developing dolichos bean seeds.

Review
Of
Literature

2. REVIEW OF LITERATURE

2.1 Dolichos bean in India

The dolichos bean or hyacinth bean (*Dolichos lablab*.L) is one of the most ancient among the cultivated plants and is densely grown throughout the tropical and sub-tropical regions of Asia, Africa and America. It is a perennial fodder legume belonging to the family *Fabaceae*. Deka and Sarkar (1990) reported that the wild forms of dolichos beans are believed to have originated in India.

In India, it is known for vegetable, fodder and pulse purpose in south, east and north-east parts of country. It is a major source of protein for South Indian diet and is rich in proteins, minerals and vitamins. Apart from its multi-utility, it has multi-benefits like drought tolerance, soil fertility enhancement by fixing atmospheric nitrogen *etc* (Raghu *et al.*, 2018). Despite of these multi-potential, dolichos bean is still considered to be an unexplored crop in terms of cultivation and efforts towards its genetic improvement.

2.2 Anti-nutritional characteristics of dolichos bean

The main limitation to the use of legumes in animal diet is the presence of anti-nutritional factors. In 1963, Schaaffhausen has proposed that leaves of dolichos bean do not contain tannins which makes them a superior feed for monogastric animals. However, seeds do contain the anti-nutritional factors like tannins, phytates and trypsin inhibitors.

According to Deka and Sarkar (1990) dolichos bean had a reasonably high content of phytic acid and the concentration among cultivars varied from 1000 to 1350mg/100g. The concentration of phytate phosphorus ranged from 282 to 380 mg/100 g constituting 73.2-93.3% of total phosphorus in the seeds. They also suggested that these values were much higher than those reported by Reddy et al. (1982) for grain legumes. They thus concluded that phytate in the dolichos bean was the main storage form of phosphorus. The concentration of tannins in dolichos bean

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ranged from 2000 to 2205mg/100g (Deka and Sarkar, 1990). They pointed out that since tannins were mostly located in the seed-coat; they were considered to be of less importance from the nutritional point of view as they were removed by cooking prior to consumption.

2.2 Phytic acid and phytases

Phytic acid (myo-inositol hexa phosphoric acid) is present in the plant seeds as a mixed salt, phytate, mainly involving Mg, Ca, Na and K (Selle *et al.*, 2000). Phytic acid contains a myo-inositol with six attached phosphate groups and is present naturally in all eukaryotes (Brown *et al.*, 1961; Sasakawa *et al.*, 1995., Giridhari, 2017). The major site of phytate deposition in most cereals is aleurone layer, whereas it is distributed uniformly throughout the kernels in dicotyledonous seeds, including oilseeds and grain legumes (Lott, 1984). Phytate salts, also called as phytates, function as sources of phosphate, myo-inositol and cations during seed germination (Reddy *et al.*, 1982). Phytate serve as a major store of phosphorous and myo-inositol for growing seedlings (Cosgrove, 1966).

Phytase (myo-inositol hexa-kis-phosphate phosphohydrolase) is involved in catalysing the hydrolysis of myo-inositol hexa-kis-phosphate (phytic acid) to inorganic monophosphate and thus lower myo-inositol phosphates (Wodzinski and Ullah, 1996). Phytic acid when degraded during seed germination by phytase enzymes liberates phosphate, inositol and mineral cations for use during seed development (Raboy and Dickinson, 1987). They also reported that phytic acid begins to deposit in seeds after the cellular phosphate levels have reached maximum level and gradually increases linearly throughout the seed development and seed filling. In many plants, microorganisms and in some animal tissues, phytate degrading activity has been detected. Several plant and microbial species phytases have also been purified and characterised. The International Union of Biochemistry (1979) recognizes two general classes of phytases, 3-phytase, 6-phytase or 5-phytases, based on the location of the phosphate group within the phytate molecule, that is hydrolysed first (Shanmugam, 2018). Based on the catalytic mechanisms, phytases are additionally classified as

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Histidine acid phosphatases (HAPs) and β- propeller phytases (BPPhys) (Lei *et al.*, 2007).

Microbial or fungal phytases hydrolyse the phosphate at the third position and plant phytase hydrolyses at the sixth position of phytin molecule. Myo-inositol is a precursor to many compounds in plants that function not only in phosphorous storage, but also in signal transduction, hormonal homeostasis, stress protection and cell wall synthesis. PA can provide resistance against various abiotic as well as biotic stresses in plants. It is also reported to give resistance against various bacterial, fungal and viral infections (Murphy *et al.*, 2008).

2.3 Biochemical properties of phytases

Most fungal and bacterial phytases are classified as monomeric enzymes (Greiner *et al.*, 1997; Wyss *et al.*, 1999). However, there are also animal phytases with multiple subunits (Mogal, 2014). Phytase accumulated during germination in maize seedlings is a dimeric enzyme made from two 38 KDa subunits (Laboure *et al.*, 1993). Phytases are classified as acid or alkaline phytases, according to the position of their initial hydrolysis of phytate Depending on optimal pH for their catalytic activity, most phytases belong to either the acid phytases or the alkaline phytases (Shanmugham, 2018). The acid phytases include histidine acid phosphatases (HAPs) from *Escherichia coli, Klebsiella terrigena* (Greiner *et al.*, 1997*), Aspergillus niger, Aspergillus fumigatus* (Mullaney *et al.*, 2000*)*, canola seeds (Houde *et al.*,1990) with pH range of 4.5-5.5. Meanwhile, *Bacillus* (Idriss *et al.*, 2002) and plant seeds like *Typha latifolia* pollen and *Lilium longiflorum* pollen (Scott, 1991) have an alkaline pH at 6.5-8.0. Most phytases have an optimal temperature of 44-60 °C (Shanmugham, 2018). In legumes, alkaline phytase activity was enriched by extraction with a non-ionic detergent, indicating that the protein is associated with the membrane (Scott, 1991). Phytic acid has 12 replaceable protons and thus can form complex with multivalent cations, hence has many forms (Oatway *et al.*, 2001). They also reported that phytate is the calcium salt of phytic acid and phytin is the Ca/Mg salt of phytic acid.

2.4 Molecular properties of phytic acid and phytases

Phytic acid was first identified in 1855 (Oatway *et al.*, 2001). Plant phytases have characteristics very similar to those of phytases of *Bacillus* (Oh *et al.*, 2001). Phytases which were isolated from maize roots (Hubel and Beck, 1996), maize seeds (Laboure *et al.*, 1993), soyabean seeds (Hegeman and Grabau, 2001) and *A. oryzae* (Shimizu, 1993) were reported to be homodimeric proteins. Phytic acid exists as a salt in nature with monovalent and divalent cations (Thorstensen and Romslo, 1990). They also reported IP^6 is a powerful iron-chelating agent which helps it to act as a low molecular weight iron transporter. A phytase that has a wide pH range and which is active in the stomach and upper intestine would be the ideal phytase for animal feed (Dersjant *et al.*, 2015).

The enzyme activity is expressed in activity units (FTU) where 1 FTU is defined as the amount of enzyme that liberates 1.0 μM inorganic orthophosphate/ min. from 0.0051 M sodium phytate/ L at pH 5.5 and temperature of 37 °C (FAO, 2012).
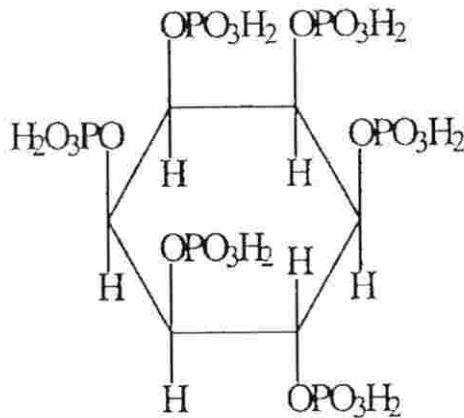
Fig 2.1: Chemical structure of phytic acid

2.5 Sources of phytic acid and phytases

Phytic acid is myo-inositol 1,2,3,4,5,6-hexakisdihydrogen phosphate (Gupta *et al.*, 2015) and it accumulates rapidly in seeds during development. In leguminous seeds and oil seeds, it is stored in the globoid crystal within the protein bodies (Erdman, 1979). Due to inefficient uptake of phosphorous by monogastric animals, about 70 per cent of total P in feed is released through their excreta (Milko *et al.*, 2008). Thus, leaching or surface run off of high levels of phytate and inorganic phosphorous can lead to algal blooms and the eutrophication of surface water (Boesch *et al.*, 2001; Milko *et al.*, 2008, Gupta *et al.*, 2015). The complete sequence of a putative phytic acid (InsP6) unigene was identified and validated in black pepper using the genome walking strategy (Giridhari, 2017).

Cereals are rich in phytates and cereal food products contain high level phytic acid (Wise, 1983). Phytate is located in the protein bodies of endosperm in legume seeds (Gupta *et al.*, 2015). Phytic acid content in whole seed ranged from 0.2 to 2.9 per cent and is >3.7 per cent in cotyledons (Harland and Prosky, 1979; Ravindran *et al.*, 1995). In animals, phytase was first reported on animal phytase in calf liver and blood by Mc Collum and Hart (1908). Gupta *et al.* (2013) proposed that more researches are yet to be developed for cost-efficient production of plant phytases which is a major problem. Microbial phytases are mostly investigated for industrial purposes than plant phytases due to their high pH and thermal stability (Bohn *et al.*, 2008).

2.6 Phytic acid biosynthesis

Phytase is the only enzyme which can initiate the phosphate hydrolysis at carbon 1, 3 or 6 in the inositol ring of phytate (Shanmugham, 2018). The conversion of D-glucose-6-phosphate to D-myo-inositol-3-phosphate by the isomerase D-myo-inositol-3-phosphate synthase (*MIPS*) is the first step in synthesis of myo-inositol (Loewus and Murthy, 2000). They also proposed that the phytic acid biosynthetic pathway which includes phosphorylation steps catalysed by one or more kinases, have not been well studied in plants.

G6P
↓ A
Ins(3)P ⇌ Ins ⟶ Ptd Ins
      B
↓
Ins(3,4)P₂
↓
Ins(3,4,6)P₃   Ins(1,3,4)P₃   Ins(1,4,5) P₃
                      E
      E   E Ins(1,3,4,5)P₄   C
↓   ↓   ↓
Ins(3,4,5,6)P₄ Ins(1,3,4,6)P₄ Ins(1,4,5,6)P₄
      E   ↓ E   C
                Ins(1,3,4,5,6)P₅
                ↓ D
                InsP₆ (Phytic acid)   (Suzuki *et al.*, 2007)
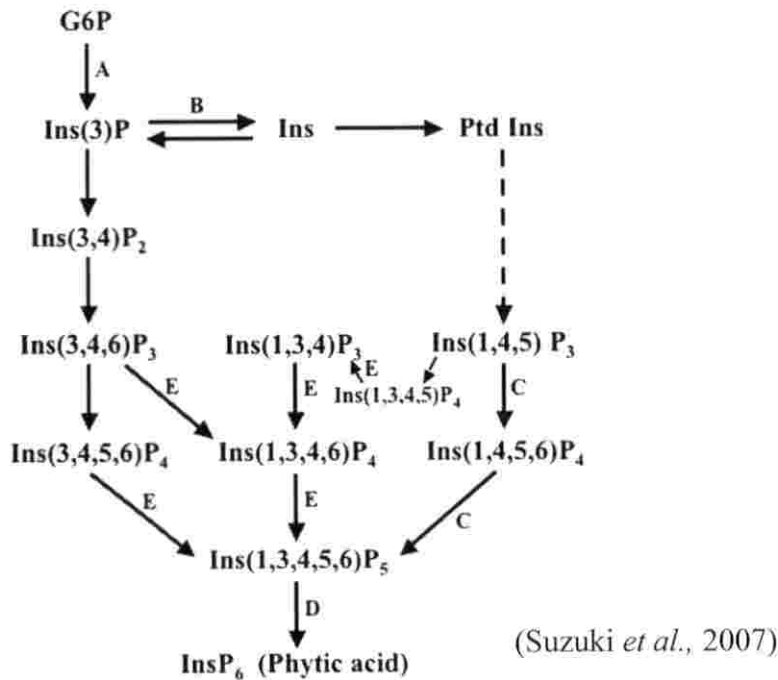
Fig 2.2 Phytic acid pathway

D-glucose-6-phosphate is converted to D-myo-inositol-3-phosphate (InsP3) by the activity of *MIPS*. It is further phosphorylated to yield myo-inositol hexa-kis-phosphate by several kinase steps. The InsP3 is dephosphorylated to free InsP by an Mg^{2+} dependent inositol monophosphate phosphatase (IMP). The enzyme myo inositol kinase (MIK) phosphorylates InsP to InsP1 and this can be further phosphorylated (Sparvolli and Cominelli, 2015).

Several genes are involved in the phytic acid biosynthesis pathway which belongs to different classes of enzymes catalysing the addition of pyrophosphates at various positions of inositol ring resulting in the production and bioconversion of various inositol phosphates. Inositol phosphate kinases (IPKs) is the first class which phosphorylates the 5-position of InsP5, InsP6 and InsP7 yielding higher InsPs (Williams *et al.*, 2015). Myo- inositol 3 phosphate synthase (*MIPS*) is the next class which convert glucose-6- phosphate to InsP3, which is the first step in InsP pathway. Inositol mono-phosphatase (IMP), inositol tris/tetra phosphate kinase (ITPK), inositol

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polyphosphate kinase (IPK2), and inositol penta-kis-phosphate 2-kinase (IPK1) are the other class of enzymes involved in the phosphorylation of inositol ring (Aggarwal *et al.*, 2015).

2.7 Roles of *MIPS1* enzyme in plants

Latrasse *et al.* (2013) reported that *MIPS1* is a protein in plants that connects cellular metabolism, pathogen response and chromatin remodelling. *MIPS1* also prevents plant cells against cell death during high light intensity or long days (Meng *et al.*, 2009). In 2010, Chen and Xiong also suggested that by the regulation of auxin distribution by *MIPS1* crucial for maintaining the phosphatidylinositol levels and pattern formation in plants. These enzymes were also found to be important for seedling development, embryo development, vascular patterning, root cap organisation, plasma membrane protein trafficking etc. Fengjie *et al.* (2010) mapped the *MIPS1* gene and developed CAPS markers for low phytic acid mutation in soyabean. Cleaved amplified polymorphic sequences (CAPS) makers specific to the *MIPS1* mutation of Gm-lpa-TW-1 (mutant line) were developed based on sequence information of all four *MIPS* genes in soyabean. In both monocots and dicots, *MIPS1* gene is found to express during the early stages of seed development, just before the accumulation of phytic acid and then decreases gradually (Fileppi *et al.*, 2010). Suppression of Inositol pentakisphosphate 2-kinase leads to drastic reduction of plant growth and even the germination itself (Kuo *et al.*, 2014). Alternatively, successful suppression of *MIPS*, which catalyzes the first step in *de novo* synthesis of myo-inositol, got the potential to generate the lines with reduced phytate content.

2.8 Physiological functions of phytic acid and its derivatives

PA is the hexa-phosphoric ester of cyclohexane (Inositol hexa-phosphoric acid, IP_6). It is usually found in seeds as a complex with essential minerals or proteins (Ahmad and Muhammed, 2013). Phytases has many applications like phytate elimination in feed and food industries, fighting environmental protection or phosphorous pollution, plant growth enhancement and the preparation of special myo-

inositol phosphatases as tools for biochemical investigations (Griener and Carlsson, 2006; Singh *et al.*, 2011).

Phytic acid assists the seedling growth by supplying the requirements of the growing tissues and hence, it is utilised during seed germination (Oatway *et al.*, 2001). The end products of phytic acid hydrolysis, particularly myo-inositol are used by the young seedlings for cell wall formation (Tsao *et al.*, 1997). The total phosphorous concentration present as phytic phosphorous increases with the concentration of P_2O_5 supplied to the plant (Oatway *et al.*, 2001).Hence, when a plant is supplied with higher doses of phosphorous than required, excess phosphorous is stored in the form of phytic acid (Oatway *et al.*, 2001). The concentration of PA changes as kernels mature and reaches to a maximum level when kernels are ripe (Pallauf and Rimbach, 1997). Phytases are activated during germination which results in increasing the availability of phosphorous to the developing embryo (Oatway *et al.*, 2001).

Phytic acid has the ability to bind either directly or indirectly to minerals, proteins and starch. The binding alters solubility, digestion, function and absorption of the food components. The phosphate groups of phytic acid may be bound with metal ions forming complexes of various solubility. Starch binding can also occur *via* hydrogen bond formation (Rickard and Thompson, 1997). Phytic acid appears to be most affected by zinc, as it can form most stable and insoluble complexes. Other minerals and nutrients like Ca, Na, Fe, Mg, Mn and Cl are also absorbed by phytic acid (Oatway *et al.*, 2001).

2.9 Medical applications of phytic acid

Oatway *et al.* (2001) has pointed out that cereals and legumes are protective foods due to their phytic acid content or due to presence of inositol phosphates. They also reported that it is able to normalize the cell division rates, even though IP_6 was not a direct antagonist to the carcinogenicity; it also reduces cell growth and enhances cancer cell differentiation.

Phytate confers antioxidant properties due to its chelating property by inhibiting the metal-catalysed free radical formation (Graf and Eaton, 1990; Shamsuddin, 1995). In addition to the suppression of colon cancer, phytic acid is proved to suppress other inflammatory bowel diseases (Reddy *et al.*, 1989). In addition, phytate may protect against lead poisoning and tooth decay (Wise, 1981; Grenby, 1972). Phytic acid lowers the rate of *in vitro* and *in vivo* starch digestion (Knuckles and Betschart, 1987). Thompson *et al.* (1987) concluded that phytic acid may have certain health benefits in blood glucose management by lowering the starch digestion rate and the blood glucose levels. Reports also shows that dietary phytates can protect against variety of cancer, kidney stone formation, diabetes mellitus, caries, atherosclerosis and coronary heart disease (Greiner *et al.*, 2006).

