SP6A (Self-Pruning) gene in Potato (*Solanum tuberosum* L.): Structural features and expression patterns

A Dissertation submitted in partial fulfillment of the requirements for the award of degree

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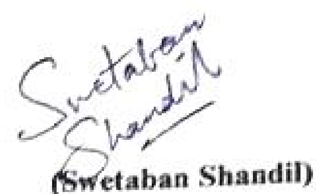
CANDIDATE'S DECLARATION

I, hereby declare that the work which is being presented in the dissertation entitled, "*SP6A (Self-Pruning) gene in Potato (Solanum tuberosum L.): Structural features and expression patterns*" in the partial fulfillment of the requirement for the award of degree of Master of Science in Biotechnology, TIET, 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, TIET, 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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This is to certify that the dissertation entitled "***SP6A (Self-Pruning) gene in Potato (Solanum tuberosum L.): Structural features and expression patterns***" submitted by Swetaban Shandil (Regd. No. 301701030) in partial fulfillment of the requirement for the award of the degree of Master in Science in Biotechnology, to TIET is a record of student's own work carried out by her under my guidance and supervision. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

The assistance and help received during the course of investigation has been fully acknowledged.

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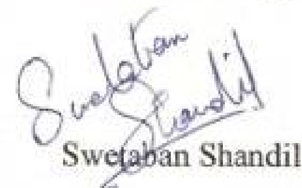

Swetaban Shandil

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LIST OF ABBREVIATIONS

Abbreviation	**Name**
BLAST	Basic Local Alignment Search Tool
BLASTp	BLAST for Proteins
Bp	Base-Pair
DEPC	Diethyl pyrocarbonate
DNA	De-oxy Ribo nucleic 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
NviiaCl	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
RNA	Ribo nucleic acid
rRNA	Ribosomal RNA
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
UTR	Untranslated region
µL	Microlitre
µg	Microgram

ABSTRACT

StSP6A gene codes for a long distance travelling RNA which acts as a mobile signal. It is expressed in both reproductive and vegetative organs in potato and other plants. It works as mobile signal which originates in leaf under short day conditions and further gets transported to the underground stolon tips to initiate tuberization. It is an important member of Self Pruning (SP) family of tomato (*Solanum lycopersicum*) and homologous to FT gene from *Arabidopsis thaliana and* CEN gene from *Antirrhinum*. Considerable progress has been made on *StSP6A* gene function associated with various stages of growth and development particularly in the *Solanacea*e family members. This study focused mainly on *StSP6A* gene since as this gene function gained importance significantly for its involvement in the process of tuberization in potato. Some *in silico* approaches were adopted to analyze the following aspects: sequence identity, sequence analysis, multiple sequence alignment, searching protein motifs, and 3-D modeling along with some other attributes. Most of the data are important as they were not reported earlier. Some experiments were also carried out to know the expression patterns of *SP6A* gene in different potato organs. Total RNA was isolated from field grown potato namely leaf, root, petiole, flower, small tuber, large tuber and tuberizing stolon. First, reverse transcription (RT) was carried out using organ specific individual total RNA samples and *SP6A* cDNA-specific oligonucleotide primer(s). Subsequently, the RT products were employed to carry out PCR. The size of the amplicon was~ 400 bp. Similar amplicon was also found using potato genomic DNA as template indicating the absence of intron sequences in the gene of study. RT-PCR data clearly indicated the presence of *SP6A* transcripts in the leaf and tuberizing stolon. However, the transcript could hardly be detected in other potato organs. In the context of Indian potato cultivars, this study would be useful particularly for studying the complex tuberization process.

Keywords: Self Pruning 6A, *Solanum tuberosum* (Potato), *Solanaceae*, Polymerase chain reaction (PCR), RT-PCR

CHAPTER-1 INTRODUCTION

1.1 About Potato (*Solanum tuberosum* L.)

Potato (*Solanum tuberosum* L.) is the largest non-grain crop in the world and is grown widely after rice, wheat and maize (Reddy et al., 2018). It is cultivated in approximately 150 nations in temperate and tropical areas, at sea level up to 4,000 m (Paul et al. 2012). It belongs to *Solanaceae* family which consists about 90 genera and 2,800 species (Spooner et al., 2014). Potatoes were brought to Spain in the end of 16th century and were spreaded across Europe in the following centuries (Hawkes et al., 1990). It is not only a commonly used vegetable but also used in starch, processed food production, alcoholic beverage production industries (Reddy et al., 2018). It is adopted for cultivation under sub-tropical conditions ((Ewing et al., 1996). It is a semi-perishable crop and can be stored for more than 6 months at 3–4 °C temperature (Zebarth et al. 2012).

1.2 Taxonomy of Potato (*Solanum tuberosum L.)*

The Solanum genus consists of approximately 2000 species, out of which more than 150 are tuber forming. Polyploidy ranges from diploids (2x) to hexaploids (6x). The prevalent potato is an autotetraploid with a 2n=4x=48 genomic make-up (Reddy et al., 2018).

1.3 History of Potato

Former research demonstrates that the center of potato origin is the central Andean region, indicating that South American Indians have been cultivating potatoes for millennia and that the tubers have been used as a prevalent food item. According to hypothesis by Hawkes (1990), potato was transported from Europe to North America via Bermuda 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. After that, Asia, Africa and Latin America have seen a drastic rise in potato manufacturing and demand.

In India, potato is cultivated with annual production of 41.55 million tons from an area of 1.97 million hectare which accounts for 25.5 percent of total production of vegetable in India 1

with 21.10 tonnes/ha of productivity. By 2020, it is estimated that, in India there will be a population of 1.3 billion and country will need potato production of around 49 million tones (Marwaha et al., 2010). No other significant food crop during this era has undergone such a change in manufacturing place. This dramatic development in developing nations reaffirms its growing significance as a food source for growing communities, rural jobs and revenue (http://www.fao.org/faostat/en/).

1.4 General Morphology

The potato is an herbaceous plant that differs from species to species in its development habit. The potato tuber is a part of a modified underground stem. Potato is reproduced primarily vegetatively by tubers and reproductively by botanical seeds, i.e. True Potato seeds (Struik 1999). In the early phase, the stem is generally erect but becomes swollen and later flat. The leaves are compound and pinnate irregularly. Buds are produced in the axil of leaves which are called as rhizomes. Potato is differentiated from all other species within the *Solanum* genus by' real potato' whose tuber is born on subterranean stolons that are real stems, not roots (Hawkes et al., 1994).

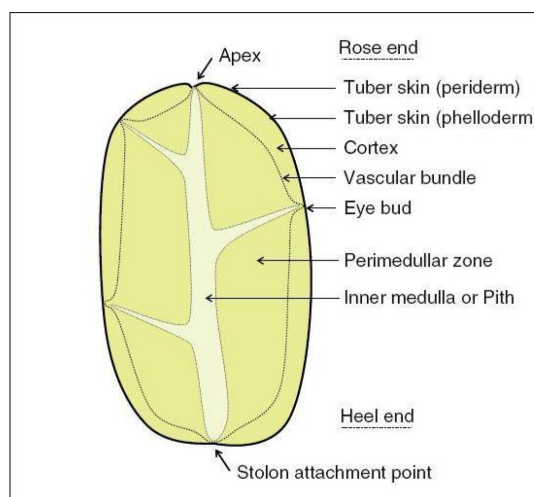


Fig. 1 Anatomy of a Tuber

(Ref: Herman J. van Eck, Potato Biology and Biotechnology: Advances and Perspectives*)*

The plant grows up to the height 0.4- 4.1m (Snooper and Knapp, 2013). The stems generally matter from hairless to compact hairy and may be purple, green or mottled green and purple. The

leaves are compound mostly with a leaflet which is a single terminal (Struik, 1999). The size of blader with the petioles ranges from 8-22×5-13 cm. The rhizomes which are often called as stolons are produced by the plants of *Solanum tuberosum* have primitive like leaves which are curved at the tip. They arise from the nodes of basal stem, which are basically beneath the ground with upto three rhizomes per nodes (Struik 1999). The tubers are spherical to ovoidical swellings of the rhizomes. 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 the surface of tuber with eyes (also called as scars of scale leaves) (Struik 1999). 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 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, 1978).

The terminal inflorescences are 5-11 cm long cymes that are usually found usually in the distal part of the plant (Spooner and Knapp 2013). The inflorescence can contain up to 25 flowers. The pentamerous flowers of 3-4 cm are all apparently ideal with the same length of styles. (Spooner and Knapp 2013). The corolla may be present in a variety of colors including white, pink, lilac, blue, violet and red violet (Spooner and Knapp 2013).The stamens have 0.1-0.2 cm long filaments and 0.3-0.8 cm long anthers (Spooner and Knapp 2013). Typically they are bright yellow or orange, except for male-sterile crops (Sleper and Poehlman 2006). The fruits are about 1-4 cm in diameter, are spherical to ovoid berries. On maturation, they become green or green tinged with white or violet bands (Spooner and Knapp 2013; Spooner and Salas 2006). There may be no seeds in the berry or it may rise to several hundred. These berries are poisonous due to presence of glycoalkaloids (Bailey et al., 1976).

