Structure and expression patterns of MADS box transcription factor, POTM1 in potato *(Solanum tuberosum* L.)

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July 2019

Candidate's Declaration

I, hereby declare that the work which is being presented in the dissertation entitled, **"Structure and expression patterns of MADS box transcription factor, POTM1 in potato** ***(Solanum tuberosum*** **L.)"** in the partial fulfillment of the requirement for the award of degree of Master of Science in Biotechnology, Thapar Institute of Engineering & Technology, Patiala, is an original record of my own research work carried out under the guidance and supervision of **Dr. N. Das**, Professor, Department of Biotechnology, Thapar institute of Engineering and technology, Patiala, India. The content in the dissertation has not been submitted to any other university or institute for award of any other degree.

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CERTIFICATE

TO WHOM IT MAY CONCERN

This is to certify that the dissertation entitled **"Structure and expression patterns of the MADS box transcription factor, POTM1 in potato *(Solanum tuberosum* L.)"** comprises research work carried out by **Ms. Kajal** (Regd. No. 301701012) under my supervision and guidance during the period between 7th January 2019 to 10th July 2019 for the partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, submitted to Thapar Institute of Engineering and technology, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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List of abbreviations

Abbreviations	Name
µg	Microgram
µL	Microlitre
BLAST	Basic Local Alignment Search Tool
BLASTn	BLAST for nucleotide
BLASTp	BLAST for protein
Bp	Base-Pair
$CaCl_2$	Calcium chloride
DEPC	Diethyl pyrocarbonate
DNA	Deoxy Ribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
HCl	Hydro chloric acid
IU/ml	International unit per Ml
Kb	Kilo Base
kDa	Kilo-Daltons
LiCl	Lithium chloride
M	Molar
mM	Mili-molar
NA	Nucleic acid
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
Nm	Nanometer
O.D.	Optical density
ORF	Open reading frame
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
Pmoles	Picomoles
RIPs	Ribosome inactivating proteins
RNA	Ribo nucleic acid
Rpm	Revolutions per minute
rRNA	Ribosomal RNA
S (60S)	Svedberg unit
SDS	Sodium dodecyl sulphate
siRNA	Short interfering RNA
T.A.E	Tris acetate EDTA
T.B.E	Tris borate EDTA
TA	Trans-acting
TE	Tris EDTA

Abstract

POTM1 is a long distance travelling RNA transcript which acts as a mobile signal. It is highly expressed in both reproductive and vegetative organs in potato and other plants. The transcripts of *POTM1* are accumulated in leaf primordial, apical and inflorescence meristem. *POTM1* is a transcription factor (M_r~28.9 kDa) with pI value of 8.77. It is an important member of MADS-box superfamily which positively regulates the expression of potato genes such as *StSP6A*. As revealed in the literature, considerable progress has been made on the MADS-box transcription factors associated with various stages of growth and development in plants including the *Solanaceae* family members. This study focused on a particular member of the MADS-box family namely *POTM1* since this gene function gained considerable importance with regard to a complex biological process like tuberization in potato. Several facile bioinformatics tools were employed in order to generate data in the following aspects: sequence identity, sequence analysis, multiple sequence alignment, searching protein motifs, and 3-D modeling along with some other attributes. Most of these features as obtained in this study were not reported earlier. Apart from *in silico* approaches, some experiments were carried out to know the expression patterns of *POTM1* gene. For this purpose, total RNA was isolated from field-grown different potato organs namely leaf, flower, tuber, and tuberizing stolon. First, reverse transcription (RT) was carried out using organ-specific individual total RNA samples and *POTM1* cDNA-specific oligonucleotide primer(s). Subsequently, the RT products were employed to carry out PCR. The size of the amplicon was ~1.0 kb**.** Similar amplicon was also found using potato genomic DNA as template. Possibly, there was no intron in the *POTM1* gene. As evident from RT-PCR data, *POTM1* transcripts were clearly detected in the tuberizing stolons and flowers. Probably this transcript level could be low or negligible in other potato organs. All these data are quiet useful and relevant with regard to the Indian potato cultivars particularly for the crop improvement aspects.

Keywords: Potato (*Solanum tuberosum* L.) cultivars; POTM1 (potato MADS-box); Transcription factors; Self Pruning (SP6A); Reverse transcription (RT); Polymerase Chain Reaction (PCR)

CHAPTER- 1 INTRODUCTION

1.1 About potato (*Solanum tuberosum* L.)

Potato (*Solanum tuberosum* L.) is a dicotyledonous, herbaceous, annual and vegetatively propagated non-grain tuberous plant; rich in carbohydrates, predominantly in the form of starch. It is an edible food crop, contributing to food and nutritional security in the world (Sarkar, 2008). It is one of the essential foods over the world ranking third in human consumption and production after the cereals species wheat (*Triticum aestivum*) and rice (*Oryza sativa*) (Birch PRJ et al., 2012). It belongs to the botanical family *Solanaceae* and genus *Solanum* (USDA, NRCS, 2010). This botanical family consists of a large diverse family of herbs, trees and shrubs that includes more than 2000 species and 90 genera. Solanum (also known as the nightshades) is one of the massive and hyper diversity genuses of Solanaceae family (Spooner et al., 2014). The other constituents of *Solanaceae* family are egg plant (*S.melongena*); tomato (*S. lycopersicum*); sweet peppers (various capsicum species); the poisonous jimsonweed (*Datura stramonium*); belladonna (*Atropa belladonna*); tobacco (*Nicotiana tabacum* and *N. rustica*), and petunia (*Petunia hybrid* L.) (Fernald, 1970; Spooner and Knapp, 2013). The clarity of proliferation of potato by vegetative amplification along with the high strengthening value of tubers are the main cause of the growing stardom of the potato in the countries (Alisdair et al., 2001).

1.1.1 History of Potato

The origin of *Solanum tuberosum* is ultimately traced to Chilean and Andean landraces. According to hypothesis of Hawkes (1990) and Laufer (1938) potato was transported to North America from Europe, may have been conveyed from England to Bermuda from England in 1913 and then from Bermuda to mainland of North American in 1621. In India the crop was introduced in 1610.In 1675 Potato was first cultivated in the gardens of Surat and Karnataka. Potato cultivation began in the Nilgiri hills in the year 1822. The commercial cultivation of potato was started at Nilgiri hills in 1822.

In India, potato is cultivated on an area of 1.97 million hectare with annual production of 41.55 million tones and accounts for 25.5 percent of total production of vegetable in India with 21.10

tonnes/ha of productivity (Anon, 2014). By 2020, it is estimated that, population will cross 1.3 billion and country will need potato production of around 49 million tonnes (Kumar and Pandey, 2008). To meet the demand of expanding population, since the population of country has crossed the mark of one billion, there is an immediate need to redesign the crop of potato and accomplish novel technologies for the improvement of quantity and quality by breaking the traditional barriers. Now, India ranks fifth in production of potato after Poland, Russia, USA and China (Krishikosh.egranth.ac.in).

Potato is considered as cold weather crop. It grows well in temperature range between 7º C to 30º C. The optimal yield is obtained when the temperature ranges from 18º C (64º F) to 20º C (68º F), while growth of tuber is inhibited or delayed when the temperature range is below 15º C (º F) and above 25º C (º F). The delay is because of low temperature, metabolism and growth slows down while due to high temperature there is stimulation of development of the plant portions above the ground which further results in the lower down the yields due to reduction in the amount of photosynthesis (Levy and Veilleux, 2007). Therefore, the period of growth of tuber is from November to March with the harvesting time in mid- March. The crop grows well in fertile soil known as Sandy Loam soil within acidic pH range 5.0-5.5.

1.1.2 Taxonomy of Potato

Potato exhibits a high level of polyploidy. It is true tetraploid and displays high degree of heterozygosity accompanied by 48 chromosomes (2n = 4x = 48) which is a combination between the diploid weed *S. sparsipilum* and diploid species *S. stentotomum* with consecutive doubling of chromosomes. There is a series of four diploid species accompanied by 24 chromosomes (2n = 2x = 24) includes *S. ajanhuiri*, *S. stenotomum*, *S. phureja,* and *S. goniocalyx*. There are 2 triploid species *S. chaucha* and *S. juzepczukii* accompanied by 36 chromosomes (2*n* = 3*x* = 36) and also one pentaploid cultivated species *S. curtilobum* with 60 chromosomes (2n = 5x = 60) (Andersson and de Vicente 2010). Out of 1500 species the genus *Solanum* L. consists of 1500 species out of which only 150 are capable to produce tubers (Poehlman and Sleper, 1995).

1.1.3 Morphology and anatomy of potato tubers

General morphology: Potato is a modified stem (stem with broadened and shortened axes) not a modified root which swells underground and enlarges to develop a storage organ or an edible

tuber (Alisdair et al., 2001). For next generation plants (so-called seed tubers), the tuber acts as a starting material and is clearly important for food and feed. During the initial phase, the stem is erect, then spreads and later become flat. The leaves of the potato plants are irregularly compound and pinnate. The tuber, are modified underground fleshy stem and carries eyes and buds like leaves in the axil at small-scale (Ewing, 1981). Due to this, from many years' tuberization, storage, and sprouting like processes have been intensively studied in potato. For transgenic approaches, potato was first accessible plant crop (Alisdair et al., 2001).

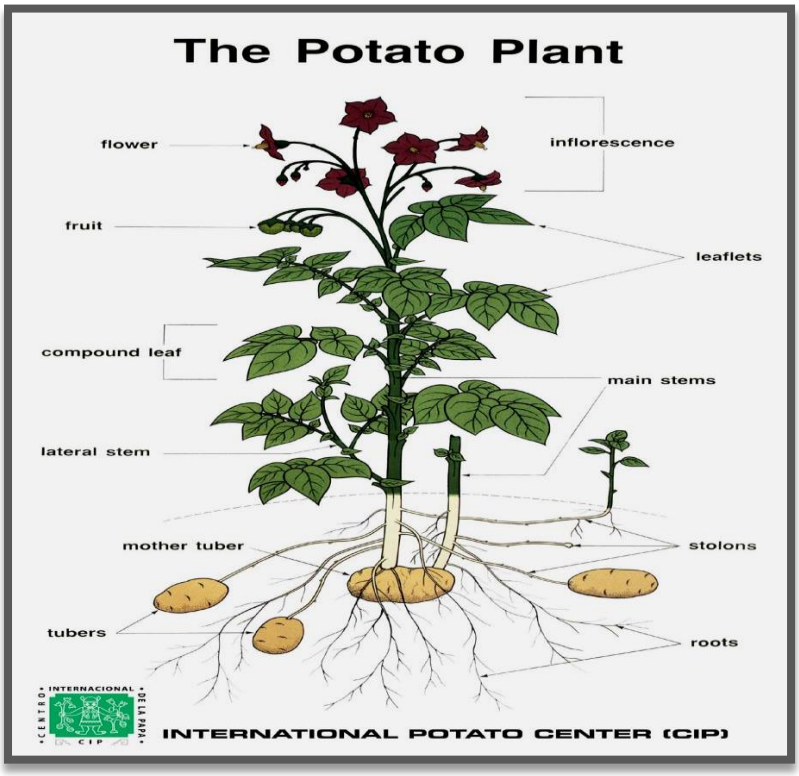

Fig. 1 Diagram showing all the parts of a potato plant
(Ref: International Potato Centre, http://cipotato.org/crops/potato)

Anatomy of potato: The plant grows up to the height of 0.4- 4.1m (Snooper and Knapp, 2013). The stems can be hairless to compact hairy and may be purple, green or mottled green and purple. The leaves are mostly pinnately compound (Struik, 2007). The size of blader with the petioles ranges from 8-22× 5-13 cm with 2-6 cm respectively and are intermediate to dark green. The rhizomes which are often called as stolons are like primitive leaves, generally curved at the tip. They arise from the nodes of basal stem, which are basically beneath the ground and

develops into spherical or ovoidal tubers (Struik, 2007). The color of tuber flesh varies from white to yellow to blue and skin from red through blue and white through yellow to tan. The texture of the tuber may also vary from creamy to russetted/nested of the surface (Spooner and Salas, 2006). The auxillary buds are on the surface of tuber with eyes (also called as scars of scale leaves) (Struik, 2007). These eyes develop into the stems when tubers are planted to form next vegetative generation. In some plants such as potato, tubers and seeds are two principle storage organs. Both tuber and seeds possess different patterns of development and anatomy. Potatoes are cultivated through tubers only and seeds are used for the purpose of genetics (Umaerus, 1987) because they prefer very tightly regulated pathway for the development which starts by the process of fertilization and finally, stops in the programmed dehydration of seeds (Cutter, 1982).

1.2 Nutritional aspects of potato

Potato is highly nutritious as it contains large amount of essential vitamins, minerals (potassium, phosphate copper, niacin, dietary fibers, vitamin C, vitamin B, and pantothenic acid) and also phytochemicals such as carotenoids and polyphenols. A mediocor sized potato along with its skin is a store house of potassium, vitamin B6, vitamin C and trace amount of iron, zinc, phosphorus, magnesium, thiamine, magnesium, riboflavin, niacin, folate. It is rich predominantly in starch. A minute but sufficient amount of this starch is resistant to enzymatic digestion in small intestine as well as in stomach, only a small amount of it reaches to large intestine in the intact form which is examined to have identical physiological effects and health benefits as fiber. This resistant fiber lowers down the plasma cholesterol as well as triglyceride concentration, produces bulk, offers sensation from insulin, favors protection against colon cancer, increases satiety and also possibly reduces the storage of fat (Shah et al., 2013). Potatoes are low in calories (110 calories in baked potato). As per 100 g of it consists of 17.9 g of carbohydrates, 85 kcal of energy, 2.6 g of proteins, 0.1 g of fat, 3.1 g of dietary fiber, 5 47 mg of potassium, 0.9 mg of iron, 0.11 mg of vitamin B1(thiamine) , 0.23 mg of vitamin B6, folate 44 µg, 14 mg of vitamin C, 1.9 mg of Vitamin K, 12 mg of calcium, 0.01 mg of vitamin E, 23 mg of magnesium, 0.153 mg of manganese, 0.296 mg of pantothenic acid, 15.44 g of starch and 75 g of water. (https://www.eufic.org)

1.3 Tuberization: An important aspect of plant life

Tuberization is a highly complex, coordinated, physio-morpholological development process that involves the formation of a stolon (an underground stem), under initiative circumstances constraints the growth and in the sub apical region it swells to form the storage organ in which starch gets accumulated in very large amount (Abelenda et al., 2011). It is an important process not just for the endurance of organisms of plants but also for the support of life of human (Aksenova et al., 2012). This process involves the large number of interactions between various factors i.e., biochemical, genetic and environmental. There are three definite processes; cellular, morphological and biochemical levels are associated with tuberization. The morphological levels include the development of underground stem (stolon) and side by side induction of tuber at the tip of stolon, at cellular levels, both cell enlargement and cell division takes place (Vreugdenhil et al. 1999), cell growth change in orientation also occurs in the stolon tip sub apical region (Xu et al., 1998b). Whereas, at biochemical levels there is synthesis of starch (Tauberger et al., 2000) and storage proteins accumulation (Taylor et al.,1992). Thus, it is a multi-stage process comprised of various successive stages that are, induction of tuberization, initiation of stolon growth and development, cessation of longitudinal growth of stolon accompanied by induction, initiation and growth of tuber.

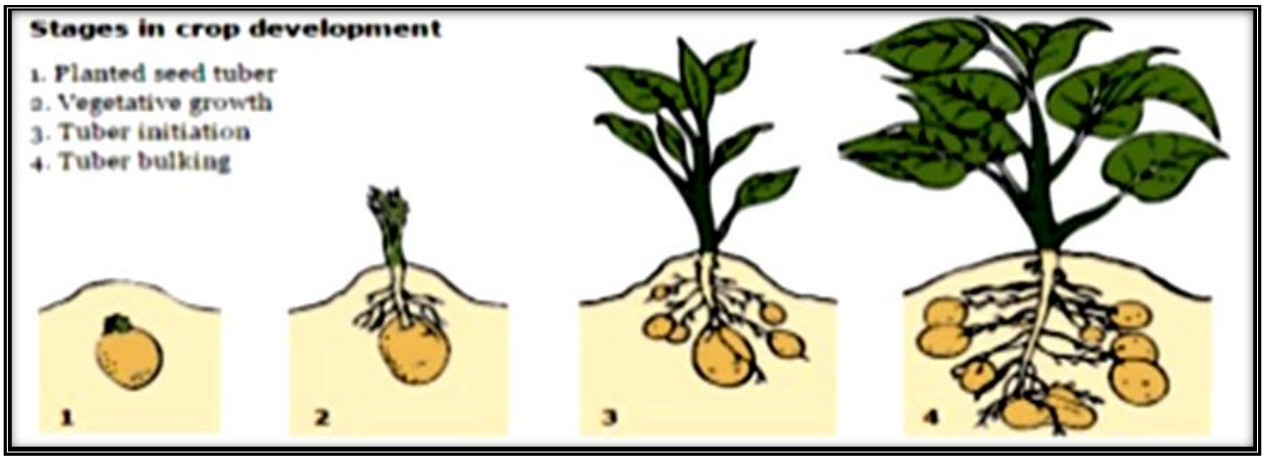

Fig. 2 Growth and development of potato plant (*Fao.org, 2008*)

The regulation and control of each stage is by an interaction of multiple genes expressed throughout the plant (Koda et al., 1988). Accumulation of large amount of carbohydrate is one of the necessary conditions for the process of tuberization. Potato tuberization is preceded by

photosynthesis activation; assimilate accumulation in stems, and their intense transport toward underground organs (Mokronosov et al., 1990).