PA is proven to inhibit cardiovascular diseases like dyslepidemia, and platelet aggregation. It can also effect the influx of extracellular calcium leading to release of insulin and it control progression of Alzheimer's disease (Anekonda *et al.*, 2011). His experiments conducted on mice model have discovered that phytic acid offered complete protection against amyloid precursor protein C- terminal fragment-induced cytotoxicity by maintaining levels of increased intracellular calcium, hydrogen peroxide, superoxide, Aβ oligomers and moderately upregulating the expression of autophagy protein (beclin-1). PA is also said to have protective effect against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) induced Parkinson's disease in mice model studies. This was connected with its anti-inflammatory effect associated with suppression of pathways that are involved in nuclear factor κB (NF-κB) and phosphorylated extracellular signal-regulated kinase (p-ERK) (Lv *et al.*, 2015).

2.10 Industrial uses of phytic acid

Phytic acid is used in treating metals to prevent rust, in etching solution for offset printing as replacement for cyanide or ammonium phosphate, in toothpastes or mouthwashes and in antifreeze/cooling water or other closed systems for corrosion protection (Reddy *et al.*, 1989). Dentistry and oral hygiene are the other two areas

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where use of phytic acid has immense potential as phytic acid can inhibit the dissolving of calcium phosphate and tooth (Graf, 1986). He also reported that in food processing industry, PA plays a major role in reducing fermentation and prevents colour changing and oxidation properties in foods and wines due to its chelating property. Phytic acid based dental cement has greater stability to acid attack with superior compressive strength, settling time and low acid erosion (Oatway *et al.*, 2001).

Phytic acid is used as an adsorbent for removal of heavy metal ions from contaminated water due to its unique binding property with different metal ions (Tsao *et al.*, 1997). Phytic acid resin could be produced by heating crop residues with phytic acid and this could be used to remove lead and other metals that contaminate water (Oatway *et al.*, 2001). In the textile industry, phytic acid relative products can be applied as anti-wrinkle catalyst for textile, prevent the fiber from static electricity and act as flame retardant for wool fabrics high char-forming ability (Cheng *et al.*, 2016).

2.11 Nutritional and anti-nutritional effects of phytic acid

The anti-nutritive effect of PA is based on its molecular structure (Pallauf and Rimbach, 1997). Bioavailability of zinc is most affected by phytic acid (Rimbach and Pallauf, 1993). Phytic acid not only depresses the bioavailability of dietary zinc but also decreases the reabsorption of endogenous intestinal zinc. High concentration of Ca further increases the anti-nutritive effect of phytic acid (PA) on bioavailability of zinc due to formation of insoluble Ca-Zn-PA complex (Flanagan, 1984). Interaction between PA and calcium (Taylor and Coleman, 1979), Magnesium (Brink *et al.*, 1991), Iron (Thompson and Erdman, 1984), Copper (Lee *et al.*, 1988) and Manganese (Davidson *et al.*, 1995) have also been described in monogastric farm animals and humans.

Phytic acid is largely indigestible by monogastric animals even-though they are important for the growth of seedlings (Simons *et al.*, 1990). This indigestibility is

due to the absence of the digestive enzyme, phytase to hydrolyse the phytic acid (Wongkaew *et al.*, 2010).

2.12 Analysis of phytic acid

There are no direct methods or no specific reagents for the detection of phytic acid or its various forms. Greiner *et al.* (2006) have reported that in the earlier times, phytate in experimental diets are found to be determined by the addition of excess Fe to precipitate phytate and further estimated by determining the inositol or phosphate contents in phytate. Phytate estimation was carried out using PAGE analysis as a preliminary confirmatory test for detection of the presence of phytate in the leaf sample of black pepper (Giridhari, 2017). PA does not possess any characteristic absorption spectrum in UV or visible light region. Thus, most analytical methods are based on the extraction of phytic acid (Oatway *et al.*, 2001). The AOAC anion-exchange method has been performed to estimate the PA content in products. But this method cannot distinguish phytic acid (IP_6) from other inositol phosphates (IP_5-IP_1) (Lehrfeld and Morris, 1992). HPLC is the primary means of separation and quantification (Lehrfeld, 1994).

2.13 Level of phytic acid in plants

Phytase activity has been identified in many plants such as maize (Laboure *et al.*, 1993), barley, rye (Greiner *et al.*, 2000), canola seed (Houde *et al.*, 1990) and lily pollen (Scott, 1991). The seeds of cereal grains and legumes show highest content of phytate among plants (Reddy *et al.*, 1989).

Phytic acid is present in globoids and are compartmentalised inside protein storage vacuoles in the seeds (O'Dell *et al.*, 1972). Reddy *et al.* (1989) showed that phytate form of phosphorous ranges from 30 per cent in roots to 80 per cent in seeds and cereals. Highest amount of phytate among cereals is in maize (0.83-2.22%) and among legumes in dolichos beans (5.92-9.15%).

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Ravindran *et al.* (1994) reported that in cereal grains, oilseeds and legumes, the major portion of total phosphorous constitutes phytate form (60.0-97.0 %) and in roots and tubers (21.0-25.0 %). Deka and Sarkar (1990) reported that dolichos bean had a fairly high content of phytic acid and its concentration among cultivars varied significantly from 1000 to 1350 mg/ 100g and the phytate phosphorous ranged from 282 to 380 mg/ 100g.

2.14 Need of phytic acid reduction

Majority of the world population is affected by micronutrient malnutrition and also of anaemia and zinc deficiency, particularly in developing countries (Jorge *et al.*, 2008). PA is a food inhibitor which chelates the micronutrients and prevent it from being available for monogastric animals, including humans as they lack the enzyme phytase in their digestive tract (Wodzinski and Ullah, 1906; Urbano *et al.*, 2000; Singh *et al.*, 2011) and is excreted. Due to inefficient uptake of phosphorous, about 70 per cent of total P in feed is released in excreta (Milko *et al.*, 2008).

Reddy *et al* (1996) showed that PA binds to minerals and prevent it to be bioavailable due to its chelating property. They also reported that PA inhibits absorption of Fe, Zn, Ca, Mg and Mn. Phytic acid chelates mineral cations, including Ca, Zn, Mg and Fe from diet and affects its bioavailability (Raboy *et al.*, 2001). Thus, many methods have been developed to minimise the PA content in food and to improve the cereal nutritional value which becomes poor due to such anti-nutrient (Nout, 1993). These include some pre-treatments like fermentation, germination, soaking and enzymatic treatment of grains with phytase enzyme as well as several genetic improvement techniques like biofortification (Gupta *et al.*, 2015).

Dephytinisation approaches include catalysing the degradation of phytic acid by use of phytic acid hydrolysing enzyme and also by use of *lpa* mutants through knock out of genes involved in phytic acid biosynthesis (Shanmugham, 2018). Holm

et al. (2002) attempted to reduce phytic acid in wheat products by introducing the *Aspergillus niger* phytase gene *phyA* into a wheat by particle bombardments of immature embryos instead of blocking its biosynthesis.

2.15 Present attempts on phytic acid reduction

Sparvolli and Cominelli (2015) reported that several low phytic acid mutants (*lpa*) were developed in many important crops like barley, maize, rice, wheat, soybean, common bean and pea. *MIPS* being the leading contributor towards the production of phytates in legumes (Loewus and Murthy, 2000), suppression of the expression of this gene can reduce the phytate content to substantial degree. RNAi is an effective post-transcriptional gene regulatory strategy in plants (Tang and Galili, 2004). In this approach, transcription of a complementary mRNA of the target gene leads to the formation of double stranded mRNA which in turn will be cut off by an RNase III, DICER (Scherer and Rossi, 2003).

Through RNAi, effective reduction in *myo-inositol phosphate synthase* expression and phytate content in the seeds of soybean has been demonstrated (Nunes *et al.*, 2006). RNAi-mediated seed-specific silencing of the IPK1 gene using the Oleosin18 promoter revealed a 3.85-fold down-regulation in IPK1 transcripts, and a significant reduction in phytate levels as well as an increase in the amount of inorganic phosphate (Pi). They could observe no negative influence on seed germination or in any other agronomic traits (Ali *et al.*, 2013). Molecular characterization, modeling and docking analysis of late phytic acid biosynthesis pathway gene, *inositol phosphate 6-/3-/5- kinase*, a potential candidate for developing low phytate crop was done (Punjabi *et al.*, 2018).

Phytic acid transport in *Phaseolus vulgaris* was studied by Cominelli *et al.* (2018). A new common bean *lpa* affecting the PvMRP1 InsP6 transporter was isolated and characterised. The bioavailability of micronutrients was improved by changing the PA content of rice grain; the manipulation techniques include the transgene approaches like suppression of phytic acid coding gene, using molecular markers in breeding and also focusing on manipulation of phytic acid and micronutrient

distributions in the endosperm and aleurone layer of rice grain (Perera *et al.*, 2018). In wheat, phytic acid levels are decreased by RNA-i mediated down-regulation of *inositol penta-kis-phosphate kinase* (IPK$_1$) (Aggarwal *et al.*, 2018). Yamaji *et al.* (2017) reported a study on reducing phosphorous deposition in rice grains with an impaired transporter in the node.

In 2015, a study was conducted by Gupta *et al.* on PA reduction and enhancement of bioavailable micronutrients in food grains. They also reported that genetic improvement and several pre-treatment methods like fermentation, soaking, germination can also improve the nutritional quality.

Seed-specific silencing of *OsMRP$_5$* (Multidrug-resistance related protein from *Oryza sativa*) resulted in reduction of seed phytic acid and weight in rice (Li *et al.*, 2014). They also reported the production of low phytic acid by hairpin RNA and artificial micro RNA – mediated silencing of OsMIK in seeds. RNA-i technology has been performed to reduce maize PA by silencing MRP$_4$ ATP-binding cassette (ABC) transporter (Gupta *et al.*, 2011). In 2011, Ye *et al.* developed a low phytic acid barley by inducing a nonsense mutation in a putative sulphate transporter gene.

2.16 Major attempts on phytic acid reduction in legumes

In faba beans, phytic acid reduction is attempted by bioprocessing which helps in improving the *in vitro* digestibility of faba bean protein (Rosa *et al.*, 2018). Influence of transcription regulatory network in low phytic acid soyabean seeds were studied by Redekar *et al.* (2017). Baghyawant *et al.* (2018) studied the impact of phytic acid on nutrient bioavailability and antioxidant properties of chickpea genotypes.

2.17 Cloning and characterization of long genes from plants

In 2016, Liu *et al.* cloned and characterised *w* gene controlling white immature fruit colour in cucumber. The *w* gene was amplified using four sets of primers inorder to get the full-length coding region which had a size of 5878 bp and were subjected

to TA cloning. Cloning and selection of genes generated by low temperature in barley shoot meristematic tissue was reported by Dunn *et al.*, 1990. In this work, cDNA library was constructed in λgt10 from mRNA extracted at low-temperature-grown (LT 6°C/2 °C grown) meristematic tissue of winter barley. From recombinant phage, the gene inserts were subcloned into the EcoRI site of M13mpl8 sequencing vector and was characterised.

Belanger and Kriz (1989) characterised *Glb1* (Globulin) gene from maize by preparing a library of sequences which had a length of 1800bp in bacteriophage λ-expression vector. The cDNA inserts were excised from LambdaZAP as recombinant pBluescript SK(-) plasmids and were subcloned into M13 mpl8 and mpl9.Cloning and characterisation of key *4-coumarate CoA ligase* gene was done in *Boehemia nivea* using pET32a plasmid as the cloning vector (Tang *et al.*, 2018). Cloning and characterization of a novel GIGANTEA gene (*IbGI*) was done in sweet potato (Tang *et al.*, 2017) which is involved in regulation of flowering, circadian rhythm and stress tolerance. The 3501 bp coding sequence of *IbGI* was amplified and subcloned into the T-blunt vector (SolGent™, South Korea) and sequenced by SolGent Company Limited (Daejeon, South Korea).

2.18 Genetic modification of phytase source

In order to reduce the PA content in cereals, genetic modification can be efficiently performed by lowering the phytase enzyme genes and by developing modified genome encoding transgenic plants for phytase enzyme (Gupta *et al.*, 2015). Vats and Banerjee (2004) has remarked that genetic modification of crop plants for heterologous phytase production will help in reducing phosphate load on agricultural ecosystem and also improves phosphate bioavailability.

A transgenic rice over-express gene encoding for phytase from *Aspergillus fumigatus*, ferretin from *Phaseolus vulgaris* and a cysteine-rich metallothionein-like protein has been developed to enhance the bioavailability from rice to humans (Gupta *et al.*, 2015). This transgenic rice was crossed with β-carotene producing rice line (Lie

and Porres, 2003). In 2004, Vats and Banerjee reported that biopharming of phytase is a cost-effective approach for phytase production. They also mentioned that in a strain improvement study of *A. niger* (NRRL 3135) by UV radiation, the phytase catalytic mutant produced 3.3-fold higher phytase (phy A) than the wild type strain.

2.19 Cloning and characterization of major genes in legumes

In the last 40 years, molecular cloning has progressed from simple isolation and joining of DNA fragments, followed by screening of potential clones to assembling of efficient DNA fragments in just few hours. The gene cloning helps in studying the structure and function of genes in detail. In medicine, cloning of bacteria helps in synthesising vitamins, hormones and antibodies. Cloning also has advantages in agriculture sector as cloning in bacteria facilitates nitrogen fixation in plants, increases the micronutrient bioavailability, reduce or lower the expression of undesired gene *etc.*

Cloning and characterization of a functional *flavanone-3ß-hydroxylase* gene from *Medicago truncatula* was reported by Shen *et al.* in 2010. This gene having a size of 1499 bp was cloned into a pMD18 vector and was sequenced. The Kunitz-type protease inhibitor (*HGPI*) gene in *Dolichos biflorus* (700 bp) was isolated, amplified and cloned in pGEMT vector (Kuhar *et al.*, 2012).

A drought tolerance gene, *MsHSP70* from alfalfa was cloned and characterised (Li *et al.*, 2017). *MsHSP70*, with an ORF of 2028-bp was discovered through homology cloning. The ORF of *MsHSP70* was amplified using cDNA of *Medicago sativa* as template with designed specific primers. The amplicon thus obtained was cloned into the pEASY-T1 vector and was confirmed by sequencing. A novel vacuolar Na^+/H^+ antiporter gene (VuNHX$_1$) for salt tolerance was cloned and characterised from cowpea (Mishra *et al.*, 2015). The full-length cDNA of VuNHX1 was obtained by RT-PCR and RACE method. The 1,981 bp cDNA of VuNHX1 thus obtained contained an ORF of 1,629 bp. The first strand cDNA synthesis and RT-PCR were performed with the pair of degenerate primers using Revert AidTM First Strand

cDNA Synthesis Kit. The amplified product was ligated into TA cloning vector, pTZR/T and sequenced.

He *et al.* (2015) conducted a study on molecular cloning and characterisation of annexin genes (*AnnAhs*) in peanut. The full-length cDNAs from isolated total RNA of *AnnAhs* were extracted using degenerate and gene specific primers for reverse transcription (RT)-PCR and rapid amplification of cDNA ends (RACE)-PCR reactions. The touchdown PCR amplifications with degenerate primers was performed using ExTaq DNA 170 polymerase. In *Phaseolus vulgaris*, ABA-specific glucosyl transferase genes were cloned and characterised (Palaniyandi *et al.*, 2015). Rani *et al.* (2015) performed cloning, *in silico* characterisation and homology modelling of *Phaseoline* gene from common bean. The full-length DNA of *Phaseoline* gene (2000bp) was isolated and amplified using specific primers. The amplified product was ligated and cloned in the pTZ57R/T cloning vector (2.8bp) with competent cell of DH5α *E. coli* strain.

2.20 Characterization of *MIPS* gene in crop plants

The *MIPS* sequences of major legumes available in Genbank are *Glycine max* (AY382834.1), *Phaseolus vulgaris* (AJ853494.2), *Vigna radiata* (XM_014653891.2), *Vigna unguiculata* (XM_028060955.1), *Cajanus cajan* (XM_020381334.1) *etc.*

Good (2001) have identified four different *MIPS* genes in soybean pant tissues – *GmMIPS1*, *GmMIPS2*, *GmMIPS3* and *GmMIPS4*. In a work carried out by Chappell et al. (2006), they characterised the soyabean *MIPS* gene family. The results indicated that *MIPS1* is highly expressed and *MIPS2*, *MIPS3* and *MIPS4* are poorly expressed in developing seeds.

12 genes corresponding to the phytic acid biosynthesis from rice were identified: *RINO1 and RINO2* are the MIPS gene, *OsIMP1* and *OsIMP2* encodes inositol mono-phosphatase, *OsIPK1* and *OsIPK2* are the IPK and *IPK2* genes respectively. It was reported that *RINO1* is expressed in developing embryos and aleurone layers, in which synthesis of phytic acid occurs whereas *RINO2* do not take

part in phytic acid biosynthesis in seeds (Suzuki *et al.* 2007). They also suggested that the timing of expression reflects the timing of phytate accumulation in these tissues.

