Molecular cloning, characterization and inhibition studies on sucrose-phosphate synthase (SPS) gene functions in the Indian potato cultivars

A thesis
submitted in fulfilment of the requirements
for the award of the degree of

Doctor of Philosophy
in
Biotechnology

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(Regn. No. 90700002)

Department of Biotechnology
THAPAR UNIVERSITY
PATIALA - 147004
PUNJAB – INDIA
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DECLARATION

I, hereby declare that the work which is being presented in the thesis, **"Molecular cloning, characterization and inhibition studies on sucrose-phosphate synthase (SPS) gene functions in the Indian potato cultivars"** submitted by me for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent an original research work under the supervision of Dr. N. Das, Professor, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

Dhakshi Taneja
(Dhakshi Taneja)

Date: 18 July 2014
Place: T.U. Patiala

CERTIFICATE

This is to certify that the thesis entitled, "**Molecular cloning, characterization and inhibition studies on sucrose-phosphate synthase (SPS) gene functions in the Indian potato cultivars**" which is submitted by Ms. Dhakshi Taneja, in fulfillment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology, Thapar University, Patiala, is a record of the candidate's own independent an original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

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Dhakshi Taneja

Table of Contents

List of Abbreviations

Name	**Abbreviation**
ADP-glucose	Adenine dinucleotide phosphate glucose
APS	Ammonium per sulfate
ATP	Adenine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
CaMV 35S	Cauliflower mosaic virus 35S
DAB	Diaminebenzidine
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	2’-deoxynucleoside-5’-triphosphate
DTT	Dithiothreitol
EBI	European Bioinformatics Institute
EDTA	Ethylenediamine-tetra acetic acid
Fru	Fructose
Fru-6-P	Fructose-6-Phosphate
FBPase	Fructose 1,6-Bisphosphatase
FW	Fresh weight
F26BP	Fructose 2,6-bisphosphate
GA_3	Gibberillic acid
GBSS	Granule-bound starch synthase
Glc	Glucose
Glc-1-P	Glucose-6-phosphate
Glc-6-P	Glucose-1-phosphate
GAP	Glyceraldehyde 3-phosphate
GUS	Glucuronidase
IAA	Indole-3-acetic acid
IgG-HRP	Immunoglobulin G-Horse raddish peroxidase
IPTG	Isopropyl-b-thiogalactoside
Kan	Kanamycin
kb	Kilo base
kJ	Kilo joule
L	Litre
LA	Luria agar
LB	Luria broth
M	Molar
mg g^{-1}	Milligram per gram
mg L^{-1}	Milligram per liter
mg min^{-1}	Milligram per minute
mg mL^{-1}	Milligram per milliliter
µg	Microgram
µg mL^{-1}	Microgram per milliliter

mL	Milliliter
mM	Millimolar
MOPS	3-(N-morpholino)-propanesulphonic acid
MS	Murashige and Skoog
NCBI	National Centre for Biotechnology Information
nm	Nanometer
NLS	Nuclear localization signals
O.D.	Optical density
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
PGI	Phosphoglucose isomerase
PGM	Phosphoglucomutase
p*I*	Isoelectric point
Pi	Inorganic phosphate
PPi	Pyrophosphate
PFK-2	Phosphofructokinase-2
PMSF	Phenylmethyl sulphonyl fluoride
RNA	Ribonucleic acid
rpm	Rotations per minute
RT-PCR	Reverse transcription polymerase chain reaction
SBRPs	Sucrose biosynthesis related proteins
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SPS	Sucrose-phosphate synthase
Suc	Sucrose
Suc-6-P	Sucrose-6-phosphate
SuSy	Sucrose synthase
SPP	Sucrose-6- phosphate phosphatase
STET	Sucrose Tris EDTA Triton X100
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
Tris	Tris-(hydroxymethyl-) aminomethane
UDP-Glc	Uridine diphosphate glucose
UGPase	UDP-glucose pyrophosphorylase
UTR	Untranslated transcribed region
v/v	Volume per volume
w/v	Weight per volume
YEM	Yeast extract mannitol

Abstract

Sucrose-phosphate synthase (SPS, EC 2.4.1.14) is a key enzyme in sucrose biosynthesis in both photosynthetic and nonphotosynthetic tissues of plants. It is encoded by different gene families. SPS exists in multiple forms which show differential distributions and functional specializations in the plant tissues. SPS activity is highly regulated by hierarchy of mechanisms including posttranslational modifications via phosphorylation, and allosteric control by the metabolic effectors, such as glucose-6-phosphate (Glc-6-P) (activator) and inorganic phosphate (Pi) (inhibitor). In this study, we report an isolation of a cDNA clone (designated SPS-C1; 3,591 bp) encoding a distinct full-length SPS A form consisting of 1,054 amino acids (designated KC-SPS1) from a commercially important Indian potato (*Solanum tuberosum* L.) cultivar, Kufri Chipsona-1 by RT-PCR approach using tuber RNA. This was first report from an Indian potato cultivar. Sequence analyses and comparison of the SPS sequences from the *Solanaceae* family members namely, potato, tobacco and tomato revealed many distinct features within and between the SPS gene families not documented earlier. Phylogenetic tree was made based on a large number of full-length SPS sequences from taxonomically different plant species. SPS expression level was studied in the leaves and tubers from some of the Indian potato cultivars by semi-quantitative RT-PCR, protein blot analyses, and assaying extractable SPS activities. The role of SPS is well-recognized in cold-induced sweetening in the potato tubers. Sucrose accumulation in the cold-stored tubers is usually correlated with higher SPS activities. For inhibition of SPS gene function, a number of antisense/sense binary genetic constructs were made using the different regions of the cDNA clone, SPS-C1 under both constitutive CaMV 35S and tuber-specific GBSSI promoters. Based on the cv. Kufri Chipsona-1, transgenic potato plants were generated using these constructs. Some promising transformant potato lines were obtained corresponding to each binary construct. Since, in the cold-stored transformed tubers, the level of soluble sugars (both sucrose and reducing sugars) was reduced by 50–80% as compared with control. In these tubers, sucrose, reducing sugar contents and extractable SPS activities were shown to be decreased significantly. Inhibition of SPS gene expression was demonstrated by semi-quantitative RT-PCR and protein blot analyses. This report would be useful for further in-depth studies on various SPS isoforms in potato and other *Solanaceae* family members. Moreover, various approaches as adopted in this study for improvement of the potato crop are quite relevant and promising.

Keywords: SPS cDNA cloning, SPS A form, Sequence analyses, Phylogenetic tree, Expression analyses, Indian potato cultivars, Constitutive and tuber-specific promoters, Antisense/sense binary constructs, *Agrobacterium*-mediated transformation, Cold-induced sweetening, Transgenic tubers

Chapter 1

Introduction

Chapter 1 Introduction

1.1. *Potato crop*

Potato (*Solanum tuberosum* L.) is a major non-grain food crop that is grown all over the world. It ranks third, and comes only after wheat and rice. Worldwide production of this crop was around 330 thousand tons in 2009 (http://faostat.fao.org/). Potato belongs to the family *Solanaceae* which consists of more than 3,000 members. Apart from potato, tomato, eggplant, tobacco, pepper and capsicum are also economically important members of the *Solanaceae* family. Among the various tuber crops such as cassava, sweet potato, yams, and taro, potato is the most important in terms of production, accounting for about 45% of the total global production of all tuber crops. Potato tubers are important dietary sources of starch, protein, antioxidants and vitamins. Therefore, this crop plays a major role with regard to global food security i.e., in fighting hunger and malnutrition. Currently, the major objectives of global potato and other crop breeding programs are optimization of production levels by producing resistant varieties towards various challenging biotic and abiotic stresses (Visser et al. 2009; Hazarika and Rajam 2011; Verma et al. 2012; Singh et al. 2014). In fact, both root and tuber crops are now considered to play pivotal role in feeding the developing world in near future. Recent trend indicates that the growth rates in tuber production are particularly high for potato. Since an annual average increase becomes 4.5 million tons per year, as compared with rice and wheat. Recent increases of potato production in the Asian continent have been noteworthy.

About 190 wild tuber-bearing species have been recognized in the section *Petota* of the genus *Solanum* reflecting very rich genetic resources of the potato crop; however, the taxonomy is currently being revised because of highly diverse landrace materials (Spooner and Hijmans 2001; Spooner et al. 2007). The cultivated *Solanum tuberosum* subsp. *tuberosum* is considered to be originated from Andean and Chilean landraces. However, the tuber-bearing *Solanum* species are widely distributed from the South Western USA to Southern Chile and Argentina, and also from sea level to the Andes Mountains. Many wild species possess many valuable traits, such as a wide range of resistances to pests and diseases, tolerances to frost and drought. As these species can be crossed directly with the common potato, therefore, they have become useful resource for breeding new cultivars (Visser et al. 2009). During the second half of the 16th century, the Spanish introduced the potato in Europe. Subsequently, this crop was introduced to many parts of Europe and throughout the world by European mariners. In India, potato was introduced by Europeans in the early 17th century.

In India, the importance of this tuber crop was realized soon after independence in 1947. The Central Potato Research Institute (CPRI), Shimla, established in 1949, adopted conventional breeding programmes for the improvement of potato crop, and released a number of high-yielding commercially important cultivars including some processing varieties. Now India ranks third in terms of area of potato cultivation, and it is the second largest country (after China) with ~34.39 million tons annual production of potato (http://www.fao.org). In the Indian subcontinent, potato is regarded as a short-duration crop with an average maturation time of 90-100 days.

Ploidy levels in potato: The most common cultivated species of potato (*Solanum tuberosum* L.) represents a hybrid between the diploid species S. *stentotomum* and the diploid weed *S. sparsipilum* with subsequent chromosome doubling (Ramanna and Hermsen 1979). The ploidy levels in potato varies from, haploid number of 12, ranging from diploid ($2n = 2x = 24$) to hexaploid ($2n = 6x = 72$) including triploids, tetraploids, and pentaploids (Spooner et al. 2005). Most of the potato cultivars are autotetraploid ($2n = 4x = 48$) and highly heterozygous. Therefore, improvement of these cultivars through classical breeding methods is an arduous task.

Morphology of potato plant: The potato, an herbaceous annual plant can grow up to 20-40 inches (50 to 100 cm) high. Plant bears alternate and irregularly pinnate compound leaves. Flowers are pentamerous, actinomorphic, perfect and have sympetalous colored corollas (white, pink, red, blue, or purple). Usually, potato plant is propagated through tubers (vegetative or asexual propagation), known as “seed tubers” or “seed potatoes”. The tubers bear lateral buds (eyes) that grow into new plants when the conditions are favourable for growth. Under certain conditions, potato plants can produce small green fruits that contain seeds, also called "true seeds" or "botanical seeds". These fruits contain large amounts of the toxic alkaloid solanine and is therefore, not suitable for consumption. Potato plants can be propagated through botanical seeds, which are known as True Potato Seeds (TPS) which not so popular for usual cultivation but very useful in conventional breeding.

Potato tuber-a modified stem: Potatoes are the somatic storage tissues, which develop by the modification of tips of the underground stems or stolons. Tuberization requires short days (small photoperiod) and long cool nights. In India, it is a winter crop except some varieties are grown in summers in hilly regions. Tuberization is the result of the activity of several interdependent processes, for example, stolon initiation and elongation, subapical swelling, cell division and the induction of specific proteins are a few of the contributing processes. The potato skin colour can vary from brownish white to deep purple. In mature tubers, cortical and pith cells accumulates large amount of starch and storage protein. Starch is the predominant storage material in potato tubers. In addition, there are also high - quality proteins, substantial amounts of essential vitamins including vitamin C, minerals, and trace elements present in potato tubers.