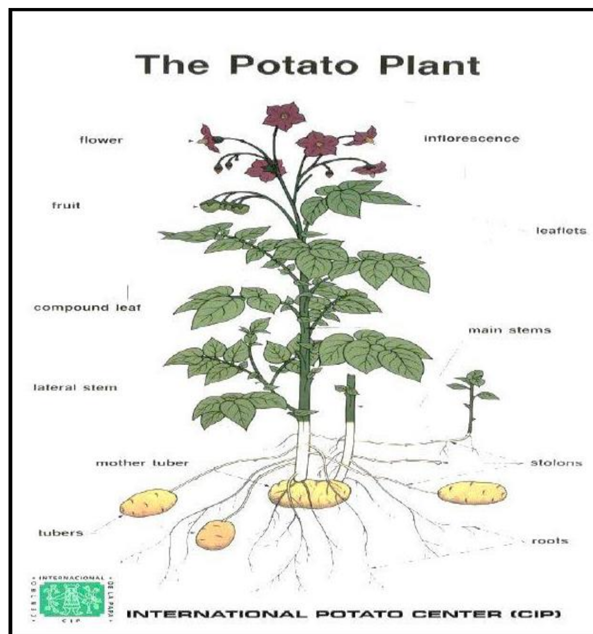


Fig. 2 Diagram showing all the parts of a potato plant

(Ref: International Potato Centre; https://cipotato.org/crops/potato/)

1.5 Economic Importance of potato

Potato is an essential component of a big percentage of the world's population's diet as a food and a crop of global significance. It is renowned for its elevated yield, high starch content, and usage as a food and for a variety of industrial applications. Potato tuber includes about 80% water and rest dry matter. Starch is dry matter significant element, accounting for about 70% of the total solids (Dutt et al., 2017).

A potato tuber's average raw material composition is 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, 547 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). Because of its elevated dietary value and manufacturing potential per unit moment, it can support the country's burgeoning population and prevent malnutrition and starvation.

1.6 Tuberization: an important part of Potato life cycle

Tuberization is complex, highly coordinated, physio-morpholological development process that involves the formation of a stolon (an underground stem). Under initiative circumstances, there is inhibition in the growth of the subapical region and further it swells to form the storage organ where starch gets accumulated (Abelenda et al., 2016). This process involves the large number of interactions between various factors at morphological, cellular, biochemical, genetic and environmental level. The morphological level 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). Whereas, at biochemical levels there is synthesis of starch (Tauberger et al. 2000) and storage proteins accumulation (Taylor et al.,1998). Thus, it is a multi-stage process comprised of various successive stages that are, induction of tuberization, initiation of stolon growth and development.

Under the influence of two specific environmental conditions potato plant starts tuberizing i.e. short day photoperiod (SD) and cool night temperature. Such optimal conditions are available in subtropical Indo-Gangetic plains only in during winter.

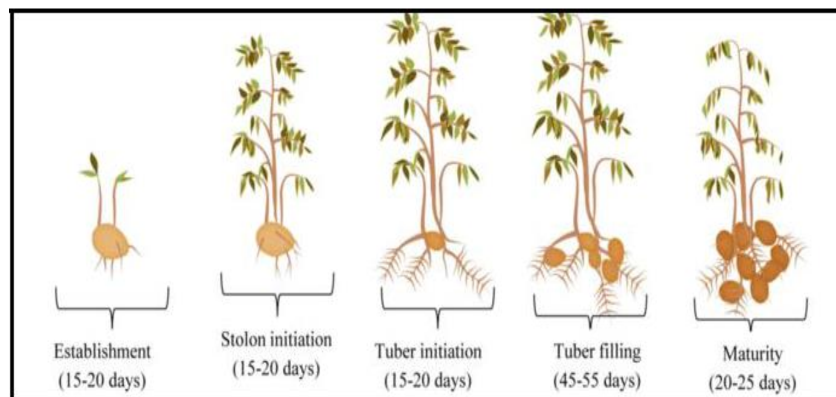


Fig. 3 Growth cycle of Potato plant (Ref.: Obidiegwu et al., 2015)

1.7 Factors Influencing Tuberization

Various external factors that strongly affect tuber formation are high irradiance photoperiod, supply of carbohydrate, nitrogen and low temperature and phytohormones promote tuber initiation and development (Vander Zaag et al., 1996). From the earlier reports, it was observed that tuber initiation was delayed with high supply of nitrogen (Krauss, 1985).

Environmental Factors: Temperature and photoperiod are two most important environmental factors affecting tuber formation in potato. It has been studied that high temperature inhibits tuber growth in potato (Gopal et al., 1998). All stages of tuberization i.e. tuber induction, tuber set, tuber bulking, tuber size and yield have been found to be affected by temperature. Optimal temperatures at which tuberization takes place are well defined and studied. At higher temperatures, tuberization is delayed or even inhibited in comparison with lower temperatures. Similarly, photoperiod plays an important role in the induction and initiation of tuber formation in the potato and has been the most intensively studied as environmental factor (Gopal et al., 1998). It has been urged that each temperature and photoperiod converge at some purpose and is most likely to regulate the process of tuberization (Dutt et al. 2017).

Other factors: There are many other factors which regulate tuberization process at the molecular level. For modulating the expression of specific target gene, various factors which are proteins in nature, plays an important role. Various transcriptional factors (TFs) such as *knox* (knotted-like homeo box) are involved during regulation of developmental events in apical meristems (Reiser et al., 2000). In specific developmental process of the shoot apical meristem (SAM), involvement of *knox* genes is supported by functional analysis and expression patterns of mutation. Also it has been reported that KNOX genes are involved in regulation of gibberellins (GA) levels. A general component of GA- signaling pathways "photoperiod responsive protein (PHOR1), standardizes the gibberellic acid (GA) and phytochrome B-mediated perception (Amador et al., 2001). Two essential phytohormones i.e. Cytokinins and Gibberellins are involved to regulate the formation of potato tuber. Gibberellic acid (GA) acts as a dominant regulator to initiate the formation of tuber, control photoperiod (Xu et al., 1998b), and monitor the sucrose and abscisic acid (ABA) levels. The role of cytokinins is to regulate the cell division, by controlling the cell cycle (Vreugdenhil et al. 1999). For the process of tuberization, there is requirement of appropriate cytokinin to auxin ratio (Sergeeva et al., 2000). Other than growth regulators, there are various genes which are involved in the several events of tuberization. *StCDF1, StBEL5* mRNA and StSP6A protein are the major mobile signals which have been identified for tuber formation (Hannapel et al., 2017). *StSP6A, StSP3D, StSP5G* and *StSP5G-like* are four FT members in potato. *StSP3D* regulates the floral transition, *StSP5G* represses *StSP6A* activity which further supresses tuberization and indicates that *StSP6A* controls tuber initiation (Abelenda et al., 2016).

SP6A gene belongs to Self Pruning family of tomato (*Solanum lycopersicum*).It is homologous to FT gene from *Arabidopsis thaliana* and CEN gene from *Antirrhinum*. It is a mobile tuber signal and plays a vital role in potato tuberization (Navarro et al., 2011). *StBEL5* functions as upstream of *StCDF1* and *StSP6A* and moved through the phloem as a full length mRNA (Hannapel et al., 2017).

In following chapter, we will be very clearly discussing about role of SP6A in tuber initiation and in long distance signaling with its mechanism. Also an explanatory note is provided on tuberization, its importance and how SP6A regulate tuberization in potato. To make it more understandable, mechanism is explained with evidence.

CHAPTER-2 REVIEW OF LITERATURE

Predominant importance of phloem transport for plant growth was sighted by plant biologists by the end of 19th century. It has been suspected that phloem mediated supply of photosynthates and other organic compounds are important for plant growth. Phloem physiology becomes well established, that allows the researcher justifiably measure the translocation and higher resolution visualization of phloem tissue.

2.1 Mobile RNA and long distance signaling in plants

In higher plants, both long as well as short distance communication pathways and coordination in the 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 (Ding et al., 2003). Several plant transcription factors have the capability to move from cell to plant plasmodesmata over a short distance such TFs are KNOTTED1-Like homeobox TF, or the APETALA1 and TF LEAFY are associated in its conservation and initiation of meristem (Sessions et al., 2000). In Arabidopsis, endogenous TA (trans-acting), si (short- interfering) RNA loci the mobile silencing signals facilitate polarization of leaf by the production of regional gradient of target gene expression. There was a surprising observation that through the phloem specific mRNAs can be even delivered to unapproachable plant organs, merely many ribonuleoproteins and RNA proteins complexes established in the translocation of phloem stem have become a fascinating candidate as an information transmitters server (Ruiz-Medrano et al., 1999). In long distance signaling some of these ribonucleoproteins and RNA proteins have a crucial role (Kehr, 2009; Opark and Cruz et al., 2000).

2.2 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 et al., 2006). Most of the RNA-binding proteins functions as chaperone and arbitrate the import of RNAs from companion cells into sieve elements. The macromolecular structure of RNA could be modified by RNA-specific chaperones so that to promote their passage through plasmodesmata and make them stabilize during translocation (Yoo et al., 2004). 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 (Zhang et al., 2009). From source to sink, RNAs seems to translocate within the sieve elements depending upon the metabolic flux. 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.

2.3 Mobile mRNA as a long-distance signal

The mobile mRNA functions only at the sites of their target are approved by eliminating 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 (Gualerzi et al., 2003). Several full-length mobile RNAs have been reported in plants (Kehr et al., 2008). However, there is very precise knowledge about how RNA sequence regulates this process. Recognition of stem and loop structures are formed by these sequences (Jansen et al., 2001). While these loop structures can be found anywhere in the transcript, they are most prevalent in the 3 ' UTR (Chartrand et al., 1999). Viroid RNA motives that facilitated selective motion of RNA through plant cells have also been recognized (Qi et al., 2004). These brief sequences most probably imitate RNA motives of endogenous plants identified by cellular transport variables (Banerjee et al., 2006).One of them is *SP6A*, a member of Self Pruning family of tomato ((*Solanum lycopersicum*) which is mobile mRNAs of potato that is believed to be originated in the leaf (Dutt et al, 2017). The recent study has shown that there is a positive correlation between *StSP6A* RNA and tuber formation. It has been found that *SP6A* functions with FT like protein in the tuber activation complex to initiate tuberization (Hannapel et al, 2017).