A clear picture of the involvement of genes like *MIK* (Myo-inositol kinase), *IMP* (Inositol monophosphate) and *IPK2* (Inositol 1,4,5-tris-phosphate kinase) during seed development in phytic acid synthesis were identified in common bean. Besides *MIPS*, *IMP* and *IPK2* also have role during seed development underlining their function in phytic acid biosynthesis. A *MIPS* gene (*PvMIPSs*) is specifically expressed during seed development and its maximum levels of expression are detected at very early stages whereas *PvIMP* and *PvIPK2* expression regulated during seed development have different timings of expression. Two *MIPS* genes, *PvMIPSs* and *PvMIPSv* were isolated from common bean. These genes isolated from leaf tissues were found to have nine introns and mainly differed in the third intron region (Fileppi *et al.*, 2010).
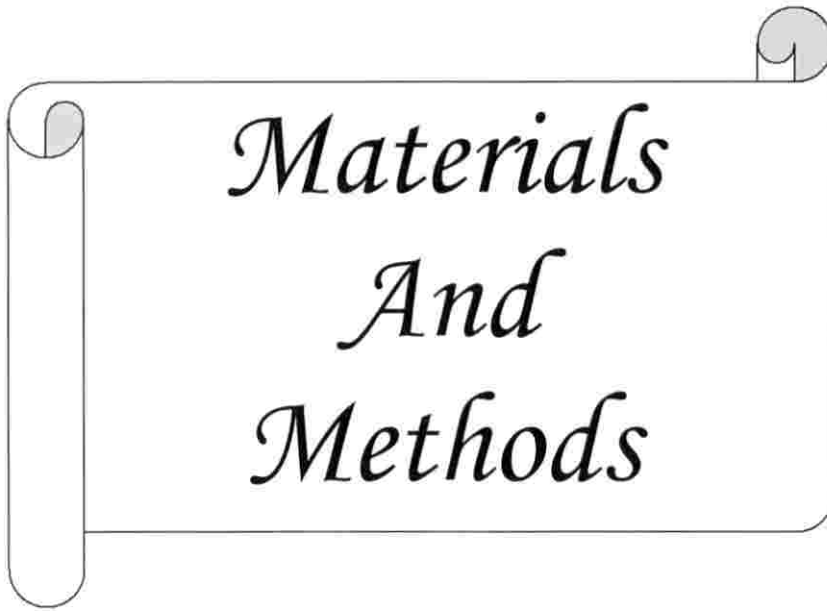
Materials And Methods

3. MATERIALS AND METHODS

The research work on 'Cloning and characterisation of *myo-inositol phosphate synthase* gene coding for phytates in *Dolichos lablab* L.' was carried out at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur. This chapter describes the materials used and the methodology followed in the research.

3.1 Materials

3.1.1 Plant materials

Seeds of dolichos bean variety Hima were procured from Department of Olericulture, College of Horticulture, Vellanikkara. At Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, the seeds were sown in earthen-pots under natural light conditions. The pods were collected at 3 to 5 days after seed set and they were immediately frozen in liquid nitrogen and kept under -80 °C until use.

3.1.2 Laboratory chemicals, glassware and equipment

In this study, AR grade chemicals from Merck India Ltd., HiMedia, Sisco Research Laboratories (SRL), Sigma-Aldrich, Thermo Scientific, Invitrogen were used. All the PCR chemicals were supplied by Invitrogen (India). The plasticware used were obtained from Tarsons India Ltd. and the glassware were from Borosilicate.

High precision electronic balance (Shimadzu), Icematic (F100 compact), Vortexer (Genei), Purelink plant RNA purification reagent (Invitrogen), RNA ZAP™ and nuclease free water (Ambion, Inc, USA), micropipettes (Eppendorf) and high speed refrigerated centrifuge (KUBOTA 3500, Japan) were used for RNA isolation. RNA quality and quantity estimations were done using NanoDrop® ND-1000 spectrophotometer. RNA quality estimation was performed using electrophoresis (BioRad), UV trans-illuminator (Wealtec) and the gel was documented using Gel Doc XR+ (Biorad, USA). For long term storage, the samples were maintained at -80 °C

(Haier Bio Medical, China) and -20 °C (Vestfrost). cDNA conversion and PCR reactions were carried out using Proflex™ PCR system (Life Technologies).

3.2 Methods

3.2.1 Collection of seed samples

The developing dolichos bean seeds were collected 3 to 5 days after seed set. The pods were wiped with RNA ZAP™ and wrapped in aluminium foil. The collected samples were immediately dipped in liquid nitrogen and proceeded with RNA isolation.

3.3 Total RNA extraction

3.3.1 General precautions for RNA extraction

Intact and high-quality RNA was obtained by careful avoiding of contamination and degradation during RNA isolation. All the materials and utensils for RNA isolation such as glassware, mortar and pestle, microtips and micro-centrifuge tubes were treated overnight with DEPC treated water (1 mL of 0.1% DEPC in 1000 mL distilled water) and then double autoclaved. TAE buffer and 75 per cent ethanol was also prepared with double autoclaved DEPC water. The electrophoresis unit was first wiped with 75 per cent ethanol or RNA ZAP™ and was washed thoroughly with DEPC treated water.

3.3.2 Isolation of total RNA

To accurately analyse RNA transcripts and for RT-PCR analysis, it is important to isolate high-quality RNA in sufficient quantity from the tissues. Total RNA was isolated from the developing seeds using Purelink® Plant RNA Purification Reagent (Invitrogen). The detailed procedure for the RNA isolation is given below.

Reagents used

1. Purelink® Plant RNA Purification reagent
2. 1.5 M NaCl
3. Chloroform

4. Ice-cold isopropanol

5. 75 per cent chilled ethanol (DEPC treated)

6. Autoclaved DEPC treated water

Procedure

1. Developing seeds (100 mg) were ground well with liquid nitrogen using DEPC treated mortar and pestle

2. One ml of plant RNA reagent was added and the homogenate was transferred to a 1.5 mL DEPC treated tube

3. Sample was homogenised by vortexing for two minutes

4. Tubes were incubated horizontally at room temperature (21 °C) for five minutes

5. The content was centrifuged at 11,400 rpm for two minutes at room temperature

6. Supernatant was transferred to a 2 mL centrifuge tube

7. To the supernatant, 100 μL of 1.5 M NaCl was added and homogenised by inverting the tube eighty times

8. Thereafter, 300μL of chloroform was added and the content was mixed by inverting eighty times

9. Tubes were then centrifuged at 11,400 rpm for 10 minutes at 4 °C

10. Upper phase was recovered and transferred to a new tube

11. To this, 300 μL chloroform was added and the above procedure was repeated

12. The final supernatant after centrifugation was extracted and 500 μL isopropanol was added and inverted eighty times

13. Tube was incubated at room temperature for 10 minutes

14. The precipitate was recovered by centrifugation at 11,400 rpm for 10 minutes at 4 °C

15. Pellet was then washed with one ml of 75 per cent ethanol by centrifuging at 11,400 rpm for 1 minute at room temperature

16. Supernatant was discarded and the pellet was dried under laminar air flow

17. Dried pellet was then dissolved in 70μL of autoclaved DEPC water or nuclease-free water

18. The tubes were later stored at -80 °C

3.4 Quality and quantity analysis of total RNA

Quality and quantity of the extracted RNA was assessed using electrophoresis and spectrophotometer.

3.4.1 Reagents and equipment

1. Agarose (1.0 %)
2. 50X TAE buffer (pH 8.0)
- Tris buffer (1M) (242.2 g/L)
- Glacial Acetic acid (57.1 mL)
- 0.5M EDTA (pH 8.0) (100 mL)
- Total volume – 1 L
3. Tracking/loading dye (6x) (GeNei)
4. Step up 1kb DNA ladder (GeNei)
5. Ethidium bromide (stock 10 mg/ mL: working concentration 0.5 µg/ mL)

Procedure

1. The gel casting tray was wiped with 70 per cent ethanol and placed appropriately in the gel caster by closing both the ends. A comb was selected and positioned on the grooves provided on the gel casting tray.
2. Gel was prepared by adding 0.6 g of agarose in 60 mL 1X TAE buffer in a glass beaker. The mixture was heated in a microwave oven till the agarose particles got dissolved completely and a clear solution was obtained.
3. The solution was allowed to cool down for few minutes at room temperature
4. Later, 3.0 µL of ethidium bromide was added and mixed well. The warm gel was slowly poured into the casting tray avoiding the air bubbles and was left to solidify for few minutes at room temperature.
5. After the solidification of gel, the comb was removed gently and the gel along with the tray was kept into the electrophoresis tank containing 0.5X TAE buffer with the wells facing negative electrode side (cathode).
6. The samples to be electrophoresed were prepared by mixing 3.0 µL of RNA sample with 2.0 µL 6X loading dye. After mixing, total volume of 5.0 µL was

loaded into the wells and 3.0 μL of DNA ladder was loaded on the sides to mark the molecular weight.

3.4.2 Gel Documentation

The electrophoresed gel was documented under UV with BioRad GelDoc™ XR+ system using PDQuest™ software.

3.4.3 Quality estimation of RNA with spectrophotometer

The quality and quantity of RNA was estimated using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). The absorption maxima for nucleic acids and proteins are at 260 nm and 280 nm, respectively, and hence the absorbance have been recorded at both the wavelengths and the purity of the samples was estimated using OD_{260}/OD_{280} ratio.

To record the absorbance, the paddle stand of the instrument was wiped properly and 1.0 μL of double autoclaved DEPC treated water was used as blank to initialize the instrument to zero. Later, 1.0 μL of sample was loaded. The RNA sample was considered to be pure if $OD_{260}/0D_{280}$ is between 1.8 and 2.0.

3.5 Primers for thermal cycling

The PCR primers were designed from the coding regions of *MIPS* sequences of *Vigna radiata* which seemed to be the closest relative of *Dolichos lablab*, retrieved from NCBI. Single set of primers for full-length gene was designed and used to amplify the gene. As cloning of a full-length gene is difficult, primers were designed as two sets of overlapping sequences of the full-length gene. The primers were then synthesised by Sigma-Aldrich.

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Table 3.1 List of primers designed from *MIPS* sequences of *Vigna radiata*

Name	Primers	Sequences (5'-3')	Annealing temperature
Set 1	Forward (MIPS VF)	GCCGATGTTCATCCAGAGTTTTAAGG	58 °C
	Reverse (M 1 R)	CCCCACAAGGAAATCTACCA	
Set 2	Forward (M 2 F)	AGATTTCATTGCTGCCAACC	55 °C
	Reverse (MIPS VR)	GCTCACTTGTACTCCAGGATCATG	

3.6 First strand cDNA synthesis and amplification of the gene

Total RNA consists of rRNA, mRNA, tRNA and other forms of RNA. For successful first strand cDNA synthesis and successful PCR amplification, an intact mRNA is essential. RT-PCR (Reverse transcription – polymerase chain reaction) is sensitive good technique for mRNA detection and quantification. Under favourable conditions, reverse transcriptase enzyme can synthesise cDNA from isolated RNA (Malek *et al.*, 2000).

SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* High Fidelity DNA Polymerase (Invitrogen) was used for sensitive, high-fidelity end-point detection and analysis of RNA molecules by RT-PCR using gene-specific primers and target RNA. The reaction was performed according to the manufacturer's protocol.

Reagents used

1.   RNA sample (1.0 pg to 1.0 µg)
2.   2X reaction mix

3. Forward primer (10 µM)

4. Reverse primer (10 µM)

5. SuperScript™ III RT/Platinum™ *Taq* high fidelity enzyme mix

6. Autoclaved distilled water

Procedure

1. The thermal cycler was programmed so that cDNA synthesis was followed immediately with PCR amplification, automatically. Two different PCRs were performed to amplify the two regions separately with respective primers designed.

Table 3.2 Thermal profile for PCR amplification of *MIPS* gene with MIPS V F and M1R primers

cDNA synthesis and pre-denaturation		Denature	Anneal	Extend	Final extension
1 Cycle		40 Cycles			1 Cycle
55 °C	94 °C	94 °C	58 °C	68 °C	68
30 minutes	2 minutes	15 seconds	30 seconds	1 minute/kb	15 minutes

Table 3.3 Thermal cycling for PCR amplification of *MIPS* gene with M 2 F and MIPS V R primers was carried out with the following program.

cDNA synthesis and pre-denaturation		Denature	Anneal	Extend	Final extension
1 Cycle		40 Cycles			1 Cycle
55 °C	94 °C	94 °C	55 °C	68 °C	68 °C
30 minutes	2 minutes	15 seconds	30 seconds	1 minute/ kb	15 minutes

1. All the reagents were thawed and added to a 0.2 ml nuclease-free, DEPC treated, thin-walled PCR tube on ice.

Table 3.4 PCR reaction mixture

Component	Volume (μL)
2X Reaction Mix	25.0
Template RNA (500 ng)	1.0
Forward primer (10μM)	1.0
Reverse primer (10μM)	1.0
SuperScript III RT/ Platinum *Taq* high fidelity enzyme mix	1.0
Autoclaved distilled water	21.0

1. All the reagents were gently mixed and spun briefly to confirm that all the components are at the bottom of the amplification tube.

2. The tubes were placed in the preheated thermal cycler programmed as described above.

3. PCR amplified products were then electrophoresed on 1 per cent agarose gel at 80 V. 1 kb DNA ladder (Banglore GeNei) was used and ethidium bromide was used for staining. The gel profile was visualised under UV and was used for further analysis.

3.7 Purification of the PCR bands from the agarose gels

The bands in agarose gel were purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's protocol.

Absolute ethanol was added to Buffer PE before use. pH indicator I was added to Buffer PB in ratio 1:250 till it becomes slight yellow in colour (pH ≤7.5).

Procedure

1. Five volumes of Buffer PB was added to one volume of PCR sample and mixed well.

2. A QIAquick spin column which was provided in the kit was placed in a 2 ml collection tube
3. Sample was applied to the column for DNA binding and was centrifuged at 13,000 rpm for one minute
4. Flow-through was discarded and the column was placed back into the same tube
5. In washing step, 0.75 mL of Buffer PE was added to the QIAquick column and centrifuged at 13,000 rpm for one minute
6. Flow-through was discarded and the column was placed back into the same tube and centrifuged additionally for one minute
7. Then, the column was placed into a clean 1.5 mL microcentrifuge tube
8. In order to elute DNA, 50 µL Buffer EB was added to the centre of the QIAquick membrane and centrifuged the column at 13,000 rpm for one minute
9. The spin column was discarded and the eluent was stored in -20 ˚C.

3.8 Molecular cloning

Cloning of the PCR product was done by using the InsTAclone PCR Cloning Kit (Thermo Scientific). The Thermo Scientific™ InsTAclone™ PCR Cloning Kit is a TA system which ensures direct one-step cloning of PCR products with 3'-dA overhangs.

The TA cloning vector pTZ57R/T (Fig. 3.1) is of high quality and is ready to use for efficient ligation with PCR products providing high cloning yields and low background. The DNA insert can be readily excised from the polylinker of pTZ57R/T and can be sequenced using M13/pUC primers.

3.8.1 Ligation

1. All the ligation reaction components, as shown in the table below, were added, briefly vortexed and centrifuged for 10,000 rpm for 3-5 seconds.

Table 3.5 Ligation mixture

Component	Volume (µL)
Vector pTZ57R/T, (0.17 pmol ends)	3.0
5X Ligation buffer	6.0
PCR product (0.52 pmol ends)	6.0 (25.0 ng)
Water, nuclease-free	(make upto 29 µL)
T4 DNA Ligase	1.0
Total volume	30.0

2. Ligation mixture was incubated at room temperature (22 °C) for 1 hour. For obtaining maximal number of transformants, the mixture was incubated overnight at 4 °C.

3. 2.5 µL of the ligation mixture was used directly for bacterial transformation.

3.8.2 Bacterial transformation

For fast and efficient preparation of chemically competent cells and fast transformation, TransformAid Bacterial Kit provided by ThermoFischer was used. Competent cells were not provided in the kit and common *E. coli* laboratory strain (*DH5α*) procured from Department of Microbiology, College of Horticulture, Vellanikkara was used for the transformation. Overnight liquid bacterial culture (Two mL of C-medium was inoculated with a single bacterial colony on the day before transformation and incubated overnight at 37 °C in a shaker was used for preparation of competent cells.

Procedure

1. On the day of transformation, culture tubes containing the required amount of C-medium (1.5 mL for each 2 transformations) were pre-warmed at 37 °C for at least 20 min. LB agar plates, supplemented with ampicillin, X-Gal and IPTG were also pre-warmed.

2. T-solution preparation: T-solution A and T-solution B were thawed (provided in the kit) and contents were mixed thoroughly. 250 µL of T-solution A and 250 µL of T-solution B were combined in a separate tube and kept on ice.

3. 150 µL of the overnight bacterial culture was added to 1.5 mL of pre-warmed C-medium and was incubated at 37 °C for 20 min. in a shaker

4. The bacterial cells were pelleted by one min. centrifugation and the supernatant was discarded

5. The cells were resuspended in 300 µL of T-solution and incubated on ice for five min.

6. Centrifuged for 1 min. in a microfuge and the supernatant was discarded.

7. The pelleted cells were resuspended in 120 µL of T-solution and incubated on ice for five min.

8. 2.5 µL of ligation mixture (containing 14 ng vector DNA) or 1 µL of supercoiled Control DNA (10-100 pg) was added into new microfuge tubes and chilled on ice for two min.

9. Then, 50 µL of the prepared cells were added to each tube containing DNA, mixed and incubated on ice for five min.

10. The mix was immediately plated on pre-warmed LB-ampicillin X-Gal/IPTG agar plates and was incubated overnight at 37 °C.

3.8.3 Analysis of Recombinant clones

Presence and orientation of the DNA insert in the plasmid was analysed in 4 to 6 white colonies and was confirmed by performing colony PCR with gene specific primer sets MIPS VF, M1R and M2 F, MIPS VR.