1.2. *Potato-an important system for basic and applied research*

All over the world, efforts are being made to produce disease-free and high-yielding potato varieties which can withstand a host of various biotic and abiotic stresses. In order to introduce these high performance attributes, many countries initiated several potato breeding programmes which are now being continued. The primary focus of applied research on potato tubers and other priority crops is mainly on disease control, starch metabolism, protein metabolism, nutritional aspects, post-harvest preservation at low temperatures, improving the yield and other processing attributes through transgene expression/inhibition of gene function, and importantly cultivation of this crop under different agro-climatic conditions (Bevan 1991; Liu et al. 2008; Bandopadhyay et al. 2010). For example, potato plants suffer from a variety of viral (such as potato viruses X and Y), bacterial (*Streptomyces scabies* and *Erwinia carotovora*), and fungal diseases (*Phythophthora infestans*), that cause serious loss to tuber yield. Various laboratories are involved in combating these problems using molecular and genetic approaches. Biochemical, molecular and cellular features of potato tuber development has proven to be an attractive model for studying underground sink organ (i.e., somatic storage tissue) formation. Many laboratories were involved in identifying a number of tuber-specific genes involved in this complex process of tuberization. Studies on starch and protein biosynthesis are important areas of tuber research. As starch is a valuable commodity in potato, many efforts are going on for manipulations of starch metabolism in terms of quality and quantity improvement. Potato contains high amount of protein. Biological values of such proteins could be improved through genetic manipulations. There is also an exciting prospect of using potato tubers as ‘factories’ for the production of novel proteins and metabolites having commercial and therapeutic importance. Therefore, in the area of 'molecular farming' potato systems are playing increasingly important role. Extent of accumulation of various soluble sugars such as sucrose, glucose and fructose along with their hexose-phosphate intermediates determine the quality of the tubers particularly under storage conditions. Moreover, interconversion between these sugars and starch, sugar-based signaling, interactions between the reducing sugars with free amino acids and proteins are interesting areas of research at biochemical level. Currently, understanding various signals, signal transduction pathways, and particularly sugar-based signalling in potato and other plants are important research areas (Koch 1996; Koch 2004; Halford et al. 2010; Swain et al. 2011; Gangappa et al. 2013).

Many desirable potato traits, such as the traits involved in tuber quality (Li et al. 2005; Menendez et al. 2002), horizontal disease resistance (reviewed by Gebhardt and Valkonen 2001), are known to be under polygenic control. For crop improvement through marker-assisted breeding or genetic modification approaches or for any biotechnology-based improvement of potato varieties, a fundamental prerequisite is the identification of the genes involved in the target traits. The allelic variations within these genes are also known to involved in the phenotypic variations for the traits (Visser et al. 2009; van de Wal et al. 2001; Draffehn et al. 2010). All the above studies were mainly based on the availability of a large number of Expressed Sequence Tags (ESTs) (Bachem et al. 2000; Rensink et al. 2005; Ronning et al. 2003) and some full-length gene sequences from potato. However, many more important gene functions involved in potato life cycle, particularly during various stages of tuber development are yet to be isolated and functionally characterized. This is only possible by exploring the rich genetic resources of different potato cultivers/clones. Despite the global importance of the potato crop, the genetics and inheritance of many important qualitative and quantitative agronomic traits are relatively poorly understood. We have limited knowledge with regard to compositional and processing traits of the potato tuber because of a) tetraploid nature of the genome, b) the high degree of heterozygosity, c) the absence of homozygous inbred lines or d) a poor collection of genetically well-defined marker stocks. It is very likely that high genetic load in potato leads to the distorted segregation ratios as observed frequently causing hindrance to the classical genetic research. However, a profound understanding of genetic composition of potato is still a basic requirement to develop efficient breeding methods. In this context, the major potato genome sequence projects are quite relevant and important (Visser et al. 2009, Genova et al. 2011).

During the last few decades considerable progress has been made on carbohydrate metabolism in potato at genetic, molecular and biochemical levels. Keeping in view with the aim of the present study, an overview of sucrose metabolism, the role of the different enzymes, and the overall role of sucrose-phosphate synthase (SPS) in the plant systems are precisely introduced in the following sections.

1.3. *Sucrose metabolism in plants*

In plants, sucrose and starch are the major photoassimilates produced during photosynthesis. Photosynthesis provides energy and carbon to both photosynthetic and nonphotosynthetic tissues for their growth and development. Therefore, translocation and partitioning of the photoassimilates are basic processes in plants where sucrose plays a very pivotal role. Sucrose (Suc), the most common naturally occurring disaccharide i.e. glucose (Glc) ($\alpha 1 \leftrightarrow 2\beta$) fructose (Fru) or Fru ($2\beta \leftrightarrow \alpha 1$) Glc. It is synthesized in the cytosol and is the main form of reduced carbon translocated from source leaves to developing tissues. During photosynthesis, starch is temporarily accumulated in the chloroplast stroma. The conversion of photoassimilates to either starch or sucrose in the photosynthetic cellular compartments is called carbon allocation. A portion of the photoassimilate is retained by the leaf to support its continued growth and metabolism; whereas, the majority is exported out of the leaf to various nonphotosynthetic organs/tissues through the vascular phloem tissue, known as translocation. Phloem translocation ensures an efficient distribution of photosynthetic energy and carbon between the different plant organs. This significant process is called carbon partitioning. This is also an important process from an agricultural perspective as it plays a crucial role in determining productivity, crop yield.

In the course of evolution, sucrose has been selected as the transport form of carbon because of its unusual linkage between the anomeric C-1 of glucose and the anomeric C-2 of fructose. This bond is not hydrolyzed by amylases or other common carbohydrate cleaving enzymes, and the unavailability of the anomeric carbons prevents sucrose from reacting nonenzymatically with amino acids. In higher plants, sucrose occupies a central position as the major product of photosynthesis and as a transport molecule for growth and development. Sucrose also fulfils many other roles in plants such as storage reserve, compatible solute under stress conditions, signal transduction, and regulation of gene expression (Huber and Huber 1996; Koch 2004; Koch 1996; Winter and Huber 2000; Halford et al. 2011). In some plant species, sucrose can also serve as direct substrate for additional biosynthetic reactions. For example, sucrose is metabolized further within the leaf to produce oligosaccharides of the raffinose family as transport sugars, or fructans as soluble carbohydrate reserves within the vacuole. (Smeekens 2000; Winter and Huber 2000; Lunn and MacRae 2003). Although sucrose biosynthesis is restricted to the cytosol, it is now believed that sucrose could be involved in regulating photosynthesis and other metabolic aspects in the plastids (Tiessen et al. 2002; Oswald et al 2001). Since the discovery of sucrose biosynthesis, considerable advances have been made in understanding its regulation. However, important aspects of sucrose metabolism still remain an enigma.

Sucrose biosynthesis: During active photosynthesis, most of the triose phosphate generated by CO_2 fixation is converted to sucrose or starch. In chloroplasts, CO_2 is fixed in the form of triose phosphate, dihydroxyacteone phosphate (DHAP) which is exported to cytosol. DHAP reversibly converted to glyceraldehyde 3-phosphate (GAP) by the action of triose phosphate isomerase. DHAP and GAP condensed to produce fructose 1, 6-bisphosphate which is then converted to fructose 6-phosphate (Fru-6-P), a reaction catalyzed by cytosolic fructose 1,6-bisphosphatase (FBPase). Glucose 6-phosphate isomerase (Phosphoglucose isomerase, PGI) catalyzes a reversible conversion of Fru-6-P to glucose-6-phosphate (Glc-6-P). Glc-6-P is interconverted to Glc-1-P by the action of phosphoglucomutase (PGM). Glc-1-P reacts with uridine triphosphate to produce uridine diphosphate glucose (UDP-Glc) and pyrophosphate, a reversible reaction catalyzed by UDP-glucose pyrophosphorylase (UGPase). UDP-Glc and Fru-6-P are the substrates for sucrose synthesis. Sucrose-phosphate synthase (SPS) catalyzes the synthesis of sucrose-6-phosphate (Suc-6-P) using the substrates UDP-Glc (an activated donor sugar) and Fru-6-P (a sugar acceptor). SPS catalyzes the transfer of glycosyl group from UDP-Glc to Fru-6-P (Huber and Huber 1996). Suc-6-P is then hydrolyzed to sucrose by sucrose-6-phosphate phosphatase (SPP). This high-activity phosphatase catalyzed reaction is essentially irreversible and displaces the reversible SPS reaction from equilibrium into the direction of net sucrose synthesis (Stitt et al. 1987). These inorganic phosphate molecules are returned to the chloroplast and used in the synthesis of more triose phosphate (Stitt and Heldt, 1985).

Regulation of sucrose biosynthesis: The partitioning of triose phosphates between sucrose and starch biosynthesis is regulated by fructose 2,6-bisphosphate (F26BP). The concentration of F26BP varies inversely with the rate of photosynthesis. Phophofructokinase-2

(PFK-2) responsible for F26BP synthesis is inhibited by dihydroxyacetone phosphate (DHAP) or 3-phosphoglycerate and stimulated by fructose 6-phosphate and Pi. During active photosynthesis, DHAP is produced and Pi is consumed resulting in inhibition of PFK-2 and lowered concentrations of F26BP. This favours greater flux of triose phosphate into fructose 6-phosphate formation and sucrose synthesis. Therefore, sucrose synthesis is favoured when the level of triose phosphates produced by the Calvin cycle exceeds the need of maintaining the operation of the cycle. Sucrose-phosphate synthase (SPS) is the key enzyme that catalyzes the formation of sucrose. Apart from F26BP, hierarchy of regulation of SPS activities at various levels in different plant tissues also play crucial role in overall sucrose biosynthesis. For further details, see the other sections related to SPS.

Translocation of sucrose: Sucrose is transported from source leaves to sinks tissues, such as tubers and seeds, through the phloem sieve elements. Some studies support the association of sieve elements with companion cells through plasmodesmata. Seive element/companion cell (SE/CC) complex works as functional unit in leaves (Oparka KJ 1986; Patrick JW 1990). Sucrose is transported from SE/CC complex to sink tissues through two pathways: a) symplastic unloading (assimilates may leave through plasmodesmata to the vascular parenchyma, and b) apoplastic unloading (directly across the plasmamembrane into the apoplast through proton-coupled sucrose transporters (SUT) (Oparka et al. 1992; Lalonde et al. 1999, 2003). During stolon elongation phase, phloem unloading predominantly follows the apoplasmic route. During initiation of tuberization, there is a shift to symplastic unloading in swollen stolons as shown by dye movement from phloem to parenchyma tissues (Viola et al. 2001). It is widely accepted that sink strength influences the distribution pattern of assimilates throughout the whole plant (Patrick JW 1990). In potato tuber, sucrose metabolism and starch synthesis in growing tubers determine its sink strength (Zrenner et al. 1995). Utilization of sucrose by sucrolytic enzymes maintains the gradient across the source and sink organs.

Sucrose breakdown and utilization in sink tissues: In the somatic storage tissues such as potato tubers, cleavage of sucrose essentially follows the mechanism of phloem unloading: such as apoplastic phloem unloading (as invertase activity is high during stolon elongation), and symplastic phloem unloading (also aided by higher sucrose synthase activity while tuberization). It may be noted here that invertases (β-fructofuranosidase) are present in the apoplast, vacuole, cytosol, plastids and mitochondria. Based on pH optima and subcellular locations, plant invertases can be classified into three types: the cell wall/ apoplast and vacuolar and neutral/alkaline invertases. Cell wall invertases and other specific stimuli favour sucrose import and amplify hexose signals, even in the presence of plasmodesmatal continuity (Sturm and Tang, 1999; Wachter et al 2003). Sucrose entering the cytoplasm frequently transported to vacuoles where it is hydrolyzed by vacuolar invertases/acid invertases. In general, hexoses produced by vacuolar invertases favor sugar signaling, cell division and expansion whereas sucrose favours differentiation and maturation (Sturm and Tang 1999; Koch 2004; Kumari and Das 2013). Cytoplasmic invertases are less active in most of the systems (Winter and Huber 2000). Sucrose synthase (SuSy) catalyzes the reversible conversion of sucrose and UDP to UDP-glucose and fructose. In plants, it usually catalyzes sucrose breakdown rather than its biosynthesis. SuSy is present in the cell wall and cytoplasm. Sucrose synthase-catalyzed reactions initiate many biosynthetic processes in the cell. For example, UDP-Glc acts as a precursor of many polysaccharides such as cellulose, pectins and arabinoxylans (Albrecht and Mustroph 2003) and synthesis of callose (Salnikov et al. 2003).