1.3.2 Factors affecting tuberization

Various external factors that strongly affect tuber formation are high irradiance, photoperiod, supply of carbohydrate, nitrogen and temperature, phytohormones. An absolute decrease of temperature up to 14-20° C, exclusively during night time, leads to sharp reduction in number and weight of growing tubers when there is an increase in temperature during night to 25-27° C (Ewing et al., 2010). Two most important phytohormones 'Gibberellins' and 'Cytokinins' are highly involved in the regulation and photoperiodic control of tuberization of potato. The dominant regulator in tuberization is "Gibberellic Acid" (Xu et al., 1998a) whereas, cytokinins plays their role in arrangement of division of cells by supervising cell cycle.

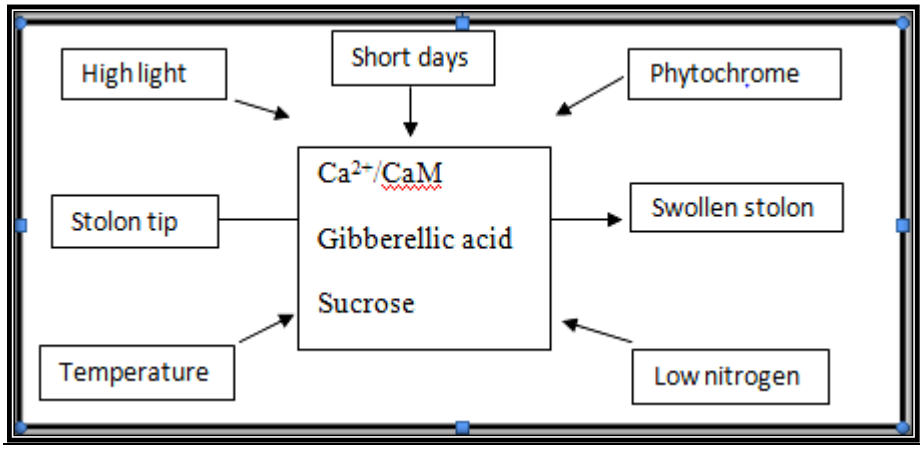

Fig. 3 Various factors affecting the process of tuberization (Jackson, 1999)

Potatoes have many advantages with a research point of view, such as they can be easily transformed and amenable to genetic manipulation. Also they can be propagated rapidly both in tissue culture and through cuttings. Moreover, genetically they are more closely related to tomato.

1.3.3 Transcription factors involved in tuberization

Transcription factors are basically proteins that bind to DNA to regulate the activity of genes and also to mediate the level of hormones. To regulate the development of meristem in potato and

also to regulate the growth of plants, including the formation of tuber there is involvement of various DNA-binding proteins (Hannapel et al., 2004). TF (Transcription factors) controls/regulates such types of events at molecular level. To modulate the specific target gene expressions, protein nature TFs by binding to the DNA acts as a developmental switches. There are numerous transcription factors (i.e. proteins) involved in order to regulate tuber formation includes POTM1 (potato MADS-box), POTH1 (potato homeobox), StSP6A, StBEL5, StPHYB, StCONSTANS and Sucrose transporters such as StSUT4, StSP5G etc (Dutt *et al.*, 2017). In order to regulate formation of tubers in potato there are certain bio-molecules that acts as an important candidates in signaling pathways. POTM1, miR172, StSP6A protein, StBEL5 RNA, GAs, molecules acts as a main candidates as a mobile signaling for formation of tuber (Dutt et al., 2017). The transcription factor encoded by *MADS*-box gene has an important role in functioning, development and also in the regulation of transcription in eukaryotes. (Riechmann et al., 1997; de Folter S et al., 2006; Gramzow et al., 2010)

POTM1 is a potato *MADS*-box gene, belongs to subfamily SQUAMOSA of *MADS*-box gene which is basically a protein (Kang and Hannapel, 1995). The expression of *POTM1-1* is highly abundant in vegetative organs, such as immature tubers, leaves, stolon and roots (Kang and Hannapel, 1995, 1996). It is a transcription factor involved in order to regulate the formation of tubers and was first isolated from an early tuber of potato cDNA library (Kang and Hannapel, 1995). It shows 97% and 91% similarity to SCM1 (*Solanum commersonii*) from a wild potato species and PEG (Petunia hybrida) from petunia respectively over the entire length of protein (Hart and Hannapel, 2002). In the following we will clearly discuss about *POTM1* and its possible roles in the long distance signaling with its mechanism. Also an explanatory role is provided as how POTM1 interact with other transcription factors and mobile signal RNAs to modulate the process of tuberization.

CHAPTER- 2 REVIEW OF LITERATURE

Long distance communication systems are unique signaling pathways, which takes advantage of vascular system. Most of these communication systems use phloem tissue to travel to the target site of action. This pathway is involved in response to abiotic stress, vehicle commander for spreading infections by the viruses, for the delivery of nutrients, and also as a regulating development (Lough and Lucas, 2006). For the growth of plants, it has been concluded that phloem negotiate the supply of many organic compounds as well as phothosynthates. To visualize the higher resolution and to measure the translocation of phloem tissue the studies on phloem became well established. For the regeneration of phloem physiology real time microscopy as well as genomic molecular biology techniques with genomics help in the manipulation of genes that specifically encodes for proteins in phloem and also helps in their identification.

2.1 RNA constituents of phloem tissues and their role in long distance signaling

In higher plants, both long as well as short distance communication pathways and coordination in their biological and metabolic activities is crucial. For the, growth of plants and to promote the process of cell differentiation at both inter and intracellular level, transport mechanism of messenger RNA is important (Haywood et al., 2002; Kim and Pai, 2009; Ding et al., 2003). Many plant transcription factors have the capability to move from cell to cell to plant plasmodesmata over a short distance. KNOTTED1-Like homeobox TF, or the APETALA1 and TF LEAFY are associated in conservation and initiation of meristem (Lucas et al., 1995; Sessions et al., 2000). In Arabidopsis, endogenous TA (transacting), si (short interfering) RNA loci the mobile silencing signals facilitate polarization of leaf by the production of regional gradient of target gene expression (Childwood et al., 2009; Dunoyer et al., 2010a). Surprising through the phloem, specific mRNAs can be even delivered to unapproachable plant organs (Medrano et al., 1999). Many ribonucleo proteins and RNA proteins complexes established in the translocation of phloem stem have became a fascinating candidate as information transmitter's servers (Medrano et al., 1999). In long distance signaling some of these ribonucleo protein and RNA proteins have a crucial role (Kehr, 2009; Opark and Cruz, 2000; Dinant and

Lemoine, 2010; Lough and Lucas, 2006). Comprehensive studies focus on phloem RNA composition, which revealed different type of RNAs.

2.1.1 Mechanism of transport and import of phloem-mobile RNAs

RNAs in the form of sieve tubes are thought to be exotic through the connecting pore-plasmodesmata units of the neighboring companion cells (Lough and Lucas, 2006). Most of the RNA-binding proteins functions as chaperone and arbitrate the RNAs import into sieve elements from companion cells. The macromolecular structure of RNA could be modified by RNA-specific chaperones to promote their passage through plasmodesmata and make them stabilize during translocation (Yoo et al., 2004; Ham et al., 2009; Lough and Lucas, 2006). Within the translocation stream, the stability of RNA does not seem to be a predominant matter, since the activity of RNase is not confronting in the sieve- elements exudates. (Sasaki et al., 1998; Gaupels et al., 2008; Zhang et al., 2009;Saad et al., 2002). From source to sink, RNAs seems to translocate within the sieve elements depending upon the metabolic flux. To facilitate the long distance transit of RNA in the phloem help must be required from several interacting proteins, mRNAs, and complex of RNP (ribonucleo-proteins) (Ham et al., 2009). Most of the plant physiologist has examined about the components of protein of long distance RNA transporting complexes and also RNA-binding protein aids exchange of companion cells into large RNP complexes and sieve tubes.

2.1.2 Full length mRNA as a long distance signals

Various protein interactions like RNA- protein, RNA-RNA, protein-protein are involved to localize mRNA during transportation (Elvira et al., 2006; Ferrandon et al., 1997). The mobile mRNA function only at the sites of their target is approved by eliminating of the process of translation (King et al., 2005). Normally, the function of UTRs (untranslated regions) is to bind the proteins that promote the migration of transcripts, such kind of migration regulates the translation efficiency and mediates the stability of RNA (Jansen, 2001; Lee and Jeong, 2006; Barreau et al., 2006; Gualerzi et al., 2003;Derriogo et al., 2000). These NA binding proteins i.e. cellular RNA molecules needs protection as they quickly fall prey to degradative process.

2.2 Factors involved in tuberization

To feed due to logarithmically expanding population of world, potato has emerged as a boon (Birch PRJ et al., 2012). As tuberization is the mechanism of survival for potato. Therefore, it is very important to identify the candidate genes that are involved in the development of tuber and tuberization so that they can be used to promote the improved yield for both traditional breeding as wells as for genetically modified crops. It's been indicated that the family of proteins involved in tuberization is ARF6 (auxins responsive factor 6), which is a key regulator of auxin responsive gene (Rampant et al., 2004). There are various transcription factors involved in RNA signaling are StBEL5 (Chen et al., 2003), POTM1 (Kang and Hannapel 1995), POTH1 (Rosin et al., 2003) and AtCO (potato orthologue Arabidopsis thaliana CONSTANS, that alone or in concert with other transcription factors regulate the potato tuber formation. The transcription factors StBEL5 and POTH1 of potato interact to repress the expression of ga20ox1 in tandem (Chen et al., 2004), thus leading to increase in the level of cytokinins and regulation of the synthesis of GA during the formation of tuber (Rosin et al., 2003). During the induction of tuber the StBEL5 work as a systemic signal in the leaf and the tip of stolon during the long distance signaling pathway (Banerjee et al., 2006). The short day length supports the transport and aggregation of StBEL5 and RNA-binding proteins helps mediates their transfer. In conjugation with KNOX, the chaperones transfers RNA transcript of StBEL5 to the tip of stolon, and regulates the process of transcription of several target genes involved in tuberization. There is need for dephosphorylation of protein in case of calcium signaling for the transcriptional activation of some of genes involved in tuberization (Raices et al., 2003). For the induction of tuber CA-binding modulator proteins and Ca^{2+} acts as a signal molecules (Jena et al., 1989). The Ca dependent protein kinase StCDPK1 is expressed in tuberizing stolon contains a highly conserved myristoylation site.

Lateral branching and apical growth processes are basic processes to determine the patterns of growth, shoot and inflorescence in plants (Rosin et al., 2003). Interactions between the signaling compounds, SAM (shoot apical meristem) and the transportation of axillary meristem from the roots are responsible for the overall branching architecture and pattern of shoots. For regulating the development of shoot apical meristem and during the process of tuberization various transcription factors of plants plays an important role. One of the examples of transcription factors which belong to highly conserved family is MADS-box genes that have distinct roles in

the development of plants, act as a regulator of vegetative and floral development. (Weigel and Meyerowitz 1994; Mao et al., 2000, Theissen, 2001), *POTM1* is a potato MADS-box gene which belongs to subfamily SQUAMOSA of MADS-box gene in plants and was first isolated from a tuber of early stage cDNA library (Kang and Hannapel 1995). It functions as a mobile signal under the conditions of short-day and plays an important role in regulating the process of tuberization (Navarro C et al., 2011). StSP3D i.e. its paralogue was required in day-neutral flowering control. MADS-box genes classical upstream regulator was FT in the ABC conservative model in the identity of organ of flower (Litt A et al., 2010). On the basis of these clues, it was sensible to believe that in the development of tuber and tuberization some of the potato MADS-box genes are involved.

POTM1 in plants: *MADS*-box gene belongs to a homeotic gene consisting of two conserved domains: one is N-terminal DNA-binding domain known as the MADS-box domain of 56 amino acids and the other is amphipathic α-helix protein interaction domain known as the K-box domain (Schwarz-Sommer et al., 1990; Ma et al., 1991). The family of *MADS*-box gene has further several subfamilies which in their domain of MADS-box has high homology (Theissen et al., 2000). The highly conserved MADS-box domain leads to cross-hybridization within the family. *POTM1* shows higher homology to gene *TM4* which is specific *MADS*-box gene for flowering than *TM3* and *TobMADS1* genes which are floral-vegetative types among the solanaceous species. In vegetative organs, *POTM1* shows no symbolic sequence homology to homeotic genes of other plants (Kang and Hannapel, 1995).

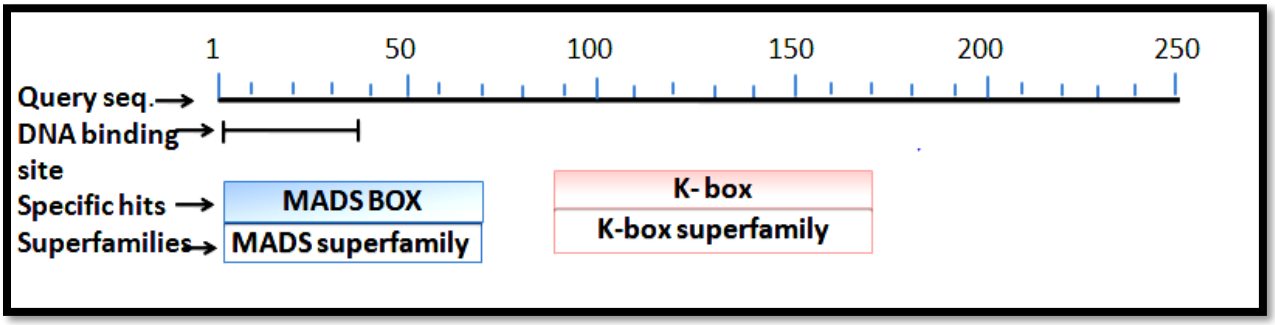

Fig. 4 POTM1 transcription factor

Expression of POTM1: *POTM1* is abundantly expressed in reproductive organs such as carpels and sepals, whereas it is poorly expressed in other vegetative organs such as leaves, stems (Jang and An, 1999). Expression of *MADS*-box gene was suppressed by BAP (6-Benzyl aminopurine) but was found to be independent of other plant hormones such as MeJA (Methyl Jasmonates), NAA, ABA (Kang and Kang, 2002).

In auxillary buds, newly formed tuber and in underground tips of stolon the transcript POTM1 levels are high, but in mature tubers the level of transcripts is low (Kang and Hannapel., 1996). During the development of auxillary buds, in a cutting system of model petiole-leaf, the POTM1-1 transcripts levels were abundant during the microtuber development, early stages and in actively growing shoots (Kang and Hannapel, 1996).

MADS-box in potato: MADS-box in potato was characterized recently, total 153 members of the family of MADS- box gene in potato was characterized. Except for the subgroups like AGL17, FLC, and TT16, most of the MADS-box genes in potato had their orthologs.

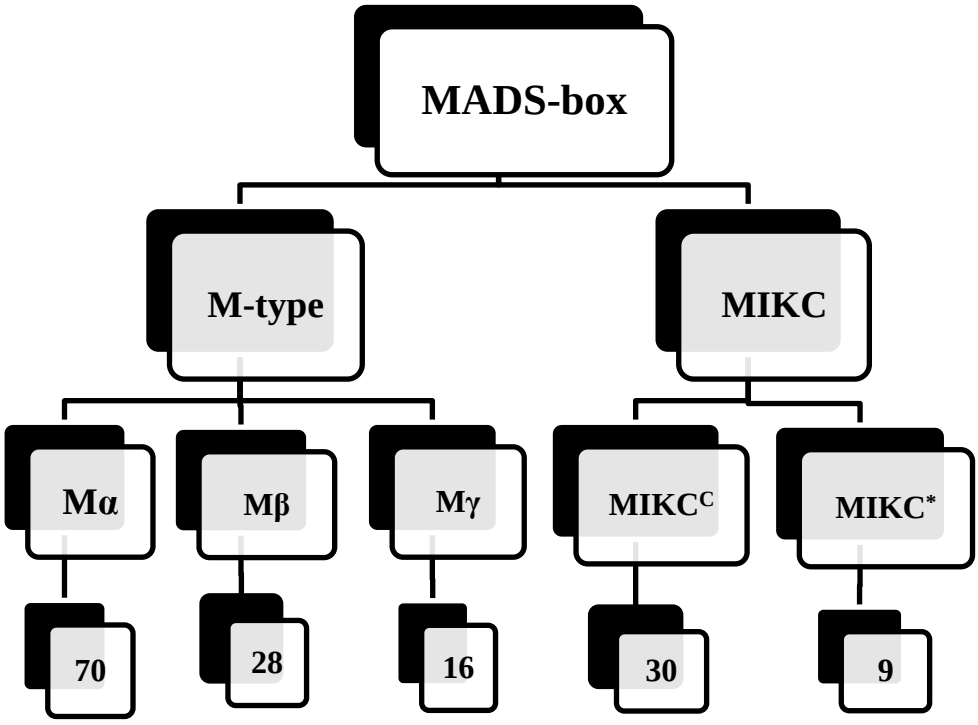

Fig. 5 Characterization of *MADS*-box gene

These proteins i.e. potato MADS-box was divided into two subfamilies M-type and MIKC. In MIKC subfamily there were 39 number of *MADS-box* gene, is further divided into two subgroups: $MIKC^{C}$ which contains 30 *MADS-box* genes and $MIKC^{*}$ which contain 9 *MADS*-box genes. In M-type subfamily there are 114 *MADS-box* genes, is further divided into three subgroups: Mα, Mβ, and Mγ contains 70, 28 and 16 members respectively (Gao et al., 2018)., The evolutionary patterns of Mα are assumed to be different, when compared with other forms (Leseberg et al., 2006; Arora et al., 2007; Wei et al., 2014; Shu et al., 2013; Grimplet et al., 2016). The large family of potato *MADS-box* gene might be due to its larger genome, which has been produced by differently genomic duplication events in different species during the course of plant evolution (Airoldi et al., 2012; Nam J et al., 2003).