2.4 Role of specific factors during tuberization:

Mechanism of tuberization has been the subject of considerable investigation by plant scientists in the past several decades. A potato tuber is a modified underground stolon or stem which is capable of producing a separate stem by means of vegetative propagation (Spooner and Salas, 2006).

Process of tuberization in potato controlled by several multiple signaling pathways such as, phytohormone signaling, RNA signaling and calcium ions signaling (Sarkar, 2008).

Phytohormone signaling involves important environmental factors such as high irradiation, short day photoperiod and low temperature which affect the potato tuberization. It has been indicated that ARF6 (auxin responsive factor 6), a key regulator of auxin responsive gene is involved in tuberization(Rampant et al., 2004). There are various transcription factors involved in RNA signaling are *StBEL5* (Chen et al., 2003), *POTM1* (Kang et al., 1995), *POTH1* (Rosin et al., 2003) and *AtCO* (potato orthologue Arabidopsis thaliana *CONSTANS*, that work alone or in concert with other transcription factors to regulate the potato tuber formation. Thus, in a long distance signaling pathway mRNA acts as a signal molecule. The transcription factors *StBEL5* and *POTH1* of potato interact to repress the expression of ga20ox1 in tandem (Chen et al., 2004), and leads 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 RNA transcript of StBEL5 is transported as a systemic signal (part long distance signaling pathway) from the leaf to the tip of stolon (Banerjee et al., 2006). The short day length supports the transport and aggregation of *StBEL5* and RNA-binding proteins. In conjugation with KNOX partners the protein chaperon accompanied the RNA of *StBEL5* to the tip of stolon, and regulates the process of transcription of several target genes involved in tuberization. Dephosphorylation of some proteins involved in the calcium signaling is also required for the transcriptional activation 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 Calcium dependent protein kinase StCDPK1 is expressed in tuberizing stolon contains a highly conserved myristoylation site. Recently it has been indicated that there is involvement of RNA signaling in tuberization. RNA signaling include several factors, such as *SP6A* (Hannapel et al., 2017) *POTM1* (Kang et al., 1995), *POTH1* (Rosin et al., 2003), *StBEL5* (Chen et al., 2003) and *Arabidopsis thaliana CONSTANS* (AtCO), independently or in concert with other regulate tuber formation in potato. Recent research firmly supported statement that — mRNA acts as a signal molecule in a long-distance signaling pathway.

2.5 Expression of the St*SP6A* gene fully correlates with tuberization initiation

Recent studies have shown that three most important signals regulate the tuberization in potato are: *CYCLING DOF FACTOR1* (*StCDF1*), *StSP6A*, and *StBEL5* (Navarro et al., 2011; Banerjee et al., 2006). *StSP6A* belongs to a family of co-regulators whereas *StCDF1* and *StBEL5* are

transcription factors. *StBEL5* mRNA and *StSP6A* protein, originate in the leaf, and work as mobile signals (Hannapel et al., 2017). Under favorable conditions, it transported from leaf to the underground stolon tip to initiate tuberization (Navarro et al., 2011).

2.6 Self-Pruning 6A (SP6A): Ortholog of Flowering locus T (FT)

StSP6A has been extensively studied and established as a candidate for the mobile tuber signal (Navarro et al., 2011). In the recent years *FT* has been proved to be the one of the best example of a long-distance mobile signal which regulates flowering. Under flowering inductive conditions, *FT* is expressed in the leaf under the control of *CO*. It moves from the leaf as a protein to the shoot apical meristem through plasmodesmata and the sieve element system (Turck et al., 2008). It belongs to phosphatidylethanolamine-binding protein (PEBP) family whose tertiary structure is similar to mammalian PEBPs (Ahn et al., 2006). Transcription is coregulated by *FT* and function with TF partner. These observations suggested that there is involvement of orthologs of *FT* as mobile signals in non-floral pathways. Utilizing heterografting experiments and transgenic analysis was carried out and experiment results revealed that silencing of *StSP6A* results in delayed tuberization under short days. Overall, it suggested that *StSP6A* is involved not in long-term tuber morphology but in the initial stage, activates tuberization (Navarro et al., 2011). Using an inducible promoter, induction of *StSP6A* led to the activation of several tuber marker genes, including *StGA2OX1*. *StSP6A* exhibited an SD-induced expression of its mRNA in leaves and stolons in a photoperiod-responsive variety (Navarro et al., 2011).

Dihydroasparagusic acid (Hd3a) is found to be involved in enhancing flower production and tuberization. It moves from a graft to wild-type potato stolons and thus tuber formation is increased (Navarro et al., 2011). It consists of three homodimers; Hd3a, OsFD, and a 14-3-3 protein and functions in a hexameric floral activation complex (FAC) that functions as a scaffold (Taoka et al., 2011). The FAC model provides interaction of *FT* with a different protein partner that regulates a unique set of target and act as molecular basis for creating multiple functions for *FT* beyond flower development (Taoka et al., 2011). It has also been found that for the activation of tuber activation complex (TAC); *StSP6A, StFD1*-like protein and a 14-3-3 protein interacts with each other (Teo et al., 2017). While activation of these *FT* genes, the floral pathway in

stolons is blocked by unknown mechanism and formation of tubers is facilitated (Navarro et al., 2011).

2.7 Roles of the St*SP6A* andSt*SP3D* genes in short day tuberization

StSP6A is repressed by *StCO* under long-term circumstances (Navarro et al. 2011). *StSUT4* impacts the accumulation of *StSP6A* mRNA and *StCO* under photoperiodic conditions. Under photoperiodic conditions, *StSUT4* induces accumulation of *StCO* which in turn inhibits accumulation of *StSP6A* and therefore inhibits tuber formation. On the other side, *StSUT4* inhibits *StCO* accumulation under short day circumstances and thus prevents StCO-mediated inhibition of *StSP6A* (Chincinska et al. 2013). This signal is enhanced in short days during transportation through a mechanism which is partially mediated by *StCO.* Activation of *StSP6A* in stolons and leaves encourages the tuberization (Navarro et al. 2011).

2.8 Evidence for transportation of *SP6A* RNA to the stolon tips

Recently, it has been found that mobile RNA signals *SP6A, StBEL5* and *POTH1* works in coordination for transportation to the stolon tip promoting tuber formation (Dutt et al, 2017). *StPHYB* suppresses the movement of *StBEL5* mRNA from the leaves to stolons under photoperiodic conditions which results in the suppression of tuberization. *StPHYB* represses the expression of *StmiR172* and thus results in non-tuberization. Similarly, role of Sucrose transporter (*StSUT4*) has been studied and it was found to induce *StCO* and inhibits tuber formation under photoperiod conditions (Dutt et al, 2017). Under short-day conditions, expression of *StSUT4* and *StPHYB* is inhibited and thus expression of *StCO* is inhibited, and thus expression of mobile tuber inducing long distance signal *StSP6A* takes place and tuber formation is induced. Further, degradation of *StRAP1* protein occurs by *StmiR172* and results in the expression of *StBEL5*. When *StPHYB* is present, *StBEL5* is transported from the leaves to stolons and biosynthesis of GA is reduced in association with *POTH1*and thus promotes tuber formation (Dutt et al, 2017).

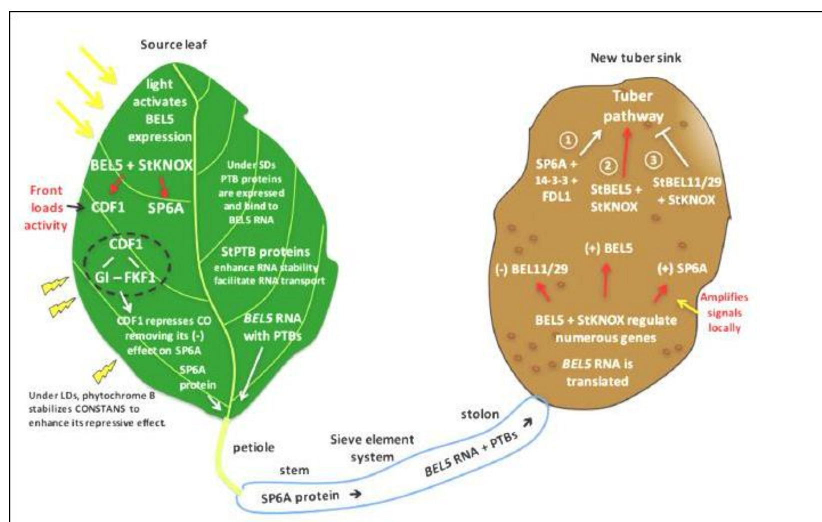


Fig. 4 Proposed Model for the tuber signaling network (ref. Hannapel et al., 2017)

2.9 Origin of the problem

Tuberization is a complex process of tuber development for regulating morphological processes occurring in underground stolon tip of potato. Being a complex process it involves many important environmental, biochemical and genetic factors, at both extrinsic and intrinsic level. So, it becomes necessary to get proper knowledge on process of tuber formation with regard to production of healthy tuber (large, mini and micro) in potato, where several mobile mRNAs play crucial roles. These mobile mRNAs are developmental switches which regulate various processes within species, therefore, detailed knowledge on mRNAs at both biochemical level and molecular level along with their expression patterns are prerequisite to know process of tuber development. Moreover, in Indian cultivars there is no report available as of now with regard to these factors associated with tuber formation process. Such types of studies are important in improving the potato crops through transgenics. One of the objectives is to standardize a suitable and easy-to-use protocol for the isolation of DNA from potato *(Solanum tuberosum)* plants so that structure and function of the genes encoding *StSP6A* TFs can be studied through PCR approach using gene-specific primers. The other objective is to know their expression patterns in different organs of potato. Indian potato cultivar namely Kufri Chipsona-1 (CS-1) and foreign exotic cultivar Désirée (De) were chosen for molecular studies. In this study, the mobile mRNA, *SP6A* was chosen because of its key feature as a long distance transport to the underground stolon tip to initiate tuberization. Keeping the above points in view, the following objectives were framed.