The regulation of these reactions is an important aspect of plant carbon metabolism because the invertase and sucrose synthase catalyzed reactions are the only known pathways of sucrose breakdown. Both these are vital processes in higher plants because it not only allocates the crucial carbon resources but also initiates hexose-based sugar signalling**.** Since inveratases produce two-fold hexoses than sucrose synthase, it involved more in altering sugar signals. Sucrose translocated from the leaf tissue to storage organs such as roots, tuber tissue, and developing seeds is commonly utilized in starch biosynthesis. Susy catalyzes the conversion of sucrose and UDP to fructose and UDP-glucose; UDP-glucose reacts with PPi to form UTP and Glc-1-P catalyzed by UDP-glucose pyrophosphorylase (UGPase). ADP-glucose pyrophosphorylase (AGPase) catalyzes the conversion of Glc-1-P and ATP to ADP-glucose and PPi. ADP-glucose is then converted to starch by starch synthase (s) (Ferni et al. 2002).

1.4. *Sucrose-phosphate synthase (SPS)*

Sucrose-phosphate synthase (SPS; EC 2.4.1.14) is the key enzyme in the process of sucrose biosynthesis. The committed pathway of sucrose synthesis involves the sequential action of following enzymes. SPS catalyzes the transfer of glycosyl group from UDP-Glc to Fru-6-P (Huber and Huber 1996). Suc-6-P is then hydrolyzed to sucrose by sucrose-6-phosphate phosphatase (SPP; EC 3.1.3.24). Sucrose-phosphate synthase (SPS), sucrose synthase (SuSy, EC 2.4.1.13) and sucrose-phosphate phosphatase (SPP) are collectively known as sucrose-biosynthesis-related proteins (SBRPs) (Salerno and Curatti 2003). SPS is expressed in photosynthetic and also in heterotrophic tissues, including potato tubers (Geigenberger et al. 1997), cotyledons (Geigenberger and Stitt 1991), fruits (Hubbard et al. 1991; Komatsu et al. 1996), roots and flowers (Fung et al. 2003), developing and germinating embryos, and cotton fibers. The metabolism of sucrose in heterotrophic tissues is characterized by a continuous process of utilization and synthesis. SPS is regulated by a multiple sophisticated mechanisms at different levels involving transcriptional regulation (Harn et al. 1993; Klein et al.1993; Chávez-Bárcenas et al. 2000), and posttranslational modifications via phosphorylation, allosteric control by the metabolic effectors, such Glc-6-P (activator) and Pi (inhibitor) (Huber and Huber 1996; Winter and Huber 2000).

SPS in photosynthetic tissues: SPS plays an important role in sucrose biosynthesis in the photosynthetic tissues. SPS undergoes diurnal changes as observed in the leaves of soybean plants (*Glycine max* L.) (Rufty et al. 1983; Huber et al. 1985) and fully-expanded leaves of cotton plant (Hendrix and Huber 1986). SPS activity showed two peaks, one in the first part of the photoperiod and second after 12 hours over the 24 hour light/dark period. SPS activity was found to be lowest in afternoon hours. During these experiments, it was observed that the rate of sucrose translocation was positively correlated to these diurnal changes in SPS activity (Huber et al. 1985). The activities of other enzymes involved in sucrose biosynthesis, UDP-glucose pyrophosphorylase, cytoplasmic fructose-1, 6-bisphosphatase, and sucrose synthase did not alter during these fluctuations. In the dark period, SPS activity remains low; interestingly, when the plants are transferred to light, increased partitioning of photosynthate into starch is favoured. Thus, SPS activity regulates the flow of carbon-flux into sucrose and partitioning of fixed carbon between starch and sucrose during daylight. Moreover, SPS maintains its endogenous rhythm even under continuous light or dark conditions. The diurnal fluctuations still persists under continuous environmental conditions as demonstrated in soybean and tomato leaves (Kerr et al. 1985; Jones and Ort 1997).

SPS in heterotrophic/sink tissues: SPS is also present in non-photosynthetic i.e. heterotrophic tissues such as potato tubers (Geigenberger et al. 1997, Reimholz et al. 1997), tomato (Miron and Schaffer 1991), banana (Hubbard et al. 1991; Nascimento et al. 1997), prunus (Hubbard et al. 1991), cotyledons of germinating *Ricinus communis* L. seedlings (Geigenberger and Stitt 1991), citrus fruits (Komatsu et al. 1996), roots and flowers (Fung et al. 2003), developing maize endosperm (KH 2004), cotton fibers (Xu et al. 2007), and alfalfa root nodules (Aleman et al. 2010), and contributes to several important processes. SPS activity is considered to be an important limiting step in source-sink relationships (Rufty and Huber 1983). Source tissues such as mature leaves and germinating seeds show high SPS activities (Avigad 1982). In sink tissues, SPS have additional roles. It is involved in a cycle of sucrose degradation and resynthesis that contributes to the regulation of sucrose import and mobilization (Dancer et al. 1990; Geigenberger and Stitt 1991, 1993), and other developmental processes such as fruit ripening (Hubbard et al. 1990; MacRae et al. 1992; Hill and apRees 1995; Roy Choudhury et al. 2009).

SPS activites under environmental stress conditions: Sucrose is synthesized under various environmental stresses, including water deficit (Morgan 1984; Quick et at. 1989; Zrenner and Stitt 1991), osmotic stress (Reimholz et al. 1994; Geigenberger et al. 1995; Geigenberger et al. 1997, 1999), and low temperature (Burton 1989). Sucrose plays important role in osmoregulation and cryoprotection in many plant tissues. Incubation of spinach leaves and potato tubers in hyperosmotic solutions of mannitol or sorbitol induced osmotic stress, resulted in increased accumulation of soluble sugars, especially sucrose (Quick et al. 1989; Zrenner and Stitt 1991; Toroser and Huber 1997). In these experiments, the overall V_{max} activity remained constant, whereas V_{sel} activity was found to be increased indicating increased activation state of the enzyme under osmotic stress. It was suggested that phosphorylation of SPS at Ser-424 was responsible for activation of enzyme during osmotic stress.

Plants can accumulate soluble sugars at low (non-freezing) temperatures. Cold stress triggers dramatic changes to plant metabolism such as general reductions in many enzyme activities and their reaction rates, appearance of new forms of enzymes, and importantly reconfiguration of the metabolome for protective functions. Apart from Suc, low-temperature exposure causes accumulation of several metabolites (known as cryoprotectants) such as trehalose, fructan, raffinose family oligosaccharides, galactinol, proline and glycinebetaine were well documented in some plant species (Pollock and Cairns 1991; Winter and Huber 2000; Cook et al. 2004; Zhu et al. 2007).

As discussed above, significant progress has been made on SPS, a key enzyme of sucrose biosynthesis, in various plant species during the last few decades. Many laboratories studied SPS at genetic, molecular and biochemical levels. SPS cDNA clones were also employed in several biotechnological applications. The next 'Review of Literature' section mainly deals with important biochemical attributes of SPS including regulation of enzyme activities, several regulatory/binding motifs; molecular cloning, characterization, expression and classification of different SPS forms in different plant species along with some promising advances on applied research relevant to the aim of present study.

Chapter 2

Review of Literature
&
Objectives

Chapter 2 Review of Literature

Biochemical and molecular studies on various SPS isoforms have been carried out in many plant species including some of the economically important members of the *Solanaceae* family such as tomato, potato, tobacco and others. The major focus areas of studies in many laboratories include: a) isolation, purification and biochemical characterization of plant SPSs and their regulation, b) molecular cloning, characterization and expression studies, c) sequence analyses and comparison, prediction of structure-function relationships, d) classification and functional specializations of the SPS forms, e) understanding the crucial biological roles of SPSs during plant growth and development, f) SPS-mediated responses to various abiotic stresses (for examples, low temperature and osmotic stress where sucrose acts as a osmoregulator and cryoprotectant), g) SPS activities and crop yield, and h) biotechnogical applications such as crop improvements through transgenics employing SPS cDNA/genomic clones. The overall progress in the aforesaid focus areas are given in the following sections.

2.1. *Biochemical studies on SPS*

Leloir and Cardini (1955) first reported that sucrose was synthesized by the enzyme SPS and SPP via the intermediate Suc-6-P. Subsequently, SPS activity and its regulation have been extensively studied in the leaves of different plant species such as spinach (Amir and Preiss 1982; Doehlert and Huber 1983), maize (Kalt-Torres et al. 1987), rice (Salerno et al. 1998). In photosynthetic tissues, it makes a significant contribution in controlling the flow of carbon flux into sucrose. In plants, SPS represents a low-abundance protein (<0.1% of the leaf soluble protein). The native SPS molecule is likely a dimer of 120-138 kDa subunits (Huber et al. 1994). SPS is allosterically regulated by glucose 6-phosphate (an activator) and inorganic Pi (an inhibitor). Fluctuation of SPS activity in leaves is a common phenomenon, which is controlled by different factors such as endogenous rhythms (i.e. diurnal changes), light/dark modulation, low-temperature stress, osmotic stress (Huber and Huber 1996; Winter and Huber 2000).

2.2. *Regulation of SPS activities*

SPS is known to be regulated by a hierarchy of several interacting mechanisms both at biochemical and molecular levels. For instance, transcription of SPS gene is developmentally regulated, and can be influenced by several environmental factors such as light, osmotic stress, exposure to low temperature. SPS is subject to covalent modifications and allosteric regulation via metabolites such as glucose-6-phosphate (Glc-6-P) and inorganic phosphate (Pi). These allosteric effectors are known to exhibit profound influence on the affinities for both the substrates, Fru-6-P and UDP-Glc. In response to different environmental conditions, SPS is regulated by reversible phosphorylation on distinct serine residues. Interestingly, the dephosphorylated form has a higher affinity for its activator Glc-6-P and a lower affinity for the inhibitor Pi. This process of dephosphorylation in SPS establishes a connection between covalent modification and allosteric regulation (Huber and Huber 1996; Toroser and Huber 1997; Lunn and MacRae 2003; Huber 2007). According to the current understanding, the phosphorylation status of SPS in the critical serine residues is the major determinant of its catalytic activity (Huber and Huber 1996; Winter and Huber 2000). In the dark i.e. in absence of active photosynthesis, SPS is phosphorylated and inactivated. Upon illumination, phosphorylated SPS is rapidly dephosphorylated, and the enzyme activity increases concomitantly with the rising rate of photosynthesis. It is also believed that even during the daytime when photosynthetic end products (i.e. Suc) accumulate within leaves, SPS is gradually phosphorylated again and thus activity declines (Stitt et al. 1988; Huber and Huber 1996). Reimholz et al. (1994) showed that the SPS was regulated in potato tubers by the allosteric effectors (such as Glc-6-P and Pi) and protein phosphorylation in an analogous manner as occurred in leaves.

2.2.1. Regulatory/binding motifs of SPS

All known SPS sequences of plant origin consist of two regions, N-terminal glucosyltranferse and C-terminal SPP-like domains connected by a chain of amino acids known as linker region. Most of the catalytic sites and regulatory/ binding sites are located at the N-terminal region. The important motifs are, UDP-glucose binding site, putative fructose-6-phosphate binding site, phosphorylation regulatory sites, Ser-158, Ser-229, and Ser-424, which are involved in light/dark regulation 14-3-3 protein binding, and osmotic stress activation, respectively. Based on the biochemical studies on spinach SPS, it is now commonly believed that there are a number of critical Ser residues in SPS that undergo reversible phosphorylation. SPS is phosphorylated at multiple seryl residues under different conditions. In spinach leaves, the three regulatory phosphorylation sites were discovered which was thought to be present in the loop regions of the enzyme (Salvucci et al. 1995).

Light/Dark modulation of SPS: In spinach, Ser-158 was identified as a major phosphorylation site responsible for light/dark modulation of SPS (McMichael et al. 1993). During dark hours, when Pi concentrations are high, SPS kinases phosphorylate the SPS at Ser-158 leading to an inactivation of the enzyme. The involvement of Ser-158 in light/dark modulation of SPS had been supported experimentally by many studies (McMichael et al. 1995; Huber et al. 1995; Weiner 1995). It is to be noted here that the putative seryl phosphorylation sites as observed/predicted in spinach SPS is not conserved exactly in most of the other plant SPS sequences available in the database. For example, in *Zea mays* and transgenic tobacco plants expressing the maize SPS gene, phosphorylation occurred at Ser-162 (Huber et al. 1995). But in other species, the regulatory phosphorylation sites are still remained to be determined experimentally. Most of the higher plant SPS sequences including monocots and dicots as reported in the literature contain this regulatory phosphorylation site.