Gao et al., 2018 investigated the effects of events of gene duplication of the *MADS-box* gene, and reported that segmental duplication basically contributes MIKC subfamily expansion, whereas M-type boom was derived mainly from tandem duplications. M-type genes are basically derived within the same chromosome from the site-specific duplications, while mainly in MIKC whole genome duplication occurs (Airoldi et al., 2012; Pinyopich et al., 2003; Edger et al., 2009; Gramzow., 2014). Moreover, rate of birth and death of MADS-box genes in different species after gene duplication were different as a result of which Nam et al., 2003; Airoldi et al., 2012 found the variable number of MADS-box genes of different species in the same subfamily. Kanno et al., 2003; Nam et al., 2003; Litt et al., 2003 reported that between M-type and $MIKC^{C}$, the intermediate was $MIKC^{*}$ in the course of evolution of plants. Wei et al., 2015 reported that M-type does not contain any introns while in case of MIKC, $MIKC^{*}$ has more number of introns.

MADS-box in tuberization: Recent studies done by Gao et al., 2018 revealed the role of *StMADS* gene in the process of tuberization. Isoforms of the *MADS-box* shows the differential expression. Out of total 153 *StMADS-box* genes, only few of the genes i.e. *StMADS1*, *StMADS3*, *StMADS11*, *StMADS13*, *StMADS16* and *StMADS29* are expressed highly in storage organs, whereas *StMADS1*, *StMADS3*, *StMADS11* and *StMADS16* are also expressed in young tubers and stolon, indicating the probable role of these genes in the development of tuber and / or in tuberization. Previous studies had shown the importance of these *MADS-box* genes in the differentiation of organs of flower in several plant models. The expression of *FT (FLOWERING LOCUS T)*, was suppressed by *FLC (FLOWERING LOCUS C)*, typical MADS-box gene bound

between the first introns and promoter in its CArG site, in the model plant Arabidopsis (Lee et al., 2000; Helliwell et al., 2006; Michaels et al., 2009). Impressively, the expressions of *MADS-box* genes along with *SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1)* and *AP1* (*APETALA 1*) were related to the promotion of flowering which was controlled by the interaction of two flower related proteins *FD (FLOWERING LOCUS D)* and *FT* (Srikanth et al., 2011). The *FT* and *MADS-box* orthologs and paralogs in monocot plants represents nearly the same regulation of transcription, moreover, a pair of *RFT1 (RICE FLOWERING LOCUS T)* and *Hd3a (Heading-date 3a)* which are a part of *FT* genes, plays an important role in the up regulation of *OsMADS15* expression, crucial for the initiation of flower (Chen et al., 2006; Schmitz et al., 2008; Tsuji et al., 2011; Hecht et al., 2011). The studies done showed that *FT* proteins have functional diversification in potato.

2.2.1 Relation of MADS-box gene with StSP6A

For the transition of flowers, gene *StSP3D* has a major role and gene *StSP6A* is mainly involved in the transition of tuberization. The *StMADS* genes (*StMADS1, StMADS3, StMADS11, StMADS12, StMADS13, StMADS17, StMADS27*) expressed in young tubers and/or in stolons are acts as possible downstream targets of *StSP6A* downstream targets. Impressively, it was found that the gene *StMADS1* and *StMADS13* have strong correlation in the expression in young tubers and/or leaves of gene *StSP6A* (Navarro et al., 2011). Moreover, the analysis of microarray data from the tissue of stolon of *StSP6A*-OX (StSP6A-overexpression) and RNAi plants, *StMADS1* and *StMADS13* expression were down regulated in *StSP6A*-RNAi plants and up regulated in *StSP6A-OX* plants. Several *MADS-box* genes and *StSP6A* may share the same map for the regulation. Gao et al., 2018 reported that *StMADS1* and *StMADS13* act as candidate genes in the downstream of *StSP6A*. Microarray studies from stolon of *St*SP6A-OX (overexpression) and RNAi plants. It was found that *StMADS1* and *StMADS13* were up regulated whereas in RNAi plants it was down regulated.

2.2.2 Model depicting: Role of POTM1 in potato tuberization

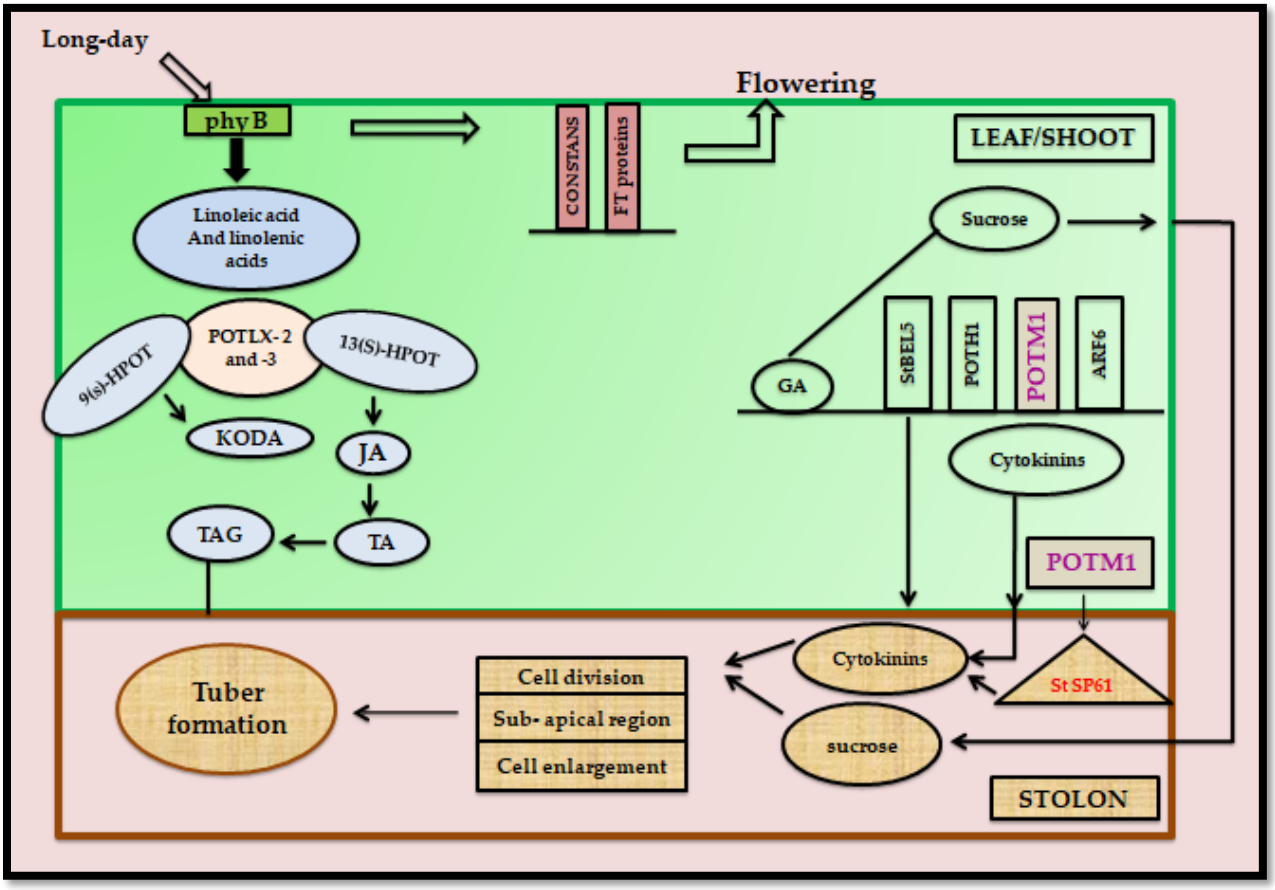

Fig. 6 Role of *POTM1* in the process of tuberization (Sarkar, 2008)

2.3 Origin of problem

Tuberization is a complex and essential process for the formation of tuber that occurs in potato in the underground stolon tip. It involves the interactions of many essential factors such as genetic, environmental, and biochemical at both intrinsic as well as extrinsic level. Therefore, with regard to the production of healthy tuber (micro, mini, and large) it is important to get detailed knowledge about the whole process of tuberization, where various TFs (Transcription factors) plays crucial roles. Within the species to regulate the diverse processes these TFs acts as developmental switches. So, detailed knowledge at both molecular as well as biochemical levels along with their expression patterns are the primary steps to know about tuberization. Moreover, in Indian cultivars with regards to the TFs involved in the process of tuberization there is no such report available as know. These kinds of studies are essential in relation to both basic and applied

aspects of research in the area of potato biotechnology; so that it would help in improving the crops of potato through the transgenic approaches. One of the objectives is to standardize easy-to-use and a suitable protocol for the isolation of DNA from potato (*Solanum tuberosum* L.) plants so that the function and structure of the genes encoding the TF POTM1 can be studied using gene-specific primers through PCR approach and the other is to know the expression patterns in different tissues of potato. The transcription factor *POTM1* was chosen in this study because of its long distance travelling RNA transcript which acts as a mobile signal. It contacts with *StSP6A* gene to regulate the formation of tuber. Keeping the points mentioned in view, the following objectives were framed.

2.3.1 Objectives of the study

- Sequence analyses, multiple sequence alignment (MSA) and motif search using the available *POTM1* sequences at both nucleotide and amino acid level
- Identification of *MADS-box* gene family members and 3-D structures of *POTM1*
- Reverse transcription (RT) using total RNA from different field-grown potato organs
- RT-PCR approach for studying expression pattern of *POTM1* in different potato organs

CHAPTER- 3 MATERIALS AND METHODS

3.1 *In silico* analysis

Structural outline for the study and identification of POTM1 of Potato (*Solanum tuberosum L.)*:

Extraction of the GenBank accession number of the POTM1 gene of Potato from NCBI database

↓

BLASTn at NCBI (http://www.ncbi.nlm.nih.gov)

↓

BLASTp at NCBI (http://www.ncbi.nlm.nih.gov)

↓

The sequences were retrieved and selected for further analysis

↓

Sequence analysis, predicting motifs and 3-dimensional structure of POTM1 gene

↓

Extraction of POTM1 protein sequence from potato gene using the resource Spud db

3.1.1 Biochemical characterization

The composition of amino acid (aa), molecular weight (MW), theoretical isoelectric point (pI), instability index and aliphaticity were determined by using EXPASY server (http://web.expasy.org/protparam/).

3.1.2 Identification of the conserved domains

The amino acid sequence of POTM1 was then uploaded to MY HITS (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and different motifs were predicted.

3.1.3 Three-dimensional model prediction

In order to predict the biological function of protein it is essential to know its structural information. To model the complete sequence of POTM1 the server I-TASSER was used. It is an approach for hierarchical protein modeling based on an iterative implementation of the TASSER (Threading ASSEmbly Refinement) program and the multiple threading alignments (Zhang Y, 2007 and 2008).

3.1.4 Extraction of POTM1 protein sequence from potato gene using the resource Spud db

The resource Spud db is used in order to extract the protein sequence of POTM1 from potato gene MADS box.

3.2 *POTM1* gene expression patterns

3.2.1 Potato plant materials

The Indian potato cultivar namely Kufri Chipsona-1 (CS-1) along with a reference Désirée (De) were routinely maintained in our tissue culture Laboratory. After acclimatization, the micro propagated plants were grown under field condition during November to March. The different propagated organs were harvested at different stages of development. They were flash frozen under liquid nitrogen and kept at -80°C for further use.

3.2.2 Other materials

Various enzymes used were purchased from Bangalore Genei Pvt.Ltd., Bangalore. The chemicals required were bought from Sigma-aldrich India Pvt. Ltd, and Himedia Pvt. Ltd, Mumbai. Primers used were synthesized by Bangalore Genei Pvt.Ltd, Bangalore. The gel extraction Qiagen kit was purchased from Genetix.

Table 1: Different chemicals and their composition

Chemicals	**Composition**	**Volume**
Gel Loading Buffer (5X)	Sucrose	35% (w/v)
	EDTA	50 mM (pH 8.0)
	Tris	25 mM
	Bromophenol Blue	0.2% (w/v)
TBE (5X) Buffer (pH 8.0)	Tris Base	54 g L^{-1}
	Boric Acid	28 g L^{-1}
	EDTA	3.8 g L^{-1}
TE Buffer	Tris HCl	10 mM (pH 8.0)
	EDTA	1 mM (pH 8.0)
DNA Extraction buffer	Tris HCl	50mM (pH 8.0)
	EDTA	50mM
	NaCl	250mM
	Sucrose	15%

Alcohol, RNase, Ethanol, Isopropanol, Potassium acetate solution (5.0 M), Sodium acetate, Chloroform

3.3 Methods

3.3.1 Isolation of total DNA from different potato cultivars

Materials required: Sterile water, Extraction buffer, 0.5% SDS, 5.0 M Potassium acetate, Isopropanol, Ethanol, TE buffer.

Procedure: Total DNA was isolated from the tissue cultured potato plantlets by a modified protocol reported earlier (Kumari et al., 2012). Plant samples were washed under tap water followed by sterile distilled water. Excess water from the plant was removed with the help of blotting filter paper. Afterwards 0.7 g of plant material was weighed and fine powder was made in the presence of liquid nitrogen with the help of mortar and pestle. The fine powder was then transferred to 20 mL tube containing 5 mL of extraction buffer and 0.5 % SDS maintained at 65°C. Contents were mixed properly with intermittent gentle shaking and incubated at 65°C for 15 min. The solution in the tube was spun at 5500 rpm for 10 minutes using centrifuge. Then 170 µL of 5.0 M potassium acetate solution was added, vigorously mixed and further incubated on ice for 20 min and solution was again centrifuged at 5500 rpm at 4°C for 15 min. Through a fine muslin cloth the supernatant was filtered and equal volume of isopropanol was added, mixed gently and incubated at -20°C for overnight. DNA was extracted by centrifugation at 12,000 rpm at 4°C for 10 min. The crude DNA pellet was washed with 70% ice cold ethanol, air dried and suspended in 100 µL of TE buffer and stored at -20°C.

3.3.2 Purification of total DNA

Materials required: Sterile water, DNase-free RNase enzyme, 3.0 M sodium acetate (pH 5.5), Ethanol, TE buffer, Extraction buffer, 8.0 M LiCl, de-ionized water.

Procedure: Further purification of total DNA was done by treatment with DNase-free RNase. 400 µL of sterile water was added into eppendorf containing DNA sample. Then 3µL of DNase-free RNase enzyme was added into solution. The solution was incubated for 45 minutes at 37°C. Equal volume of phenol and chloroform (200 µL each) was added into the solution and mixed properly for 10-15 minutes by gently inversion mixing. Then the sample was centrifuged at 8000

rpm for 10 minutes. Followed by DNA was precipitated using 0.1 volume of 3.0 M sodium acetate (pH 5.5) and 2.0 volumes of ethanol. DNA was finally recovered by dissolving the pellet in 50 µL of TE buffer and stored at (-20°C). The quality and quantity of DNA was checked spectrophotometrically by measuring the A_{260}/A_{280} ratio.

3.3.3 Isolation of total RNA from potato tissues

Materials required Lithium chloride, RNA extraction buffer, Tris-HCL (100mM) at pH8.0, EDTA (10mM) at pH 8.0, 1.0% SDS, 0.2% (β-mercaptoethanol), RNase-free DNase, RNase-free deionized water.

Table 2: Stock and working solution for RNA isolation

Solution	**Stock solution**	**Working solution**
Tris buffer	0.5M (pH 8.0)	100Mm (pH 8.0)
LiCl	8M	10Mm (pH 8.0)
EDTA	0.5M (pH 8.0)	10mM (pH 8.0)
SDS	10%	1%
β mercaptoethanol	0.2%	0.1ml

Procedure: Plant tissue contain high amount of polysaccharides, phenolics, nucleases and other storage material. Therefore, isolation of RNA from plant material in terms of intactness and quality is relatively difficult. For, that number of methods is reported in literature. Here we used, SDS-Phenol method described by Gilman (1987) which was used as such or with some modifications depending upon the plant material. The plant materials (0.2 to 1.0 g) were frozen and were pulverized into a fine powder using liquid nitrogen. The powder was further mixed in a buffer containing lithium chloride and SDS (RNA extraction buffer, 100mM tris-HCL pH 8.0, 10mM EDTA pH8.0, 1.0% SDS, 0.2% (β-mercaptoethanol) followed by direct extraction with phenol: chloroform (1:1), under ice-cold conditions, 8.0 M LiCl (one third volume of the aqueous solution) was added to the supernatant and incubated for minimum 2 hours for selective

precipitation of RNA. The crude RNA was further clarified by solvent extraction and ethanol precipitation. After that RNA was dissolved in RNase-free deionized water, and kept in aliquots at -70°C for further use. The quality of the RNA samples were checked by regular and formaldehyde agarose gel electrophoresis along with RT-PCR using different potato gene specific primer.

3.3.4 Purification of total RNA

Materials required: RNase-free DNase enzyme, Phenol: Chloroform, Ethanol, RNase-free deionized water.

Procedure: The crude RNA was purified by treatment with RNase free DNase followed by solvent extraction and ethanol precipitation. RNA was dissolved in RNase-free deionized water and aliquots were stored at -70°C for further use. The spectrophotometric analysis using A_{260}/A_{280} ratio of the RNA samples were also measured to check the quality.