Procedure

1. Colony grid was prepared and incubated overnight at 37 °C in a shaker

2. Single colonies were suspended in 20 µL water and denatured it at 98 °C for 10 min.

3. The tubes were then centrifuged at 10,000 rpm for one min.

4. The supernatant was taken and used further in colony PCR reaction instead of autoclaved distilled water

5. PCR master mix was prepared for the number of colonies analysed. Each 20 µL reaction mixture had the components as follows

Table 3.6 PCR reaction mixture for Colony PCR

Component	Quantity (µL)
Taq buffer without $MgCl_2$(10X)	2.0
dNTPs (10mM)	2.0
$MgCl_2$(25mM)	1.2
Forward Primer (10 µM)	0.6
Reverse Primer (10 µM)	0.6
Taq DNA polymerase	0.1
Total volume	20.0

6. PCR mix was aliquoted to the PCR tubes on ice

7. Volume in each tube was made up to 20 µL by adding the supernatant from the above- mentioned step

Thermal profile for colony PCR consisted of :

- Initial denaturation   - 94 °C for 2 min.
- Denaturation   - 94 °C for 30 sec.
- Primer annealing   - 58 °C/55 °C for 30 sec.   } 30 cycles
- Primer extension   - 72 °C for 1 min.
- Final extension   - 72 °C for 10 min.
- 4 °C - hold the sample.

8. PCR products were analysed on 1.0 per cent agarose gel

3.8.4 Plasmid Isolation

Plasmid isolation was done using PureLink® Quick Plasmid Miniprep Kit (Invitrogen), which used PureLink® spin columns made of silica membrane to achieve high yields of up to 40 μg of sequencing-grade plasmid DNA.

Components

- Resuspension buffer (R3; 50 mM Tris-HCl, pH 8.0; 10 mM EDTA)
- RNase A (20 mg/ mL in Resuspension Buffer R3)
- Lysis buffer (L7; 200 mM NaOH, 1% w/v SDS)
- Precipitation buffer (N4)
- Wash buffer (W9)
- Wash buffer (W10)
- TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA)
- Wash and recovery tubes
- Spin columns

Procedure

1. One to five mL of the overnight grown LB culture was centrifuged at 10,000 rpm for 10 min. and media were removed.
2. Resuspension buffer (R3) of 250 μL was added with RNase A to the cell pellet and pellet was resuspended until it became homogeneous.
3. Lysis buffer (L7) of 250 μL was added and mixed gently by inverting the capped tube until the mixture was homogeneous. The tube was incubated at room temperature for five min.
4. Precipitation buffer (N4) of 350 μL was added and mixed immediately by inverting the tube, until the mixture became homogeneous. Then, the lysate was centrifuged at $12,000 \times g$ for 10 min.
5. The supernatant from step 4 was loaded onto a spin column in a 2-mL wash tube and the column was centrifuged at $12,000 \times g$ for 1 minute. Flow-through was discarded and the column was placed back into the wash tube.

6. 700 µL Wash buffer (W9) was added with ethanol to the column and centrifuged the column at 12,000 × g for 1 minute. The flow-through was discarded and column was placed into the wash tube. Column was centrifuged at 12,000 × g for 1 min. additionally and the wash tube along with the flow-through were discarded.

7. The Spin Column was placed in a clean 1.5-mL recovery tube. 75 µL of preheated (65 °C) TE buffer (TE) was added to the center of the column and the column was incubated for 1.0 minute at room temperature.

8. Column was centrifuged at 12,000 × g for 2 min. Recovery tube contained the purified plasmid DNA and the column was discarded. The plasmid DNA was stored at 4 °C (short-term) or at −20°C (long-term).

3.8 Sequencing of *MIPS* gene DNA fragment

The isolated plasmid was sent to or AgriGenome, Cochin for Sanger sequencing.
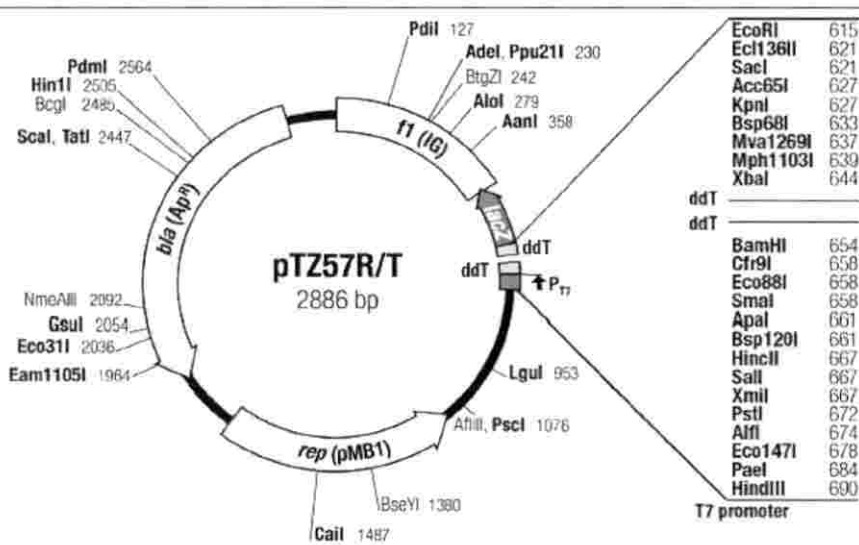
Fig. 3.1 Map of the pTZ57R/T cloning vector. Unique restriction sites are indicated
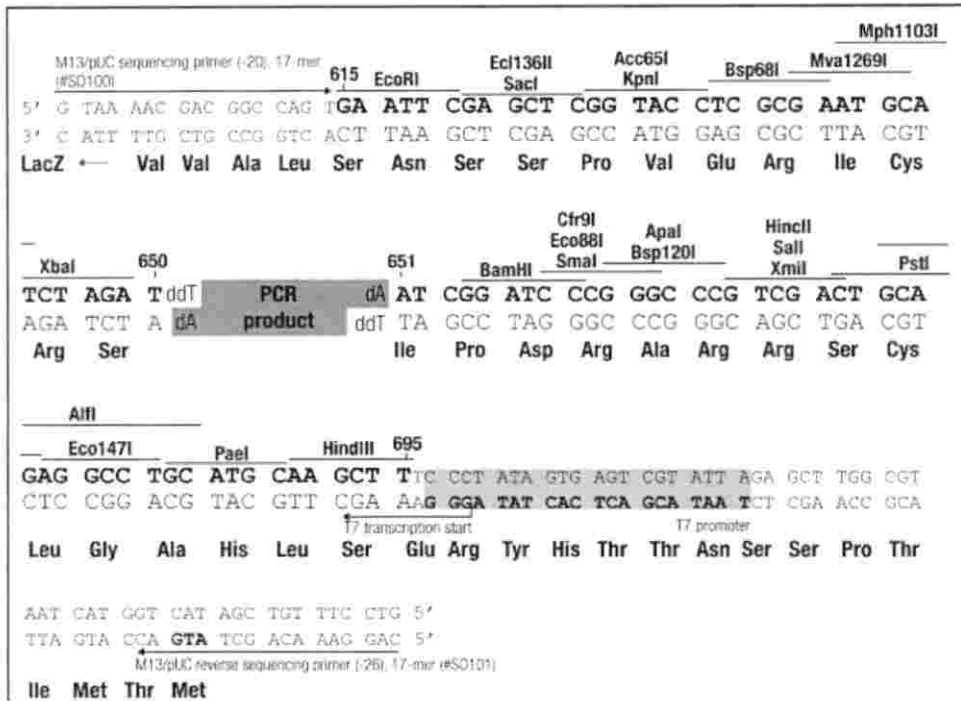
Fig. 3.2 DNA sequence of multiple cloning site region in vector

3.10 Sequence analysis

The forward and reverse sequences for both the regions of *MIPS* gene were aligned using CAP3 and a final contig of the gene was synthesised. BLASTn was performed to confirm the sequences. The longest ORF in the sequence was identified using ORF Finder at GenBank webpages.

3.11 Phylogenetic analysis

The *MIPS* sequence of dolichos bean and the nucleotide sequences of *MIPS* from other 16 plants were obtained from GenBank and aligned using MAFFT software. Phylogenetic analysis was conducted with MEGA (Molecular Evolutionary Genetic Analysis) using version 10.0.5 software (Tamura *et al.*, 2007). A neighbor-joining algorithm was used for constructing the phylogenetic tree, and bootstrap values were computed with 100 replicates to evaluate support for the groupings. Similar phylogenetic analysis was also performed for *AdMIPS* (Cui *et al.*, 2013) and *VrMIPS* (Wongkaew, 2010).

3.12 Homology modelling

The secondary structure of *MIPS* gene of *Dolichos lablab* was constructed by homology modelling using the MODELLER 9.14 software. The deduced protein sequences of *MIPS* used as a query in BLASTp search showed high similarity of 49.5 percent with A chain of *MIPS* sequence of *Caenorhabditis elegans* (1VKO A). The constructed structure was further analysed in Ramachandran plot using the RAMPAGE server. The three-dimensional structure of the *MIPS* protein of *Dolichos lablab* was visualised using the software RASMOL.

The three-dimensional *MIPS* protein structure of *Vigna unguiculata* and *Phaseolus vulgaris* were also constructed using the MODELLER software using the same template of 1VKO A to compare the active site residues in these three related legumes. The use of MODELLER software to construct a comparative model for a protein with unknown structure was reported by Eswar *et.al.* (2008). The active site residues in the constructed *MIPS* structures of dolichos bean, *Vigna unguiculata* and *P.vulgaris* were identified using COACH server of I-TASSER software. The protein structures bound with amino acid residues were visualised using PYMOL software.

Results

4. RESULTS

The results of the study on 'Cloning and Characterisation of *myo-inositol phosphate synthase* gene coding for phytates in *Dolichos lablab* L.' carried out at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, during 2017-2019 are presented in this chapter.

4.1 Isolation and quantification of total RNA

A good concentration of total RNA was isolated from developing seeds (3-5 days after seed set) of dolichos bean using Purelink® Plant RNA Purification Reagent (Invitrogen). The RNA samples were quantified spectrophotometrically in NanoDrop® spectrophotometer ND 1000.

RNA isolation was performed using Trizol reagent but, distinct bands were not obtained. Good quality RNA was obtained by using the Plant RNA Purification Reagent protocol proposed by Silva *et al.* (2011) with some adaptations described by Pereira *et al.* (2017).

The ratio of OD_{260}/OD_{280} for the samples were quantified spectrophotometrically and was greater than 1.8, indicating pure RNA. The ratio of OD_{260}/OD_{230} was greater than 1.0 indicating that the samples were free from polysaccharides and polyphenols and quality was confirmed through 1 per cent agarose gel electrophoresis. Three intact bands were obtained corresponding to 28S, 18S and 5S rRNA + tRNA which marked the quality of isolated RNA (Plate 4.1).

Table 4.1 Quality estimation of isolated RNA using NanoDrop spectrophotometer

Sl. No.	Seed Sample (RNA)	OD_{260}/OD_{280}	OD_{260}/OD_{230}	Concentration (ng/ μL)
1.	R1	1.85	1.06	2730.7

4.2 First strand cDNA synthesis and amplification of the gene

The synthesis of DNA from an RNA template through reverse transcription produces complementary DNA (cDNA). The cDNA was then used as a template for Quantitative Reverse transcription PCR (RT-qPCR). This PCR can be performed in a one-step or a two-step assay. In this study, one-step assay was followed using SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* High Fidelity DNA Polymerase (Invitrogen) in which reverse transcription and PCR occurs in a single tube. One-step RT-qPCR used overlapping gene-specific primers which were designed from the similar sequences of *Vigna radiata*. The final extension temperature was extended to 15 min. from five min. to ensure efficient 3'-dA tailing of the PCR product. This helped in yielding higher number of recombinant clones. The amplified product was analysed on 1.0 per cent agarose gel and presence of 1779 bp band in agarose gel electrophoresis confirmed the amplification of MIPS gene from cDNA produced (Plate 4.2). Later, two sets of overlapping primers were designed as cloning was difficult with longer genes (>1kb). The isolated RNA was amplified with these primers using SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* High Fidelity DNA Polymerase (Invitrogen) and the expected band size was obtained. (Table 4.2).

Table 4.2 Expected band sizes of PCR products with the respective primer sets

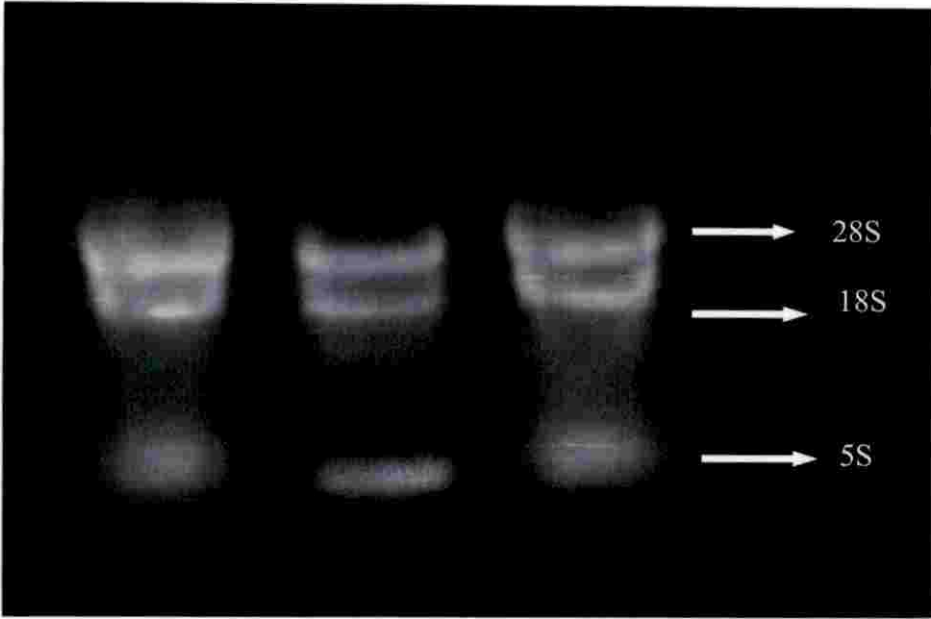| Sl.no	Primers	Product size obtained (bp)
1	MIPS VF, M1R	880
2.	M2F, MIPS VR	900

Plate 4.1 Gel documentation of total RNA isolated from developing seeds of *Dolichos lablab*
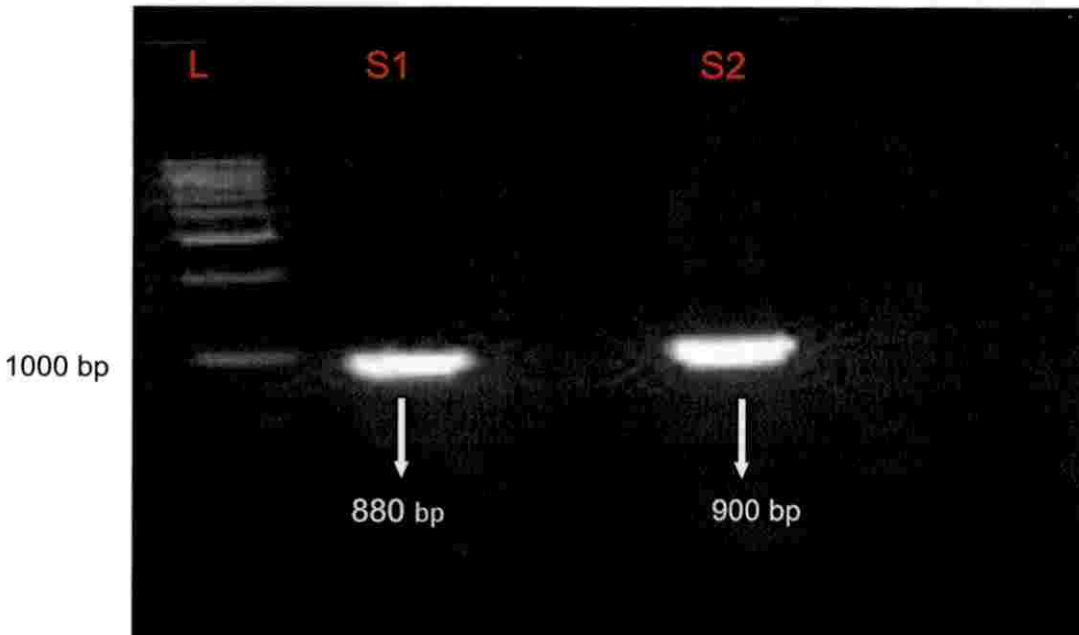

Plate 4.2 Gel documentation of PCR amplification of *MIPS* gene from *Dolichos lablab*

L – ladder (1 kb), S1 – PCR product of MIPS VF and M1R, S2 – PCR product of M2F and MIPS VR

4.3 Purification of the PCR amplified fragments

The DNA sample obtained after PCR amplification was purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacture's protocol. The quantity estimated was in between 25-27 ng/ μL based on the absorbance at 260 and 280 nm and stored at -20 °C and purified samples were outsourced for sequencing (AgriGenome Labs Pvt. Ltd., Kochi).

Table 4.3 Concentration of purified PCR samples

Sl. No.	PCR samples (purified)	Concentration (ng/ μL)
1.	Sample 1 (with MIPS VF and M 1 R)	24.8
2.	Sample 2 (with M 2 F and MIPS VR)	27.7

4.3 Molecular cloning

Molecular cloning was performed to insert the desired DNA fragment into the vector by using InsTAclone PCR Cloning Kit (Thermo Fisher Scientific). The vector used for cloning was pTZ57R/T which was provided in the kit. For efficient ligation, optimal insert/vector ratio is required. The product (insert) size ranged from 880 bp and 900 bp. Thus, 170 ng was taken for the ligation reaction (as recommended in the cloning kit by Thermo Fisher Scientific company). Cloning procedure was performed according to the manufacture's protocol. Cloning of the full length 1779 bp was failed and couldn't observe any recombinant colonies. Hence, cloning was performed separately for each fragment.