2.9 .10 Objectives of the study

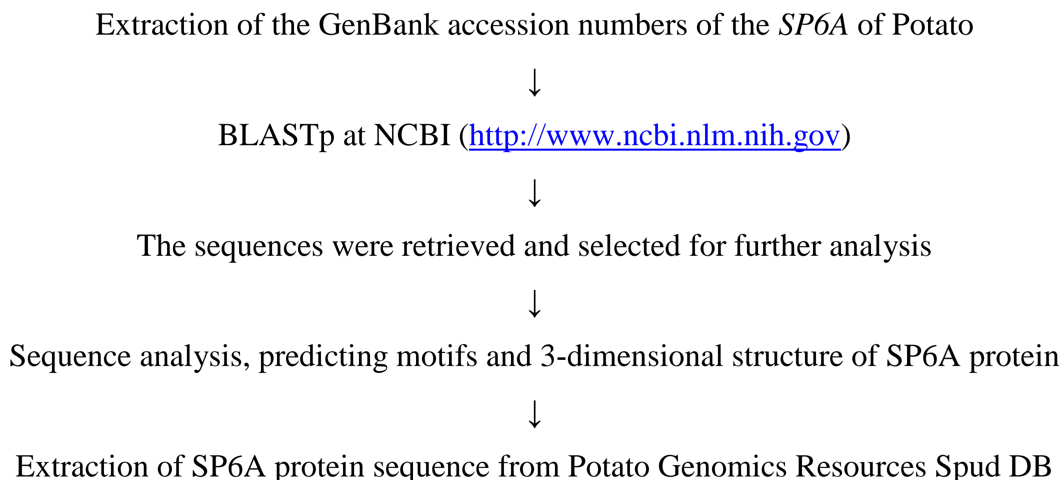
Molecular cloning and thorough sequence analysis are prerequisites for recombinant expression of *SP6A*. Keeping in view the overall progress so far in the laboratory, this thesis work focused mainly on the following aspects:

- Sequence analyses, multiple sequence alignment (MSA) and motif search using the available *SP6A* gene sequences at both nucleotide and amino acid level
- Prediction of various protein motifs and 3-D structural features of SP6A protein
- Analysis of amplicon profile in the different Indian potato cultivars using their total DNAs and *SP6A*-specific oligonucleotide primers
- Expression patterns/occurrence of *SP6A* transcripts in potato by RT-PCR approach

CHAPTER-3 MATERIALS AND METHODS

3.1 *In-silico* analysis on StSP6A

A flowchart of strategies for the identification and study of *SP6A* of Potato (*Solanum tuberosum*):



- *Biochemical characterization:* The theoretical pI, amino acid composition, aliphaticity and molecular weight were determined by using EXPASY server (http://web.expasy.org/protparam/).

- *Identification of conserved domains*: The SP6A protein sequences were then uploaded to MY HITS (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and different sites and motifs were predicted.

- *In silico* characterization**:** Phyre2 tool (http://www.sbg.bio.ic.ac.uk/~phyre/) was used for secondary structure prediction and protein fold recognition.

- *3-D modelling***:** SWISS model web tool (https://swissmodel.expasy.org/) depicts the 3D protein structure of the protein model. SWISS model gave reliable protein models, which were selected on the basis of X-ray/NMR region.

3.2 *SP6A* gene expression pattern:

3.2.1 Potato plant materials:

Commercial Indian Potato Cultivar Kufri Chipsona-1 (CS-1) and foreign exotic reference (Desiree) were used in this study. After acclimatization, the plants where grown into field in the starting month of November to March, which grows under cold temperature and short day length light conditions. Then they were at different stages of their development. They were stored at -80°C for further studies.

3.2.2 Other requirements

Enzymes were purchased from Bangalore Genei Pvt. Ltd., Bengaluru. Chemicals required were bought from Sigma-aldrich India Pvt. Ltd., and Himedia Pvt. Ltd., Mumbai. Primers used were synthesized by Eurofilms Genomic Pvt. Ltd., Bengaluru. Other chemicals were prepared in laboratory given in **Table 1**.

Table 1 Different chemicals and their composition

Chemicals	Composition	Volume
Gel Loading Buffer	Sucrose	35% (w/v)
(5X)	Tris EDTA	50 mM (pH 8.0)
	Bromophenol blue	25 mM
		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)
Extraction Buffer	Tris HCl	50 mM (pH 8.0)
	EDTA	50 mM
	NaCl	250 mM
	Sucrose	15%

Ethanol, Isopropanol, 5M Potassium acetate solution (pH-5.5), Sodium acetate, DNA free RNase, Alcohol, Chloroform

3.3 Methods:

3.3.1 Isolation of total DNA from different potato cultivars

Procedure: Total DNA was isolated from the tissue cultured potato plant by a modified protocol reported earlier (Kumari et al. (2012). Plant samples were washed under tap water followed by sterile distilled water. To remove the excess water on the plant, blotting filter paper was used. 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 (250µL of 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 machine. Then 170µL of 5.0 M potassium acetate solution was added, mixed vigorously and incubated further 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 ice cold 70% ethanol, air dried and suspended in 100µL 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, 8M Licl, DEPC-treated water, Sodium acetate

Procedure: Further purification of DNA was done by treatment with DNase-free RNase. 400µL of sterile water was added into eppendrof containing 50µL 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 (200µL 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 50µL of TE buffer and stored at (-20°C).

3.3.3 Isolation of total RNA from potato organs:

Materials Required: Lithium chloride, RNA extraction buffer, Tris-HCL (100mM) at pH8.0, EDTA (10mM) at pH8.0, 1.0% SDS, 0.2% (β-mercaptoethanol), RNase-free DNase, RNase-free DEPC (Diethyl pyrocarbonate) deionized water.

Table 2 Stock and working solution for isolation of total RNA

Solution	Stock Solution	Working Solution
Tris Buffer	0.5M (pH 8.0)	100mM (pH 8.0)
LiCl	8M	100Mm
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 the 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 pulverized in the liquid nitrogen to a fine powder. The content were mixed in a buffer containing lithium chloride and SDS (RNA extraction buffer, 100mM tris-HCL pH-8.0, 10mM EDTA pH-8.0, 1.0% SDS, 0.2% (β-mercaptoethanol) followed by direct extraction with phenol:chloroform in 1:1 ratio, under ice-cold conditions, 8.0M LiCl (one third volume of the form aqueous solution) was added to the supernatant and incubated for minimum 2 hours for selective precipitation of RNA. The crude RNA was further purified followed 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.

3.3.5 Purification of total RNA

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.

3.3.6 Designing of oligonucleotide primers

The following oligonucleotide primers were designed based on the available genome sequence corresponding to *StSP6A* coding region gene in GenBank database (GenBank ID: AY186737.1).The *SP6A* coding region sequence of 3463 bp is comprised of following features: molecular type of this sequence is mRNA means all information is provided at cDNA level. Total length of CDS region is 421 bp, starting from 412th nucleotide to 606th as first exon, then from 1379th to 1440th as 2nd exon, from 1960th to 2000th as 3rd exon and 2238th to 2362nd as fourth exon. No 5'-UTR is observed as the codon starts from the first nucleotide and the last codon ends at 2362nd nucleotide. mRNA joins from 412 to 606 (exon 1), 1379 to 1440 (exon 2), 1960 to 2000 (exon 3)and 2238 to 2362 (exon 4). Oligonucleotide primers should be 10-24 nucleotides long. By keeping the above parameters in mind following primers were designed:

- GC content should ranges from 40 to 60%.
- The primer should not be complementary to any other primer or self-complementary 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 lesser than the melting temperature.
- Long run sequences of a single nucleotide should be avoided.
- Primers with significant structures are avoided.

Details of designed primers are given below in **Table 3**:

Table 3 Details of designed primers

S. No.	Name of Oligomers	Description of the Oligomers	Length
1.	SPF1-0358	5'-AATATCCTAGCATATTGTCA-3'	20 bp
2.	SPF2-0404	5'-ATATTATCATGCCTAGAGTTG-3'	21 bp
3.	SPR1-2378	5'-AGCAAAGTCTCTTGTGTTAG-3'	20 bp
4.	SPR2-2455	5'-CGACGTCCTCCAGTACCACTC-3'	21 bp

3.3.7 Polymerase Chain Reaction (PCR)

Materials required: Template DNA (prepared from total DNA of different potato cultivars), Primers (Resuspended in sterile distilled water), Buffer (usually 10X), Taq polymerase, dNTPs (2mM stock), sterile dd H2O

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 thermo stable DNA polymerase. Composition of reaction mixture of PCR reaction is mentioned in table 4. Thermal cycling parameters for 30 cycles are given in table 5.