3.3.5 Designing of oligonucleotide primers

The following oligonucleotide primers were designed based on the available sequence corresponding to POTM1 gene in GenBank database (GenBank ID: U23757). Total length of CDS region of this POTM1 gene is 752bp, starting from 58th nucleotide to 810th nucleotide. Where, 5′-UTR is 57 bp long and 3′-UTR is 257 bp long starting from 811th nucleotide to 1068th nucleotide. The whole sequence is 1068 bp and ORF length is 752 bp with include 250 codons (including stopcodon). The various parameters kept in mind while designing the primers are:

- Oligonucleotide primers should be 10-24 nucleotides long.
- GC content should be 40-60%.
- The primer should not be self-complementary or complementary to any other primer to form primer-dimer or hair pin.
- Melting temperatures of primer pairs should not differ by more than 5ºC, so the GC Content and length must be chosen accordingly.
- The annealing temperature should be about 5°C lower than the melting temperature.
- Long run sequences of a single nucleotide should be avoided.

- Primers with significant structures are avoided.

Keeping all these parameters in mind following primers were designed using cDNA sequences of POTM1 present in database are given in **Table 3.**

Table 3: Details of designed primers of POTM1

Sr. No.	Name of Oligomers	Description of the Oligomers	Length
1.	PMF-0057	5′-AATGGGAAGAGGAAGAGTCC-3′	20bp
2.	PMR1-0935	5′-ATTGTGGCATCAATATAGAG-3′	20bp
3.	PMR2-0982	5′-ATCCATCTATATTGTGGCATG-3′	21bp

3.3.6 Polymerase Chain Reaction (PCR)

PCR was used to amplify a specific DNA sequence in a simple, rapid and automated manner using forward and reverse primer. PCR is repeated cycling of three steps: heat denaturation of template DNA (94°C); annealing of primers to the complementary sequences in template DNA (55°C); extension of annealed primers by a thermo stable DNA polymerase (72°C). Thermal cycling parameters for 30 cycles are given in **Table 5.**

Materials required: Template DNA (prepared from genomic DNA of different potato cultivars), Primers (Resuspended in sterile TE or water), Buffer (usually 10X), Taq polymerase, dNTPs (2mM stock), and Sterile ddH_2O.

Procedure: Polymerase Chain Reaction amplifies the DNA sequence using reverse and forward primers. PCR occurs in following steps: heat denaturation of template DNA, annealing of oligonucleotide primers to complementary sequences in single strand template DNA, and extension of annealed primers by a thermostable DNA polymerase. The compositions of reaction mixture and thermal cycling parameters for 30 cycles of PCR reaction are given in **Table 4** and **Table 5** respectively.

Table 4: Composition of reaction mixture (volume 50µL) of PCR reaction

PCR component	50µl reaction	Final concentration
Template	Variable	<1,000ng
10X Taq reaction buffer	5µl	1X
10 µ mole Forward primer	2µl	0.2 µM (0.05-1 µM)
10 µ mole Reverse primer	2µl	0.2 µM (0.05-1 µM)
10 mM dNTPs	2µl	200 µM
Taq polymerase	0.7µl	1.2units/50µl PCR
Distilled water	To make up to 50µl	

Table 5: The Thermal cycling parameters of PCR

Steps	Temperature	Time for genomic (min)	Time for RT (min)
Pre-heating	95° C	3	1
Denaturation	94° C	1	1
Annealing	55° C	2	2
Polymerization	72° C	3	1
Holding	4° C	Variable	Variable

Reaction was optimized and reaction mixture was prepared by standard reagents in a tube. The master mix was prepared by aliquoting all reagents except template. Followed by, a gentle mixing with the help of vortex is given and briefly centrifuges to collect all components to the bottom of the tube. Afterwards master mix was distributed equally to the tubes along with template to be amplified. Finally amplification parameters were optimized depending upon primers and template and repeated run of 30 cycles are given in a thermal cycler.

3.3.7 Reverse Transcription -Polymerase Chain Reaction (RT-PCR)

Materials required: DEPC water, Oligo dT primer, 10 mM dNTPs, RNase inhibitor, Reaction buffer, RNA sample.

Procedure: First strand cDNA was synthesized using Revert Aid H minus M-MuLV reverse transcriptase. The enzyme lacks ribonuclease H activity specific to RNA in RNA-DNA hybrids. Therefore degradation of RNA does not occur during first strand cDNA synthesis, resulting in higher yields of full-length cDNA from long templates up to 13 kb. Reverse transcription (RT) was performed using the Revert Aid TM H Minus first strand cDNA synthesis kit from Fermentas Life Sciences containing M-MuLV reverse transcriptase and the gene-specific reverse primer, PMF-0057 and PMR-0935. For each RT reaction, approx. 2.0 µL of total RNA either from tuber or leaf from Indian potato cultivars was used as template. All the steps of reverse transcription were carried out according to the manufacturer‘s instructions. In order to isolate full length cDNA PCR was carried out using the individual RT product as template. 6 µL DEPC water was taken and RNA sample was added. Followed by Oligo dT primer was added and mixed properly and eppendorf were kept at 65 ºC for 5 min and then at room temperature for 5 minutes. Reaction buffer (4 µL), RNase inhibitor (1 µL) were added and 10 mM DNTP was added. The tubes were incubated at 42 ºC for 1 hour. After initial denaturation at 94ºC for 1 min 30 sec, the thermal cycling parameters during PCR were: denaturation at 94ºC for 1 min, annealing at 55ºC for 2 min; polymerization at 72ºC for 3 min for 30 cycles followed by final extension at 72ºC for 5 min.

3.3.8 Agarose gel electrophoresis

Materials required: Agarose (Hi media), 0.5 X TBE buffer, Ethidium bromide dye (0.5 µg mL^{-1}), sterile water, DNA samples and amplicons, Bromophenol blue dye, Gel Electrophoresis instrument, UV Transilluminator, Gel documentation system (BIO-RAD)

Procedure: Agarose gel electrophoresis was performed using standard methods (Sambrook- a laboratory manual). 1.0 % agarose gel was made in 0.5X TBE buffer and ethidium bromide dye (0.5µgmL^{-1}) was added to it. Gel was then casted in the casting tray. The DNA/ RNA samples were loaded in wells after the solidification of gel. Electrophoresis was carried out in 0.5X TBE

(running buffer) at 2-5 volt per cm till the tracking dye covered two-third of the gel length. Finally the bands were visualized under UV light.

CHAPTER-4 RESULTS AND DISCUSSION

4.1 *In silico* analysis: Sequence analysis, motif search, 3-D structure on POTM1

POTM1 is a transcription factor which plays very important role in tuberization. Comprehensive report on *in-silico* analysis is not available in the database with regard to biochemical and structural attributes of *POTM1*. So, efforts have been made to find some biochemical attributes and structures of *POTM1* isoforms. This study focused on the *POTM1* gene and it's isoforms from Potato.

4.1.1 Salient sequence features of POTM1 gene and POTM1 *protein from potato*

POTM1 gene: In this study, we followed that POTM1 protein consists of 250 amino acids encoding 1068 bp gene sequence from Potato (GenBank ID: U23757). This is basically a transcription factor involved in the process of tuberization in potato. This 1068 bp *POTM1* gene consists of 5′ flanking region (including promoter) from 1st to 57th bp, the coding sequence from 58th to 810th bp and the remaining portion refers to 3′ flanking region from 811th to 1068th bp of this gene. Size of the ORF in *POTM1* gene is 752 bp (58 to 810) including stop codon.

POTM1 *protein:* The polypeptide of POTM1 (Protein id: AAA92839) consists of 250 amino acids which includes various sites such as DNA-binding site [nucleotide binding] on amino acids 2..4,6,8,13,15,19..20,23..24,26..27,30..31,33..34,38; Dimerization interface site [polypeptide binding]: on amino acids 21,28..29,32..33,35..36,39,44,46,48,54,56,63,66..67,70,73..74; Putative phosphorylation site [posttranslational modification]: on amino acid 59; MADS-box domain from amino acid 2-74 and K-box domain from amino acid 83-173.

4.1.2 Sequence analysis of the different regions of POTM1 by BLASTn

5′-flanking region as a query sequence: The nucleotide sequence of *POTM1* gene was analyzed by NCBI BLAST tools. When BLASTn was done for 5′ flanking region of *POTM1* gene, it shows largest promoter part in all potato genes available in database. It shows divergence in distal part of promoter region of 5′ flanking region of POTM1 gene. The 57-bp 5′-flanking region of *POTM1* gene was found to be close (100 % sequence identity) to a gene copy in *Solanum tuberosum* transcription factor (*POTM1-1*) mRNA, complete cds (GenBank ID: U23757.1). However, sequence divergence was prominent if compared with some known

POTM1 genes from potato, since the sequence identity values were 98.02% (GenBank ID: NM 001288213.1), 97.60% (GenBank ID: AF002666.1), 93.31% (GenBank ID: XM_015224062.2), 92.27% (GenBank ID: XM_026031307.1), 93.15% (GenBank ID: AY098732.1), 87.91% (GenBank ID: NM_001247244.2), 88.18% (GenBank ID: BT013224.1). Query coverage ranged from 100% to 73% in the BLAST search data for these sequences.

Coding region as a query sequence: When BLASTn was done for coding region of *POTM1-1* gene; it showed significant homology with other gene sequences of *POTM1-1* genes. The 1068 bp coding region was found to be identical (100% sequence identity with 100% query coverage) to a gene copy in *Solanum tuberosum* transcription factor (POTM1-1) mRNA, complete cds (GenBank ID: U23757.1), 98.02% sequence identity with 97 % query coverage to a gene copy in *Solanum tuberosum* transcription factor (*POTM1-1*), mRNA (GenBank ID:NM 001288213.1), 97.60% sequence identity with 92% query coverage to a gene copy in *Solanum commersonii* MADS box transcription factor (SCM1) mRNA, complete cds (GenBank ID:AF002666.1), 93.31 % sequence identity with 80% query coverage to a gene copy in PREDICTED: *Solanum pennellii* agamous- like MADS-box protein AGL8(LOC107023380), transcript variant X1, mRNA (GenBank ID: XM_015224062.2), 92.27% sequence identity with 77% query coverage to a gene copy in PREDICTED: *Solanum lycopersicum* TDR4 transcription factor (FUL1), transcript variant X2, mRNA (GeneBank ID: XM_026031307.1), 93.15% sequence identity with 73% query coverage to a gene copy in *Lycopersicon esculentum* TDR4 transcription factor mRNA, complete cds (GeneBank ID: AY098732.1), 87.91% sequence identity with 96% query coverage to a gene copy in Solanum lycopersicum agamous-like MADS-box protein AGL8 homolog (FUL1),mRNA (GeneBank ID: NM_001247244.2), 88.18% sequence identity with 94% query coverage to a gene copy *Lycopersicon esculentum* clone 134464F, mRNA sequence (GenBank ID: BT013224.1)

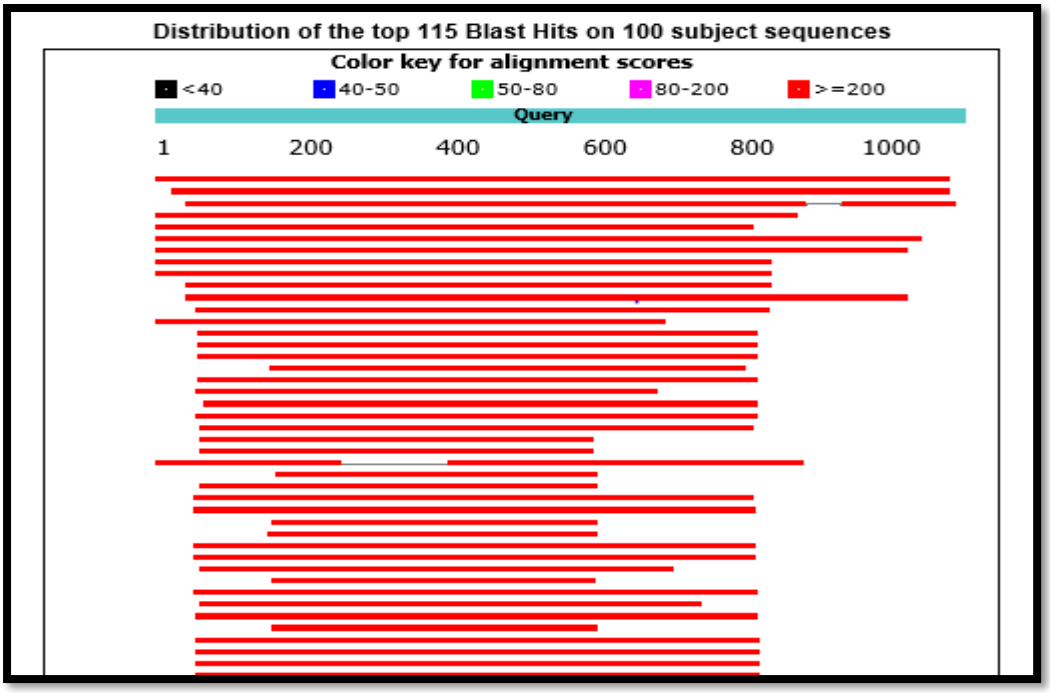

Fig.7 BLASTn analysis of *POTM1*

Table 6: Details of homology with other gene sequences of *POTM1* genes

Species name	**Accession no.**	**ORF length (in base pairs)**	**Identity %**	**Query coverage %**	**Total no. of base pairs (bp)**
Solanum tuberosum	NM 001288213.1	752(36-788)	98.02%	97%	1062
Solanum commersonii	AF002666.1	752(32..784)	97.60%	92%	1079
Solanum pennellii	XM_015224062.2	752(182..934)	93.31%	80%	1153
Solanum lycopersicum	XM_026031307.1	734(142..879)	92.27%	77%	1046
Lycopersicon esculentum	AY098732.1	737(25..762)	93.15%	73%	1072
Solanum lycopersicum	NM_001247244.2	737(107..844)	87.91%	96%	1086
Lycopersicon esculentum	BT013224.1	998(43..1041)	88.18%	94%	1082

4.1.3 BLASTp analysis of POTM1

When BLASTp was done for POTM1 protein (GenBank protein ID: AAA92839); it showed significant homology with other forms of POTM1 proteins. The 250-amino acid sequence was found to be identical (100% sequence identity with 100% query coverage) to amino acid sequence in agamous-like MADS-box protein AGL8 homolog (*Solanum tuberosum*) (GenBank protein ID: NP_001275142.1), 94.38 % sequence identity with 99 % query coverage to the amino acid sequence in agamous-like MADS-box protein AGL8 homolog (*Solanum lycopersicum*) (GenBank protein ID: NP_001234173.2), 93.98 % sequence identity with 99% query coverage to the amino acid sequence in TDR4 transcription factor (*Solanum lycopersicum*) (GenBank protein ID: AAM33098.1), 90 % sequence identity with 100% query coverage to the amino acid sequence in Agamous –like MADS-box protein AGL8- like protein (*Capsicum chinense*) (GenBank protein ID: PHU15370.1), 90% sequence identity with 100% query coverage to the amino acid sequence in PREDICTED: agamous-like MADS-box protein AGL8 homolog isoform X1(*Capsicum annuum*) (GenBank protein ID: XP_016555896.1), 83.40% sequence identity with 100% query coverage to the amino acid sequence in MADS box transcription factor (Petunia x hybrid) (GenBank protein ID: AAF19721.1), also 84.8% sequence identity with 99% query coverage to the amino acid sequence in PFFUL1 (*Physalis pubescens*) (GenBank ID: AGT21642.1).

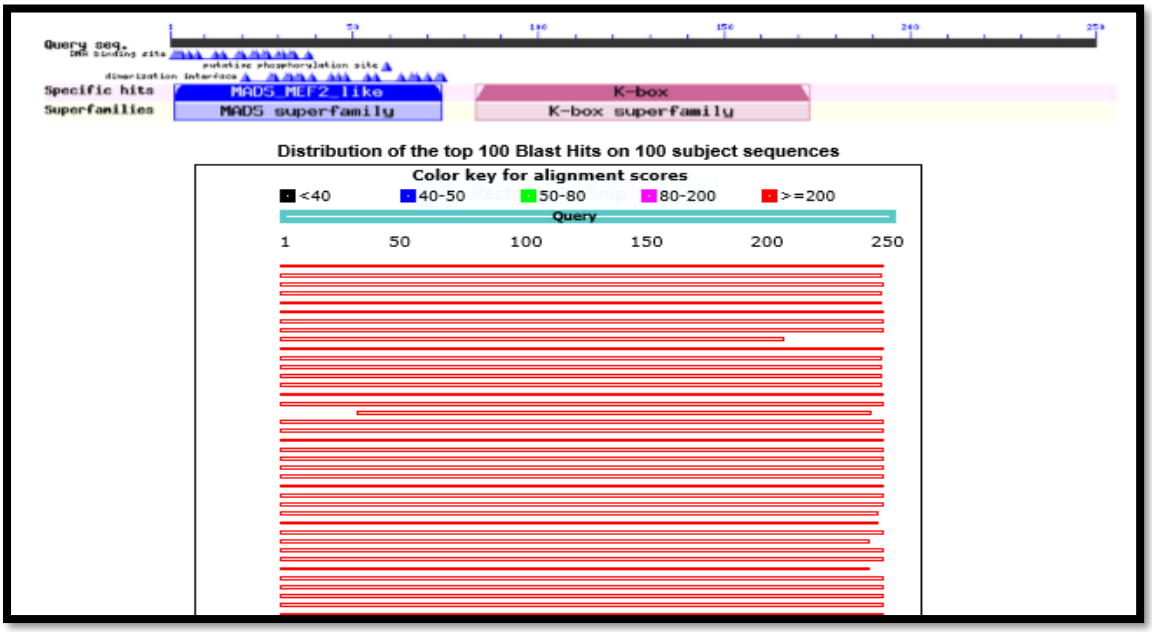

Fig. 8 BLASTp analysis of POTM1

Table 7: Details of some homologous POTM1 sequences as available in the database

Species name	**Accession no.**	**Max. score**	**Identity %**	**Query coverage %**	**Amino acids**
Solanum tuberosum	NP_001275142.1	516	100%	100%	250
Solanum lycopersicum	NP_001234173.2	483	94.38%	99%	245
Solanum lycopersicum 2	AAM33098.1	481	93.98%	99%	245
Capsicum chinense	PHU15370.1	430	90.44%	100%	251
Capsicum annuum	XP_016555896.1	427	90.04%	100%	251
Petunia x hybrid	AAF19721.1	420	83.40%	100%	246
Physalis pubescens	AGT21642.1	412	84.80%	99%	237

4.1.4 Multiple sequence alignment using of different POTM1 of potato (Solanum tuberosum L.) using Constraint-based Multiple Alignment Tool (COBALT)

Multiple sequence alignment was performed for total of six MADS box sequence from potato (*Solanum tuberosum*) using GenBank ID: U23757, i.e., *St*MADS1, *St*MADS2, *St*MADS3, *St*MADS4, *St*MADS5 and *St*MADS6 using multiple sequence alignment tool COBALT as shown in **Fig. 9**. MSA reveals the variations in both N and C-terminus. Here, Query refers to StMADS-box.