4.3.1 Transformation

Efficient competent cell preparation and quick transformation was obtained by the Transform Aid Bacterial Transformation Kit. The competent cells used

were *E. coli* laboratory strain DH5 alpha and were transformed using the recombinant plasmid pTZ57R/T which was having ampicillin resistance. Control experiment was also set up. Transformed cells were selected using blue-white screening (Plates 4.3, 4.4, 4.5). Control plate had more of blue colonies than white ones which indicated the absence of DNA insert. About 10-13 white colonies were observed separately in both the plates with respective primer sets confirming the presence of DNA insert. For the maintenance of transformed colonies, colony grid was prepared from the selected white colonies from both plates with LB ampicillin media separately (Plates 4.6, 4.7).

4.3.2   Detection of the insert (recombinant clones)

The white colonies indicating the recombinant clones were analysed and single colony from each plates of different fragments were subjected to colony PCR for confirmation with the designed sets of forward and reverse overlapping primers (MIPS VF, M1R and M2F, MIPS VR). The PCR products were checked in 1.0 per cent agarose gel and bands with 880 bp and 900 bp length amplicon was observed in the respective colonies (Plate 4.8) selected which confirmed the presence of insert after the cloning process. The transformed colonies were picked up and were cultured by incubating overnight at 37 °C in tubes containing LB media. Isolation of plasmid DNA was done by PureLink Quick Plasmid DNA Miniprep Kit, Invitrogen, separately for both the fragments. The isolated DNAs were tested for the presence of insert DNA and a band of 880 bp and 900 bp were obtained.

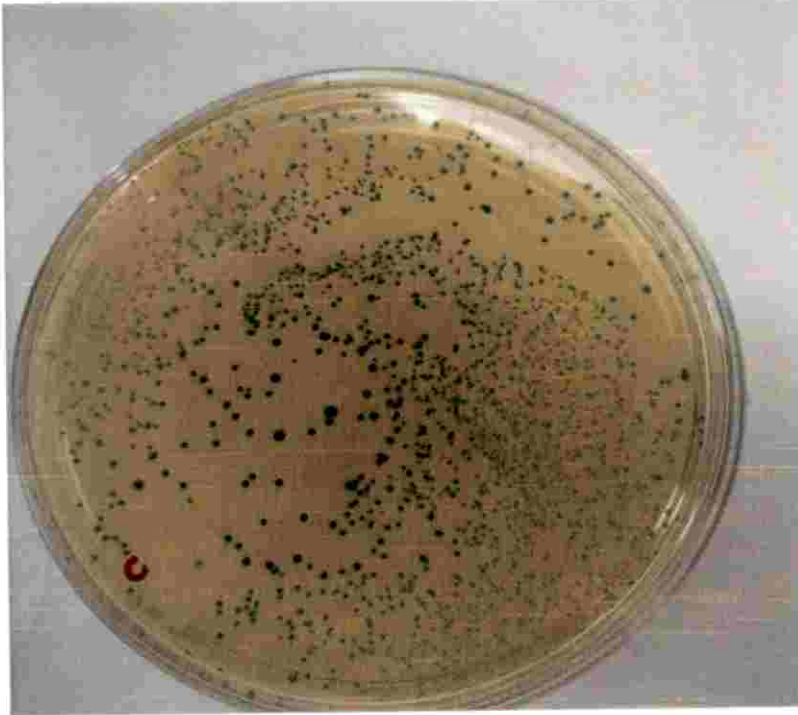Plate 4.3 Blue-white screening of control DNA 1 (vector without insert)

Plate 4.4 Blue-white screening of sample 1 (S1- with primers MIPS VF and M1R)

S6.a

Plate 4.5 Blue-white screening of sample 2 (S2- with primers M2F and MIPS VR)
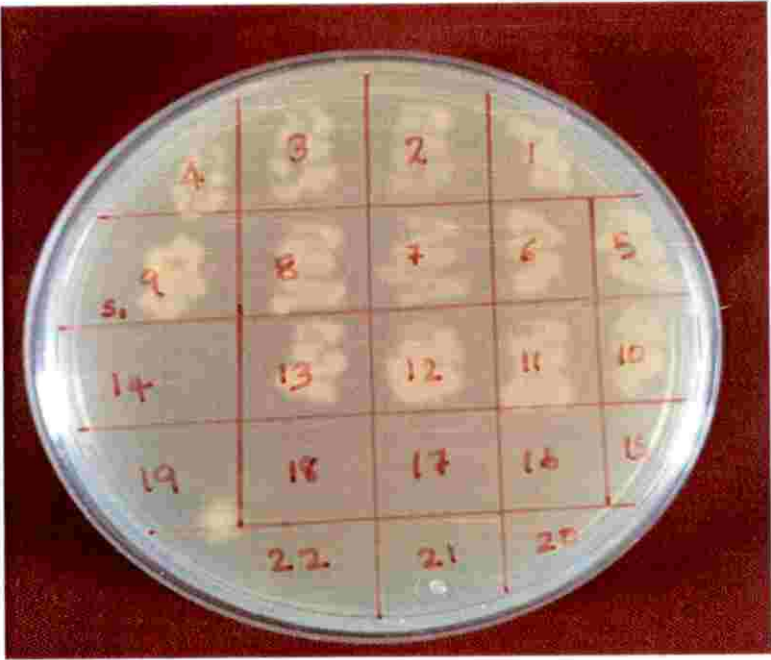

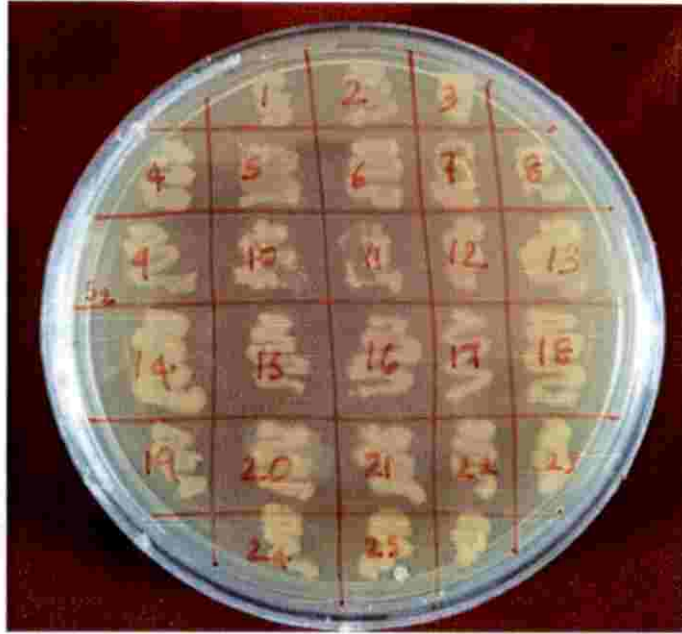Plate 4.6 Colony grid from white colonies of S1

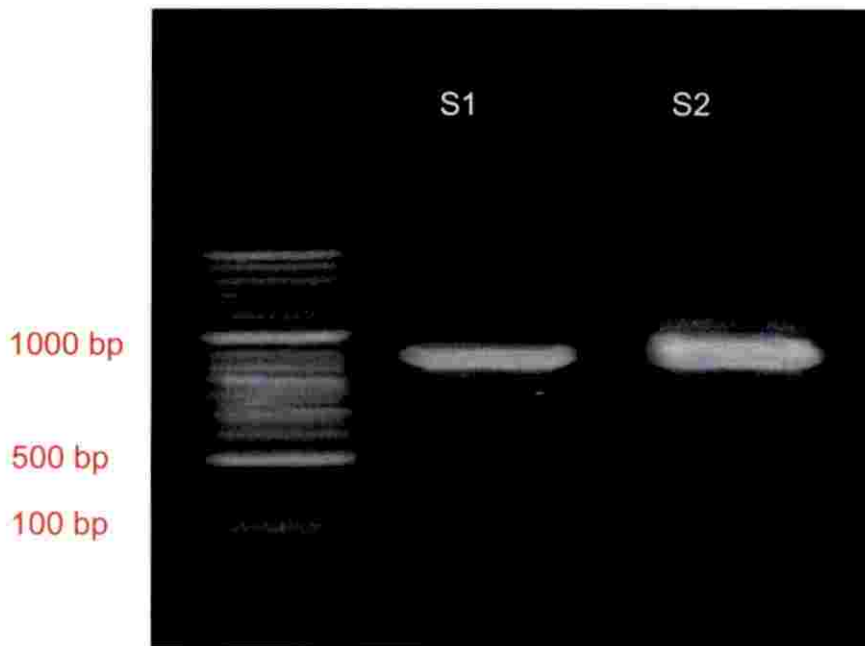Plate 4.7 Colony grid of white colonies from S2

Plate 4.8 Gel documentation of Colony PCR products (S1 – 880 bp and S2 – 900 bp)

4.4   *In silico* analysis of the sequences

Both the regions of *MIPS* were subjected to paired-end sequencing separately and the forward and reverse sequences from both the regions were aligned using CAP3 programme to generate the contig. Total length of the gene obtained was 1776 bp.

4.4.1 Homology

The assembled contig was compared with the sequences available in GenBank, using BLASTn. The *MIPS* sequence generated from lablab had 100 per cent coverage with 96 per cent homology with *MIPS* gene of *Vigna unguiculata* (Figure 4.5). This indicated that isolated *MIPS* gene belonged to a legume family and was more similar to *Vigna unguiculata.*

Sequence of *MIPS* gene from *Dolichos lablab* variety Hima

5'ACTCGTTCTGTGACATGTGAAGAAGCATACTATTGAGCTTCGTCTATATATTGAGGTTATGCGC
TACGTGAAACTGCACACAACCACCCATCAGCTTCTTCTTCTTCCTAAGAGAGTTTTTTCTTTCCACC
TTTGGAAGCAAGAAAAATGTTCATCCAGAGTTTTAAGGTTGAGAGTCCCAACGTGAAGTACAGA
GAGGGTGAGATTGAGTCCGTGTACAACTATGAAACCACTGAACTTGTTCACGAGAACAAGAATG
GCAGTTACCAGTGGGTTGTGAAGCCCAAAACTGTGAAATATGAATTTAAAACTAACACTCATGTT
CCTAAACTAGGGGTAATGCTTGTGGGGTGGGGTGGAAACAATGGCTCAACCCTCACCGGTGGT
GTTATTGCTAACAGAGAGGGTATTTCATGGGCAACAAAGGACAAGATTCAACAGGCCAATTACT
TTGGGTCCCTCACCCAGGCATCAGCCATCAGAGTGGGGTCTTTCCAAGGAGAGGAAATATATGC
CCCATTCAAGAGCCTCCTTCCAATGGTAAACCCTGATGATATTGTATTCGGGGGATGGGATATCA
GTGACTTGACCCTGGCTGATGCCATGGCCAGGGCCAAGGTGTTTGACATCGACCTGCAGAAACA
GTTGAGGCCTTACATGGAATCCATGACTCCACTCCCTGGAATCTATGACCCCGATTTCATTGCTG
CCAACCAAGGAGACCGTGCCAACAACGTCCTCCAGGGCACAAAGAAAGAACAAGTTCAACAAA
TCATCAAAGACATTAAGGAGTTTAAAGAAAAGAGCAAGGTTGACAAGGTGGTTGTCCTCTGGAC
AGCCAACACAGAGAGGTACAGCAACGTAGTTGTGGGATTAAACGACACCGTAGAAAACCTCTT
GGCTTCCTTGGACAGGAACGAGGCTGAGATTTCCCCTTCCACCTTGTATGCCATTGCTTGTGTCA
TGGAAAATGTTCCCTTCATCAATGGAAGCCCTCATAACACCTTTGTCCCAGGGCTGATTGATCTG
GCCATCAAGAGGAACAGTCTGATAGGAGGAGATGACTTTAAGAGTGGTCAGACCAAAATGAAA
TCTGTGTTGGTGGATTTCCTTGTCGGGGCAGGCATCAAGCCAACATCAATAGTGAGTTACAACC
ATCTGGGAAACAATGATGGTATGAATCTCTCAGCCCCTCAAACCTTCCGTTCTAAGGAAATCTCC
AAGAGCAATGTTGTTGACGACATGGTCAATAGCAATGCCATCCTCTATGAACCTGGCGAACATC

44

CTGACCATGTTGTTGTTATTAAGTATGTGCCTTACGTTGGGGACAGCAAGAGAGCAATGGATGA
GTACACTTCAGAGATATTCATGGGCGGAAAGAACACAATAGTGTTGCACAACACCTGTGAGGAT
TCACTGTTAGCTGCTCCTATCATCTTGGACCTGGTCCTTCTTGCTGAGCTTAGCACTCGAATCCAG
TTTAAAGCTGAAGATGAGGGCAAGTTTCACTCATTCCACCCGGTTGCCACCATCCTCAAAGCTGA
AGATGAGGGCAAGTTTCACTCTTTCCACCCGGTTGCCACCATCCTCAGTTACCTGACCAAGGCCC
CTTCTTGTTCCACCTGGTACCCCAGTGGTGAATGCATTATCAAAGCAACGAGCTATGCTGGAAAA
CATCATGAGGGCTTGTGTTGGATTAGCTCCGGAGAACAACATGATCCTGGAGTACAAGTGAGCA
ATCGGATCCCGGGTAACGATTTGGGATAGTTTGGTTAA 3'

4.10 Assembly and analysis of the sequence

The assembled sequence had a length of 1776 bp and was analyzed by
BLAST homology search which showed similarity to *MIPS* genes of different
crop species (Fig 4.6). Analysis by ORF Finder for the coding region showed that
the nucleotide produced 14 different open reading frames (ORFs) and the longest
was ORF1 with 1620bp, coding for 539 amino acids. The ORF belonged to 5'-3'
frame 1 with the start and end site at 148 and 1767 bp respectively (Fig 4.7).

The deduced *D. lablab MIPS* amino acid sequence used as a query
sequence in a BLASTp search showed the top scoring results with *inositol-3-
phosphate synthase gene* (*Caenorhabditis elegans*) with 49.5 per cent identity (Fig
4.8). The protein structure of the *MIPS* protein of *Caenorhabditis elegans*
(Accession No: 1VKO A) was downloaded from Protein Data Bank (PDB). A 3D-
protein structure of the deduced *MIPS* sequences of *D.lablab* was constructed by
homology modelling using MODELLER 9.14 . Analysis of the constructed model
in Ramachandran plot using RAMPAGE server showed that 490 residues were
present in the favoured region and only 4 residues were present in the outlier
region (Fig 4.9). The constructed protein structure was visualised in RASMOL
(Fig 4.10).

A 3D-structure of the *MIPS* protein of *Vigna unguiculata* and *Phaseolus
vulgaris* were also constructed with MODELLER using *C.elegans* as the template.
The active site prediction and structure-function annotation of the constructed
MIPS structures of dolichos bean (Fig 4.12), *V. unguiculata* (Fig 4.14) and

P.vulgaris (Fig 4.16) were performed by I-TASSER software. The results revealed that about 26 active site residues were predicted in the *MIPS* structure of all the three legumes but differed in the position and amino acid attached. The active site residues of dolichos bean (Fig 4.11) were bound by the amino acids like Val-63, Gly-64, Gly-66, Gly-67, Asn-68, Asn-69, Asp-139, Ile-176, Arg-189, Thr-226, Ala-227, ASN-228, Thr-229, Gly-277, Ser-278, Asp-302, Phe-303, Ser-305, Asn-337, Asp-338, Lys-351, Ile-417, Ala-421, Lys-463. Similarly, the residues of *V. unguiculata* (Fig 4.13) were attached with amino acids namely Val-112, Gly-113, Gly-115, Gly-116, Asn-117, Asn-118, Asp-188, Ile-189, Gly-224, Ile-225, Arg-238, Thr-275, Ala-276, Asn-277, Thr-278, Gly-326, Ser-327, Asp-351, Phe-352, Ser-354, Asn-386, Asp-387, Lys-400, Asp-467, Ala-471, Lys-513 and of *P.vulgaris* (Fig 4.15) were Val-67, Gly-68, Gly-70, Asn-72, Asn-73, Asp-143, Ile-180, Arg-193, Thr-230, Ala-231, Asn-232, Thr-233, Gly-281, Ser-282, Asp-306, Phe-307, Ser-309, Asn-341, Asp-342, Lys-355, Asp-422, Ala-426, Lys-468.

4.11 Phylogenetic analysis

In the BLAST homology search, *MIPS* of dolichos bean showed similarity with *MIPS* gene sequences of 100 different crops with an identity ranging from 96 to 77.4 percent and query coverage in the range of 100 to 77 percent. From this aligned sequence, those showing an identity of 87 percent or more and a query cover of at least 78 percent were selected for phylogenetic analysis (Table 4.4).

Table 4.4 List of nucleotides used for phylogeny tree construction

Organism	Accession No.
Vigna unguiculata	XM 028060955.1
Phaseolus vulgaris	FJ 647022.1
Phaseolus vulgaris	XM 007159658.1

Phaseolus vulgaris (MIPSV)	FN 356964.1
Vigna radiata	XM 014653891.2
Vigna radiata	XM 014657812.2
Vigna angularis	XM 017553278.1
Cajanus cajan	XM 020381334.1
Glycine max	NM 001248320.2
Glycine soja	XM 028387992.1
Glycine max (MIPS4)	XM 003531313.3
Glycine soja	XM 028377349.1
Glycine max	AK 287037.1
Glycine max	XM 003525017.4
Cucumis sativus	XM 004143430.2
Papaver somniferum	XM 026573704.1

Phylogenetic analysis of the aligned nucleotide sequences deduced from MIPS of *Dolichos lablab* and other major MIPS genes was performed using MEGA (Molecular Evolutionary Genetic Analysis) version 10.0.5 software. A neighbour-joining algorithm was used for constructing the phylogenetic tree, and bootstrap values were computed with 100 replicates to evaluate support for the groupings.