Osmotic stress activation: A second SPS phosphorylation site, such as Ser-424 in spinach was shown to undergo reversible phosphorylation in response to osmotic stress; however, it is not present in all SPS proteins (Toroser and Huber 1997). Incubation of spinach leaves and potato tubers in hyperosmotic solutions of mannitol or sorbitol increased the V_{sel} activity of SPS, whereas V_{max} activity remained constant. In other words, osmotic stress led to altered kinetic properties of SPS (Huber et al. 1999). Protein kinase PK_{IV} was thought to be involved in phosphorylation of Ser-424 under stress conditions.

14-3-3 binding site: In spinach SPS, Ser-229 refers to a phosphorylation site that provides putative interaction site for 14-3-3 proteins (Toroser et al. 1998; Moorhead et al. 1999). Huber et al. (1998) showed that 14-3-3 proteins can bind or interact with phosphorylated SPS at Ser-229. It is believed that such type of protein-protein interactions could influence SPS activities. This partly explains why SPS activity was inhibited both in selective and non-selective assays. It was demonstrated that addition of a synthetic phosphopeptide based on SPS–Ser-229 recognition site disrupted the SPS-14-3-3 complex thereby stimulating the SPS activity (Toroser et al. 1998). In spinach, RQVSAP is the recognition sequence as it contains Ser-229, whereas the conserved motif RSXpSXP (where X is any amino acid and pS is phosphoserine) is found in other SPSs where 14-3-3 proteins can bind. The significance of 14-3-3 binding still remains to be elucidated.

2.2.2. Regulation of SPS by protein kinases

SPS protein kinases: In spinach, two types of protein kinases are known to be involved in phosphorylation of SPS: PK_{III} (Mr ~150 kD), a calcium independent protein kinase, and PK_{I} (Mr ~45 kD) a calcium dependent protein kinase. PK_{III} is consisdered as the major protein kinase that phosphorylates Ser-158 (McMichael et al. 1995). The catalytic subunit of PK_{III} (Mr ~65 kD) was found to show the properties of SNF1-like kinases that are often regulated by phosphorylation (Douglas et al. 1997). The basic residues at -3 and -6 positions and hydrophobic residue at -5 position in the flanking regions of Ser-158 were shown to be targeted sites for PK_{III} (McMichael et al. 1995; Toroser et al. 1998). In maize, only calcium dependent protein kinase had been identified. The basic residues at -3 and -6 positions and a hydrophobic residue at -5 position are the 'recognition residues' for PK_{I}.

Metabolic regulation of protein kinases: Glu-6-P was thought to regulate PK_{III}-catalyzed phosphorylation of SPS (Weiner et al. 1992). PK_{III} is allosterically inhibited by Glu-6-P; whereas, SPS is allosterically activated by Glu-6-P. In potato tubers, a positive correlation was observed between the *in vivo* Glu-6-P content and activation state of SPS that may explain the metabolite regulation of protein kinase (Geigenberger et al. 1998). Calcium-dependent, PK_{I} is the only protein kinase that was isolated from maize leaf (Huber et al. 1995). This report indicated that cytosolic calcium ion concentrations may also regulate sucrose biosynthesis in some species. SPS is activated *in vivo*, during light conditions when the calcium ion concentration in cellular pools was observed to be lower as compared with dark.

SPS protein phosphatases: Type 2A SPS protein phosphatase (SPS-PP) dephosphorylated phospho-SPS thereby releasing the SPS into an active state. Dephosphorylation of phospho-SPS is inhibited by Pi. Such phosphatase activity is also regulated under light/dark conditions. The enzyme isolated from illuminated spinach leaves showed slightly higher activity and reduced sensitivity to Pi (Huber and Huber 1992).

2.2.3. *SPS-SPP complex*

Soluble enzymes often occur in discrete complexes with other enzymes of the same pathway as evident from a variety of systems (Srere 1987). The concept of the 'metabolic unit' is now recognized in both prokaryotes and eukraryotes (Huber and Huber 2000). Suc-6-P is hydrolyzed to sucrose by the enzyme SPP. Some SPS forms contain an SPP-like domain at the carboxy terminus. Molecular cloning studies revealed that SPS contains an SPP-like domain at the carboxy-terminus, to which SPP can bind through protein-protein interactions. Therefore, SPS-SPP complex formation could be one of the control points of sucrose biosynthesis in plants (Lunn et al. 2000, Lunn and MacRae 2003). Sequence comparison revealed that the members of plant sucrose-phosphatase (SPP) and haloacid dehalogenase (HAD) superfamily of phosphatases/hydrolases consisted of three conserved motifs, Motif I, Motif II, and Motif III (Lunn et al. 2000). Motif I is associated with the active site having consensus DXDX (T/V), in which the first D residue forms an acylphosphate intermediate during catalysis (Collet et al. 1998). Only Motif I and Motif III could be predicted in the SPP-like domain of different SPS family members. As compared to B and C family members, Motif I in A members was found to be very close to the consensus.

2.3. *Studies on SPS at molecular level*

2.3.1. Molecular cloning studies

Significant advances have been made on SPS at molecular level covering different plant species. Worrell et al. (1991) first reported the isolation of SPS cDNA from maize. Since after maize, a number of cDNA/genomic sequences encoding different SPS isoforms from different plant species including economically important crops covering both monocots and dicots have been submitted in the databases and most of them reported in the literature, such as spinach (Klein et al. 1993; Sonnewald et al. 1993), sugarbeet (Hesse et al.1995), faba bean (Heim et al. 1996), citrus fruit (Komatsu et al. 1996; Komatsu et al. 1999), rice (Valdez-Alarco´n et al. 1996 & Yu et al. 2005), sugarcane (Sugiharto et al. 1997), resurrection plant (*Craterostigma plantagineum*, Ingram et al. 1997), potato (Krause et al. 1998), *Arabidopsis thaliana* (Theologis et al. 2000), Kiwifruit (Langenkämper et al. 2002), tobacco (Chen et al. 2005), Poplar (*Populus trichocarpa*, Tuskan et al. 2006), grape (Jaillon et al. 2007), Japanese pear (Itai and Tanahashi 2008), sorghum (Paterson et al. 2009), muskmelon (Tian et al. 2010), alfalfa (Aleman et al. 2010), tomato (GenBank ID: NP_001234839, AFD64638). All these molecular studies contributed significantly in understanding structure-function relationships of the predicted SPSs, their classification, phylogenetic relationships, importantly their expression patterns and functional specializations in plants.

2.3.2. Classification of SPS forms

Molecular cloning studies clearly indicated that higher plants contained more than one gene encoding SPS. Langenkämper et al. (2002) analyzed a number of known SPS genes.

Based on phylogenetic analyses they proposed that SPS genes in higher plants could be separated into three distinct families, designated A, B, and C. For example, the genomic sequences of *Arabidopsis thaliana* revealed the presence of four distinct SPS genes representing at least one member in each family; more particularly, two genes on chromosome 5 belong to Family A, one gene on chromosome 1 to Family B, and one gene on chromosome 4 to Family C. Similarly, each of three *Citrus* genes belongs to one of the three families (Komatsu et al. 1996). Lunn and MacRae (2003) proposed that at least one representative for each family was present in the genome of a given dicot species and that a member of each family was expressed. Analyses of genomic and EST sequence data from rice and maize revealed the presence of five unique full-length SPS genes in the rice genome, and at least seven SPS genes could be found in maize (Lutfiyya et al. 2007). Based on the phylogenetic analyses, currently three SPS gene families are recognized in dicotyledonous plant species; while in the monocotyledonous Poaceae species such as wheat, maize and rice, there are five gene families (Castleden et al. 2004, Sharma et al. 2010).

2.3.3. Differential expression and functional specializations of the SPS forms

Differential expression of the members of *SPS* gene families was studied in some plant species. Three unique SPS genes namely CitSPS1, CitSPS2, and CitSPS3 were isolated from citrus fruit, and their expression was found to vary between the tissues (Komatsu et al. 1996). CitSPS1 and CitSPS2 genes were found to be expressed in almost all plant organs such as leaf, flower and fruit. However, CitSPS1 was highly expressed in mature leaves and fruits and its level was very low in young leaves, flowers and immature fruits. Higher levels of CitSPS2 expression were found in flowers and mature fruits. CitSPS3 was expressed in young and mature leaves. Three SPS genes encoding three different isoforms were isolated and characterized from alfalfa (*Medicago sativa* L.). One of them, i.e. MsSPSA belongs to class A, while other two memebers, MsSPSB and MsSPSB3 belong to class B. In alfalfa, enhanced expression of MsSPSA could be noticed in the nodule, whereas both MsSPSB genes showed enhanced expression in the leaves. Moreover, leaf and nodule SPS enzymes showed differences in chromatographic and electrophoretic mobilities, and also found to vary with regard to their $V_{\max}$ and allosteric regulation (Aleman et al. 2010). Considerable progress was also made in monocots. For example, in the rice genome, five SPS genes (designated as *OsSPS1*, *OsSPS2, OsSPS6*, *OsSPS8*, and *OsSPS11*) were detected (Sakamoto et al. 1995; Castleden et al 2004;

Okamura et al. 2011). Okamura et al. (2011) demonstrated that the rice SPS genes, particularly *OsSPS1*, were preferentially expressed in source tissues; whereas *OsSPS2*, *OsSPS6*, and OsSPS8 were expressed in both source and sink tissues in a comparable manner. They also showed that the expression of all the SPS genes, particularly that of *OsSPS1* and *OsSPS11*, were relatively higher at night when the activation state of the SPS proteins was low, and the intracellular mRNA levels of *OsSPS1* and *OsSPS6* were negatively correlated with sucrose content. Very recently, Yonekura et al. (2013) made a significant contribution on transcriptional control of SPS genes in rice. They reported that the promoter activities of rice SPS genes namely *OsSPS1* and *OsSPS11* were controlled by light and circadian clock. Sucrose could not exhibit significant effect on the expression of these genes. All these studies clearly indicate that the SPS genes are under complex transcriptional controls and show differential expression patterns in the plant tissues.

Considerable progress has been made on SPS in some of the commercially important members of the *Solanaceae* family. In tobacco, at least three different SPS genes, designated as *NtSPSA*, *NtSPSB*, and *NtSPSC* representing one member of each of the three SPS gene families. Each SPS isoform had a distinct but overlapping expression pattern. For example, NtSPSA (the A-family member) was expressed in all tissues but in a varying manner. Expression of the B isoform (NtSPSB) was confined to the reproductive organs, and NtSPSC mRNA was exclusively detected in mature source leaves. A-family members have been the subject of most expression studies and most of the expressed sequence tags (ESTs) examined belong to the A family, implying that A-family genes are more abundantly expressed than those belonging to other families (Chen et al. 2005). Based on cDNA cloning studies and Documentation of NCBI's Annotation Process, a number of sequences encoding full-length SPS from different tomato cultivars were submitted to the database under the following GenBank IDs: AY726439 (3373 bp; protein_id AAU29197, 1054 aa), AF071786 (3337 bp; protein-id AAC24872, 1050 aa), NM_001246991 (3488 bp; protein_id NP_001233920, 1053 aa), JQ086765 (3551 bp; protein_id AFD64637, 1045 aa), JQ086766 (3600 bp; protein_id AFD64638, 1064 aa), and XM_004250713 (3344 bp; protein_id XP_004250761). The expression patterns of the corresponding SPS genes in different tomato tissues are still not known since most of these sequences are unpublished. However, all these full-length SPS sequences from tomato could be grouped into three SPS gene families: A, B, and C as evident from sequence comparison and phylogenetic analysis. Out of the above six SPS sequences in tomato, the first four represent SPS A family members, and each of them appeared to be distinct variant in terms of total size and amino acid substitutions; the fifth belongs to SPS B family, and the last one being a member of SPS C family.