Fig. 9 Multiple sequence alignment of amino acid sequences of six different forms of MADS protein. The alignment is based on COBALT tool.

4.1.5 Significant protein motifs in POTM1 (GenBank Protein ID: AAA92839)

The predicted amino acid sequence of *POTM1* was examined for the prediction of some protein motifs using the following program MY HITS: (http://myhits.isbsib.ch/cgibin/motif_scan). A number of motifs were predicted as shown in **Fig. 10**

MGRGRVQLKR IENKINRQVT FSKRRSGLLK KAHEISVLCD AEVGLIVFST KGKLFEYAND 60 SCMERLLERY ERYSFAERQL VPTDHTSPGS WTLEHAKLKA RLEVLQRNQK HYVGEDLESL 120 NMKELQNLEH QLDSALKHIR SRKNQLMHES ISVLQKQDRA LQEQNNQLSK KVKEREKEVA 180 QQNQWDQQNH EINSSTFVLP QQLDSPHLGE AYQNTNVVDN GEVEGGNSSQ QQGAANNTVM 240 PQWMLRHLNG 250

Fig. 10 Amino acid sequence of POTM1 (GeneBank protein id: AAA92839). Some protein motifs are highlighted using different colors.

The amino acid sequence and position of the individual protein motifs are presented in **Table 8.**

Table 8: Position and motif sites of POTM1

S. No.	Sites	Sequence	Amino acid position	Color
1.	N-glycosylation site	NDSC (green)	59-62	Orange
		NSST	193-196	
		NSSQ	227-230	
		NNTV	236-239	
2.	CAMP- Phosphorylation site	KRRS	23-26	Purple
3.	Casein kinase- □□ phosphorylation site	SCME	61-64	Dark blue
		SFAE	74-77	
4.	N-myristoylation site	GGNSSQ	225-230	Grey
		GAANNT	233-238	
5.	Protein kinase C phosphorylation site	SKR	22-24	Olive green
		STK	49-51	
		SRK	141-143	
		SKK	169-171	
6.	MADS-box domain	RGRV..……LFEY	3-57	Red
		MGRGR……YANDS	1-61	
7.	Bipartite nuclear localization signal	KRIENKINRQVTFSKRRS	9-26	Green
		KRIENKINRQVTFSKRR	9-25	
8.	K-box domain profile	PGSW……REKE	88-178	Yellow
		FAERQ.....KVKE	75-174	
9.	SRF-type transcription factor (DNA-binding and dimerisation domain)	KRIEN……FEYAN	9-59	Light blue

In the above POTM1 sequence mainly 20 protein motifs were found. Four motifs (NDSC, NSST, NSSQ, and NNTV) were found on N- glycosylation site which are highlighted with orange color.

The functions of N-Glycolysation sites are to modify appropriate residues of asparagine of proteins with oligosaccharides structures, to influence their bioactivities and properties. The biosynthesis of N-glycoprotein involves a glycosyltransferases, multitude of enzymes, and glycosidases, encoded by distinct genes. One motif (KRRS) was found on cAMP-Phosphorylation site which are highlighted with purple color. From Casein kinase II phosphorylation site, two motifs (SCME and SFAE) were found which are highlighted by dark blue color. The transfer of phosphate to substrate peptide is catalyzed by Casein Kinase Ⅱ. Two N-myristoylation sites (GGNSSQ and GAANNT) were found which are grey in colored. In the modification of lipidation the site N-myristoylation helps i.e., in attachment of amide bond to alpha-amino group of N-terminal residue of glycine. It plays role in signal transduction and membrane targeting in plant which are responsible to environmental stress. From Protein kinase C phosphorylation site four motifs (SKR, STK, SRK, and SKK) were found which are highlighted by olive green color. The enzyme proteinase kinase C are cascade of signal transduction which are involved in controlling other proteins functions through the hydroxyl group phosphorylation of amino acid residues serine and threonine. There were also two motifs (RGRV…..LFEY, MGRGR…...YANDS) found at MADS- box domain signature which was colored red. . The MADS-box domain represents the DNA-binding domain; has functions in the dimerisation of protein and nuclear localization (Treisman, 1995). Also two motifs (KRIENKINRQVTFSKRRS, KRIENKINRQVTFSKRR) were found at Bipartite nuclear localization signal which was highlighted with green color words. Three motifs (PGSW…REKE, FAERQ…KVKE) were found at K-box region highlighted with yellow color. Other than these, one motif (KRIEN…FEYAN) was also found at SRF- type transcription factor (DNA- binding and dimerisation domain) highlighted light blue in color.

4.1.6 Salient biochemical attributes of POTM1 protein (GenBank Protein ID: AAA92839)

The entire 250 amino acids sequence of POTM1 protein was analyzed by using protparam tool of ExPASy resource portal under Swiss Institute of Bioinformatics (SIB) which revealed, some of the important biochemical attributes. The calculated molecular weight (MW) of the POTM1 was found to be 28.922kDa with a predicted isoelectric point (pI) of 8.77. Predicted formula of POTM1 protein was $C_{1247}H_{2010}N_{386}O_{391}S_8$. Out of its total 250 amino acids, 35 were strongly

basic (+) (Arg + Lys), 32 were strongly acidic (-) (Asp + Glu) hence, giving positive charge to protein. The instability index (II) was computed as 52.70, which classified the protein as unstable. The amino acid composition data revealed that some of the amino acids such as Asn (7.6%), Ile (2.8%), Lys (7.2%), Phe (2.0%), Ser (7.6%), Tyr (2.0%), Val (6.4%), Ala (4.8%), Arg (6.8%), Asp (3.6%), Cys (0.8%), Gln (10.0%), Glu (9.2%), Gly (5.2%), His (4.0%), Met (2.4%), Pro (2.0%), Thr (3.2%), Trp (1.2%) occurred more frequently as compared to their average occurrence; whereas the amino acids namely Arg (3.9%), Cys (0.4%), Glu (4.8%), Gly (6.4%), Leu (8.7%), Met (3.5%), Pro (4.2%) and Thr (6.1%) occurred less frequently (Doolittle 1989). The estimated half life of POTM1 protein is 30 hours (mammalian reticulocytes, in vitro), 20 hours (yeast, in vivo), 10 hours (Escherichia coli, in vivo) as predicted in this analysis and Grand average of hydropathicity (GRAVY) was found to be -0.923.

Hydropathy plot of POTM1

The hydropathy profile was generated for POTM1 protein using the ProtScale tool based on the Kyte- Doolittle scale.

Hydropathy plot of POTM1 protein revealed that amino acid sequence shared an alternative pattern of hydrophobicity and hydrophilicity. For example: amino acids 5-34 were hydrophilic whereas amino acids 35-51 were hydrophobic followed by hydrophilic at 52-100 and again hydrophobic trend was maintained from 101-102, hydrophilic at amino acids 103-148 followed by hydrophobic at 149-151, again hydrophilic at 152-194 and hydrophobic at 195-196, hydrophilic at 197-198 and hydrophobic at 199-201 and again hydrophilic trend to 202-236 followed by hydrophobic at 237, hydrophilic at amino acids 238-240 and hydrophobic at 241-242 and again trend to hydrophilic from amino acids 243-246 in polypeptide POTM1.

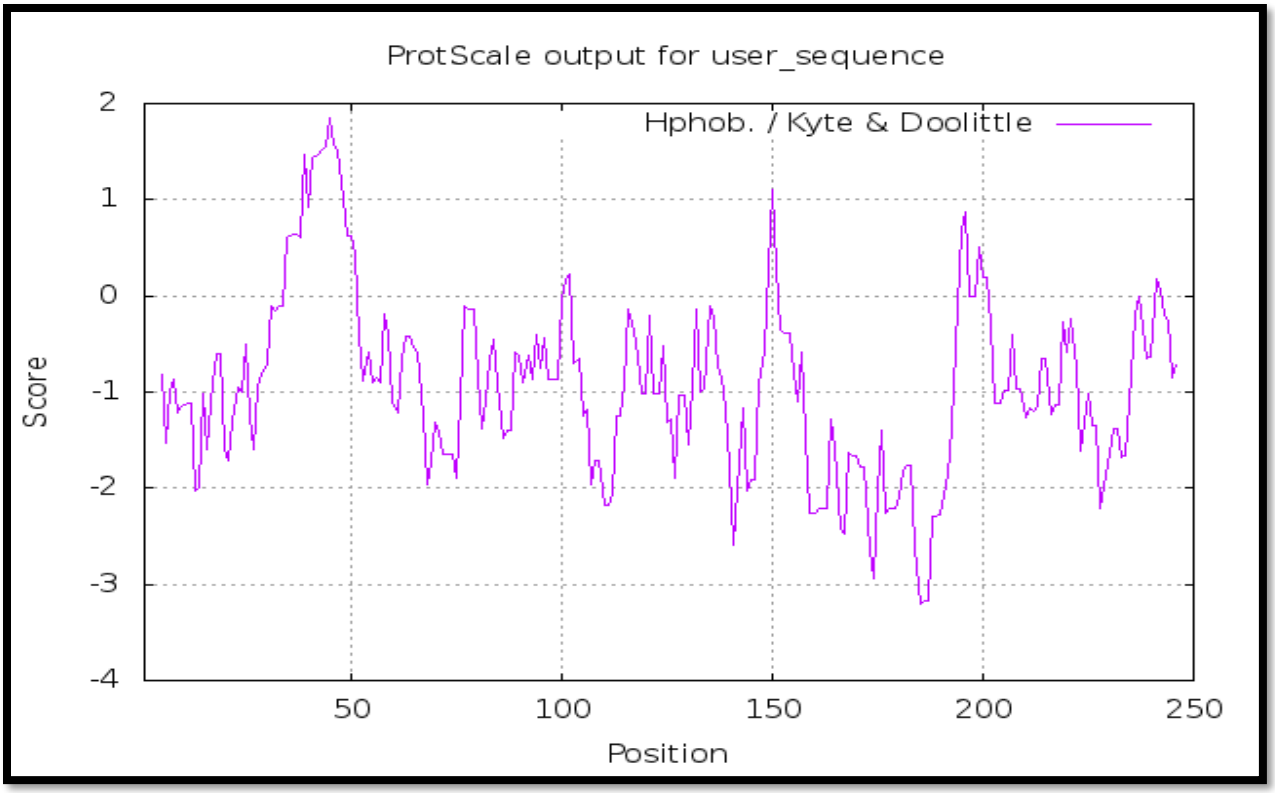

Fig. 11 Hydropathy plot of POTM1 protein.

4.1.7 Amino acids composition analysis in the POTM1

Almost all the standard amino acids are present in the POTM1 in varied amounts. The composition of amino acid of POTM1 was inspected and are presented in **Table 9** which revealed deviation if compared with their average occurrence in the natural proteins. The increase or decrease in the amount of amino acid in a protein changes its structure and functionality. For example, Histidine and Cysteine occur relatively at less frequency in POTM1; and some hydrophobic amino acids such as Alanine, Asparagine, Leucine, Serine, Valine and Isoleucine. Alanine and Valine occur more in these isoforms. All these significant changes in the amino acid composition could have profound effects on the structure and function of the POTM1 isoforms.

Table 9: Analysis of some amino acid composition (in %).

Amino acids	Average occurrence	Occurrence of POTM1
Alanine (A)	3	8.4%
Arginine (R)	5.1	2.9%
Asparagine (N)	4.3	7.1%
Aspartic acid (D)	9	5.5%
Cysteine (C)	1.9	0.6%
Glutamine (Q)	4	4.2%
Glutamic acid (E)	6	5.2%
Glycine (G)	8	4.5%
Histidine (H)	2.3	0.6%
Isoleucine (I)	5.3	7.1%
Leucine (L)	12	8.1%
Lysine (K)	5.9	7.4%
Methionine (M)	4	1.9%
Phenylalanine (F)	3.9	5.5%
Proline (P)	5.2	2.9%
Serine (S)	6.8	8.1%
Threonine (T)	10	4.9%
Tryptophan (W)	2	1.9%
Tyrosine (Y)	3.2	4.5%
Valine (V)	6.6	8.4%

4.1.8 Comparison between the different isoform of POTM1

POTM1 have some biochemical attributes which play a very important role in the functioning of the proteins. The comparison between different isoforms of POTM1 as reference is given in **Table 10**. The instability index gives the estimation of protein stability in test tube. A weight value comes from this technique which tells about the stability of the protein. Proteins with instability index less than 40 are considered to be stable and the proteins with instability index

above 40 are unstable. Here, POTM1 ranges from 86.7 to 22.68, telling that all the polypeptides of POTM1 are not stable.

The aliphatic index occupied by aliphatic side chains (A, V, I, L) is considered as positive factor and is defined as relative volume of a protein for the increase of thermal stability of globular proteins. In POTM1 it was found to be in the range 112.93 – 30.54 shown in **Table 10.**

Table 10: Comparison of different isoform of POTM1

Sr. No.	Name	Transcript ID	Molecular weight (in KDa)	No. of amino acids	Total number of atom	Instabity index	pI value	Aliphatic index	Gravy
1.	StMADS1	PGSC0003DMT400010451	28.93	250	4045	50.86	8.77	77.96	-0.923
2.	StMADS2	PGSC0003DMT400017416	32.46	287	4589	61.58	5.88	85.61	-0.757
3.	StMADS3	PGSC0003DMT400000028	25.88	224	3655	54.77	9.08	76.52	-0.820
4.	StMADS4	PGSC0003DMT400000383	28.88	255	4043	47.31	8.74	79.18	-0.619
5.	StMADS5	PGSC0003DMT400068325	27.31	234	3837	59.22	9.68	73.72	73.72
6.	StMADS6	PGSC0003DMT400073134	28.58	248	4001	56.87	9.46	73.55	-0.925
7.	StMADS7	PGSC0003DMT400009112	26.20	225	3680	58.36	9.16	78.84	-0.881
8.	StMADS8	PGSC0003DMT400003485	28.35	247	3959	62.70	9.44	73.40	-0.917
9.	StMADS 9	PGSC0003DMT400003483	28.49	246	3990	43.84	8.24	82.03	-0.719
10.	StMADS10	PGSC0003DMT400062319	14.02	119	1977	45.06	9.81	78.66	-0.484

11.	StMADS11	PGSC0003DMT400083739	25.25	221	3563	50.54	8.74	83.39	-0.748
12.	StMADS12	PGSC0003DMT400039313	23.31	200	3338	64.74	9.91	98.50	-0.704
13.	StMADS13	PGSC0003DMT400063313	28.72	248	4006	58.08	9.11	70.40	-0.948
14.	StMADS14	PGSC0003DMT400063315	28.41	246	3972	50.55	8.84	79.35	-0.661
15.	StMADS15	PGSC0003DMT400009510	26.36	228	3723	40.28	9.18	88.03	-0.708
16.	StMADS16	PGSC0003DMT400024247	26.52	235	3776	44.92	6.50	90.51	-0.503
17.	StMADS17	PGSC0003DMT400072891	27.91	242	3903	55.40	8.34	78.97	-0.783
18.	StMADS18	PGSC0003DMT400072888	28.61	244	4018	58.40	8.30	83.93	-0.848
19.	StMADS19	PGSC0003DMT400056363	27.56	241	3863	43.39	8.99	79.75	-0.765
20.	StMADS20	PGSC0003DMT400065060	12.44	111	1772	37.53	10.22	89.46	-0.161
21.	StMADS21	PGSC0003DMT400013248	26.91	232	3791	58.73	9.31	82.03	-0.776
22.	StMADS22	PGSC0003DMT400044552	28.57	254	3997	54.55	9.22	75.63	-0.822
23.	StMADS23	PGSC0003DMT400019070	24.65	210	3453	48.42	8.48	79.81	-0.812