The phylogenetic tree was clustered into three main groups (Fig 4.17). Group I included sequences of *Vigna radiata, Vigna angularis, Vigna unguiculata, Phaseolus vulgaris, Dolichos lablab* where in *D.lablab* was clustered into a separate sub group I b and the remaining members

belonged to subgroup I a. Group II included sequences of *Cajanus cajan, Glycine max and Glycine soja. Cucumis sativus* and *Papaver somniferum* which were present as an out group was clustered into group III.

The phylogenetic tree indicated that *MIPS* of *Dolichos lablab* was similar to the *MIPS* of *Vigna radiata, Vigna unguiculata, Phaseolus vulgaris* and the result was in line with that obtained in BLASTn analysis.
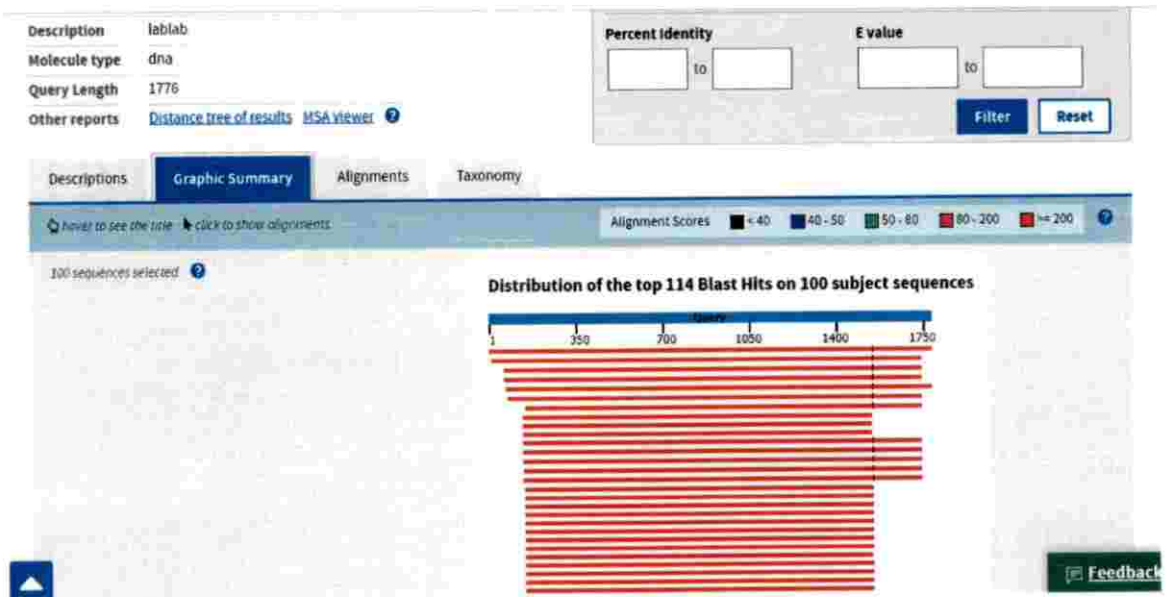
Fig 4.5 Graphical summary of BLASTn search of nucleotide sequence of *MIPS* gene in *D.lablab*

Fig 4.6 Description of alignment result using BLASTn of *MIPS* sequences of *D,lablab*
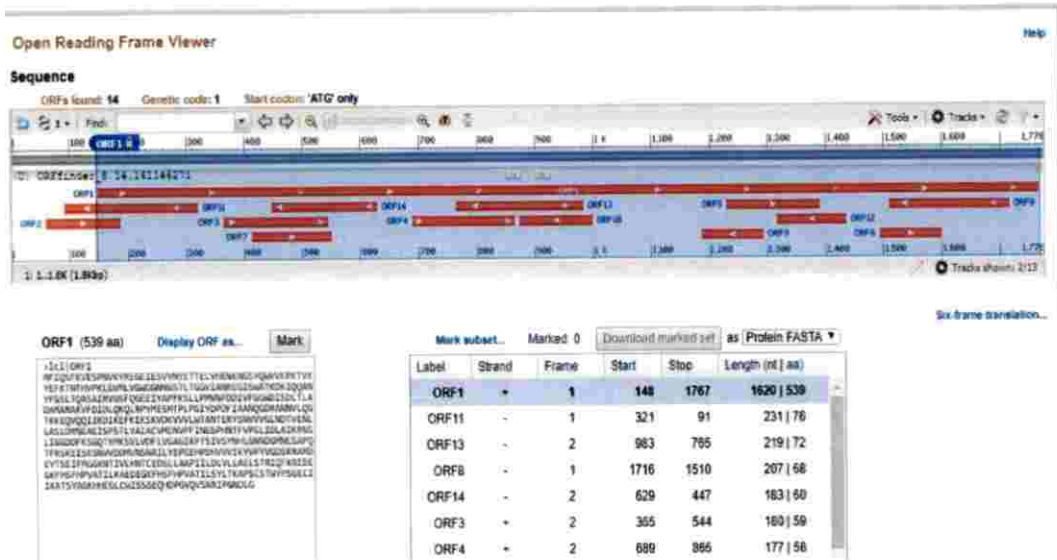
Fig 4.7 ORF of *Dolichos lablab MIPS* sequence
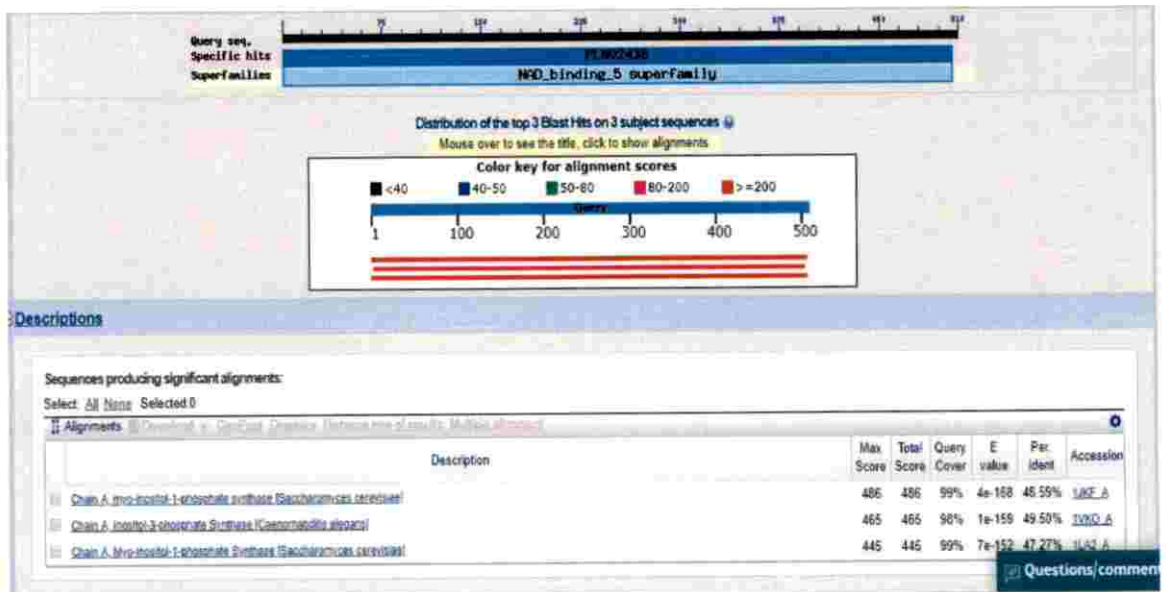
Fig 4.8 BLASTp search of amino acid sequence from *MIPS* gene of *D.lablab*

Evaluation of residues

```
Residue [  37 :GLN] ( -47.79, 164.26) in Allowed region
Residue [  98 :PHE] ( -44.03, 145.89) in Allowed region
Residue [ 192 :ASN] (-132.38,  80.17) in Allowed region
Residue [ 240 :ASN] (-158.31,  31.17) in Allowed region
Residue [ 253 :ASN] (  72.81,  40.65) in Allowed region
Residue [ 256 :GLU] ( -71.05,  16.26) in Allowed region
Residue [ 271 :ASN] (  79.34,  16.34) in Allowed region
Residue [ 283 :PHE] ( -73.00,  20.00) in Allowed region
Residue [ 284 :VAL] ( -39.53, 154.92) in Allowed region
Residue [ 295 :ASN] (  69.10,  41.80) in Allowed region
Residue [ 389 :TRP] (  69.48,-129.55) in Allowed region
Residue [ 443 :GLU] (-160.06,  86.09) in Allowed region
Residue [ 494 :LEU] (-130.56,  77.31) in Allowed region
Residue [ 498 :ASN] ( -47.49, -22.81) in Allowed region
Residue [  91 :LYS] ( 171.36,-119.23) in Outlier region
Residue [ 302 :ASP] (  88.03, 118.03) in Outlier region
Residue [ 306 :GLY] (-161.30, -68.81) in Outlier region
Residue [ 496 :PRO] (-116.80, 178.15) in Outlier region
Number of residues in favoured region    (~98.0% expected) :  498 ( 96.5%)
Number of residues in allowed region      ( ~2.0% expected) :   14 (  2.8%)
Number of residues in outlier region                        :    4 (  0.8%)
```

RAMPAGE by Paul de Bakker and Simon Lovell.

Please cite: S.C. Lovell, I.W. Davis, W.B. Arendall III, P.I.W. de Bakker, J.M. Word, M.G. Prisant, J.S. Richardson and D.C. Richardson (2002) Structure validation by Calpha geometry phi,psi and Cbeta deviation. *Proteins: Structure, Function & Genetics.* **50**: 437-450

Fig 4.9 Analysis of residues in Ramachandran plot
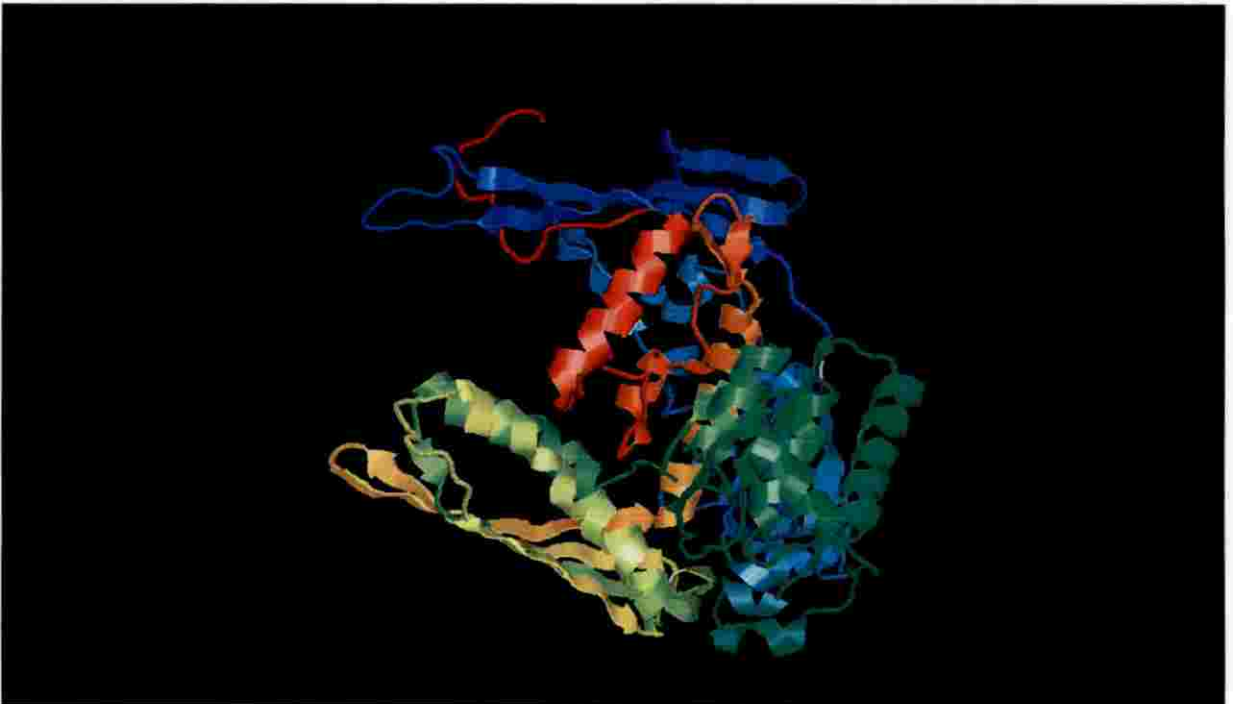
Fig 4.10 Three-dimensional representation of the *MIPS* gene of *Dolichos lablab* using software RasMol Version 2.7.5.2.

Fig 4.11 COACH results for *MIPS* of *Dolichos lablab*

Fig 4.12 *MIPS* structure of *Dolichos lablab* showing active site residues (represented as spheres)

Click to view	Rank	C-score	Cluster size	PDB Hit	Lig Name	Download Complex	Consensus Binding Residues
●	1	0.96	69	1vkoA	NAD	Rep, Mult	112,113,115,116,117,118,188,189,224,225,238,275,276,277,278,326,327,351,352,354,386,387,400,467,471,513
○	2	0.39	22	3qvtA	KPG	Rep, Mult	354,355,356,357,358,381,383,387,391,400,404,431,441,467,513
○	3	0.29	24	1p1jA	PO4	Rep, Mult	354,355,356,357,404,441,513
○	4	0.05	4	1rm0A	MN	Rep, Mult	117,118,385,439,467,468
○	5	0.01	2	1h6dA	NDP	Rep, Mult	475,478,480,482,508,509
○	6	0.01	2	3qvsA	PO4	N/A	506,521,522,523
○	7	0.00	1	4xogA	1PS	Rep, Mult	296,314,315,318
○	8	0.00	1	1f32A	NMD	Rep, Mult	507,510
○	9	0.00	1	4xr4B	1PS	Rep, Mult	367,371,372,373
○	10	0.00	1	4dpkA	PO4	Rep, Mult	114,187,188,189,190,221

Download the residue-specific binding probability, which is estimated by SVM.
Download the predicted bound ligands and detailed prediction summary.
Download the templates clustering results.
(a) C-score is the confidence score of the prediction. C-score ranges [0-1], where a higher score indicates a more reliable prediction.
(b) Cluster size is the total number of templates in a cluster.
(c) Lig Name is the name of possible binding ligand. Click the ligand name to view its information in the BioLiP database.
(d) Rep is a single complex structure with the most representative ligand in the cluster, i.e., the one listed in the Lig Name column.
Mult is the complex structures with all potential binding ligands in the cluster.

Fig 4.13 COACH results for *MIPS* of *Vigna unguiculata*
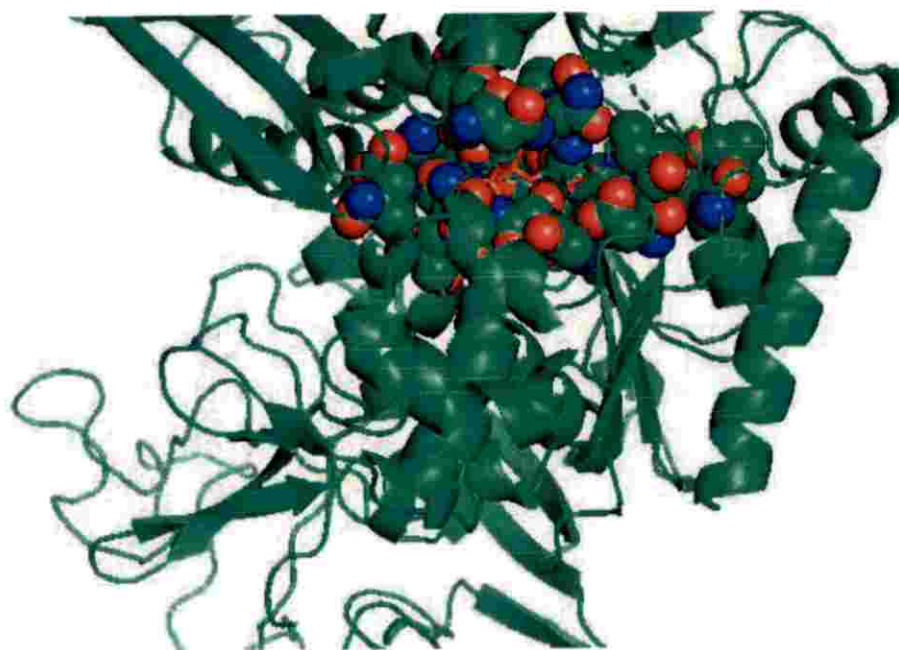
Fig 4.14 *MIPS* structure of *Vigna unguiculata* showing active site residues (represented as spheres, ligand as sticks)

Click to view	Rank	C-score	Cluster size	PDB Hit	Lig Name	Download Complex	Consensus Binding Residues
●	1	0.97	69	1vkaA	NAD	Rep, Mult	67,68,70,71,72,73,143,144,179,180,193,230,231,232,233,281,282,306,307,309,341,342,3...
	2	0.48	21	3qvsA	KPG	Rep, Mult	309,310,311,312,313,336,338,342,346,355,359,386,396,422,468
	3	0.27	24	1pjjA	PO4	Rep, Mult	309,310,311,312,359,396,468
	4	0.06	4	1rm0A	MN	Rep, Mult	72,73,340,394,422,423
	5	0.01	2	4xqgA	1PS	Rep, Mult	302,436,437,440,441
	6	0.01	3	1B3A	TND	Rep, Mult	68,71,143,144,145,179,180,181,231,233
	7	0.01	3	3cInA	MG	N/A	470,472,473,475
	8	0.00	1	1ml3B	CYX	Rep, Mult	310,311,337,387
	9	0.00	1	3qvsA	PO4	Rep, Mult	461,478,479
	10	0.00	1	1rysD	NDP	Rep, Mult	433,435,437,463,464

Download the residue-specific binding probability, which is estimated by SVM.
Download the predicted bound ligands and detailed prediction summary.
Download the templates clustering results.
(a) C-score is the confidence score of the prediction. C-score ranges [0-1], where a higher score indicates a more reliable prediction.
(b) Cluster size is the total number of templates in a cluster.
(c) Lig Name is the name of possible binding ligand. Click the ligand name to view its information in the BioLiP database.
(d) Rep is a single complex structure with the most representative ligand in the cluster, i.e., the one listed in the Lig Name column.
Mult is the complex structures with all potential binding ligands in the cluster.