In potato, apart from photosynthetic tissues, sucrose metabolism is known to play important role in both growing and cold-stored tubers. Reimholz et al. (1994) first isolated phosphoglucose isomerase (PGI)-free SPS from the potato tubers. Subsequently, considerable progress has been made in understanding the biochemical roles of SPS and other SBRPs in both growing and cold-stored potato tubers (Zrenner et al. 1995; Hill et al. 1996; Krause et al. 1998). Based on the immunoreaction techniques, Reimholz et al. (1997) showed the presence of multiple SPS forms in the cold-stored potato tubers. The immunopositive polypeptides were tentatively termed as SPS-la, SPS-lb, SPS-2 and SPS-3 having approx. molecular weights of 125, 127, 135 and 145 kDa, respectively. All these isoforms were also found to vary in tissue distribution patterns in potato suggesting their involvement in growing and mature tissues, in flower parts, and in acclimation to low temperature. As evident in the database, SPS-P4 refers to only one known full-length potato SPS cDNA clone from the reference cultivar Desiree (GenBank ID: X73477) which encodes a polypeptide of 1,053 amino acids. This is a representative of SPS A family in potato. SPS-1a and SPS-1b appeared to be close to the SPS-P4 encoded predicted polypeptide. SPS protein and activity in potato tubers, young and source leaves were found to be significantly reduced by antisense inhibition and co-suppression using the full-length 3,159-bp coding sequence of SPS-P4. Interestingly, the expression patterns of several forms of SPS were not reduced uniformly in the transgenic potato plants indicating significant sequence variations between them (Reimholz et al. 1997; Krause et al. 1998).

2.4. *Some advances on SPS with regard to applied research*

SPS activity and crop yield: Considerable progress has been made in understanding sucrose metabolism and SPS at genetic level. Plant growth and productivity could be correlated with SPS activity in some important crop plants like maize, rice and sugarcane. Sucrose accumulation in sugarcane stems has been correlated with SPS activity (Grof et al. 2007; Zhu et al. 1997; McIntyre et al. 2006), whereas in maize SPS activity could be correlated with vigour and biomass yield (Causse et al. 1995a; Causse et al. 1995b). Genetic studies in maize revealed that grain yield QTL (quantitative trait loci) are linked to SPS activity QTL, and ADP-glucose pyrophosphorylase activity QTL (Sarquis et al. 1998; Prioul et al. 1999). Altering SPS activity could influence several traits in plants as demonstrated by transgenic approaches. Plant height QTL in rice appeared to coincide with that of OsSPS1, and the transgenic rice plants with increased SPS activity were found to be taller than the control (Ishimaru et al. 2004). Roy Choudhury et al. (2009) showed differential transcriptional responses of *SPS* gene under different stimuli such as ethylene, auxin, wounding, low temperature and different photoperiods during ripening of banana fruit. Yonekura et al. (2013) made significant observations. They reported that the promoter activities of rice SPS genes namely *OsSPS1* and *OsSPS11* are controlled by light and circadian clock, but not by sucrose.

Overexpression and inhibition SPS activities through transgenics: Apart from investigating the role of SPS in the photosynthetic and nonphotosynthetic tissues in different plant species, overexpression and silencing of SPS genes through transgenics have become the target of active research in many laboratories. There is growing interest in many laboratories in improving plant productivity through over-expression of SPS genes. The rationale was to ensure increasing sucrose export from leaves to other parts of the plants. These studies were not only relevant in terms of the applied aspects i.e. industrial utility of the plant matter but also helped significantly in understanding the functional specialization of the SPS isoforms. A few examples are given here. Over-expression of maize SPS gene had profound influence on photosynthesis, assimilate partitioning, and growth in tomato *(Lycopersicon esculentum* var UC82B) (Galtier et al. 1993). Nguyen-Quoc et al. (1999) demonstrated that over-expression of SPS could enhance sucrose unloading in transformed tomato fruit. Over-expression of *Arabidopsis* SPS A gene led to altered growth and fibre development in tobacco (Park et al. 2008). Upregulation of the sucrose metabolism genes namely UDP-glucose pyrophosphorylase, sucrose synthase, and sucrose-phosphate synthase (SPS) led to altered sucrose metabolism which had direct impact on primary growth and biomass production in tobacco (Coleman et al. 2010). In most of the cases, associated phenotypes of SPS over-expressing transformed plants were increased fruiting and freezing tolerance (Lunn and MacRae 2003). SPS activity was found to be increased significantly in correlation with high-rate cellulose synthesis for secondary wall deposition in various heterotrophic systems such as cultured mesophyll cells of *Zinnia elegans* L. var. Envy, etiolated hypocotyls of kidney beans (*Phaseolus vulgaris*), and cotton (*Gossypium hirsutum* L. cv Acala SJ-1) fibers (Babb and Haigler 2001). SPS antisense or co-sense suppressed transgenic lines were generated in potato (*Solanum tuberosum* L.), rice (*Oryza sativa* L.) and *Arabidopsis thaliana* (L.) (Geigenberger et al. 1999; Geigenberger and Stitt, 2000; Ono et al. 1999; Strand et al. 2000). All these transformed plants supported the view that SPS plays a pivotal role in controlling the carbon flux through sucrose biosynthesis pathway in both photosynthetic and non-photosynthetic tissues. In tobacco, silencing of NtSPSA mRNA did not influence carbohydrate metabolism whereas NtPSPSC-silenced plants showed considerably increased starch content which was due to impaired starch mobilization (Chen et al. 2005). Antisense repression of SPS gene under the constitutive CaMV 35S promoter resulted in decreased plant growth and fruit yield in the muskmelon (Tian et al. 2010).

2.5. *Cold-induced sweetening in potato-an undesirable problem*

What is cold-induced sweetening? Potato being a perishable commodity and its harvest time (March/April) coincides with the rise in temperature, therefore a major portion of the harvested potato tubers need to be stored at low temperatures (2–4^0C) for certain period of time to prevent damage caused by sprouting, weight loss and rotting of the tubers. This also helps to overcome pathogen problems. Potato tubers accumulate soluble sugars namely sucrose, glucose and fructose at low, non-freezing temperatures, referred to as cold-induced sweetening first observed by Mullar-Thurgau in 1882. Although the process of cold-induced sugar accumulation has eco-physiological relevance with regard to chilling tolerance/cold acclimation, but it is an undesirable trait in the potato crop because of economic significance. Sugar accumulation in the cold-stored tubers makes them unsuitable not only for the production of crisps and chips but also in terms of consumer acceptance as vegetables (Burton 1989). Production of the processed potatoes at high frying temperature causes a nonenzymatic Maillard reaction between free aldehyde groups of reducing sugars and free α-amino groups of amino acid and proteins to form adducts, which results in a dark and unpalatable product (Dale and Bradshaw 2003; Kumar et al. 2004). During this process, a side reaction between asparagine and reducing sugars leads to the production of acrylamide, a potent neurotoxin and carcinogen (Mottram et al. 2002; Stadler et al. 2002). The amount of acrylamide in the fried potato products depends on the reducing sugar content of the tubers, and is increased by prior low temperature storage of the potatoes (Olsson et al. 2004, De Wilde et al. 2005, Williams 2005). Potato cultivars/clones differ significantly with regard to their glucose forming potential (GFP; μmol glucose/μmol sucrose) during low temperature storage. For example, GFP value of the clone Picasso was around 500-fold higher than that for the clone ND3828-15 L(Sowokinos 2001). Menéndez et al. (2002) reported that Quantitative Trait loci (QTL) were linked to the genes encoding invertase, sucrose synthase, sucrose-phosphate synthase, ADP-glucose pyrophosphorylase, sucrose transporter 1, and a putative sucrose sensor. Their results suggested that allelic variants of enzymes involved in carbohydrate metabolic pathways contributed to the genetic variations in cold sweetening.

Plausible mechanisms of cold-induced sweetening: It is commonly believed that during storage at low temperature, an imbalance between starch degradation and glycolysis, leads to the accumulation of sucrose, which is then converted into glucose and fructose. Some possible mechanisms are precisely described here. a) Starch degradation may be catalyzed by either amylases (hydrolytic route) or starch phosphorylase (phosphorolytic route). Claassen et al. (1993) showed that increased starch phosphorylase activity triggered sugar accumulation in cold stored potato tubers. Increased activities of hydrolytic enzymes, α-amylase and β-amylase, were also reported during low temperature (Cottrell et al. 1993). Hexose phosphates (hexose-P) produced by starch breakdown either hydrolytically or phosphorolytically, are exported from the amyloplasts to cytosol via the glucose phosphate-phosphate translocator (Weber 2004; Smith et al. 2005). During cold sweetening, starch is mainly degraded through phosphorolytic route rather than hydrolytic (Morrell and ap Rees, 1986a). Glucan-water dikinase (GWD) i.e., starch granule-bound R1 protein was found to be involved in starch phosphorylation which influences overall starch structures. It was demonstrated that overall secondary structure of starch was responsible for its degradability (Lorberth et al. 1998; Rommens et al. 2006). b) Starch breakdown products along with the accumulated hexose-phosphates lead to sucrose biosynthesis in the cytosol via UDP-glucose pyrophosphorylase followed by SPS and SPP-catalyzed reactions (Sowokinos 2001). (c) Sucrose may be further hydrolyzed by invertases to reducing sugars i.e., glucose and fructose. It was reported that the activity of vacuolar acid invertase increased in the tubers stored at low temperatures, and the level of activity varied between the potato cultivars (Isherwood 1973; Pollock and Rees 1975; Richardson et al. 1990; Zrenner et al. 1996).

Although a number of gene functions of diverse carbohydrate metabolic pathways are possibly involved in the cold sweetening process, but the enzymes namely UDP-glucose pyrophosphorylase, sucrose-6-phosphate synthase, sucrose-6-phosphate phosphatase, and acid invertase gained considerable importance because of their roles both individually and collectively in controlling the flux of carbon to free sugars. Therefore, inhibition of the genes encoding the above enzymes through transgenics refer to direct approaches for preventing cold sweetening process in potatoes (Sowokinos 2001).

2.6. *SPS and cold-induced sweetening in potato*

The increased Suc accumulation was correlated with increased SPS activity in both photosynthetic (Guy et al. 1992; Holaday et al. 1992) and nonphotosynthetic tissues, such as potato tuber (Geigenberger et al. 1995). Under stress situation (i.e. drought and cold), altered kinetic properties of SPS were thought to be important in controlling the flux of carbons to free sugars (Hill et al. 1996; Geigenberger et al. 1997, 1999). The onset of cold sweetening in potatoes was associated with the following conspicuous changes such as activation of SPS, appearance of a new form of this enzyme, increased SPS activity, stimulation of sucrose synthesis, and the decline of hexose phosphates (Hill et al. 1996; Reimholz et al.1997; Deiting et al. 1998). As described earlier, the presence of multiple forms of SPS in the cold-stored potato tubers was demonstrated by Reimholz et al. (1997). During cold exposure, a reversible increase of intracellular SPS-1b level could be correlated with a change in the kinetic properties of SPS. V_{sel} activity of SPS was found to be increased, whereas V_{max} activity remained constant. The changes in kinetic properties of SPS probably explain the enhanced Suc synthesis in cold-stored tubers under limiting substrate (i.e., hexose-phosphates) concentrations (Hill et al. 1996; Krause et al. 1998).

Krause et al. (1998) employed a 3·6 kb DNA fragment having the entire ORF of a full-length SPS cDNA clone i.e. SPS-P4 (GenBank ID: X73477), and a 2·0 kb DNA derived from 3741-bp partial SPS cDNA clone i.e. SPS-P1 in making antisense and sense genetic constructs under the constitutive CaMV 35S promoter. These SPS-antisense and sense (co-suppression) potato lines of cv. Desiree were used to analyze the contribution of SPS in controlling the cold sweetening process in potato. Antisense transformants with ~75% reduction in SPS expression showed a reproducible but non-proportional (10 to 40%) decrease of soluble sugars in cold-stored tubers. The results indicated that SPS expression was not solely responsible for Suc accumulation in potato. Out of the multiple forms, only SPS-la and SPS-1b forms were decreased in the antisense transformants suggested that they were encoded by the same gene. Similar results on SPS inhibition were also reported in the potato tubers under water deficit conditions (Geigenberger et al. 1999).