24.	StMADS24	PGSC0003DMT400058560	25.89	224	3714	48.62	9.33	89.20	-0.688
25.	StMADS25	PGSC0003DMT	24.85	214	3509	57.96	8.22	84.21	-0.641
26.	StMADS26	PGSC0003DMT400060995	25.79	229	3636	50.66	5.85	87.64	-0.571
27.	StMADS27	PGSC0003DMT400041773	27.17	238	3800	56.90	5.50	79.03	-0.873
28.	StMADS28	PGSC0003DMT400065614	27.06	232	3806	56.16	9.28	80.78	-0.833
29.	StMADS29	PGSC0003DMT400048352	12.98	116	1862	35.39	9.45	91.64	-0.412
30.	StMADS30	PGSC0003DMT400004875	21.99	188	3143	48.30	9.19	109.89	-0.136
31.	StMADS31	PGSC0003DMT400050265	16.05	142	2297	52.50	9.83	95.42	-0.208
32.	StMADS32	PGSC0003DMT400089046	98.44	84	1422	82.55	10.16	90.60	-0.177
33.	StMADS33	PGSC0003DMT400086843	96.09	82	1400	86.70	9.88	112.93	-0.254
34.	StMASD34	PGSC0003DMT400094997	17.85	158	2473	55.72	5.80	74.11	-0.669
35.	StMADS35	PGSC0003DMT400096271	16.88	151	2360	49.19	6.61	76.95	-0.548
36.	StMADS36	PGSC0003DMT400067385	17.44	155	2452	56.42	8.73	81.81	-0.463
37.	StMADS37	PGSC0003DMT400067393	10.59	94	1497	57.19	7.83	79.79	-0.511

38.	StMADS38	PGSC0003DMT400045797	12.40	108	1774	43.62	10.39	91.94	-0.278
39.	StMADS39	PGSC0003DMT400045802	25.94	225	3654	36.29	9.04	88.80	-0.267
40.	StMADS40	PGSC0003DMT400088968	12.84	110	1820	41.56	8.76	86.91	-0.482
41.	StMADS41	PGSC0003DMT400095957	15.11	129	2103	34.45	4.74	74.81	-0.685
42.	StMADS42	PGSC0003DMT400029458	34.14	303	4734	48.90	4.62	75.25	-0.470
43.	StMADS43	PGSC0003DMT400029433	47.07	421	6295	43.27	4.60	57.43	-0.657
44.	StMADS44	PGSC0003DMT400016092	21.61	197	3038	47.23	6.84	79.29	-0.569
45.	StMADS45	PGSC0003DMT400092385	22.03	197	3099	55.93	8.76	76.35	-0.702
46.	StMADS46	PGSC0003DMT400097135	21.15	189	2968	46.59	8.91	73.81	-0.623
47.	StMADS47	PGSC0003DMT400000210	15.99	143	2260	49.38	5.32	84.48	-0.397
48.	StMADS48	PGSC0003DMT400091428	20.07	172	2805	42.29	6.76	71.45	-0.717
49.	StMADS49	PGSC0003DMT400057855	18.55	164	2596	44.29	8.96	69.51	-0.599
50.	StMADS50	PGSC0003DMT400057853	17.32	155	2449	47.13	9.60	72.26	-0.496
51.	StMADS51	PGSC0003DMT400088168	17.22	155	2408	34.46	9.52	65.29	-0.585

52.	StMADS52	PGSC0003DMT400057849	17.25	154	2420	45.97	9.30	65.13	-0.642
53.	StMADS53	PGSC0003DMT400057844	18.22	163	2550	35.80	6.83	76.50	-0.360
54.	StMADS54	PGSC0003DMT400095091	17.53	155	2463	47.27	9.55	67.81	-0.674
55.	StMADS55	PGSC0003DMT400092429	17.078	155	2414	39.45	9.57	76.00	-0.458
56.	StMADS56	PGSC0003DMT400085569	12.59	116	1778	57.91	9.90	68.79	-0.428
57.	StMADS57	PGSC0003DMT400013105	24.26	213	3390	46.86	6.20	74.18	-0.611
58.	StMADS58	PGSC0003DMT400094816	25.05	221	3525	42.14	9.49	82.49	-0.456
59.	StMADS59	PGSC0003DMT400013136	21.99	197	3108	41.27	9.13	81.12	-0.404
60.	StMADS60	PGSC0003DMT400097081	11.67	101	1642	44.25	8.50	59.90	-0.858
61.	StMADS61	PGSC0003DMT400089159	18.84	164	2663	49.22	6.25	74.88	-0.806
62.	StMADS62	PGSC0003DMT400033861	21.07	182	3008	33.21	9.30	81.92	-0.703
63.	StMADS63	PGSC0003DMT400090024	18.91	171	2660	38.53	6.61	85.03	-0.352
64.	StMADS64	PGSC0003DMT400092871	15.75	140	2239	27.15	9.64	80.86	-0.554
65.	StMADS65	PGSC0003DMT400087813	20.64	182	2897	37.19	7.80	72.36	-0.665

66.	StMADS66	PGSC0003DMT400095980	20.09	173	2827	58.30	8.74	76.65	-0.701
67.	StMADS67	PGSC0003DMT400097080	17.74	158	2510	59.00	9.49	77.03	-0.561
68.	StMADS68	PGSC0003DMT400087767	20.09	175	2864	39.36	6.76	91.31	-0.501
69.	StMADS69	PGSC0003DMT400050830	97.92	90	1365	27.72	5.66	67.22	-0.714
70.	StMADS70	PGSC0003DMT400090655	19.96	174	2797	33.75	4.90	89.66	-0.456
71.	StMADS71	PGSC0003DMT400091464	19.79	176	2798	39.75	8.47	87.56	-0.335
72.	StMADS72	PGSC0003DMT400086589	40.93	501	5276	38.21	5.23	30.54	0.736
73.	StMADS73	PGSC0003DMT400094942	14.99	131	2126	53.67	9.44	92.21	-0.302
74.	StMADS74	PGSC0003DMT400091054	12.71	114	1805	34.06	9.91	93.07	-0.171
75.	StMADS75	PGSC0003DMT400085650	19.64	168	2775	50.42	9.97	77.74	-0.673
76.	StMADS76	PGSC0003DMT400086435	11.83	105	1704	45.46	10.64	92.76	0.044
77.	StMADS77	PGSC0003DMT400085261	19.06	164	2701	46.19	10.49	80.79	-0.720
78.	StMADS78	PGSC0003DMT400090556	19.30	167	2740	42.20	9.92	83.41	-0.470

79.	StMADS79	PGSC0003DMT400095668	13.60	122	1948	32.16	10.03	95.00	-0.225
80.	StMADS80	PGSC0003DMT400093117	20.28	180	2839	40.23	6.44	78.67	-0.501
81.	StMADS81	PGSC0003DMT400095190	20.64	180	2888	42.05	6.98	73.22	-0.626
82.	StMADS82	PGSC0003DMT400089582	18.56	161	2607	25.29	4.97	88.39	-0.492
83.	StMADS83	PGSC0003DMT400088010	18.48	161	2591	22.68	4.79	91.99	-0.446
84.	StMADS84	PGSC0003DMT400063794	25.60	231	3554	42.08	6.75	71.00	-0.463
85.	StMADS85	PGSC0003DMT400091172	18.75	162	2663	57.47	6.99	80.06	-0.651
86.	StMADS86	PGSC0003DMT400091277	20.89	180	2912	39.07	6.14	66.11	-0.811
87.	StMADS87	PGSC0003DMT400094024	20.84	183	2910	59.63	5.60	84.10	-0.542
88.	StMADS88	PGSC0003DMT400011427	19.84	171	2789	42.12	5.73	75.20	-0.782
89.	StMADS89	PGSC0003DMT400074941	17.40	150	2462	31.87	9.13	76.67	-0.784
90.	StMADS90	PGSC0003DMT400089966	19.69	170	2762	45.03	4.94	74.59	-0.729
91.	StMADS91	PGSC0003DMT400086554	16.51	144	2333	34.08	7.84	66.39	-1.042
92.	StMADS92	PGSC0003DMT400093647	16.73	144	2339	42.80	5.04	72.36	-0.653

93.	StMADS93	PGSC0003DMT400088611	18.83	165	2617	49.39	4.64	70.24	-0.748
94.	StMADS94	PGSC0003DMT400094043	17.17	149	2405	40.34	4.81	78.52	-0.744
95.	StMADS95	PGSC0003DMT400090714	11.64	101	1641	60.51	9.36	63.76	-0.760
96.	StMADS96	PGSC0003DMT400087801	16.59	144	2366	56.18	9.52	75.21	-0.724
97.	StMADS97	PGSC0003DMT400049905	19.56	173	2771	50.89	5.74	82.37	-0.664
98.	StMADS98	PGSC0003DMT400094062	23.70	204	3319	39.06	8.86	75.00	-0.577
99.	StMADS99	PGSC0003DMT400085930	12.13	106	1739	41.84	9.24	86.42	-0.433
100	StMADS100	PGSC0003DMT400093319	20.53	178	2884	34.03	7.02	77.70	-0.494
101	StMADS101	PGSC0003DMT400090103	20.48	178	2880	32.57	6.91	76.07	-0.452
102	StMADS102	PGSC0003DMT400086619	20.95	186	2944	41.21	6.84	76.51	-0.513
103	StMADS103	PGSC0003DMT400097714	16.74	150	2354	56.27	9.62	74.07	-0.639
104	StMADS104	PGSC0003DMT400090264	17.13	152	2405	49.45	9.36	71.84	-0.729
105	StMADS105	PGSC0003DMT400094407	23.68	211	3306	41.53	5.56	78.58	-0.711
106	StMADS106	PGSC0003DMT400088636	13.10	117	1832	37.05	9.60	72.48	-0.609
107	StMADS107	PGSC0003DMT400002920	20.31	176	2892	65.03	9.45	80.85	-0.615

108	StMADS108	PGSC0003DMT400094886	20.67	180	2897	44.77	6.92	74.22	-0.639
109	StMADS109	PGSC0003DMT400097162	18.78	165	2651	50.01	9.51	76.79	-0.728
110	StMADS110	PGSC0003DMT400097165	21.11	181	2962	47.19	5.05	75.41	-1.057
111	StMADS111	PGSC0003DMT400087771	21.13	181	2958	56.40	5.13	72.76	-1.066
112	StMADS112	PGSC0003DMT400064120	37.21	320	5132	39.92	7.59	56.66	-0.800
113	StMADS113	PGSC0003DMT400064083	15.86	139	2250	55.97	8.60	75.04	-1.011
114	StMADS114	PGSC0003DMT400015303	19.64	168	2814	33.16	7.67	100.30	-0.577
115	StMADS115	PGSC0003DMT400085892	20.77	181	2889	31.67	4.85	78.12	-0.675
116	StMADS116	PGSC0003DMT400019861	18.33	157	2582	31.15	8.79	74.46	-0.803
117	StMADS117	PGSC0003DMT400047919	24.93	219	3467	29.03	5.18	75.71	-0.753
118	StMADS118	PGSC0003DMT400091840	23.33	200	3323	29.60	8.86	90.15	-0.585
119	StMADS119	PGSC0003DMT400097491	27.23	235	3816	45.45	5.24	78.85	-0.799
120	StMADS120	PGSC0003DMT400068356	14.96	131	2135	51.64	8.61	84.73	-0.816
121	StMADS121	PGSC0003DMT400083172	14.98	131	2131	47.76	8.29	81.83	-0.874
122	StMADS122	PGSC0003DMT400071682	35.37	299	4938	45.73	7.69	72.34	-0.759

123	StMADS123	PGSC0003DMT400071001	44.33	388	6103	35.58	6.10	60.03	-0.787
124	StMADS124	PGSC0003DMT400085676	26.19	228	3640	36.97	5.34	77.81	-0.621
125	StMADS125	PGSC0003DMT400069110	15.08	130	2138	61.31	7.63	80.92	-0.952
126	StMADS126	PGSC0003DMT400086663	14.65	128	2097	53.99	7.59	89.06	-0.783
127	StMADS127	PGSC0003DMT400095005	19.63	172	2814	46.38	8.35	99.07	-0.422
128	StMADS128	PGSC0003DMT400094660	19.31	165	2785	37.77	9.19	99.27	-0.604
129	StMADS129	PGSC0003DMT400085905	25.13	219	3566	47.46	5.74	86.85	-0.663
130	StMADS130	PGSC0003DMT400085037	23.42	202	3352	43.71	8.50	92.23	-0.598
131	StMADS131	PGSC0003DMT400096078	25.18	219	3569	42.24	5.26	85.94	-0.686
132	StMADS132	PGSC0003DMT400094714	24.82	219	3500	38.39	6.77	81.55	-0.661
133	StMADS133	PGSC0003DMT400043360	14.98	131	2126	55.50	7.60	78.09	-0.974
134	StMADS134	PGSC0003DMT400043366	15.24	131	2169	56.23	8.91	80.23	-1.001
135	StMADS135	PGSC0003DMT400014105	20.53	177	2914	39.06	5.62	90.90	-0.736
136	StMADS136	PGSC0003DMT400090410	18.94	164	2707	41.41	5.06	104.57	-0.481

137	StMADS137	PGSC0003DMT400014107	20.55	179	2937	29.86	5.88	95.31	-0.605
138	StMADS138	PGSC0003DMT400086956	17.44	147	2480	52.29	9.33	78.23	-0.919
139	StMADS139	PGSC0003DMT400061724	42.88	383	6014	56.74	5.27	79.92	-0.409
140	StMADS140	PGSC0003DMT400061726	36.79	327	5168	53.89	6.27	72.45	-0.496
141	StMADS141	PGSC0003DMT400089454	28.31	245	3956	40.10	5.73	78.69	-0.637
142	StMADS142	PGSC0003DMT400077258	43.48	390	6123	58.49	6.48	78.46	-0.411
143	StMADS143	PGSC0003DMT400085256	42.46	378	5993	54.52	6.86	79.39	-0.343
144	StMADS144	PGSC0003DMT400088489	24.24	213	3404	60.27	5.76	74.93	-0.487
145	StMADS145	PGSC0003DMT400090953	38.42	336	5389	50.44	7.02	74.17	-0.535
146	StMADS146	PGSC0003DMT400095755	39.39	344	5526	53.61	7.02	74.13	-0.505
147	StMADS147	PGSC0003DMT400095426	40.58	349	5743	56.04	9.74	77.82	-0.586
148	StMADS148	PGSC0003DMT400095062	32.55	282	4602	61.13	9.19	75.96	-0.521
149	StMADS149	PGSC0003DMT400089787	20.45	174	2899	30.19	9.39	77.18	-0.798
150	StMADS150	PGSC0003DMT400091650	30.12	259	4194	27.36	9.40	64.29	-0.816

151	StMADS151	PGSC0003DMT400088654	37.45	327	5256	49.91	8.33	73.52	-0.557
152	StMADS152	PGSC0003DMT400090586	26.88	235	3802	49.62	8.49	77.96	-0.549
153	StMADS153	PGSC0003DMT400073719	30.11	261	4224	53.72	8.29	69.46	-0.642

4.1.9 Three-dimensional model prediction

To annotate the biological function of protein it is essential to know its 3D-structure complete. To model the complete sequence of POTM1 the server I-TASSER was used. It is an approach for hierarchical protein modeling based on an iterative implementation of the TASSER (Threading Assembly Refinement) program and the multiple threading alignments (Zhang Y (2008); Zhang Y (2007). The amino acid FASTA sequences from NCBI were taken and uploaded in the tool, many PDB files were generated to identify the suitable templates for modeling of POTM1, but it was inspected that no model were able to satisfy the query coverage. For that reason, I-TASSER server (http://zhang.bioin-formatics.ku.edu/I-TASSER) was used to predict the best model. I-TASSER algorithm complete methodology has been described in details in the following papers (Wu S, Skolnick J, Zhang Y (2007); Zhang Y (2009)). Restraints from templates which are identified by multiple threading programs were used by I-TASSER to build a full length model. The TM-score (template modeling score) calculations were implemented.

Quality estimation

The judgment of the quality of threading alignment is usually on the basis of Z-score of the alignment. It is defined as the energy score in units of standard deviation relative to the statistical average of all alignments. For the quality estimation of threading alignment in I-TASSER normalized Z-score parameter is implemented. Normalized Z-score < 1 depicts the better alignment. The top ten threading templates used by I-TASSER along with normalized Z-score are listed in **Table 11**.

Table 11: Threading templates along with normalized Z-score of the threading alignments.

Rank	PDB hit	Norm. Z-Score
1.	1n6Ja	1.13
2.	4ox0A	1.61
3.	1n6Ja	1.83
4.	1n6Ja	1.28
5.	6aayA	1.38
6.	1hbx	5.27
7.	4ox0A	1.30
8.	1hbx	3.75
9.	4ox0A	1.80
10.	1n6jA	1.06

Structural similarity

The structural similarity of protein is measured by global distance test score, root mean squared deviation and TM-score. The score of TM stays ranges from 0-1 where the higher value represents a greater similarity between the structures (Mughda *et al*., 2012). The top ten PDB structures of similar topology are listed in **Table 12** along with their TM-score.