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

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

Table 5 The thermal cycling parameters of PCR

Steps	Temperature	Time
Initial Denaturation	94°C	2 min
Denaturation	94°C	1 min
Annealing	55°C	2 min
Polymerization	72°C	2 min
Holding	4°C	Variable

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

Material Required: DEPC water, Oligo dT primer, 10 mM dNTP, RNase inhibitor, Reaction buffer, RNA sample.

Procedure: Reverse transcription: Revert Aid H Minus M-MuLV reverse transcriptase enzyme was used to synthesize first strand cDNA. Ribonuclease H activity specific to RNA in RNA-DNA hybrids is not present in this enzyme; therefore RNA degradation does not occur. Lack of RNA degradation leads to higher yields of full-length cDNA from long templates upto 13kb.
RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas Life Sciences) containing M- MuLV reverse transcriptase and the gene-specific reverse primers, SPF2-0404 and SPR1-2378. Template used for each RT reaction was sample of leaf, tuberizing stolon, tuber and petiole (approx. 2.0 µg) from Kufri Chipsona 1. According to manufacturer's instruction the steps for RT were performed. Isolation of full length cDNA was done by performing PCR. PCR mixture was made by using RT product as template, and adding 6 µl DEPC water, RNA sample ((2 µg) and oligo dT primer (2 µl). The mixture was properly mixed and eppendorfs were kept at 65°C for 5 minutes followed by incubation at room temperature for 5 minutes. Reaction buffer (4 µl), RNase inhibitor (1 µl), 10 mM dNTPs were added to the mixture and the eppendorfs were incubated at 42°C for 1 h and 65°C for 5-7 min.
RT-PCR: For RT-PCR, cDNA was used as template and following thermal cycling parameters were followed: 94ºC for 1 min, annealing at 55ºC for 2 min; polymerization at 72ºC for 1 min for 30 cycles followed by final extension at 72ºC for 5 min.

3.3.9 Agarose gel electrophoresis

Material Required: Agarose (Sigma), 0.5X TBE buffer, Ethidium bromide dye (5 mg/mL), Sterile water, DNA samples, Bromophenol blue dye, Gel electrophoresis apparatus, UV transilluminator, Gel-Documentation system.

Procedure: Standard method (Sambrook- a laboratory manual) was followed to perform Agarose gel electrophoresis. 0.8% agarose gel was weighed and mixed in 0.5X TBE buffer and ethidium bromide dye was added to it. Gel was then casted in tray and allowed to polymerize. The DNA samples were loaded in the wells by mixing them with Bromophenol blue. Electrophoresis was carried out in 0.5X TBE (running buffer) at 2- 5 volt till the tracking dye covered 3/4th length in agarose gel. The DNA bands were then visualized under UV light.

CHAPTER-4 RESULTS & DISCUSSION

4.1 *In silico* analysis

StSP6A has been established as a candidate for the mobile tuber initiation. It is found that very less work has been done in the literature for the *in silico* analysis. So, efforts have been made to find the structure and other biochemical attributes of SP6A protein.

This study focused on the Sequence analysis, comparison and motif search in SP6A protein from Potato (*Solanum tuberosum*).

4.1.1 Salient sequence features

SP6A gene: *SP6A* protein consisting of 140 amino acids encoded by a 3463 bp gene sequence from Potato (GenBank Acc. No.: AY186737.1). The coding sequence is from exons starting from 412 to 606, 1379 to 1440, 1960 to 2000 and 2238 to 2362. The size of ORF in *SP6A* gene is 421 bp including stop codon.

SP6A Protein: The SP6A Protein (GenBank protein ID: AAO31794) consists of 140 amino acids which includes various sites such as Phosphatidylethanolamine-binding protein (PEBP); Substrate binding site (chemical binding), whereas the substrate binding site is on amino acid 69, 83, 85, 109-112, 116, 118.

Coding region as a query sequence: BLASTn was done for coding region of *StSP6A* gene which showed more homology with other gene sequences of *SP6A*-like genes. The 3043-bp coding region was found to be identical (100% sequence identity with 100% query coverage) to a gene copy in *Solanum lycopersicum* Chromosome ch05, complete genome (GenBank ID: HG975517.1), 100% sequence identity with 100 % query coverage to a gene copy in *Solanum lycopersicum* chromosome 5 clone C05HBa0142A20, complete sequence (GenBank ID: AC238789.2), 99.97 % sequence identity with 100% query coverage to a gene copy in *Solanum lycopersicum* cultivar I-3 chromosome 5 (GenBank ID: CP023761.1), also 91.71 % sequence identity with 99% query coverage to a gene copy in *Solanum pennellii* chromosome ch05, complete genome GenBank ID: HG975444.1).

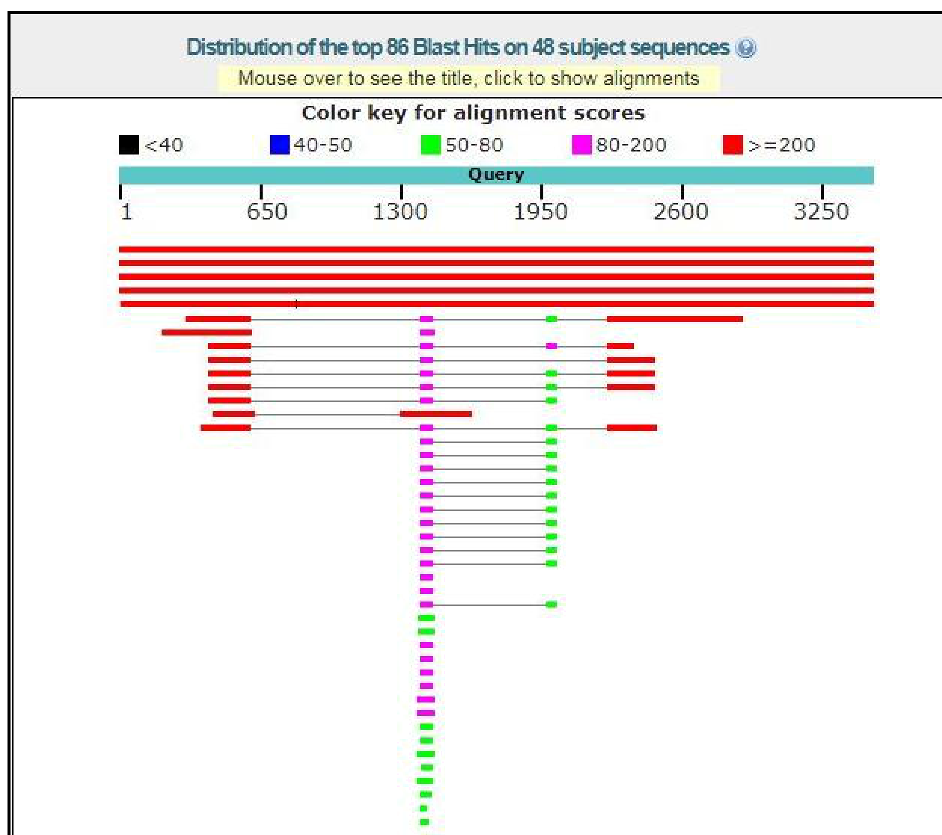


Fig. 5 BLASTn analysis of SP6A gene

The SP6A (GenBank protein id: AAO31794) sequences were used as reference sequences for protein modeling. Amino acids were retrieved in FASTA format from NCBI and submitted in BLASTp server of NCBI. BLASTp gave the homologous sequences with maximum identity and query coverage. The homologous sequences were used for comparison, characterization and validation of protein models.

4.1.2 BLASTp analysis using the sequences of SP6A Protein

When BLASTp was done for SP6A protein (GenBank protein ID: AAO31794); it showed more homology with other forms of SP6A proteins. The 140 amino acid sequence was found to be identical (100% sequence identity with 100% query coverage) to amino acid sequence in protein SELF PRUNING 6A of *Solanum lycopersicum* (GenBank protein ID: NP_001316376.1), 97.86 % sequence identity with 100 % query coverage to the amino acid sequence in protein FLOWERING LOCUS T-like of *Solanum pennellii* (GenBank protein ID: XP_015074525.1), 90.71 % sequence identity with 100% query coverage to the amino acid sequence in PEBP

protein of *Solanum tuberosum* (GenBank 85.71% sequence identity with 100% query coverage to the amino acid sequence in *Nicotiana sylvestris* (GenBank protein ID: AVG70961.1). 81.56% sequence identity with 100% query coverage to the amino acid sequence in *Betula platyphylla* (GenBank protein ID: AFR31531.1). Details of some homologus SP6A sequences are provided in Fig 6 and Table 6:

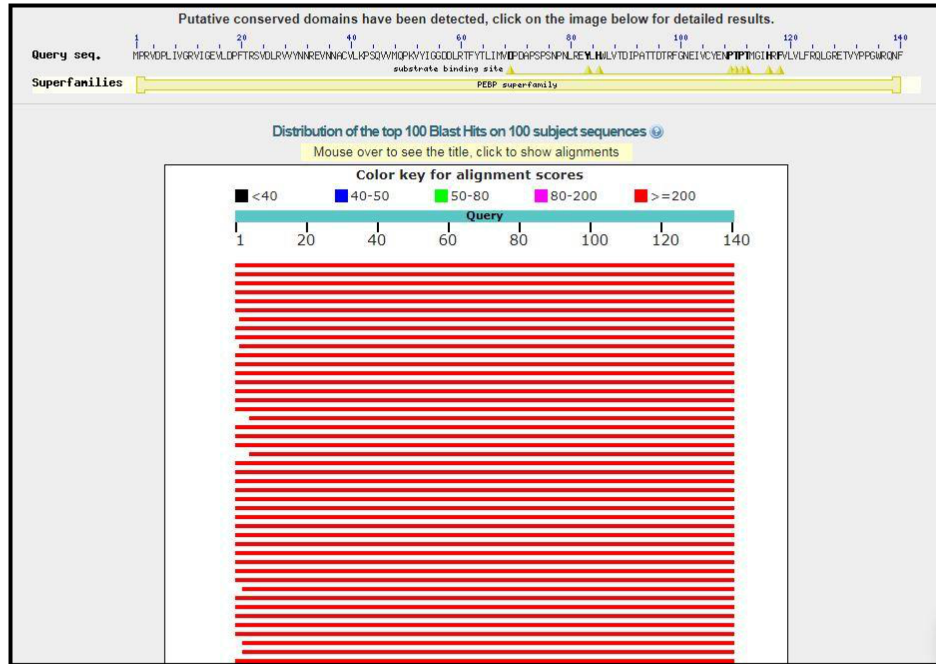


Fig. 6 BLASTp analysis of SP6A gene

Table 6 Details of homologous sequences of SP6A Protein

Protein Name	Accession No.	Amino acids	Max. Score	% Identity	Query Cover %
SP6A	NP_001316376.1	140	295	100	100
Flowering Locus T like	XP_015074525.1	173	290	97.86	100
PEBP protein	BAV67095.1	173	271	90.71	100
Flowering Locus T Beta	AVG70958.1	173	268	87.14	100
Flowering Locus T-c	AVG70961.1	174	262	85.71	100
Floweing Locus T protein *(Betula platyphylla)*	AFR31531.1	174	244	81.56	100

4.1.3 Multiple sequence alignment using Constraint-based Multiple Alignment Tool (COBALT)

For multiple sequence alignment (MSA), the amino acid sequences of SP6A and its six isoforms were taken which included SP6A1, SP6A2, SP6A3, SP6A4, SP6A5, SP6A6, using multiple sequence alignment tool COBALT as shown in Fig. . MSA reveals that N terminus and C terminus show variation. Comparison between the sequences showing variation due to mutations, deletions or insertions are in colored letter in the **fig 7**.

```
SP6A            ------MPRVDPLIVGRVIGEVLDPFTRSVDLRVVYNN-REVNNACVLKPSQVVMQPKVYIGGDDLR-TFYTLIMV   68
SP6A1           -------------------------------MSVVYNNNKHVYNGHEFFPSSVTSKPRVEVHGGDLR-SFFTLVMI   44
SP6A2      [89]AYGSPVPPLPPPPVTMLTLNSGPEFHFFSTLNPIRQTQQKNISNEGLASPAPGRKRHPESNNNNSGD-QKSKRMIK   164
SP6A3      [83]A-ASPVSA-----TTMLNLNSVPELHFFD--NPLRQ-------NSILHQP--------------------------   118
SP6A4           MSIDQELPQELLEKKLPYIKVITSSNDDEDDQYI---NCQTPKSSQYLIPKILSCPPAPKKPKRVSScSSCKRKLI   73
SP6A5           MSTDLQFPQDLFEINSPNINSSQRCINNNINIIINKDDCKTPKSSPFLIPKILKCPAAPKKPKRVIS--SCKRKL-   73
SP6A6           MVKDLQTPKDFQENQSPNINSPP------------PNECKTPKSPSCKIPKAVNCPGAPKKPKRANR-SSCKRRLR   63

SP6A    69      DPDAPSPSNPNLREYLHWLVTDIpaTTDTRFGNEIVCYENPTPTMGIHRFvLVLFRQLGRETVYP-PG [5]  140
SP6A1   45      DPDVPGPSDPYLREHLHWIVTDIpgTTDCSFGREVVGYEMPRPNIGIHRFvFLLFKQKKRQTISSaPV[36]  148
SP6A2   165     NRESAARSRARKQAYTNELEMEV-----ANLMEENARLKRQQQQLCLASAgALLPKMKSLNRTSTaPF      227
SP6A3   119     ------------NAYLNELETEV-----AHLVEENARLKKQQQQLRLAAA-IQVPKKNSLHRTSTaPF      168
SP6A4   74      DEFKFFDEQEEIESFFRFVDV------NSTKKR-RRCLV*----------------------------      106
SP6A5   74      -QFVEIVASKEVESFFRILDDDIvaSSNASKKI-----------------------------------      105
SP6A6   64      FEIVVMVAEEEIDSFFRNAED----ANNGCNKIMKRRRSM----------------------------      99
```

Fig 7 Multiple sequence alignment of the SP6A sequences from potato and other plants. Sequence alignment was based on Clustal Omega tool along with some minor manual adjustments.

4.1.4 Searching protein motifs

The amino acid sequence of SP6A was analysed for prediction of some important protein necessary for protein function motifs using the tool MY HITS as shown in **Fig 8**.

```
 1 MPRVDPLIVG RVIGEVLDPF TRSVDLRVVY NNREVNNACV LKPSQVVMQP KVYIGGDDLR

61 TFYTLIMVDP DAPSPSNPNL REYLHWLVTD IPATTDTRFG NEIVCYENPT

PTMGIHRFVL 121 VLFRQLGRET VYPPGWRQNF
```

Fig. 8 Prediction of protein motifs in StSP6A

Amino acid sequence of SP6A (GenBank protein id: AAO31794). Some protein motifs are highlighted using different colors.

The position and amino acid sequence of the protein motifs are presented in **Table 7.**

Table 7 Motif sites and position of SP6A Protein

S.No.	Motif Site	Motif	Position	Color Code
1.	Tyrosine kinase	RFG…VCY	98-106	Blue
2.	Phosphatidylethanolamine-binding protein family signature	YTL…YLH	63-85	Purple
3.	Chorismate mutase domain profile.	MPR…NRE	1-34	Orange
4.	Methyl CpG binding domain profile	VLF…QNF	122-140	Olive Green
5.	Phosphatidylethanolamine binding protein (PEBP)	DPF…QNF	18-140	Yellow

In the above StSP6A sequence mainly 5 motifs were found. One motif (**RFG…VCY**) was found on Tyrosine kinase site which is highlighted with blue color. It plays an important role in signaling of different biological processes i.e. cell growth and differentiation. From Phosphatidylethanolamine-binding protein family signature site, one motif (**YTL…YLH**) was found which is highlighted by purple colour. PEBP act as promotor as well as repressor in flowering during long day conditions. From Chorismate mutase domain profile site one motif (**MPR…NRE**) was found which is highlighted by orange color. This motif is responsible for biosynthesis of Phenylalanine and Tyrosine.Methyl CpG binding domain profile (**VLF…QNF**) site colored olive green plays a vital role in gene expression, gene organization and in plant growth. Phosphatidylethanolamine binding protein (PEBP) (**DPF…QNF**) which is highlighted in yellow color words was found to play a role in lipid binding and inhibition of serine protease.

4.1.5 Salient biochemical attributes of SP6A protein (GenBank Protein ID: AAO31794)

The sequence of SP6A consisting of 140 amino acids was analysed 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 of the SP6A was found to be 160.06 kDa with a predicted isoelectric point (pI) of 6.08. Predicted formula of SP6A protein is $C_{725}H_{1129}N_{197}O_{204}S_6$. Out of its total 140 amino acids, 14 were strongly basic (+) (Arg + Lys), 27

15 were strongly acidic (-) (Asp + Glu). The instability index (II) was computed as 33.16, which classified the protein as stable. The amino acid composition data revealed that some of the amino acids such as Arginine (8.6%), Asparagine (6.4%) Phenylalanine (4.3%), Proline (10.0%), Tyrosine (4.3%) Valine (12.9%) occurred more frequently as compared to their average occurance; whereas the amino acids namely Alanine (2.1%), Aspartic acid(6.4%), cysteine (1.4%), Glutamine (2.9%), Glutamic acid(4.3%), Glycine (5.7%), Isoleucine (5.0%), Histidine (1.4%), Leucine (8.6%), Lysine (1.4%), Methionine (2.9%), Serine (2.9%) and Threonine (7.1%) Tryptophan (1.4%) occurred less frequently (Doolittle 1989). The estimated half-life of SP6A protein is 30 h (mammalian reticulocytes, in vitro), >20 h (yeast, in vivo), >10 h (Escherichia coli, in vivo) as predicted in this analysis and Grand average of hydropathicity (GRAVY) is -0.172

Hydropathy plot of StSP6A: The hydropathy profile was generated for SP6A protein using the ProtScale tool based on the Kyte- Doolittle scale. Hydropathy plot of SP6A protein revealed that the amino acid sequence share an alternative pattern of hydrophilicity and hydrophobicity For example: amino acids from 5 to 17 were hydrophobic whereas amino acids 18 to 19 are hydrophilic in nature followed by the amino acid 20 hydrophobic in nature and again amino acid 21 to 23 are hydrophilic in nature, amino acid 24 to 28 are hydrophobic whereas 29 to 36 are hydrophilic, 37 to 39 are hydrophobic and 40 to 41 are hydrophilic, 42 to 45 are hydrophobic and 46 to 47 are hydrophilic, again 48 to 51 are hydrophobic and 52 t 61 are hydrophilic, 62 to 70 are hydrophobic whereas 71 to 83 are hydrophilic, amino acid 84 is hydrophobic while 85 to 86 are hydrophilic, 87 to 91 are hydrophobic and 92 to 100 are hydrophilic, ad 101to 103 are hydrophobic. After that 104 to 114 are hydrophilic and 115 to 124 are hydrophobic and in the end 125 to 136 are again hydrophilic. It followed a travelling wave like pattern. Generally it is a hydrophlic protein as observed by the **fig 9**.