2.7. *Relevance of the present study*

Some of the current focus areas of sucrose metabolism in plants include isolation of cDNA/genomic clones, sequence analyses/comparison of the SBRPs for better understanding the structure-function relationships and evolutionary relatedness, gaining knowledge on a variety of intracellular and extracellular factors involved in the transcriptional controls (that influence the expression of the concerned genes expression). Moreover, significance of post-translational modifications on the enzyme activities, understanding the roles of these enzymes in plant growth and development, sugar signaling, various sugars and SBRP-mediated responses to the abiotic stresses such as low temperature and osmotic stresses, and phenotypic traits associated with altered expression levels through genetic manipulations represent some of the important focus areas of research. In SBRPs, the biochemical role of SPS is well-recognized and linked to the most of the important aspects of sucrose metabolism.

The members of the *Solanaceae* family namely tobacco and tomato contain multiple SPS forms belonging to three different SPS families as evident in the published research reports. Potato is also an important member of the *Solanaceae* family. Most of the potato cultivars are autotetraploid ($2n = 4x = 48$) and highly heterozygous. High level of DNA polymorphism in the potato (*Solanum tuberosum)* genome of is well known. Multiple allelism and natural allelic variations because of cumulative mutations are common in potato (van de Wal et al. 2001; Draffehn et al. 2010). Therefore, it is likely that more than one SPS form corresponding to each class of SPS gene family could exist in potato, another important member of the *Solanaceae* family, depending on the cultivar genotype. Literature survey and database search clearly indicated that in potato, only one cDNA (designated SPS-P4) encoding full-length SPS was reported from the cv. Desiree (reference potato cultivar). Therefore, many more genes encoding distinct SPS isoforms are yet to be isolated and characterized from potato. Such exercise is a prerequisite for in-depth understanding of the gene functions involved in sucrose metabolism at molecular level. In this context, different potato cultivars/clones would be quite useful in exploring their rich genetic resources. There are number of high-yielding Indian potato cultivars suitable to different agro-climatic zones of the Indian subcontinent. These potato cultivars vary with regard to genetic make-up, maturation time, tuber dry matter, starch quality (i.e., amylose to amylopectin ratio, glucan chain lengths) and sugar content. Some of them include processing varieties such as Kufri Chipsona-1, Kufri Chipsona-2. No report was available on SPS in these potato cultivars at molecular and biochemical level till date.

In India, the potato is regarded as short-duration crop with an average growing period of 90-100 days. Approx. 44 Indian potato cultivars developed through conventional breeding and released by CPRI, Shimla during the last few decades. The processing varieties should have the high dry matter content (22-23%) and low reducing sugar level (below 250 mg/100 g fresh tuber weight). Freshly harvested tubers of only a few of the Indian processing varieties more or less fulfil the above criteria. In order to avoid sprouting and other undesirable processes during summers, potatoes are stored in refrigerated cold stores at 2–4°C. The prolonged storage of tubers at such low temperatures leads to the accumulation of reducing sugars-an undesirable process known as 'cold-induced sweetening' or 'Low-temperature sweetening' as mentioned earlier. Varietal differences were there with regard to the extent of accumulation of soluble sugars (both reducing and nonreducing) during low temperature storage. It is now believed that SBRPs together with other proteins involved in carbohydrate metabolism are implicated in the above undesirable phenomenon. This cold sweetening phenomenon appeared to be a common problem even in the processing varieties. Therefore, inhibitions of several gene functions including the genes encoding SPS through transgenic approaches are considered to be promising to circumvent such undesirable process in these commercially important potato cultivars. Keeping the above-mentioned points in view, a few objectives were framed for the present study (see the section 2.8 for 'Objectives').

2.8 *Objectives*

Based on the relevance of present study as described in the previous section (vide section 2.5), the following objectives were framed:

- **Molecular cloning and characterization of cDNA and genomic clones corresponding to SPS in the Indian potato cultivars**

- **Making various Ti-plasmid based antisense & sense genetic constructs by using different regions of SPS cDNA/genomic clones under constitutive and tuber-specific promoters**

- ***Agrobacterium*-mediated genetic transformation of potato plants with the above genetic constructs followed by screening of the transgenic potato lines**

- **Biochemical studies on sucrose-phosphate synthase (SPS) activity and the level of sugar accumulation in the transgenic potato tubers at various experimental temperatures**

Chapter 3

Materials and Methods

Chapter 3 Materials and Methods

3.1. Materials

3.1.1. *Procurement of plant and other materials*

The germplasm of some high-yielding, commercially important Indian potato cultivars such as Kufri Chipsona-1 (KC-1), Kufri Chipsona-2 (KC-2), Kufri Chandramukhi (KCM), Kufri Jyoti (KJ), Kufri Ashoka (KA) and Kufri Pukhraj (KP) were procured from Central Potato Research Institute (CPRI), Shimla, India. For convenience, short name of each cultivar is shown within parenthesis.

Chemicals/biochemicals: Various chemicals/biochemicals/molecular biology items were procured from different sources. The chemicals were purchased from Sisco Research Laboratory Pvt. Ltd. Mumbai, Qualigens Fine Chemicals, Merck, CDH Pvt. Ltd., New Delhi, and HiMedia Laboratories Mumbai. Various enzymes used were purchased from Bangalore Genei Pvt. Ltd., Bangalore and Amersham Biosciences Ltd., Hongkong. The oligonucleotide primers used in the study were synthesized from Bangalore Genei Pvt. Ltd., Bangalore. All salts and additives were purchased from HiMedia Labs Limited, India and growth hormones from sigma chemicals, USA. The gel extraction Qiagen Kit was purchased from Genetix. Glasswares and Plasticwares were purchased from Borosil and Tarsons Products Pvt. Ltd.

3.1.2. *Maintenance of potato germplasm*

The potato cultivars, KC-1, KC-2, KCM, KJ, KA and KP as used in the study vary with regard to their genetic make-up, maturation time and growth in different agro-climatic zones of the Indian subcontinent. The cultivars KCM and KA are early maturing, whereas the remaining cultivars are medium maturing. All these cultivars along with Desiree (a late maturing exotic cultivar) were routinely micropropagated in our laboratory under controlled conditions (16 h light/8 h dark, 25-27ºC, and 70% relative humidity) for four to five weeks on MS basal medium.

3.1.3. *Bacterial strains and vectors*

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

Agrobacterium tumefaciens (*LBA4404) strain:* LBA4404 (Ach5 pTiAch5) Sm/Sp(R) in the virulence plasmid (from Tn904); all T-DNA of pTiAch5 eliminated in pAL4404 (Hoekema et al. 1983). LBA4404 strain was maintained on YEM medium containing rifampicin (15 µg mL^{-1}) and streptomycin (50 µg mL^{-1}). During triparental mating, *E. coli* pRK2013 and *Agrobacterium tumefaciens* LBA4404 strains were used as helper and recipient, respectively.

Cloning vector: pUC19 Vector: pUC19 (GenBank ID: X02514; 2686 bp) is high copy number commonly used plasmid cloning vector in *E.coli* (Yanisch-Perron et al. 1985). This plasmid vector contains 54-bp multiple cloning site having unique sites for a number of different hexanucleotide-specific restriction endonucleases.

Binary vectors: pBI121 is a binary vector (also a shuttle vector as it contains both the *E. coli* and *A. tumefaciens*-specific *ori*) having CaMV 35S-GUS fusion (Chen et al. 2003; GenBank ID: AF485783); and GBSSI-GUS gene fusion was made based on pBI121 (Bansal et al. 2012).

3.1.4. *Oligonucleotide primers*

The following oligonucleotide primers were designed and the details of the different primer sequences are described below:

The following oligonucleotide primers were used based on SPS-P4 cDNA sequence (GenBank ID: X73477): the forward primer D20-SPS, 5′-CTAAGTTCTCTCTCGCT GTC-3′ (corresponding to the bases 11-30); SPS-F2447, 5′-CAGTGGATTGTGATGCT AGC-3′ (corresponding to the bases 2447-2466) and three reverse primers were, F20-SPS, 5′-TTGCTCGTCAATCTCCTGTC-3′ (complementary to the bases 1274-1293); SPS-3601, 5′-ATAATGCCTTCCGATGGATG-3′ (complementary to the bases 3582-3601) and E20-SPS (Reverse Primer, 5′-ATGACAATTTCGGAAGCATC-3′complementary to the bases 1243-1262).

GBSSI gene-specific primers: The different primers were designed based on the available granule-bound starch synthase (GBSSI) gene sequence in the database (GenBank ID: X58453) corresponding to the wild type monoploid potato clone, AM79.7322. Forward primers: GB1-F01, 5′-AATGCAACAGTATCTTGTAC-3′ corresponding to the bases 54-73 and GB1-F02, 5′-AGACATAGGAATGTCAAGTG-3′ corresponds to the bases 514-533; reverse primers: GB1-R01, 5′-AAGAACATCACCTAGTCCAC-3′ complementary to the bases 1363-1382 and corresponds to the N-terminus of mature peptide; and GB1-

R02, 5′-CTTGTTGAGCTGTGTGAGTG-3′ complementary to the bases 785-804 and consists of the predicted transcription start site (TSS) region.

Constitutive gene-specific primers: Actin (Mr ~41,800) is widely distributed in eukaryotic cells, often being the most abundant protein and commonly making up of 10% of the total cell protein. The primers designed for amplification of actin gene (Dohmann et al. 2005) were: forward primer FW-ACT2**,** 5′-ATTCAGATGCCCAGAAGTCTTGTTC-3′ and reverse primer RV-ACT2, 5′-GCAAGTGCTGTGATTTCTTTGCTCA-3′.

Binary vector specific primers: The different primers were designed based on the available pBI121 binary vector sequence in the database (GenBank ID. AF485783). The details are given below:

CaMV 35S promoter-specific primers: Forward primer AF35-01, 5′-ATTCAAATAGAG GACCTAAC-3′ corresponding to the bases 5291-5310, and reverse primer BR35-02, 5′-CCGTGTTCTCTCCAAATGA-3′complementary to the bases 5791-5809.

GUS gene-specific primers: Forward primer US-F5832 5′-TGGTCAGTCCCTTATG TTAC-3′ corresponding to the bases 5832-5851 and two reverse primers US-R6747, 5'-CAAGTCCGCATCTTCATGAC-3′ complementary to the bases 6728-6747 and US-R7719, 5′-TTCGAGCTCGGTAGCAATTC-3′ complementary to the bases 7700-7719.

3.2. Methods

3.2.1. *Genomic DNA isolation from potato cultivars*

Approximately 0.2 g to 2.0g of plant material was used for total DNA isolation based on the protocol described by Kumari et al. (2013). Potassium acetate used in this process removes carbohydrates and other bulk impurities from the solution. Plant material (~2.0 g) was frozen in liquid nitrogen, pulverized to fine powder and mixed with 15 mL extraction buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0, 250 mM NaCl, 15% sucrose) maintained at 65^{o}C. The contents were incubated at 65^{o}C for 20 min with intermittent gentle shaking. 5.0 mL potassium acetate solution (5.0 M) was added, mixed well and incubated further on ice for 20 min. Then the solution was centrifuged at 4000g, 4^{o}C for 20 min and the supernatant was filtered through two layers of fine muslin cloth. Isopropanol (0.70 vol) was added to the supernatant, mixed gently and incubated at -20^{o}C for 4-5 hrs. Then centrifuged at 10000g, 4^{o}C for 15 min and the supernatant was decanted. The DNA pellet was washed with ice cold 70% ethanol, air dried and dissolved in 500 μL of TE buffer (10 mM Tris-HCl, pH 8.0 and 1.0 mM EDTA, pH 8.0). The crude DNA was further purified through DNase-free RNase treatment followed by solvent extraction twice using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1), followed by DNA precipitation using 0.1 vol of 3.0 M sodium acetate (pH 5.5) and 2.0 vol of ethanol and incubated at -20°C for 4-5 hr. After high speed centrifugation, DNA was washed with 70% ethanol and dissolved in 200-250 µL of TE buffer. For mini scale isolation (for 0.1 to 0.2 g plant materials), the same protocol was scale down for the necessary steps. The quality and quantity of DNA samples were checked by agarose gel electrophoresis and spectrophotometric analysis by measuring the A_{260}/A_{280} ratio and absorbance at 260 nm, respectively.