Table 12: PDB structures structurally closest to model along with TM-score of the structural alignment

Rank	PDB hit	TM score
1.	1ciiA	0.442
2.	2ycuA	0.438
3.	6bcuA	0.426
4.	1rqgA	0.426
5.	4phuA	0.424

6.	3h0Ga	0.422
7.	4xnvA	0.421
8.	4ib4a	0.416
9.	1js4A	0.416
10.	1h7Xb	0.413

Confidence-score (C-score)

C-score (Confidence-score) is for the quality estimation of predicted models by I-TASSER. C-score of best model of POTM1 was -4.65. Lower the score; better is the model. **Fig. 12** depicts the 3D structure of POTM1

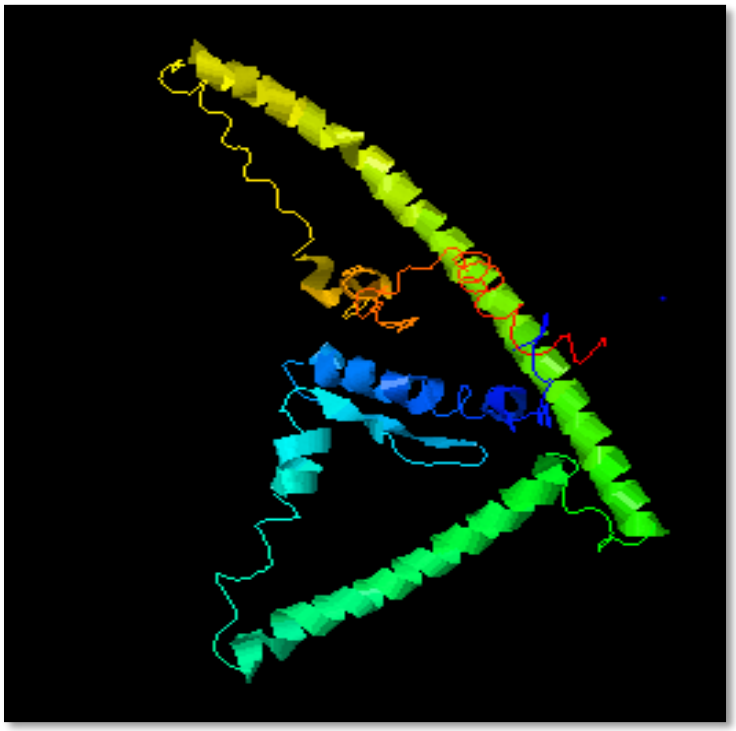

Fig. 12: Schematic representation in the predicted 3-D structure of POTM1 showing the arrangements of regions: α-helices, β-sheets, and loop.

Model quality

To estimate the improved model quality QMEAN (Quality model energy analysis) model quality estimation was employed. It is a composite scoring function which involves linear combination of four terms having statistical potential covering the major aspects of stability of protein. QMEAN Z-score by relating the absolute quality of a model to reference structures of similar size deposited in PDB i.e. model size +/- 10% provides an estimate. **Fig. 13** shows the plot for the estimation of quality of model by comparing the model of POTM1 with non-redundant set of PDB structures.

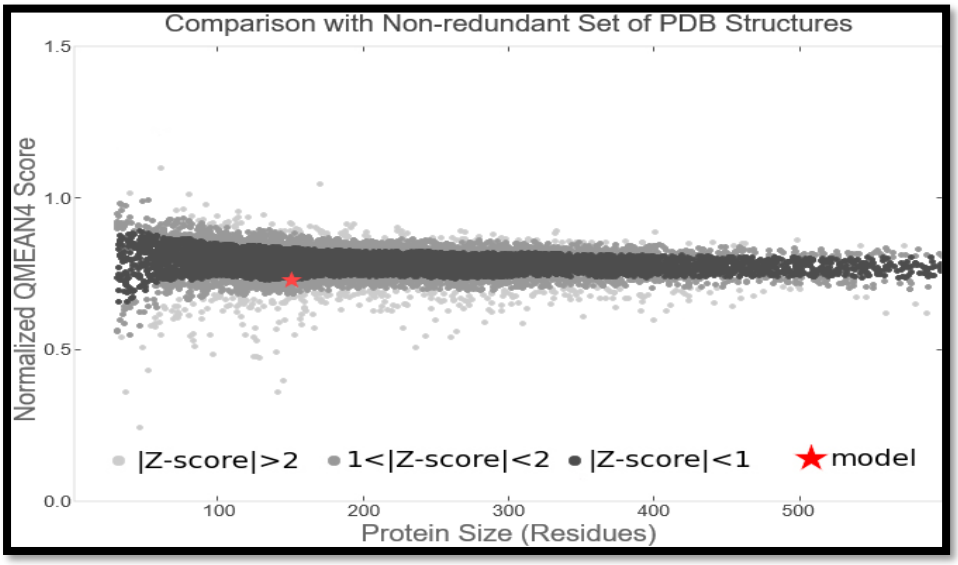

Fig. 13: The estimation of quality of model obtained by QMEAN server. The area built by different gray colored shades circles in the plot represents the reference structures from the PDB QMEAN score.

B-factor profile

B-factor is a value to show the extent of the inherent thermal mobility of atoms/ residue in proteins. This value is deduced from threading template proteins template proteins from the PDB in I-TASSER in combination with the sequence profiles derived from database. The reported B-factor profile in the figure below corresponds to the normalized B-factor of the target protein. An important way of validation of protein model is based on the energy level as shown in **Fig. 14**.

If the energy level of the sequences is less than 0 then it is considered to be the best model

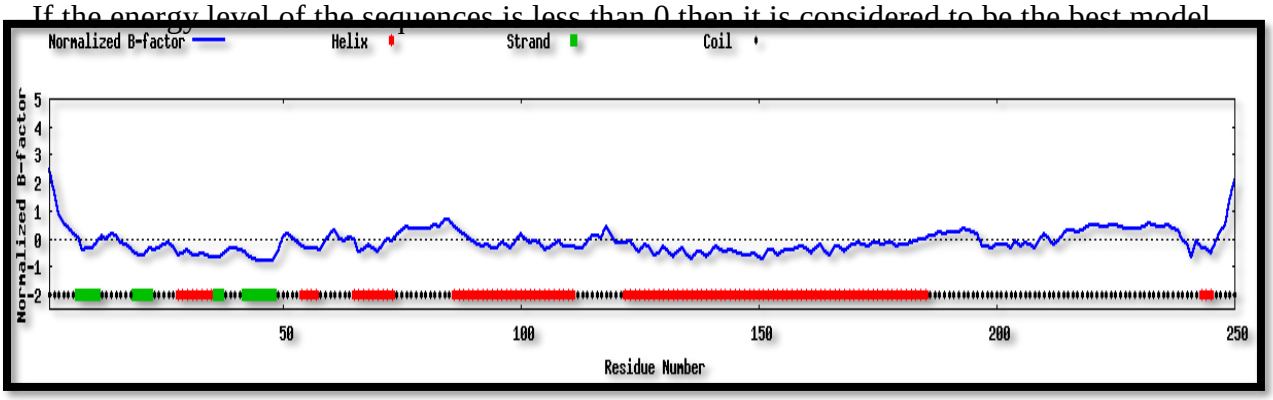

Fig. 14: B-factor profile for the predicted model.

4.2 Molecular studies on the *POTM1* gene in potato

In depth understanding of structure and function of *POTM1* gene and there expression patterns are important areas of research with regard to understand the complex mechanism of tuberization. Potato is an important system to carry out molecular biology works. In this, some efforts are made towards isolation of *POTM1* genes mainly, through PCR approach as presented in the following sections.

4.2.1 Isolation of total DNA from the potato cultivars

Total DNA was isolated from different organs of field grown Indian potato cultivar namely Kufri Chipsona-1 (CS-1) and foreign exotic reference Désirée (De). The quality of total DNA was checked by agarose gel electrophoresis as shown in following **Fig. 15**

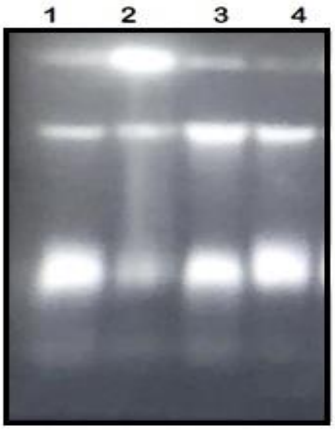

Fig. 15 Total DNA from the potato cultivars. Lane 1 and 2 –CS-I, Lane 3and 4- De

Above amplicon profile shows good amount of isolated DNA from different potato cultivars. Here we can see CS-1 and De in lane 1, 3 and 4 indicate prominent intensity of bands, whereas, band observed in CS-1 is lane 2 is less intense.

4.2.2 Purification of potato DNA

Total DNA was purified by DNase-free RNase treatment. Purified DNA samples were checked by Agarose gel electrophoresis as shown in **Fig. 16.** The distinct bands show the quality of the purified DNA present in CS-1 and De potato cultivar.

Fig. 16 Purified DNA from different potato cultivars. Lane 1-CS-1; Lane 2- De

4.2.3 Isolation of total RNA from different Potato organs

Total RNA was isolated from different organs of potato i.e., Flower, Leaf, Tuber, and Tuberizing Stolon. The crude RNA samples were checked by agarose gel electrophoresis as shown in **Fig. 17**. Ribosomal RNA bands were distinct indicating the intactness of total RNA preparations. Some of the RNA samples had genomic DNA impurities.

Fig. 17 Total RNA from various potato tissues. In Fig 13 Lane1- Leaf, Lane2- Tuberizing stolon, Lane3-Flower, Lane4- Tuber

4.2.4 Purification of total RNA

Total RNA was purified by RNase-free DNase treatment followed by the method of solvent extraction. Purified RNA samples were checked by agarose gel electrophoresis as shown in **Fig. 18**. The distinct bands show the inherent quality of the purified RNA present. Nanodrop was used to find A_{260}/A_{280} ratio to access the concentration of the prepared RNA samples as shown in **Table 13**.

Fig. 18 Purified RNA from different Potato organs. Lane 1- leaf, Lane 2-Tuberizing stolon, Lane 3-Tuber, Lane 4-Flower

Table 13: Quantification of concentration of RNA of different organs of Potato using Nanodrop Spectrophotometer

Potato organs	Volume of RNA soln (µL)	Concentration (µg/mL)
Leaf	1 µL	180 µg
Tuber	1 µL	710 µg
Tuberizing stolon	1 µL	640 µg
Flower	1 µL	230 µg

4.3.5 PCR using primers specific to POTM1 gene

Efforts were made to amplify *POTM1* gene(s) from a few potato (*Solanum tuberosum L.*) varieties by PCR under different conditions using specific primer pairs. For this purpose, total DNA preparations from the following potato (*Solanum tuberosum L.*) cultivars namely Kufri Chipsona-1 (CS-1), and Désirée (De) were used as template during PCR. For each specific primer pair, PCR was carried out under annealing temperature 55°C. Individual primer pair-specific amplicon profiles are shown in the following sections.

PCR amplification results using different potato genomic DNAs and the primer pair, PMF-0057, PMR1-0935 are shown in **Fig. 19**. Amplification occurred in both potato cultivars namely CS-1

and De where the size of the amplicon was around ~0.9 kb. The result suggest that *POTM1* gene is conserved in these cultivars, however, there might be minor sequence divergence. A single intact band was observed in the case of cultivar CS- I; whereas in case of De, two intact bands ~0.9 kb and 2.0 kb were observed. Very likely, 2 kb amplicon was a non specific product. The amplicon of 0.9 kb clearly suggest that it is devoid of introns(s).

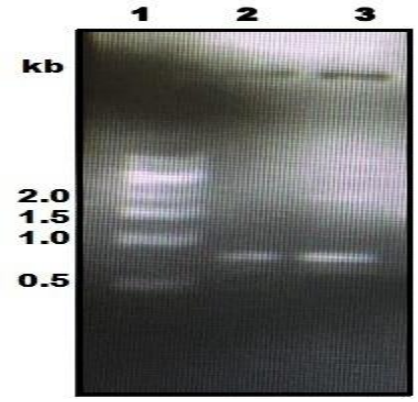

Fig. 19 PCR using different Indian Potato cultivars. Lane1- 500bp Ladder, Lane2-CS-1, Lane3- De

4.2.6 RT PCR using specific primer set of POTM1 in different potato organs

RT-PCR was carried out using total RNA from different potato tissues and the primer pair PMF-0057 with PMF-0935. The RT-PCR data clearly indicated that amplification occurred only in the case of 2 potato organs namely tuberizing stolon and Flower shown in **Fig. 20.** In Tuberizing stolon and Flower, the size of the amplicon was around 935 bps which was in the expected size range.

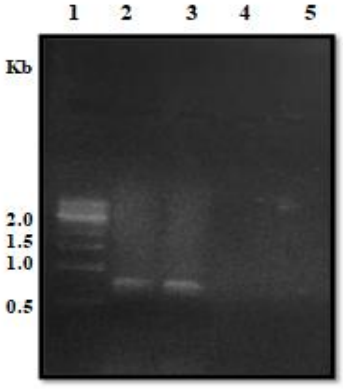

Fig. 20 PCR using different potato organs. Lane1- 500bp Ladder, Lane2- Tuberizing stolon, Lane3- Flower, Lane3- Leaf, Lane4- Tuber

4.2.7 Semi Quantitative RT PCR using a constitutive Actin gene

Actin is 43 kDa most abundant proteins which contribute to the contractile property and muscle cells. It is a major part of plant cytoskeleton which maintains internal architecture of the cell. It has three main isoforms (α-actin, β-actin and γ-actin). It participates in more protein-protein interactions than any other known proteins.

A semi quantitative RT PCR was done using a constitutive actin gene. PCR amplification was found in all the tissues indicate that it is a constitutive gene expressed in all organs more or less uniformly.

As evident from results, *POTM1* transcripts have been detected in tuberizing stolons and flowers which have been widely reported in literature. But we failed to detect any transcripts from roots and stems, which infers that *POTM1* may have role in development of reproductive organs. Efforts need to be made for cloning of the RT-PCR product in a suitable vector for sequencing and characterization. Moreover, such type of molecular approach could be employed for identification of various isoforms of *POTM1* gene in potato.

CONCLUSIONS

POTM1 belongs to MADS-box superfamily, which is considered to be a long distance travelling transcript. The transcripts of POTM1-1 are accumulated in leaf primordial and apical meristem and are transferred to tuberizing stolon and inflorescence meristem after getting the appropriate signal. Literature survey revealed that there was no research report on these transcription factors with regard to Indian potato cultivars.

- Although there are some *POTM1* sequence data available in the database at both nucleotide and amino acid level, but the salient sequence features and comparison were not highlighted in the previous research reports. Keeping this in mind, efforts are made on sequence analysis and comparison using the available *POTM1* sequence at both nucleotide and amino acid level. This exercise provided a clear and comprehensive idea about the sequence relatedness between the *POTM1* sequences.
- Now, we know the salient sequence features, conserved amino acids and some protein motifs in the *POTM1* sequences. Such type of information is not only helpful in understanding the evolutionary consequences but also important with regard to gene manipulation and protein engineering.
- Our study also revealed the crucial attributes of potato MADS-box genes, which would be useful to study the evolutionary history. Several bioinformatics tools were employed in order to study 3-Dimensional structure of *POTM1*.
- The good quality genomic DNA was isolated from two important potato cultivars.
- The different *POTM1* gene-specific oligonucleotide primers were used in PCR using genomic DNA as template to know the variation between the different cultivars. The cultivar-specific amplicons could be further studied in detail at molecular level.
- Total RNA was isolated and purified from different field-grown potato organs.
- Efforts were made to study the expression patterns/occurrence of *POTM1* transcripts in different organs of potato. This transcript was detected clearly in tuberizing stolons and flower as evident from the RT-PCR data.
- In conclusion, the thesis work made a consolidated base for further studying *POTM1* in the Indian potato cultivars at both biochemical and molecular level.