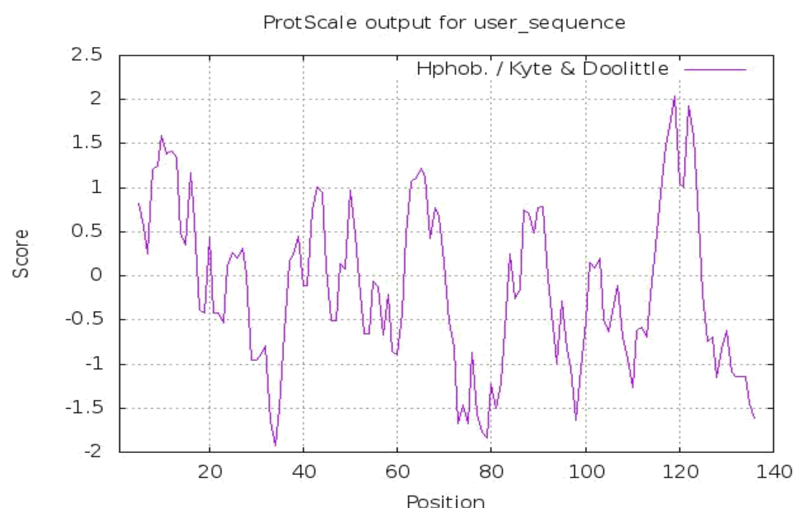


Fig. 9 Hydropathy plot of StSP6A protein

4.1.6 Amino acids composition analysis in the SP6A protein:

The amino acid composition of SP6A was closely inspected. The composition of some of the amino acids is presented in Table 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: Arginine, Asparagine Phenylalanine, Proline, T yr o s i n e , Valine occurred more frequently as compared to their average occurance; whereas the amino acids namely Alanine, Aspartic acid, cysteine, Glutamine, Glutamic acid, Glycine, Isoleucine, Histidine, Leucine, Lysine, Methionine, Serine, Threonine, Tryptophan occurred less frequently in these isoforms (Doolittle 1989). All these significant changes in the amino acid composition could have profound effects on the structure and function of the SP6A protein.

Table 8 Analysis of amino acid composition (%)

Amino Acid	**Average occurrence**	**SP6A**
Alanine (A)	3	2.1%
Arginine (R)	5.1	**8.6%**
Asparagine (N)	4.3	6.4%
Aspartic acid (D)	9	6.4%
Cysteine (C)	1.9	1.4%
Glutamine (Q)	4	2.9%
Glutamic acid (E)	6	4.3%
Glycine (G)	8	**5.7%**
Histidine (H)	2.3	1.4%
Isoleucine (I)	5.3	5.0%
Leucine (L)	12	8.6%
Lysine (K)	5.9	**1.4%**
Methionine (M)	4	2.9%
Phenylalanine (F)	3.9	4.3%
Proline (P)	5.2	**10.0%**
Serine (S)	6.8	**2.9%**
Threonine (T)	10	**7.1%**
Tryptophan (W)	2	1.4%
Tyrosine (Y)	3.2	4.3%
Valine (V)	6.6	**12.9%**

4.1.7 3-D modeling studies

3-dimensional protein structures of SP6A protein was predicted by using Swiss model tool .The amino acid FASTA sequences from NCBI were taken and uploaded in the tool, to find the best model. The protein models are given in **Fig. 10**.

- **Model structure**

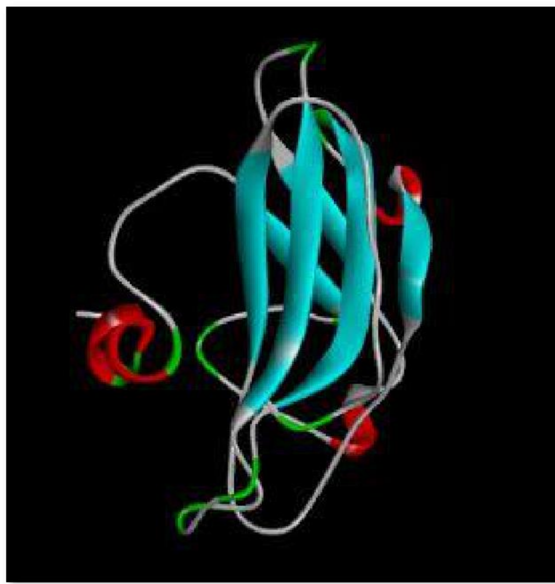


Fig. 10 Model Structure of SP6A

4.1.8 X-ray/NMR structure:

Using ProSA-web tool the protein model was validated. For validation, the PDB file was uploaded on ProSA tool and the following data was obtained as given in **Fig.11**. It depicts the position of protein in the X-ray/NMR region. The protein is shown by the black colored dot (.) if the protein falls in the region then it is considered as the best model.

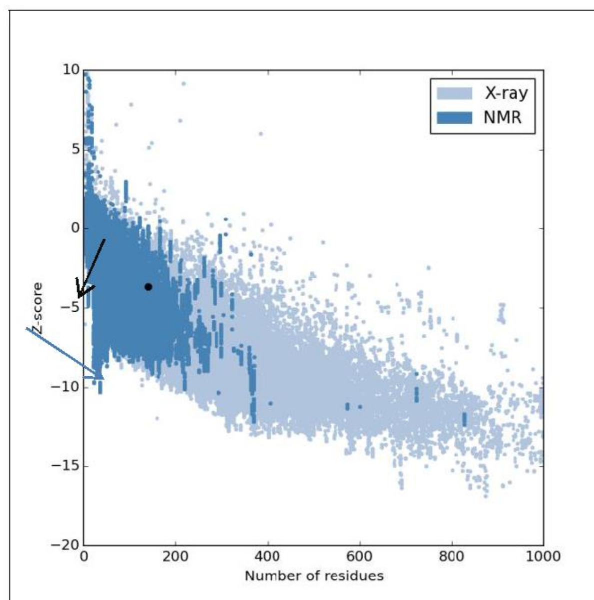


Fig. 11 X-ray/NMR structure of SP6A

4.1.9 Ramachandran analysis of curcin protein as visualized by SWISS Model tool:

Ramachandran plot is a way to visualize energetically allowed regions for backbone of amino acid residues in protein structure as indicated by dots in **Fig.12**. The plot was obtained by uploading the protein sequence to SWISS Model tool. In the Ramachandran plot of SP6A, the dot indicates the residues of proteins lying in the favored, allowed and outlier region as described in **Table 9**. There is almost similarity in the values of the proteins lying in allowed region but are very less number of proteins present in the negative region.

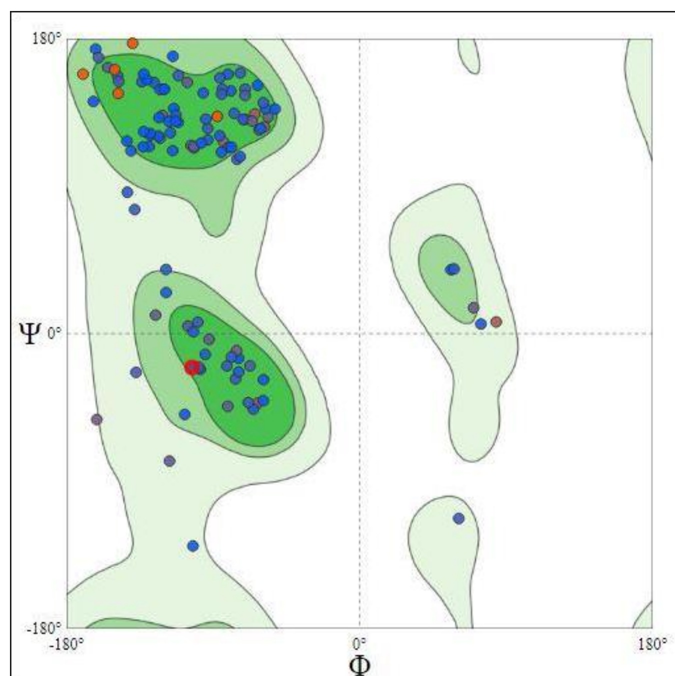


Fig. 12 Ramachandran Analysis of SP6A

Table 9 Ramachandran plot values of SP6A

MolProbity Score	1.45
Clash Score	0.46
Favoured	93.98%
Outliers	1.5% A103 ILE, A5 ASP, A81 ARG, A87 LEU
C-Beta Deviations	1 A94 THR
Bad Angles	A71 ASP, A116 HIS, A36 ASN, A69 ASP, (A5 ASP-A6 PRO), A5 ASP, A85 HIS, (A91 ILE-A92 PRO), (A108 ASN-A109 PRO)
Cis Non-Proline	(A81 ARG-A82 GLU)
Cis Prolines	(A72 ALA-A73 PRO)

4.1.10 Comparison between different isoforms of SP6A:

SP6A isoforms have some biochemical attributes which play a very important role in the functioning of the proteins. The comparison between different isoforms of SP6A 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, SP6A ranges from 33.6 to 107.98, which shows that SP6A is stable whereas all other isoforms are unstable.

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 SP6A, it was found to be in the range of 63.30 to 92.86.