3.2.2. *Agarose gel electrophoresis*

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

3.2.3. *Restriction digestion of DNA*

Restriction endonucleases recognize short DNA sequences and cleave the double-stranded DNA at specific sites within or adjacent to the recognition sequences. Restriction digestion was carried out in a reaction volume of 20 µL for 3-4 hrs at specified temperatures. The main components of the reaction are: DNA sample (0.5 -1.0 µg), Restriction enzyme (1-5 units), Specific Buffer (1X), BSA (1X), and the final volume was made with sterile distilled water.

3.2.4. *RNA Isolation from potato tissues*

Plant tissues contain high amount of polysaccharides, phenolics, nucleases and other storage material. Therefore, isolation of RNA from plant materials interms 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 pulverized in the liquid nitrogen to a fine powder. The contents were mixed in a buffer containing lithium chloride and SDS (RNA extraction buffer: 100 mM LiCl, 100 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 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 two hours for selective precipitation of RNA. The crude RNA was further purified by RNase-free DNase treatment followed by solvent extraction and ethanol precipitation. After that, RNA was dissolved in RNase-free deionized water, and kept in aliquots at -70^{0}C for further use. The quality of RNA samples were checked by regular and formaldehyde agarose gel electrophoresis along with RT-PCR using different potato gene-specific primers. The spectrophotometric analysis, A_{260}/A_{280} ratio of the RNA samples were also measured to check the quality.

3.2.5. *Formaldehyde agarose gel electrophoresis*

RNA samples were denatured by treatment with formamide and separated by electrophoresis through agarose gel containing formaldehyde (Sambrook et al. 1989). For 100ml agarose gel (1.5%) : 1.5 g of agarose in 62 mL of sterile water was boiled and cooled up to 55ºC. 20 mL of 5X MOPS buffer and 18 mL of deionized formaldehyde was added. The gel was casted in gel casting apparatus and was allowed to set for one hour at room temperature. The gel was submerged in the 1X MOPS electrophoresis buffer. RNA samples were prepared as, 10.0 µL of RNA, 2.0 µL of 5X MOPS electrophoresis buffer, 3.0 µL of formaldehyde, 7.0 µL formamide and 1.0 µL ethidium bromide (200 µg mL^{-1}) were mixed. The samples were incubated at 65ºC for 15 min and immediately chilled on ice. 3.0 µL of formaldehyde gel loading buffer was added and loaded in the gel and electrophoresis was carried out at 4-5 V cm^{-1}. Composition of 5X MOPS Buffer: 0.1 M MOPS (pH 7.0), 40 mM Sodium acetate, 5 mM EDTA (pH 8.0); Formaldehyde Gel Loading Buffer: 50% glycerol, 1.0 mM EDTA (pH 7.5), 0.25% Bromophenol Blue.

3.2.6. *Reverse Transcription*

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 upto 13 kb. In the reaction mixture, 1.0-2.0 µg of total RNA was mixed with 1.0 µL of oligo $(dT)_{18}$ or gene specific reverse primer and made the reaction volume 10.0 µL in ice. Then the mixture was incubated at 70ºC for 5 min and quickly chilled on ice. 4.0 µL of reaction buffer (5X), 1.0 µL of RiboLock Ribonuclease inhibitor and 2.0 µL of 10 mM dNTP mix were added and mixed well. Then reaction mixture was incubated at 37ºC for 5 min. 1.0

µL of Revert Aid H Minus M-MuLV reverse transcriptase was added and incubated at 39ºC for 60 min. The reaction was stopped by heating at 70ºC for 10 min and then quickly chilled on ice.

3.2.7. *Polymerase Chain Reactions (PCR)*

PCR consisted of three cycling steps, heat denaturation of DNA template, annealing of oligonucleotide primers to single stranded DNA templates, and extension of the annealed primers by a thermostable DNA polymerase. The PCR was usually set in 50 µL reaction volume as: 5µL of 10X PCR buffer, 0.5-1.0 µg Template DNA, 10 pmoles forward primer, 10 pmoles reverse primer, 2.5 µL of 2.5 mM dNTP mix, 1.0 µL (1U μL^{-1}) *Taq* DNA polymerase and finally the volume was made up to 50 µL with sterile water. After initial denaturation at 94ºC for 1 min 30 s, the thermal cycling parameters were: denaturation at 94ºC for 1 min, annealing at 55ºC or 50ºC for 2 min; polymerization at 72ºC for 2 min or 1 min for 30 cycles followed by final extension at 72ºC for 5 min.

3.2.8. *Treatment with Klenow enzyme*

Generally, the PCR-amplified DNA products are not truly blunt-ended as the enzyme *Taq* DNA polymerase has a tendency to add an extra 'A' residue at the 3' end of both the strands. Klenow treatment polished the DNA removing the extra 'A' residue at the 3' ends and filling up the recessed 3' termini. For this purpose, 25 µL of amplified DNA products i.e. the inserts were dissolved in minimum volume of water. In the same tube, a 40 µL of reaction volume was set up by adding required amount of 10X Klenow enzyme buffer, dNTP-mix and finally 1-2 unit of Klenow enzyme. The reaction was kept at 28°C for 40 min and then terminated by incubating at 65°C for 5-7 min.

3.2.9. *Purification of DNA fragments*

For purification, the following methods were adopted:

Electro-elution: Dialysis membranes were cut into pieces of required length and washed with distilled water thoroughly. Then these were boiled in 2% w/v sodium bicarbonate, 1 mM EDTA for 10 min. Membranes were again thoroughly washed with distilled water to remove bicarbonate and were then boiled for few minutes in distilled water. Membranes were again boiled in 400 mL of 1 mM EDTA for 10 min. The desired DNA bands were first resolved in 0.8 % agarose gel prepared in 1X TAE buffer. With the help of a sterile blade a slice of agarose gel containing the desired band was excised. This agarose gel slice was transferred to a prepared dialysis bag and submerged in 0.6-0.7 mL 1X TAE buffer followed by electrophoresis for one to two hours. Eluted DNA sample was transferred to a fresh microfuge tube and then solvent extraction was done with saturated phenol twice followed by mixture of phenol and chloroform (1:1). DNA was precipitated by addition of 1/10th volume of 3M sodium acetate (CH_3COONa) and 2.5 volumes of ethanol. The tubes were mixed well and kept at -20°C. After high speed centrifugation, the DNA pellet was washed with 70 % ethanol, air-dried and finally dissolved in 13-15 μL of TE buffer and stored at -20°C for subsequent use.

Elution of DNA bands using Qiagen kit: QIAEX II agarose gel extraction protocol was followed according to manufacturer's instructions. For purification of DNA fragments, DNA samples were run on 0.8 % agarose in 1X TAE buffer. The DNA bands were excised carefully without UV exposure. The gel slices were weighed separately in 1.5 mL microfuge tube and three volumes of Buffer QX1 was added to one volume of gel. QIAEX II solution was resuspended by vortexing for 30 seconds and 20 μL of it was added to the sample. The samples were incubated at 50°C for 10 min (with intermittent vortexing after every 2 min to keep QIAEX II in suspension) to solubilize the agarose. The samples were centrifuged for 30 seconds at high speed and the supernatant was carefully removed with a pipette. The pellet was washed with 500 μL of Buffer QX1. For this, the pellet was first resuspended by vortexing and then the sample was centrifuged for 30 seconds followed by removal of supernatant. Similarly, the pellet was washed with 500 μL of PE Buffer twice, supplied with the kit. The pellet was air dried until it appeared powdery white. The pellet was resuspended in ~14 μL of TE buffer and incubated at 50°C for 10 min and then centrifuged for 30 seconds. The supernatant containing the purified DNA was carefully transferred into a fresh microfuge tube. The above two steps were repeated to increase the yield. The eluates were combined and the yield checked on 0.8 % agarose gel.

3.2.10. *Ligation reaction*

A ligation reaction was set up in order to ligate the purified DNA fragment into the vector, using the enzyme T4 DNA ligase. It catalyzes the formation of phosphodiester bond between the juxtaposed 5'-phosphate and 3'-OH termini in the duplex DNA. The main components of a ligation reaction are: vector (~0.3 μg), insert (~0.3 to 0.6 μg) and T4 DNA Ligase enzyme (1-10 units). T4 DNA Ligase buffer containing PEG 8000 was used in case of blunt end ligation. The reaction volume was made up to 15 μL and the reaction was set up at ~15°C for cohesive end ligation and at ~20°C for blunt end ligation. Ligation reaction was carried out for a period of 10-12 h.

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

E.coli DH5α was transformed with ligated DNA samples using the $CaCl_2$ method (Mandel and Higa 1970). *E.coli* culture was inoculated in 25 mL of Luria broth and incubated at 125 rpm at 37°C for overnight. An overnight-grown culture (~100µL) was re-inoculated in 25 mL of Luria broth and then incubated at 37°C with shaking for 2-3 h (O.D. 0.4-0.6 at 590 nm). The culture was kept on ice to minimize the metabolic activities of cells. The cells were pelletized and washed with ice-cold 100 mM $CaCl_2$. The cells were resuspended in 1.0 mL of ice-cold 100 mM $CaCl_2$ and kept in ice for 2½ hrs in order to develop competence. The ligation mix (~5 µL) was added to competent cell suspension (100 µL), mixed well and kept at 4°C for 30 min. Heat shock was given at 42°C for 2 min, followed by the addition of LB (1 mL) and incubation at 37°C for 1½ hrs. *E.coli* transformants were screened by spreading the cell suspension on selective LA medium (50 µg mL^{-1}ampicillin/ kanamycin). The plates were incubated at 37°C for 16-18 h. Recombinant pUC19 clones were screened based on blue/white selection.

3.2.12. *Plasmid isolation*

Two methods were followed for plasmid isolation: alkali lysis and boiling methods.

Alkali lysis method (Birnboim and Doly 1979): 1.5 mL overnight grown culture of transformant *E. coli* was pelletized in microfuge tube. The cells were mixed with 200 µL of Solution I and 30 µL lysozyme (stock 10 mg mL^{-1}). Then 400 µL of Solution II was added and mixed followed by addition of 300 µL of Solution III (curdy white precipitate was formed). The microfuge tubes were kept in ice for 30 min and centrifuged at high speed for 15 min. The supernatants were treated with 2.0 µL of DNase free RNAse (10 mg mL^{-1}) followed by phenol and chloroform mixture (1:1) extraction. To the aqueous layer, equal volume of isopropanol was added and kept at 4ºC for 20 min. The DNA was pelletized at high speed centrifuge (12,000 rpm) for 15 min. The pellet was washed with 70% ethanol, air-dried and dissolved in 30 µL of TE buffer and stored at -20ºC.

Boiling method (Holmes and Quigley 1981). 1.5 mL overnight grown culture of transformant *E. coli* was harvested in microfuge tube. The cells were resuspended in 800 µL of STET buffer and 30 µL of lysozyme was added to the bacterial suspension. The microfuge tube was kept in boiling water bath for 90 seconds. After cooling down to room temperature, high speed centrifugation (12,000 rpm) was carried out for 15 min. The supernatants were treated with 2.0 µL of DNase free RNAse (10 mg mL^{-1}) followed by phenol and chloroform mixture (1:1) extraction. To the aqueous layer, 1/10th volume of

3M sodium acetate (CH_3COONa) and equal volume of isopropanol was added and kept at 4ºC for 30 min. The DNA was pelletized at high speed centrifuge (12,000 rpm) for 15 min. The pellet was washed with 70% ethanol, air-dried and dissolved in 30 µL of TE buffer and stored at -20ºC.