REFERENCES

- Airoldi CA, Davies B (2012) Gene duplication and the evolution of plant MADS-box transcription factors. *J Genet Genomics*.39:157–65
- Aksenova NP, Konstantinova TN, Golyanovskaya SA, Sergeeva LI, and Romanov GA (2012) Hormonal Regulation of Tuber Formation in Potato Plants. Russian Journal of *Plant Physiology* Vol. 59, 451-466
- Alisdair R. Fernie and Lothar Willmitzer (2001) Molecular and Biochemical Triggers of Potato Tuber Development. *Plant Physiology*. Vol. 127
- Arora R, Agarwal P, Ray S, Singh AK, Singh VP, Tyagi AK et al. (2007) MADS-box gene family in rice: genome- wide identification, organization and expression profiling during reproductive development and stress. BMC *Genomics*. 8:242
- Banerjee AK, Chatterjee M, Yu Y, et al. (2006) Dynamics of a mobile RNA of potato involved in a long-distance signaling pathway. *Plant Cell*. 18:3443-3457
- Barreau, C., Paillard, L., & Osborne, H. B. (2005). AU-rich elements and associated factors: are there unifying principles?. *Nucleic acids research*, *33*(22), 7138-7150.
- Birch PRJ, Bryan G, Fenton B, E.M. Gilroy, I. Hein, J.T. Jones, and I.K. Toth (2012) Crops that feed the world 8: Potato: are the trends of increased global production sustainable? *Food Security* 4:477-508
- Carrera E, Jackson SD, Prat S (1999) Feedback control and diurnal regulation of gibberellins 20-oxidase transcript levels in potato. *Plant Physiol* 119:765-773
- Chen H, Banerjee AK, Hannapel DJ (2004) The tendem complex of BEL and KNOX partners is required for transcriptional repression of GA20ox1. *Plant J*. 38:276-284
- Chen M, Ni M (2006) RFI2, a RING-domain zinc finger protein, negatively regulates CONSTANS expression and photoperiodic flowering. *Plant J*.46:823–33
- Cutter, E. (1982). Structure and morphology of the potato plant. In TTie Potato Crop, The Scientific Basis of Improvement, ed. Harris, P.M. Chapman
- De Folter S and Angenent GC (2006) Trans meets cis in MADS science. *Trends Plant Sci*. 11:224-31
- Derrigo, M. A. R. C. E. L. L. O., Cestelli, A. L. E. S. S. A. N. D. R. O., Savettieri, G. I. O. V. A. N. N. I., & Di Liegro, I. (2000). RNA-protein interactions in the control of stability and localization of messenger RNA. *International journal of molecular medicine*, *5*(2), 111-134.
- Dinant S, Lemoine R (2010) The phloem pathway: new issues and old debates. *Comptes rendus biologies*, 333(4), 307-319

- Doering-Saad C, Newbury HJ, Bale JS, Pritchard J (2002) Use of aphid stylectomy and RT-PCR for the detection of transporter mRNAs in sieve elements. *J. Exp. Bot*. 53:631–37
- Duan W, Song X, Liu T, Huang Z, Ren J, Hou X, et al. (2014) Genome-wide analysis of the MADS-box gene family in Brassica rapa (Chinese cabbage). *Mol Gen Genomics.* 290:239–55
- Dunoyer P, Brosnan CA, Schott G, Wang Y, Jay F, Alioua A, Himber C, Voinnet O (2010a) An endogenous, systemic RNAi pathway in plants. *EMBO J* 29:1699–1712
- Dutt S, Manjul AS, Raigond P, Singh B, Siddappa S, Bhardwaj V, Kawar PG, Patil VU, and Kardile HB (2017) Key players associated with tuberization in potato: potential candidates for genetic engineering. *DOI:10.1080/07388551.2016.1274876*
- Edger PP, Pires JC (2009) Gene and genome duplications: the impact of dosagesensitivity on the fate of nuclear genes. *Chromosom Res.*17:699–717
- Elvira, G., Massie, B., & DesGroseillers, L. (2006). The zinc- finger protein ZFR is critical for Staufen 2 isoform specific nucleocytoplasmic shuttling in neurons. *Journal of neurochemistry*, *96*(1), 105-117.
- Ewing, E. E. (1981). Heat stress and the tuberization stimulus. *American Potato Journal*, *58*(1), 31.
- Faivre-Rampant O, Cardle L, Marshall D, et al. (2004) Changes in gene expression during meristem activation processes in Solanum tuberosum with a focus on the regulation of a auxin response factor gene. *J Exp Bot*. 55:613-622.
- Fernald, M. L. 1970. Gray's maual of botany. A handbook of the flowering plants and ferns of the central and northeastern United States and adjacent Canada. 8 ed. (edition) American Book Company, New York. 1251 pp. (pages)
- Ferrandon D, Koch I, Westhof E, Nüsslein–Volhard, C (1997) RNA–RNA interaction is required for the formation of specific bicoid mRNA 3′ UTR–STAUFEN ribonucleoprotein particles. *The EMBO journal*, 16(7), 1751-1758
- Gao H, Wang Z, Li S, Hou M, Zhou Yao, Zhao Y, Li G, Zhao H and Haoli Ma (2018) Genome-wide survey of potato MADS-box genes reveals that StMADS1 and StMADS13 are putative downstream targets of tuberigen StSP6A. *Genomics* 19:726
- Gaupels, F., Buhtz, A., Knauer, T., Deshmukh, S., Waller, F., Van Bel, A. J., ... & Kehr, J. (2008). Adaptation of aphid stylectomy for analyses of proteins and mRNAs in barley phloem sap. *Journal of experimental botany*, *59*(12), 3297-3306.
- Gramzow L, Ritz MS, Theiβen G (2010) On the origin of MADS-domain transcription factors. *Trend Genet.* 26:149-53

- Gramzow L, Weilandt L, Theißen G(2014) MADS goes genomic in conifers: towards determining the ancestral set of MADS-box genes in seed plants. *Ann Bot.* 114:1407–29
- Grimplet, J., Martínez-Zapater, J. M., & Carmona, M. J. (2016). Structural and functional annotation of the MADS-box transcription factor family in grapevine. *BMC genomics*, *17*(1), 80.
- Gualerzi, C. O., Giuliodori, A. M., & Pon, C. L. (2003). Transcriptional and post-transcriptional control of cold-shock genes. *Journal of molecular biology*, *331*(3), 527-539.
- Ham, B. K., Brandom, J. L., Xoconostle-Cázares, B., Ringgold, V., Lough, T. J., & Lucas, W. J. (2009). A polypyrimidine tract binding protein, pumpkin RBP50, forms the basis of a phloem-mobile ribonucleoprotein complex. *The Plant Cell*, *21*(1), 197-215.
- Hannapel DJ, Chen H, Rosin FM, Banerjee AK, and Davies PJ (2004) Molecular controls of tuberization. *Am J Potato Res.* 81: 263-274
- Hart JK, Hannapel DJ (2002) In situ hybridization of the MADS-box gene POTM1 during potato floral development. *J Exp Bot* 53: 465-471
- Hawkes, J. G. 1990. The potato: Evolution, biodiversity & genetic resources. Smithsonian Institution Press, Washington, D.C. (district of columbia)
- Haywood V, Kragler F, Lucas WJ (2002) Plasmodesmata: Pathways for protein and ribonucleoprotein signaling. *Plant Cell* 14 (suppl.), S:303–S325
- Hecht V, Laurie R, Vander Schoor J, Ridge S, Knowles C, Liew L, et al. (2011) The pea GIGAS gene is a FLOWERING LOCUS T homolog necessary for graft transmissible specification of flowering but not for responsiveness to photoperiod. *Plant Cell.*23:147–61
- Helliwell CA, Wood CC, Robertson M, James Peacock W, Dennis ES (2006) The Arabidopsis FLC protein interacts directly in vivo with SOC1 and FT chromatin and is part of a high-molecular-weight protein complex. *Plant J.*46:183–92
- Jackson SD (1999) Multiple signaling pathways control tuber induction in potato. *Plant Physiol* 119:1–8
- Janssen R-P (2001) mRNA localization: message on the move. *Nat. Rev. Mol. Cell Biol.* 2:247–56
- Jena PK, Reddy ASN, Poovaiah BW (1989) Molecular cloning and sequencing of a cDNA for plant calmodulin: signal-induced changes in the expression of calmodulin. *Proc Natl Acad Sci USA* 86: 3644-3648
- Jose A Abelenda, Cristina Navarro and Salome Prat (2011) From the model to the crop: genes controlling tuber formation in potato. *Science Direct* 22: 287-292
- Kang S-G, Hannapel DJ (1995) Nucleotide sequences of novel potato (*Solanum tuberosum* L.) MADS-box cDNAs and their expression in vegetative organs. *Gene* 166: 329-330

- Kang S-G, Hannapel DJ (1996) A novel MADS-box gene of potato (Solanum tuberosum L.) expressed during the early stages of tuberization. *Plant Mol Bio* 31: 379-386
- Kang S-G, Kang H (2002) Characterization of potato vegetative MADS-box gene, POTM1-1, in response to hormone applications. *Journal of Plant Biol*. 45(4): 196-200
- Kanno A, Saeki H, Kameya T, Saedler H, Theissen G (2003) Heterotopic expression of class B floral homeotic genes supports a modified ABC model for tulip (Tulipa gesneriana). *Plant Mol Biol*. 52:831–41
- Kaufmann K, Melzer R, Theiβen G (2005) MIKC-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. *Gene.* 347:183-98
- Kehr J (2009) Long-distance transport of macromolecules through the phloem. *F1000 Biol Rep*, 1, 31
- Kim I, Pai HS (2009) Mobile macromolecules in plant development. *Journal of Plant Biology*, 52(3), 186-192
- King, M. L., Messitt, T. J., & Mowry, K. L. (2005). Putting RNAs in the right place at the right time: RNA localization in the frog oocyte. *Biology of the Cell*, *97*(1), 19-33.
- Koda, Y, E.A. Omer, T. Yoshihara, H. Shibata, S. Sakamura & Y. Okazawa (1988) Isolation of a specific potato tuber-inducing substance from potato leaves. *Plant and Cell Physiology* 29: 1047-1051
- Laufer, B. 1938. The American plant migration: Part I (1): The potato. Publications of the Field Museum of Natural History Anthropological Series 28(1):1-132.
- Lee H, Suh SS, Park E, Cho E, Ahn JH, Kim SG, et al. (2000) The AGAMOUS-lIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis. *Genes Dev*.14:2366–76
- Leseberg CH, Li A, Kang H, Duvall M, Mao L (2006) Genome-wide analysis of the MADS-box genes or RT-qPCR analysis in potato under abiotic stress. *Plant Methods*. 13:85
- Levy, D., and R.E. Veilleux (2007) Adaptation of potato to high temperatures and salinity- a review. *American Journal of Potato Research* 84: 487-506
- Litt A, Kramer EM (2010) The ABC model and the diversification of floral organ identity. *Semin Cell Dev Biol*. 21:129–37
- Lough TJ, Lucas WJ (2006) Integrative plant biology: Role of phloem long-distance macromolecular trafficking. Annu. Rev. *Plant Biol.* 57:203–232
- Lucas WJ, Bouche-Pillon S, Jackson DP, Nguyen L, Baker L, Ding B, Hake S (1995) Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. *Science* 270:1980–1983

- Lucas WJ. 1995. Plasmodesmata: intercellular channels for macromolecular transport in plants. Curr. Opin. Cell Biol. 7:673–80
- Ma H, Yanofsky MF, Meyerowitz EM (1991) *AGL*1-*AGL*6, an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. *Genes Devel* 5:484-495
- Mao L, Begum D, Chuang H-W, Budiman MA, Szymkowiak EJ, Irish EE, Wing RA (2000) JOINTLESS is a MADS-box gene controlling tomato flower abscission zone development. *Nature*.406: 910-913
- Michaels SD (2009) Flowering time regulation produces much fruit. *Curr Opin Plant Biol*.12:75–80
- Mokronosov, A.T., Chailakhyan, and M.Kh. (1990) Tuberization and Sink–Source Relations in Potato, Regulation of Potato Growth and Development. Eds., *Moscow: Nauka* 6–12
- Mugdha Srivastava, Gupta S.K., Abhilash P.C., Singh N. (2012) Structure prediction and binding sites analysis of curcin protein of Jatropha curcas using computational approaches. *J. Mol Modeling. PP* 2971-2979
- Nam J, DePamphilis CW, Ma H, Nei M (2003) Antiquity and evolution of the MADS-box gene family controlling flower development in plants. *Mol Biol Evol*.20:1435–47
- Navarro C, Abelenda JA, Cruz-Oro E, Cuellar CA, Tamaki S, Silva J, et al. (2011) Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. *Nature*. 478:119-22
- Ng, M. and Yanofsky, M. F. (2001) Function and evolution of the plant MADS-box gene family. *Nat. Rev. Genet.* 2, 186− 195
- Oparka KJ, Santa Cruz S (2000). The great escape: phloem transport and unloading of macromolecules. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 51:323–47
- Pinyopich A, Ditta GS, Savidge B, Liljegren SJ, Baumann E, Wisman E, et al. (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development. *Nature*.424:85–8
- Poehlman, J. M., & Sleeper, D. A. (1995). Breeding Hybrid Cultivars. Breeding Field Crops. USA, Forth Edition. *Relation to Diallel Crossing Sys-tems. Australia, J. Bio. Sci*, *9*, 463-493.
- Raices M, Macintosh GC, Ullao RM, et al. (2003) Sucrose increases calcium-dependent protein kinase and phosphatase activities in potato plants. *Cell Mol Biol* (Noisy-le-grand) 49:959-964
- Riechmann JL and Meyerowitz EM (1997) MADS domain proteins in plant development. *Biol Chem*. 378:1079-101
- Rosin FM, Hart JK, Horner HT, Davies PJ, Hannapel DJ (2003) Overexpression of a knox gene of potato alters vegetative development bydecreasing gibberellin accumulation. *Plant Physiol* 132: 106– 117

- Ruiz-Medrano R, Xoconostle-Cazares B, Lucas WJ (1999) Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. *Development* 126:4405–19
- Sarkar D (2008) The signal transduction pathways controlling in planta tuberization in potato: an emerging synthesis. *Plant Cell Rep* 27:1-8
- Sasaki T, Chino M, Hayashi H, Fujiwara T. 1998. Detection of several mRNA species in rice phloem sap. *Plant Cell Physiol.* 39:895–97
- Schmitz RJ, Tamada Y, Doyle MR, Zhang X, Amasino RM. Histone H2B (2008) Deubiquitination is required for transcriptional activation of FLOWERING LOCUS C and for proper control of flowering in Arabidopsis. *Plant Physiol.1*49:1196–204
- Schwarz-Sommer Z, Huijer P, Nacken W, Saedler H, Sommer H (1990) Genetic control of flower development by homeotic genes in Antirrhinum majus. *Science* 250: 931-936
- Sessions A, Yanofsky MF, Weigel D (2000) Cell-cell signaling and movement by the floral transcription factors LEAFY and APETALA1. *Science* 289:779–82
- Shu Y, Yu D, Wang D, Guo D, Guo C (2013) Genome-wide survey and expression analysis of the MADS-box gene family in soybean. *Mol Biol Rep*.40:3901–11
- Spooner D.M. and Knapp, S. (2013) *Solanum tuberosum* (http://solanaceaeesource.org/content/solanum-tuberosum). [12 December 2014]
- Spooner, D. M., & Salas, A. (2006). Structure, biosystematics, and genetic resources of potato. *Handbook of Potato Production, Improvement and Post-Harvest Management*, 1-39.
- Srikanth A, Schmid M (2011) Regulation of flowering time: all roads lead to Rome. *Cell Mol Life Sci*.68:2013–37
- Struik, P. C. (2007). Above-ground and below-ground plant development. In *Potato Biology and Biotechnology* (pp. 219-236). Elsevier Science BV.
- Tauberger E, Fernie AR, Emmermann M, Renz A, Kossmann J, Willmitzer L, Trethewey RN (2000) Antisense inhibition of plastidial phosphoglucomutase provides compelling evidence that potato tuber amyloplasts import carbon from the cytosol in the form of glucose-6-phosphate. *Plant J* 23:43-53
- Taylor MA, George LA, Ross HA, Davies HV (1998) cDNA cloning and characterization of an α-glycosidase gene from potato (Solanum tuberosum L.), *Plant J* 13:419-425
- Theissen G (2001) Development of floral organ identity: stories from the MADS house. *Curr Opin Plant Biol* 4:75-85
- Theissen, G., Becker, A., Di Rosa, A., Kanno, A., Kim, J. T., Munster T, Winter, K. U., and Saedler, H. (2000) A short history of MADS-box genes in plants. *Plant Mol. Biol.* 42, 115−149.
- Tian Y, Dong Q, Ji Z, Chi F, Cong P, Zhou Z (2015) Genome-wide identification and analysis of the MADS-box gene family in apple. *Gene*.555:277–90

- Tsuji H, Taoka KI, Shimamoto K (2011) Regulation of flowering in rice: two florigen genes, a complex gene network, and natural variation. *Curr Opin Plant Biol.*14:45–52
- Umaerus, M., 1987. True potato seed. Proceedings 10th Triennial Conference European Association for Potato Research, Aalborg, Denmark, pp. 72-102.
- Verhees J, van der Krol AR, Vreugdenhil D, van der Plas LHW (2002) Characterization of gene expression during potato tuber development in individuals and populations using the luciferase reporter system. *Plant Mol Biol* 50:653–665
- Vishal Vilas Shah, Nutan Dhanpal Shah, Prasad Vasantrao Patrekar (2013) Medicinal Plants from Solanaceae Family. *Research J. Pharm. And Tech.* 6(2): 143-151
- Vreugdenhil D, Xu X, Jung CS, van Lammeren AAM, Ewing EE (1999) Initial anatomical changes associated with tuber formation on single-node potato (Solanum tuberosum L.) cuttings: a re-evaluation. *Ann Bot* 84:675-680
- Wei B, Zhang RZ, Guo JJ, Liu DM, Li AL, Fan RC, et al. (2014) Genome-wide analysis of the MADS-box gene family in Brachypodium distachyon. *PLoS One.* 9:e84781
- Wei X, Wang L, Yu J, Zhang Y, Li D, Zhang X (2015) Genome-wide identification and analysis of the MADS-box gene family in sesame. *Gene*.569:66–76
- Weigel D and Meyerowitz EM (1994) The ABCs of floral homeotic genes. *Cell* 78:203-209
- Wu, S., Skolnick, J., & Zhang, Y. (2007). Ab initio modeling of small proteins by iterative TASSER simulations. *BMC biology*,*5*(1), 17.
- Xu X, van Lammeren AAM, Vermeer E, Vreugdenhil D (1998a) The role of gibberellins, Abscisic acid and sucrose in the regulation of potato tuber formation in vitro. *Plant Physiol* 117: 575-584
- Xu X, Vreugdenhil D, van Lammeren AAM (1998b) Cell division and cell enlargement during potato tuber formation. *J Exp Bot* 49:573-582
- Xu Z, Zhang Q, Sun L, Du D, Cheng T, Pan H, et al. (2014) Genome-wide identification, characterization and expression analysis of the MADS-box gene family in Prunus mume. *Mol Gen Genomics*.289:903–20
- Yoo BC, Kragler F, Varkonyi-Gasic E, Haywood V, Archer-Evans S, et al. (2004) A systemic small RNA signaling system in plants. *Plant Cell* 16:1979–2000
- Zhang S, Sun L, Kragler F (2009). The phloem-delivered RNA pool contains small noncoding RNAs and interferes with translation. *Plant physiology*, 150(1), 378-387
- Zhang, Y. (2008). Progress and challenges in protein structure prediction. *Current opinion in structural biology*, *18*(3), 342-348.
- Zhang, Y. (2009). Protein structure prediction: when is it useful?. *Current opinion in structural biology*, *19*(2), 145-155.