Reset to initial orientation   Spin On Off

Fig 4.15 COACH results for *MIPS* of *Phaseolus vulgaris*

Fig 4.16 *MIPS* structure of *Phaseolus vulgaris* showing active site residues (represented as spheres, ligand as sticks)

Fig 4.17 Phylogenetic analysis of *Dolichos lablab MIPS* and other *MIPS* sequences retrieved from Genbank.

Discussion

5. DISCUSSION

The *Dolichos lablab* L. is one of the under exploited legumes in tropics and subtropics and has multiple uses. In developing countries with low intake of a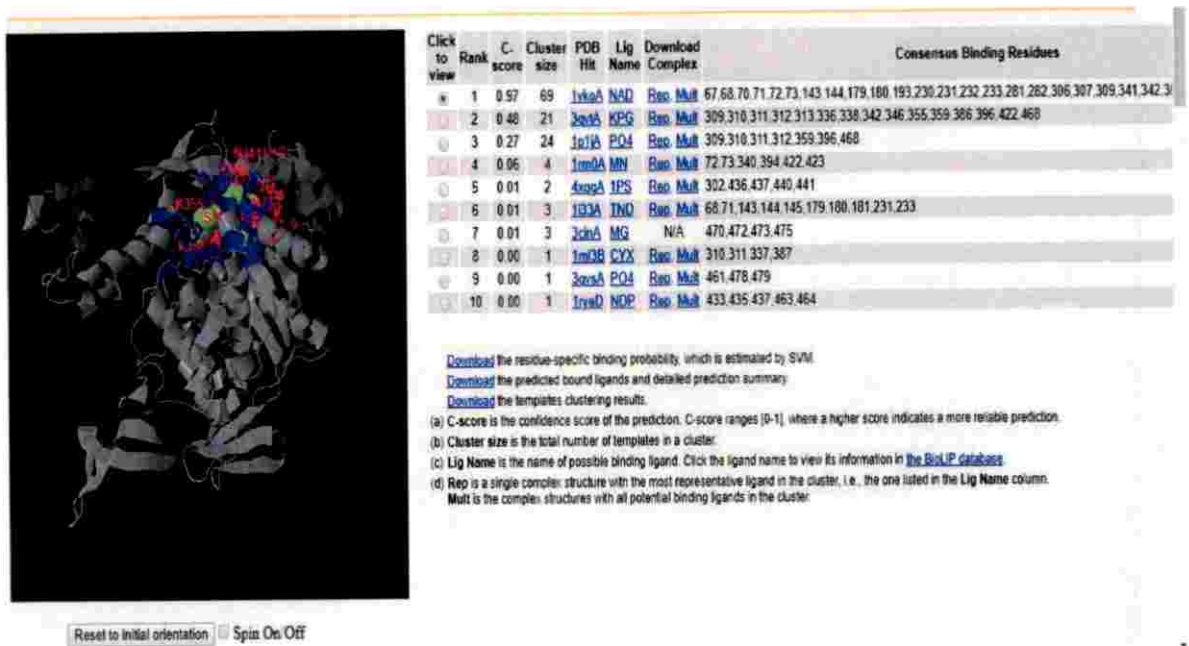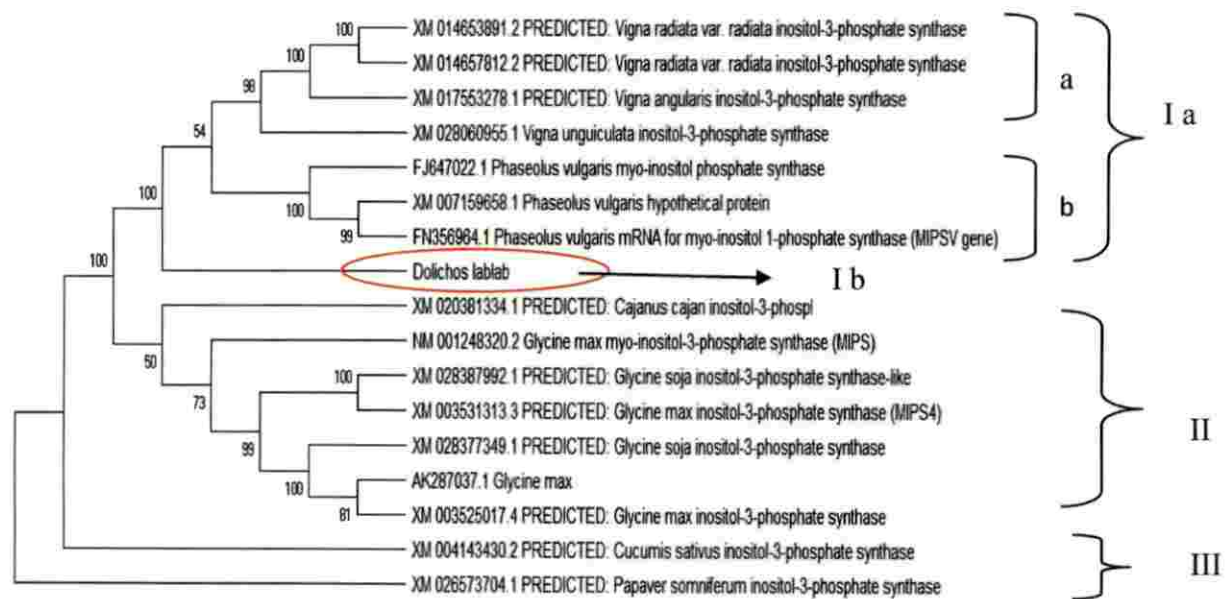nimal protein, legumes are the major source of protein. However, the presence of anti-nutritional factors such as trypsin inhibitors, phytins, saponins is reported to be high in legumes. Phytic acid (PA) is the major storage form of phosphorus in mature cereal and legume seeds (Lott, 1984). Dolichos bean with phytic acid content of 1000 to 1350 mg/ 100g (Deka and Sarkar, 1990) has less nutritional value due to low availability of minerals.

Phytic acid is present naturally in all eukaryotes with a myo-inositol ring attached to six phosphate groups (Brown *et al.*, 1961; Sasakawa *et al.*, 1995., Giridhari, 2017). It is considered to be the major storage form of phosphorous in storage organs such as seeds, tubers, and roots and helps in germination and growth by acting as a source of phosphorus (Morton and Raison, 1963; Aberoumand, 2011). According to Raboy (1997), about 65 per cent of the phosphatic fertilizers applied are converted to phytic acid and this account for 65-80 per cent of total seed phosphorus. PA is able to provide resistance against abiotic as well as biotic stresses in plants. It is also reported to give resistance against various bacterial, fungal and viral infections (Murphy *et al.*, 2008).

In legumes, gene *MIPS* is understood to play the major role in phytate synthesis (Loewus and Murthy, 2000). As monogastric animals including humans cannot digest the phytase complexes, reducing the phytate level in legumes can increase their nutritional value. Investigations have shown that all PA in seeds are not necessary for seedling establishment and therefore low phytic acid (*lpa*) mutants of barley (Larson *et al.*, 1998; Dorsch *et al.*, 2003), maize (Shi *et al.*, 2003), soybean (Hitz *et al.*, 2002), wheat (Guttieri *et al.*, 2004), rice (Larson *et al.*, 2000; Ali *et al.*, 2013; Kuwano *et al.*, 2009), common bean (Campion *et al.*, 2009) and *Arabidopsis*

(Stevenson-paulik *et al.*, 2005) were developed, with no reduction in seedling growth.

Lowering of phytates can be achieved by silencing one or more genes through RNAi approach, for which the mRNA sequence is essential. As there were no references on identification and characterisation of *MIPS* gene coding for phytates in dolichos bean, this study was conducted to sequence and annotate *MIPS* gene from the cDNA of developing dolichos bean seeds. The results of cloning and characterisation of *MIPS* gene in dolichos bean are discussed in this chapter based on the available literature and earlier reports.

5.1   Isolation and quantification of RNA

Qualitative and quantitative differences in composition of plant phenolics and polysaccharides in different plant tissues significantly affected the efficiency of nucleic acid extraction and purification procedures (Kansal *et al.*, 2008). It has been laborious to extract good quality total RNA from plant parts (such as seeds) containing high levels of phenolic compounds, carbohydrates, and other compounds that bind with the RNA (Kansal *et al.*, 2008).

In this study, seeds at developing stages (3-5 days after seed set) were used for total RNA extraction. The maximal transcript levels of *VrMIPS1*(*MIPS* of *Vigna radiata*) was observed between 7 and 9 DAF (Wongkaew, 2010). In soybean, *GmMIPS1* expresses early at the cotyledonary stage of seed development and reached the peak at 2 to 4 mm seeds (Hegeman *et al.*, 2001). In yellow passion fruit, the *PeMIPS1* transcript was maximum at 9 days after pollination (Abreu and Aragao, 2007). Earlier expression of this gene indicates that the conversion of G-6-P to MIP occurs earlier during seed development (Abreu and Aragao, 2007). In rice, the relationship between *MIPS* gene expression and phytic acid synthesis in developing seeds has been reported by Yoshida *et al.*, 1999. According to Wongkaew (2010), the highest level of *VrMIPS1* transcripts was detected during 7 and 9 DAF in developing seeds.

In soybean, *MIPS1* was highly expressed in developing seeds, but was minimally expressed in flowers, leaves, and roots (Chappell *et al.*, 2006). But, the highest level of *SeMIPS1* transcripts in sesame was observed in leaves rather than stems and roots. (Chun *et al.*, 2003). In yellow passion fruit, *PeMIPS1* gene was noticed in ovules, pollen grains, developing seeds, and leaves (Abreu and Aragao, 2007), whereas the transcript of *RINO1* gathered at high levels in developing rice seeds, but was undetectable in leaves, roots, or flowers (Yoshida *et al.*, 1999). These results indicated that *MIPS* gene expression was observed in most of the tissues and the degree of expression varied in different species. In this study, dolichos seeds were collected during 3-5 days after seed set and good amount of transcript was obtained.

Many attempts were made to isolate RNA from seeds of dolichos beans by using available RNA isolation protocols. In this study, most of the protocols failed to give high quality and intact RNA from the seeds. The protocol proposed by Silva *et al.* (2011) with a few modifications described by Pereira *et al.* (2017) was successful to yield high quality RNA. Pereira *et al.* (2017) reported that as this protocol used many organic solvents such as phenol and chloroform in addition to many mixing steps (essential for homogenisation), it could remove many insoluble materials (polysaccharides and proteins).

The $A_{260/280}$ ratio obtained was adequate (~2) and also had good concentration. Plant RNA reagent is optimised to have less sample DNA contamination, twice as effective as Trizol reagent and it also follows a simplified RNA extraction protocol for minimised time and labour. β-mercapto ethanol, a reducing agent is present in the reagent which inhibits the polyphenolic oxidation. Samples were crushed in liquid nitrogen and extracted RNA using Plant RNA reagent suggested by Silva *et al.* (2011) and Pereira *et al.* (2017). Addition of NaCl helps in precipitating RNA from other cellular components. Chloroform wash helped in removal of polysaccharides and traces of phenolic contaminants in the extract. If the time gap is reduced, single chloroform wash itself yields a good quality RNA. Over-drying of the pellets, after ethanol wash has reduced the

integrity of RNA. Vigorous tapping of the tubes during the dissolution of pellets can lead to shearing of the RNA. Wiping of pestle, mortar, scalpel, gloves with RNase Zap™ can substitute the use of DEPC.

The overall quality of total RNA was assessed by 1.0 per cent agarose gel electrophoresis. It is recommended to follow denaturing formaldehyde agarose gel electrophoresis for RNA, since under normal conditions, RNA forms secondary structure that prevent its mobility in the gel under native condition, leading to poor separation in ordinary agarose gel (Bryant and Manning, 2000). However, this study was followed by normal agarose gel electrophoresis for RNA assessment using TAE buffer (freshly prepared with DEPC/ double autoclaved distilled water). This method proves to be easier and safer method as the formaldehyde is carcinogenic. The RNA analysed in the agarose gel was having 3 intact (28S rRNA, 18S rRNA and 5S rRNA + 5.8S rRNA + t-RNA) bands which confirmed the purity of RNA with no DNA or protein contamination. TAE/ TBE – agarose gels can be used for routine analysis of RNA for determining its quantity and integrity (Rio *et al.*, 2010).

5.2 cDNA conversion and amplification of the gene

The most essential element determining the efficiency of PCR is the specific sequence and optimal concentration of primer and there are numerous bioinformatics tools that efficiently help in primer designing and analysis (Abd-Elsalam, 2003). The primer sequences for PCR amplification of *MIPS* was derived through sequence alignment of deduced *MIPS* proteins from plant species (Kumari and Sachdev, 2013).

Two *MIPS* genes were found in *Arabidopsis* (Johnson and Sussex, 1995), three in sesame (Chun *et al.*, 2003), seven in maize (Larson and Raboy, 1999), four in soybean (Chappell *et al.*, 2006), and three in mung bean (Wongkaew *et al.*, 2010). Sequence similarity of the *MIPS* gene was confirmed before designing the primers by carrying out BLASTn homology search with available sequences in the database and it was showing similarity with *MIPS* gene from many different crops. As no references of sequences in dolichos bean was available, primers were designed

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based on the consensus regions in the multiple sequence alignment/ pair wise alignment. Ovcharenko *et al.* (2005) have proposed that multiple-sequence alignment analysis is a powerful approach for detecting functional regulatory elements and understanding phylogenetic relationships and for annotating the genes. Brodin *et al.*, (2013) also reported that it is important to locate primers in relatively conserved regions bounding the region of interest, and to design primers. Hence, primers for the confirmation of sequence information from the cDNA of dolichos bean was designed based on the available *MIPS* sequence of *Vigna radiata* by Primer3 software.

The isolated RNA was first subjected to cDNA conversion using RevertAid First strand cDNA synthesis kit. As no results were obtained after the PCR amplification of the cDNA, many trials were performed with cDNA conversion by changing the incubation temperature from 42 °C to 45 °C as the primer sets were rich in GC content (as per manufacture's protocol). Still, no results were observed. Many thermal cycling reactions were also performed with different gradients of annealing temperatures with AccuTaq LA DNA polymerase high-fidelity *Taq* enzyme but did not produce any results. This might be due to that the gene was of larger size (1779 bp) and efficiency of the reverse transcriptase enzyme would have been inefficient to transcribe such a longer target.

Hence, high-fidelity reverse transcription was performed with one-step RT-PCR system. As gene specific primers typically anneal at higher temperatures than random or oligo(dT) primers, higher temperature of 55 °C was set for cDNA synthesis. The full length of the gene was tried to get amplified using a single primer set and could obtain positive result but it was hard to clone the longer fragment. So, to clone efficiently, two sets of overlapping primers were designed. OligoCalc software was used for checking the quality requirements of the primers like length of the sequence, GC content, melting temperature and presence of any hairpin loops (Giridhari, 2017).

The first strand of dolichos *MIPS* cDNA was synthesised from mRNA followed by its amplification by one-step RT-PCR amplification method using SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* High Fidelity

DNA Polymerase (Invitrogen). According to the manufacturer, this system contains two major components: SuperScript III RT/Platinum *Taq* High Fidelity Enzyme Mix and 2X Reaction Mix. The enzyme mix combines SuperScript III Reverse Transcriptase and Platinum *Taq* DNA Polymerase High Fidelity, which is an enzyme mixture composed of recombinant *Taq* DNA polymerase, Pyrococcus species GB-D polymerase, and Platinum *Taq* antibodies, which helps in blocking polymerase activity at ambient temperatures enabling hot start PCR. The proprietary buffer system in 2X Reaction Mix is optimized for reverse transcription and PCR amplification. In this method, all components for cDNA synthesis and PCR amplification are combined in a single tube with gene-specific primers and target RNA. Reverse transcription was automatically followed by PCR cycling without any additional steps.

The cDNA conversion using Revert Aid First Strand cDNA Synthesis Kit (ThermoFischer) was found to be less efficient compared to the results reported in *Vigna radiata* (Wongkaew, 2010), kiwi fruit (Cui *et al*., 2013), soyabean (Kumar *et al*., 2012). The 1776 bp cDNA sequence contained an ORF of 1620 bp coding for 539 aminoacids. Wongkaew (2010) reported that 1779 bp *MIPS* cDNA sequence of *Vigna radiata* contained 1533 bp of ORF that code for 510 amino acids. The same results were observed in *GmMIPS* of soybean (Kumar *et al*., 2012) and *AdMIPS* of kiwi-fruit (Cui *et al*., 2013). The 3' and 5' end of *MIPS* cDNA from an internal fragment of the *MIPS* gene was amplified using RACE PCR in *Vigna radiata* (Wongkaew, 2010) whereas Phusion TM high-fidelity PCR Kit (Finnzymes) was used for long PCR amplification in soybean (Kumar *et al*., 2012).