Table 10 Biochemical attributes of different isoforms of SP6A

S.No.	Name	Transcript Id	Molecular Formula	No.of Amino Acids	Molecular Weight (kDa)	Stability Instability Index	pI Value	Aliphatic Index	GRAVY
1.	SP6A1	PGSC0003DMT400018307	$C_{758}H_{1161}N_{217}O_{217}S_5$	148	169.4	51.5	9.03	67.0	-0.47
2.	SP6A2	PGSC0003DMT400061403	$C_{1064}H_{1719}N_{325}O_{345}S_{10}$	227	249.0	64.9	9.71	63.3	-0.79
3.	SP6A3	PGSC0003DMT400009393	$C_{823}H_{1278}N_{246}O_{257}S_4$	168	188.5	69.1	6.87	70.3	-0.68
4.	SP6A4	PGSC0003DMT400060379	$C_{546}H_{877}N_{145}O_{167}S_6$	105	123.3	80.3	8.26	74.1	-0.73
5.	SP6A5	PGSC0003DMT400070298	$C_{524}H_{863}N_{143}O_{156}S_5$	105	118.2	76.7	9.33	92.8	-0.31
6.	SP6A6	PGSC0003DMT400070301	$C_{479}H_{793}N_{151}O_{145}S_9$	99	112.7	108.0	9.80	48.2	-1.05

4.2 Expression Pattern Studies of *StSP6A*

4.2.1 Isolation of total DNA from the potato cultivars:

Total DNA was isolated from commercial Indian cultivars namely Kufri Chipsona-1 (CS-1), and foreign exotic reference cultivar Désirée (De) from the different organs of potato, collected from the field grown plant. The quality of DNA was checked by agarose gel electrophoresis as shown in following (data not shown).

It shows good amount of isolated DNA from different potato cultivars. We see CS-1 and De prominent intensity of bands, whereas, band in CS-1 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. 13.** The distinct bands show the quality of the purified DNA present in CS-1 and De potato cultivar.

Fig. 13 Purified DNA from different potato cultivars. Agarose gel electrophoresis of total purification from two potato cultivars are indicated lane wise: Lane 1-CS-1; Lane 2- De

4.2.3 Isolation of total RNA from different organs of Potato:

Total RNA was isolated from different organs of Potato, i.e., leaf, stem, tuberizing stolon, tuber, and petiole. The crude RNA samples were checked by agarose gel electrophoresis. Ribosomal RNA bands were distinct indicating the intactness of total RNA preparations. The distinct bands show the quality of the RNA present as shown in **Fig. 14.**

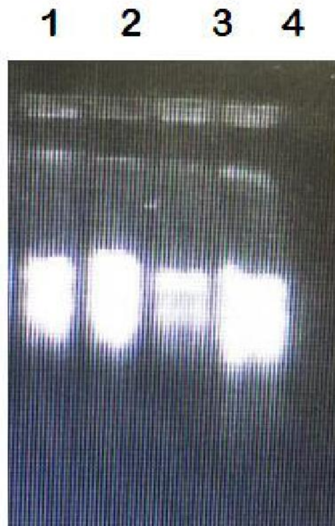


Fig. 14 Total RNA from various potato organs. Lane1- Leaf, lane 2- Tuberizing stolon, lane 3- Tuber, lane 4- Petiole

4.2.4 Purification of total RNA:

Total RNA was purified by RNase-free DNase followed by solvent extraction and dissolving the pellet in DEPC water. Purified RNA samples were checked by Agarose gel electrophoresis as shown in **Fig 15**. The distinct bands show the quality of the purified RNA present. Nanodrop spectrophotometer was used to find A260/A280 ratio to access the quality and quantity of the prepared RNA samples as shown in **table 11**.

Fig.15 Purified RNA from different Potato organs. Lane 1- leaf, Lane 2-tuberizing stolon, Lane 3-tuber, Lane 4- petiole

Table 11 Quantification of RNA using nanodrop spectrophotometer

Potato Organs	**Vol. of RNA soln. (µL)**	**Conc. (µg/mL)**
Leaf	1	180 µg
Tuber	1	710 µg
Tuberizing stolon	1	640 µg
Petiole	1	610 µg

4.2.5 PCR using primer specific to SP6A gene:

Efforts were made to amplify *SP6A* gene from few potato varieties (*Solanum tuberosum*) by PCR approach using specific primer pairs. For this purpose, total DNA preparations from the following potato (*Solanum tuberosum*) cultivars namely Kufri Chipsona-1 (CS-1) and Désirée (De) were used 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 section.

Using PCR amplification results using different potato total DNAs using the primer pairs SPF2-0404, SPR1-2378 is shown in **Fig 16**. Amplification occurred in both potato cultivars namely CS-1 and DE where the size of the amplicon was around 400 bp in each case which was in expected range indicating that no inrons were present. Single band was observed in case of CS-1 whereas in case of the reference cultivar De, multiple bands were observed.

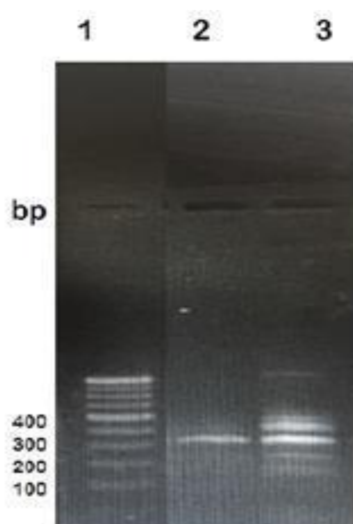


Fig. 16 PCR products using different potato cultivars, Lane 1- 100 bp ladder; Lane 2- CS-1; Lane 3- De

4.2.6 RT PCR using specific primer set of *StSP6A* in different potato organs

Results of PCR: RT-PCR was carried out using total RNA from different potato organs using the primer pair SPF2-0404, SPR1-2378. The RT-PCR data clearly indicated that amplification occurred only in the cases of 2 potato organs namely leaf and tuberizing stolon **(Fig. 17)**. In leaf and petiole, the size of the amplicon was around 400 bp which was in the expected size range. In case of tuber and petiole, no amplification was observed.

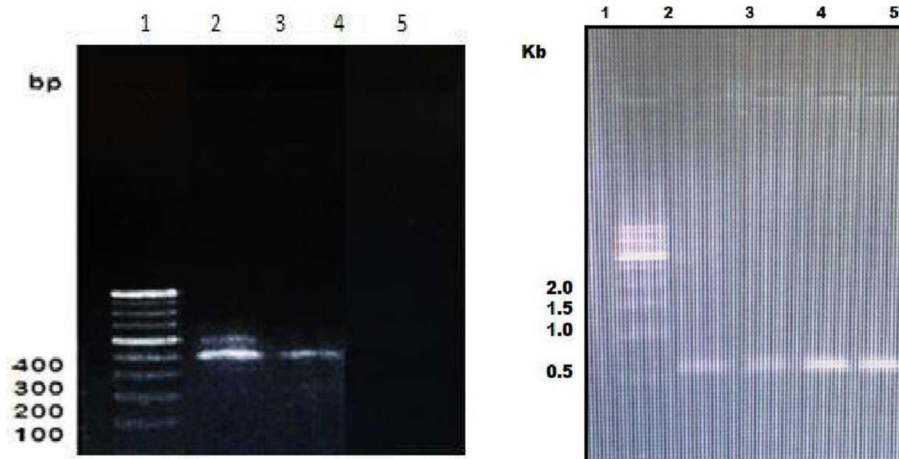


Fig. 17 Showing RT PCR product using gene specific primers and the other one showing Semi-quantitative RT PCR using a constitutive Actin gene. Lane 1- 1 kb Lane 2- leaf, Lane 3-tuberizing stolon, Lane 4-tuber, Lane 5-petiole (In both figures)

A semi quantitative RT PCR was done using a constitutive actin gene. PCR amplification was of 0.6 kb in all the four potato organs as mentioned above. It means that it is a constitutive gene expressed in all organs whereas amplification occurred only in the 2 cases of potato organs namely leaf and tuberizing stolon where the size of the amplicon was around 400 bp which was in the expected size range. In case of tuber and petiole, no amplification was observed shown in **fig 17**.

Actin is most abundant protein of 43 kDa which contributes 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 isotypes (α-actin, β-actin and γ-actin). It usually participates in more protein-protein interactions than any other known proteins.

CONCLUSIONS

- *StSP6A* belongs to SELF PRUNING family, which is also considered to be an ortholog of FT. It works as mobile signal and is transported from leaves to underground stolon tip to initiate tuberization. Literature survey revealed that considerable progress has been made on *SP6A* gene associated with growth and development in plants including *Solanaceae* family.

- Although there are some *SP6A* sequence data available in the database at both nucleotide and amino acid level, but the salient sequence features and comparison were not available in a comprehensive manner in the previously publish reports. Keeping this in mind, efforts are made on sequence analysis and comparison using the available *SP6A* sequence at both nucleotide and amino acid level. This exercise provided a clear and comprehensive idea about the sequence relatedness between the *SP6A* sequences.

- Now, we know the conserved amino acids along with some protein motifs in SP6A. Such type of information is not only helpful in understanding the evolutionary consequences but also important with regard to gene manipulation and protein engineering.

- Total DNA was isolated from different commercially important Indian potato cultivars, and both quality and quantity were assessed by spectrophotometric analysis.

- The different *StSP6A* gene-specific oligonucleotide primers were used in PCR 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 organs of potato under field conditions.

- Efforts were made to study the expression patterns of *StSP6A* in different organs of potato using RT-PCR approach. Transcripts were detected only in the leaves and tuberizing stolon as evident from the RT-PCR data

- It is very likely that differential expression patterns of StSP6A in potato could be associated with organ-specific specialized functions.

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