5.3 Molecular cloning of the PCR product

Molecular cloning refers to the creation of recombinant DNA molecules. It includes the insertion of the isolated DNA sequence into a vector for propagation, without alteration of the original DNA sequence. These clones can be used to generate many copies of the DNA for analysis of the gene sequence (Tirabassi and Bio, 2014). The amplicon obtained was cloned in pTZ57R/T vector using *E.coli* DH5α cells as competent cells. In soybean, the PCR product was cloned into

*p*Bluescript SK+ vector by blunt end ligation (Kumar *et al.*, 2012). The pGEMT vector (Promega) was used for cloning *AdMIPS* in kiwi fruit as reported by Cui *et al.*, 2013. A background test for blue/white colour selection was done by plating ligated mixture on plates containing IPTG and X-Gal. After incubating the plates at 37 °C for overnight, blue and white colonies appeared which represented non-recombinants and recombinants respectively. Since the *MIPS* gene was inserted within the structural gene of β-galactosidase, recombinants were unable to produce colour as synthesis of β-galactosidase was disrupted, but non-recombinants with functional β-galactosidase was able to hydrolyse X-Gal to produce blue colonies. The white colonies which were spotted on LB agar plates supplemented with ampicillin (100µg/ ml) along with X-Gal and IPTG were again plated and a colony grid was prepared. About 10-13 white colonies were able to be spotted from both the plates which indicated the presence of recombinant DNA. This is based on the principle of α-complementation of the β-galactosidase gene. This phenomenon of α-complementation was first demonstrated by Agnes Ullmann. The colonies thus produced were confirmed using colony PCR and the sequence was deduced by Sanger sequencing.

The deduced nucleotide sequence of dolichos *MIPS* gene was compared with those of other plants and the homology revealed a high degree of sequence identity of 96.0 per cent similarity to the *inositol-3-phosphate* gene of *Vigna unguiculata* and had 100 per cent coverage. The *VrMIPS1* appeared in closer relationship to *P. vulgaris MIPS1* than to that of *G. max MIPS1* (Wongkaew., 2010). *GmMIPS* also showed 95 % similarity to *P. vulgaris* (Kumar *et al.*, 2012). A drought tolerance gene, *MsHSP70*, with an ORF of 2028-bp from alfalfa was cloned into the pEASY-T1 vector and was characterised (Li *et al.*, 2017). Cloning, *in silico* characterisation and homology modelling of *Phaseoline* gene (2000 bp) was performed in common bean. The amplified product was cloned in the pTZ57R/T cloning vector (2.8bp) using a competent strain of *DH5α E. coli* strain (Rani *et al.*, 2015).

5.3 Phylogenetic analysis

Previous studies of the nucleotide sequences and protein structures of *myo-inositol-1-phosphate synthase* have provided supporting evidence that *MIPS* is a highly conserved enzyme throughout the eukaryotic and prokaryotic phyla (Majumder *et al.*, 2003). The nucleotide sequence of *MIPS* gene was subjected to phylogenetic analysis to know the evolutionary relationship with other crops. In the present study, *MIPS* sequence of dolichos bean was subjected to phylogenetic analysis using selected *MIPS* gene sequences from BLAST search to understand the evolutionary relationships among the selected crop species. The evolutionary tree analysis showed that *MIPS* sequence of *Dolichos lablab* was similar to the *MIPS* sequence of *Vigna radiata* and *Phaseolus vulgaris,* justifying that it is a highly conserved enzyme. Even though the nucleotide sequence of *Vigna unguiculata* showed higher similarity in BLAST search, it was grouped between *Phaseolus vulgaris* and *Vigna radiata*. The close proximity in the positioning of different species of phylogenetically related crops like *Vigna radiata, Vigna angularis, Vigna unguiculata* as well as *Glycine max* and *Glycine soja* in the tree constructed indicate that the clustering was done accurately. This evolutionary analysis has helped in identifying the crops with more similarity to the sequence of *MIPS* of dolichos bean and therefore provides a closer reference of sequences for further molecular studies. Even though similar works were conducted in the isolation and characterization of *MIPS* gene of different crops, the evolutionary analysis of the gene was not carried out by many.

Wongkaew (2010) have carried out phylogenetic study of *Vigna radiata MIPS* gene (*VrMIPS1*) with amino acid sequences of other monocot and dicot *MIPS* genes. The results showed monocot *MIPS1* and dicot *MIPS1* were in separated clusters. He also reported that *VrMIPS1* appeared in the dicot cluster with firm relationships *to P. vulgaris MIPS1* than to that of *G. max MIPS1*.

The amino acid sequence of *GmMIPS* showed a high degree of homology and was clustered firmly in a subgroup belonging to Fabales species in a constructed phylogenetic tree. High degree of sequence identity was shown by *Glycine max MIPS* protein with *MIPS* of different plant sources from both monocots as well as

dicots. It shared 96% identity with *Vigna radiata*, 95% with *Phaseolus vulgare*, 94 % with *Cicer arietinum*, 93 % with *Nicotiana tabacum*, 92 % with *Sesamum indicum*, 89 % with *Triticum aestivum*, 88 % with *monocots Zea mays, Oryza sativa* and 87 % with *Arabidopsis thaliana* which was also established by phylogenetic analysis (Kumari and Sachdev, 2013).

Cui *et al.* (2013) conducted a phylogenetic analysis with *AdMIPS* (*Actinidia deliciosa*, kiwi fruit) and protein sequences of *MIPS* from 20 organisms and showed that *AdMIPS* was most closely similar to that from *Citrus paradisi*, a tropical fruit. Interestingly, he could also observe that *Triticum aestivum* grouped with dicotyledons than with other monocotyledons such as *Zea mays*. These results were on par with the analysis carried out in the present study for *MIPS* sequence of dolichos bean. The results therefore confer the fact that the *MIPS* gene family expresses a similar evolutionary relationship with each other.

5.4 Homology modelling
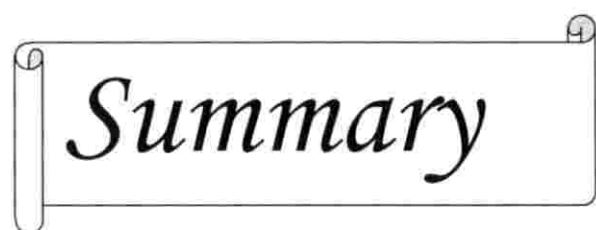
The secondary structure prediction of *Dolichos lablab MIPS* gene showed similarity to *inositol-3-phosphate synthase* protein structure of *C. elegans* (1VKO A) which was retrieved from PDB. The three-dimensional structure of *MIPS* of dolichos bean was constructed and the active sites were predicted. The secondary structure of *MIPS* of *V. unguiculata* and *P. vulgaris* were also constructed using the same template as of *C. elegans* and active site predictions were performed since the protein structures of *MIPS* of any of the legumes were not available in PDB. The active site prediction of the three structures exhibited 26 consensus ligand binding residues to NAD ligand as predicted by the software itself. The COACH results showing highest C-score was accepted since C-score indicates the confidence score of prediction. The highest C-score indicates more reliable prediction.

Even though the BLAST result of *MIPS* of *D.lablab* showed 96 per cent similarity to *V. unguiculata* and 94.9 per cent similarity to *P. vulgaris,* the ligand binding residues predicted in both the structures were different with that of *D. lablab*. This may be due to the difference in length of *MIPS* protein sequence of both the legumes.

According to Yang *et al.* (2013), COACH is a meta-server approach to protein-ligand binding site prediction. From the submitted structure of target proteins, COACH will generate complementary ligand-binding site predictions using two methods, TM-SITE and S-SITE, which could accept ligand-binding templates from the BioLiP protein function database by binding-specific substructure and sequence profile differentiations.

The information of active site residues on the *MIPS* protein structure will help us in bioengineering and biofortification of crop plants thus increasing their nutritional value. Prediction of ligand binding sites is crucial to elucidate protein functions and is helpful for drug design (Hu *et al.*, 2016). Bioengineering of plants will also assist in the production of therapeutically important proteins and molecules that can be utilized in the manufacturing of pharmaceutical drugs. Chaudhary *et al.* (2018) has reported about bioengineering of crop plants for improved tetrahydrofolate production. The *de novo* synthesis of folates in plants was considered to be tightly regulated by feedback-regulation of certain biosynthetic pathway catalysts at rate-determining steps. He suggested that this challenge could be solved by bioengineering the crop plants by overexpression of evolutionary diverged key folate biosynthetic enzymes having different allosteric sites, which prevent the feedback regulation. The increased production of folates in the transgenic lines was analogous with differential allosteric regulatory cavities accessible at *E. coli* dihydroneopterin aldolase (*Ec*DHNA) surface having critical amino-acid differences as Ile 64 (His-63), Val 70 (Phe-69), His 75 (Arg-78) and Arg 79 (Glu-72). He thus concluded that the biotechnological approach of these diverse allosteric sites were exploited for improved folate production in plants.

The *MIPS* sequence of dolichos bean deduced in this study could be directly used in RNAi and similar cisgenic or gene editing strategies to generate lablab accessions with reduced phytic acid content. The information generated on the protein structure and active site variations in the protein sequence could assist in bioengineering and bio-fortification of the crop thereby increasing its nutritional quality.

Summary

6. SUMMARY

Dolichos bean (*Dolichos lablab* L.) is one of the major legumes among cultivated plants. It is an ancient domesticated pulse and multi-purpose crop. Dolichos bean has a high content of phytic acid (1000-1350 mg/ 100g). Phytic acid (PA) is the major phosphorus storage compound of most seeds and cereal grains. PA has been reviewed as an antinutrient due to its inhibitory effect on mineral bioavailability. The most striking chemical impact of PA is its strong chelating ability with multivalent cations, especially di and trivalent cations, to form cation-PA complexes. Thus, phytates are considered as the characteristic anti-nutrients, rendering the minerals unavailable.

The development of low phytate crops is considered as a salient objective in plant transgenic programs aimed at improving nutritional quality as well as at expanding environment friendly and sustainable production. A substitute to phytase engineering for decreased seed phytic acid levels is the reduction of the expression of enzymes in the biosynthetic pathway of phytic acid. Down regulation of the *MIPS* (*myo-inositol phosphate synthase*) gene encoding the first and rate-limiting enzyme in the biosynthetic pathway offers a budding approach for the development of low-phytic acid crops. To silence any gene through RNAi strategy, precise mRNA sequence information is mandatory and the objective to clone, sequence and annotate *myo-inositol phosphate synthase* gene from the cDNA of developing dolichos bean seeds. The present study on 'Cloning and characterisation of *myo-inositol phosphate synthase* gene coding for phytates in *Dolichos lablab* L.' is summarised in this chapter.

1. RNA isolation from the developing seeds of dolichos bean was carried for the molecular studies.

   - Good quality RNA was isolated using Purelink® Plant RNA Purification Reagent (Invitrogen) from developing seeds of dolichos bean (3-5 days after seed set).

- Quantification of RNA using spectrophotometer gave an absorbance ($A_{260/280}$) value of 1.8 indicating good quality RNA.
- Concentration of RNA was 2730 ng/ μL dissolved in 70 μL of nuclease free water.

2. cDNA synthesis and amplification of the *MIPS* gene

- Primers for the full-length coding sequence (1776 bp) of *MIPS* were designed based on the available *MIPS* sequence of *Vigna radiata*.
- As cloning of full-length gene is difficult, two sets of overlapping primers were designed.
- Two sets of primers (MIPS VF/M 1 R, M 2 F/MIPS VR) were designed based on the *MIPS* sequence of *Vigna radiata* retrieved from Genbank.
- The isolated RNA was converted to cDNA and both the primer sets produced amplification at 880 bp and 900 bp respectively.

3. Cloning and sequencing of the PCR product

- The amplicon obtained was cloned in pTZ57R/T vector using *E.coli* laboratory strain *DH5 α* cells as competent cells.
- Transformed colonies were selected using blue-white screening. About 10-13 white colonies were observed in both plates with respective primer sets which indicated the presence of DNA insert.
- Transformation was confirmed by colony PCR and the specific bands of 880 bp and 900 bp were seen in the white colonies.
- The plasmid DNA was isolated and sequenced using Sanger platform.
- The obtained MIPS sequence had a size of 1776 bp cDNA sequence contained an ORF of 1620 bp coding for 539 aminoacids.

4. Phylogenetic analysis was conducted to understand the evolutionary relationship of *MIPS* of dolichos bean with *MIPS* sequences of other crops.

- The analysis showed that MIPS of *Dolichos lablab* was more similar to MIPS of *Vigna radiata, Vigna unguiculata, Phaseolus vulgaris*

5. Homology analysis of *MIPS* of dolichos bean using BLAST to understand the degree of similarity with other *MIPS* sequences
   - The homology search revealed that the *MIPS* sequence of dolichos bean was more similar to that of *Vigna unguiculata* with 100 per cent coverage and 96 per cent similarity.

6. The three-dimensional structure of *MIPS* protein of *Dolichos lablab* was constructed using MODELLER software

   - The deduced *MIPS* amino acid sequence of dolichos bean was used as a query in BLASTp search which showed top scoring results with inositol-3-phosphate synthase gene of *Caenorhabditis elegans* with 49.5 per cent identity.
   - 3D-structure of the *MIPS* of dolichos bean was constructed by homology modelling and was visualised in RASMOL software.
   - Analysis of the constructed model in RAMPAGE server showed that 490 residues were present in the favoured region and only 4 residues were present in the outlier region.

7. Active site residues were identified in the MIPS sequences of *Dolichos lablab, Vigna unguiculata* and *Phaseolus vulgaris* and were compared each other

   - The 3D-structure for the *MIPS* sequences of *Dolichos lablab, Vigna unguiculata* and *Phaseolus vulgaris* were constructed using MODELLER.
   - The active site residues in the constructed structures were predicted with I-TASSER software.

- 26 ligand binding residues were identified in both the protein structures but were not similar due to the difference in length of the *MIPS* protein sequences.

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Cloning and characterisation of *myo-inositol phosphate synthase gene* coding for phytates in *Dolichos lablab* L.

by

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ABSTRACT
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Abstract

ABSTRACT

Hyacinth bean or dolichos bean (*Dolichos lablab* L.) is a major vegetable legume grown in the tropical and subtropical regions of the world. Legumes are considered as the major source of protein for countries having short supplies of animal protein. The major storage form of phosphorus (P) in mature cereal and legume seed is phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate, $InSP_6$) which strongly binds metallic cations such as calcium, zinc, magnesium and iron to form a mixed salt called phytate. Thus, the phytates are characteristic anti-nutrients, rendering the minerals unavailable. Dolichos bean has a high content of phytic acid (1000-1350 mg/ 100 g). The *Myo-inositol phosphate synthase* (*MIPS*) is the major gene for phytate synthesis in legumes. Suppression of *MIPS* will help in generating lines with reduced phytate content, for which, sequence characterization of *MIPS* is mandatory. Thus, this research work was carried out with the objective to sequence and annotate *myo-inositol phosphate synthase gene* from the cDNA of developing dolichos bean seeds.

Good quality total RNA from the developing seeds (3-5 days after seed set) of *Dolichos lablab* (var. Hima) was isolated using Purelink® Plant RNA Purification Reagent (Invitrogen). The total RNA was subjected to cDNA synthesis and PCR amplification using SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* High Fidelity DNA Polymerase (Invitrogen) in which reverse transcription and PCR occured in a single tube. The PCR primers were designed from the coding regions of *MIPS* sequence of *Vigna radiata*, retrieved from NCBI. As cloning of a full-length gene is difficult, primers were designed as two sets of overlapping sequences of a full-length gene, namely MIPS V F - M1R (880 bp) and M2F – MIPS V R (900 bp) combinations. The purified PCR products were ligated into pTZ57R/T and cloned in *E. coli* DH5α. The recombinant clones were analyzed by blue or white screening. The transformation was confirmed by colony PCR, plasmid was isolated and used for sequencing.

The DNA sequence was subjected to homology search using BLASTn and it had showed 96 per cent similarity and 98 per cent query coverage with the *MIPS* sequence of *Vigna unguiculata*. The 1776 bp cDNA with 1620 bp coding sequence encoded for a protein of 539 amino acids as predicted by ORF FINDER software. The prediction of a 3D- protein structure of the deduced *MIPS* sequences of *D. lablab*, *V.unguiculata and P.vulgaris* were performed by MODELLER 9.14 and was visualized using RASMOL. Similarly, the prediction and comparative analysis of the active sites of enzyme from *D. lablab, V.unguiculata and P.vulgaris* had shown that the ligand binding residues varies among different species of the same protein. The deduced protein sequences of *MIPS* used as a query in BLASTp search showed similarity of 49.5 per cent with A chain of *MIPS* sequence of *Caenorhabditis elegans* (1VKO A). The constructed structure was further analyzed in Ramachandran plot using the RAMPAGE server and the results showed that 490 residues were present in the favoured region and only 4 residues were present in the outlier region. The phylogenetic relationship of the lablab *MIPS* sequences with that of other legumes was analyzed by neighbor-joining method using MEGA.v.10.0.5. It was closer to the *MIPS* gene of *Vigna radiata*, *Vigna unguiculata* and *Phaseolus vulgaris*, which was in line with the BLASTn results.

This is the first study in dolichos bean to characterize its *MIPS* gene. The information generated in this study could be directly used in RNAi and similar cisgenic or gene editing strategies to generate lablab accessions with reduced phytic acid content.