3.2.13. *Sequence analyses and phylogenetic tree*

The nucleotide sequence of the SPS cDNA was analyzed by NCBI Blast tools. The amino acid sequence was predicted by the open reading frame (ORF) finder available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). For calculating the theoretical molecular weight, isoelectric point (p*I*), and amino acid composition of the predicted protein, the ProtParam tool of ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB; URL:http://expasy.org/tools/) was used. The different ProtScale tools of ExPASy were used for prediction of the hydrophobic character (Kyte and Doolittle 1982), and the various secondary structures such as α-helix, β-sheet, β-turn, and random coil. G+C content analysis was carried out by DNADynamo software (http://www.bluetractor software.co.uk/). Isochore plots were generated by another EMBL-EBI sequence analysis tool (http://www.ebi.ac.uk/Tools/ emboss/Isochore/). For multiple sequence alignment, both the Clustal Omega (an EMBL-EBI sequence analysis tool; http://www.ebi.ac.uk/Tools/) and the *MultAlin* software (http://www.multalin.toul- ouse.inra.fr/multalin/; Corpet 1988) were used. In order to construct phylogenetic tree, multiple sequence alignment was done by the *MultAlin* software, followed by the neighbor-joining method (with bootstrap consensus) using MEGA 5.0 software (Saitou and Nei 1987; Tamura et al. 2011). For this purpose, a total of 43 predicted full-length SPS sequences from the plant species of different taxonomic groups were used.

3.2.14. *Genetic transformation of Agrobacterium strain*

Agrobacterium strain was transformed with binay genetic constructs by two methods: Electroporation and Triparental mating.

Electroporation: *Agrobacterium tumefaciens* strain LBA4404 was grown in 20 mL of YEM broth for overnight at 28ºC/160 rpm. The cells were pelletized and washed thrice with ice cold 10% glycerol (10-15 mL). Then resuspended in the mixture of 30 µL of 1M sorbitol and 90 µL of 10% glycerol. Electrocompetent cells were kept in ice for immediate use or stored at -20ºC for further use. The plasmid DNA (1.0-3.0 µg) was mixed to an aliquot of electrocompetent cells (20 µL) under ice-cold conditions. The mixture was added to the cuvette and an electric pulse was given for few seconds. The cuvette was removed from the chamber and immediately YEM broth was added in the cuvette to transfer the cells to test tube containing 1 mL of YEM broth and incubated for 3-5 hrs at 28°C at 160 rpm. For the screening of transformed cells, the cells were spreaded on YEM (rif^{15} + kan^{50}) plates.

Triparental mating (TPM): Ti plasmid based-binary constructs were transferred from donor strain (E. coli) to the recipient *Agrobacterium* with the help of helper strain (*E. coli* harboring broad host range plasmid pRK2013). and a recipient *Agrobacterium* strain (harboring *vir* plasmid). The Ti plasmid in *E. coli* was mobilized to recipient *Agrobacterium* strain due to mobilization function of pRK2013 (broad host range plasmid). The transformed *A. tumefaciens* strains were screened on the medium containing antibiotics for which resistance is provided by genetic markers unique to that recipient *Agrobacterium* and Ti plasmid vector. For TPM, three strains were grown on their respective agar media. The recipient *Agrobacterium tumefaciens* strain LBA4404 was grown on Luria agar medium containing rifampicin (15 µg mL^{-1}) at 28ºC. The donor *E. coli* strain harboring engineered Ti plasmid and conjugal helper *E. coli* strain (pRK2013) were grown on Luria agar medium containing kanamycin (50 µg mL^{-1}) at 37ºC. The three cultures were mixed with sterile loop on Luria agar plate and incubated at 28ºC for 24 hrs. The small portion of the patch was picked and serially diluted in 0.9% saline. The cell suspensions were spread on Luria agar containing antibiotics rifampicin (15 µg mL^{-1}) and kanamycin (50 µg mL^{-1}) and incubated at 28ºC. Further transformed *Agrobacterium* were maintained on YEM medium containing rifampicin (15 µg mL^{-1}) and kanamycin (50 µg mL^{-1}).

3.2.15. *Agrobacterium-mediated potato transformation*

Inter-nodal stem segments of five to six weeks old potato plantlets (Kufri Chipsona-1), grown in MS medium, were co-cultivated with the above individual *Agrobacterium* transformants following the steps as described by Beaujean et al. (1998). In order to obtain kanamycin resistant complete potato plantlets, the composition of shoot regeneration and rooting media were essentially kept unaltered as described by Bansal et al. (2012).

Briefly, the transformed *Agrobacterium* strain (corresponding to individual genetic constructs) was co-cultivated with internodal stem segments of five to six weeks old potato plantlets (Kufri Chipsona-1). The single colony of transformant strain was grown in YEM broth for 24 hrs up to 0.4-0.5 O.D and then 1mL of culture was diluted in 10 mL of

MS basal medium. Inter-nodal stem segments were co-cultivated with diluted culture for 10 min and placed horizontally on MS basal medium after blotting on sterile filter paper; incubated in dark for 48 hrs in growth room. Internodal stem segments were washed in cefotaxime (250 mg L^{-1}) and shifted to the selective shoot regeneration medium (MS medium containing Zeatin 2.5 mg L^{-1}, GA_3 3.0 mg L^{-1}, IAA 0.01 mg L^{-1} supplemented with kanamycin 80 mg L^{-1} and cefotaxime 250 mg L^{-1}) for regeneration and primary selection of the transgenic potato lines. The regenerated shoots were further transferred to the rooting media i.e., MS medium supplemented with IAA (0.05 mg L^{-1}), kanamycin (80 mg L^{-1}) and cefotaxime (250 mg L^{-1}), to obtain complete potato plantlets.

3.2.16. *Production of mini tubers, and storage at different experimental temperatures*

Transgenic (corresponding to each genetic construct) and nontransgenic (control) potato plantlets were grown in polybags in the restricted experimental plots, after proper hardening and acclimatization. Mini tubers were harvested after 90–100 d of cultivation under field conditions (mid of November to the end of February). After harvesting, the tubers were properly cleaned with tap water and air dried. Weight of the tubers and other morphological features were noted. A portion of freshly harvested tubers from each variety was used immediately and the rest were transferred to different experimental temperatures (25ºC and 4ºC).

3.2.17. *Semi-quantitative RT-PCR*

For semi-quantitative RT-PCR, reverse transcription was carried out in a reaction volume of 20 μL using 2.0 μg of total RNA, oligo $(dT)_{18}$ primer and cDNA Synthesis Kit from Fermentas Life Sciences. 3.0 μL of each RT mixture was used as template in PCR (50 μL reaction volume) using the SPS cDNA specific forward and reverse primers, D20-SPS and F20-SPS, respectively and 1.0 unit of *Taq* DNA polymerase (Bangalore Genei). During PCR, the thermal cycling parameters were kept same as mentioned earlier except polymerization at 72ºC for 2 min. As control, the primers, Actin-FW and Actin-RV were used to amplify ~250 bp fragment using the same 3.0 μL individual RT mixture as template. Polymerization step at 72ºC was kept 1 min in each thermal cycle in this case. The SPS and actin-specific RT-PCR products were resolved in 0.8% and 1.2% agarose gel electrophoresis, respectively. The relative expression levels between the potato cultivars were assessed by the quantification tool of the gel documentation system (Bio-Rad, USA).

3.2.18. *Extraction of soluble sugars*

Total soluble sugars were extracted from the freshly harvested, cold-stored and warm-stored tubers of transgenic and control plants based on the protocol described by Deiting et al. (1998). Briefly, approx. 200 mg of tuber slice was crushed thoroughly using mortar and pestle to make fine paste, and then extracted with 1.0 mL of 80% ethanol at 80ºC for 45 min, then extracted with 1.0 mL 50% ethanol at 90ºC for 30 min, and finally extracted with 1.0 mL water at 90ºC for 30 min. The supernatants were combined and kept at 65ºC for 2-3 hrs to ensure complete evaporation of the solvents; then dissolved in 1.5 mL water, and stored at -20ºC. Soluble sugar extract was stored at -20ºC for determination of total soluble sugars by Anthrone method and reducing sugars by Nelson-Somogyi's Method as described by Sadasivam and Manickam (1996).

3.2.19. *Determination of reducing sugars by Nelson-Somogyi's Method*

The reducing sugars reduce the copper from Cu^{2+} to cuprous oxide form when heated with alkaline copper tartrate. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. The blue color developed is compared with standards and the intensity of color is read at 620 nm. The color is stable for at least 18 hrs.

$$CuSO_4.5H_2O + \text{reducing sugar} \rightarrow Cu_2O + \text{sugar (oxidized)}$$

$$Cu_2O + \text{arsenomolybdate reagent} \rightarrow \text{blue colored complex} + CO_2$$

Standards were prepared by taking different aliquots of D-glucose (stock 0.1 mg mL^{-1}). The soluble sugar extract was 100 µL and 200 µL aliquots of each taken in clean test tubes. The volume in each of the samples was made up to 2.0 mL with water followed by addition of 1.0 mL of alkaline copper tartrate reagent. (Alkaline copper tartrate: Solution A- 2.5 g of anhydrous Na_2CO_3, 2.0 g $NaHCO_3$, 2.5 g sodium-potassium tartrate and 20.0 g anhydrous Na_2SO_4 dissolved sequentially in 80.0 mL water and finally volume made up to 100 mL. Solution B - 15.0 g of $CuSO_4$ was dissolved in a small volume of distilled water having a drop of conc. H_2SO_4 and volume made up to 100 mL with water. 4.0 mL of solution B and 96 mL of solution A were mixed well before use). All the tubes were kept in boiling water bath for 10 min using marbles on top to prevent evaporation loss. Cooling of the tubes to room temperature, followed by addition of 1.0 mL arsenomolybdic acid reagent (Arsenomolybdate reagent: 2.5 g of ammonium molybdate was dissolved in 45.0 mL water. To it 2.5 mL conc. H_2SO_4 was then added. 0.3 g disodium hydrogen arsenate was dissolved in 25 mL water. Finally the above solutions were mixed and then incubated at 37ºC for more than 24 hrs). The volume in each tube made up to 10 mL by adding water followed by incubation for 10 min at room temp. Absorbance was taken at 620 nm immediately after vortexing.

3.2.20. *Sucrose estimation by modified anthrone method*

Sucrose was determined by modified anthrone method as described by Van Handel (1968). The method is based on the destruction of reducing sugars with hot alkali followed by by determination of fructosyl moiety of sucrose by anthrone reagent under ice-cold conditions. Sugar sample (70 µL) was treated with 30% KOH (70 µL) and boiled for 10 minutes. To the mixture, 1mL of 0.14% anthrone reagent was added followed by incubation at 40ºC for 20 minutes. Samples were read at 620nm. Standards were prepared by taking different concentrations, 10-150µg of ultra pure sucrose (stock 0.1 mg mL^{-1}).

3.2.21. *Determination of total soluble sugars by anthrone method*

Anthrone method can be used for estimation total sugars (both reducing as well as non-reducing sugars) in samples. In this method, the sugars in presence of conc. sulphuric acid get dehydrated and produce furfural (from pentoses) or 5-hydroxymethylfurfural (from hexoses) which when react with anthrone to produce a colored compound with λmax of 630 nm. Pentoses, hexoses, heptoses and their derivatives yield a colored product; whereas trioses, tetroses and amino sugars do not yield any colored product.

25 µL and 50 µL aliquots of each soluble sugar extract were taken in clean test tubes. Standards prepared by taking different aliquots D-glucose (stock 0.1 mg mL^{-1}) so that the tubes contained the following amounts 10, 20, 40, 60, 80, 100 µg along with blank. The volume in each of the sample was made up to 1.0 mL with distilled water followed by addition of 5.0 mL anthrone reagent (200 mg anthrone was dissolved in 100 mL of ice cold 95% H_2SO_4). The contents in each tube cooled prior to addition of anthrone reagent. All the tubes were kept in boiling water bath for 10 min using marbles on top to prevent evaporation loss. Absorbance was taken at 630 nm after cooling to room temperature.

3.2.22. *Protein extraction from different plant materials*

Approx. 200 mg of potato tissue sample (tissue cultured plantlets, leaves and tubers) was crushed to fine powder using liquid nitrogen. 1.0 mL of protein extraction buffer [50 mM MOPS-NaOH (pH 7.5), 10 mM $MgCl_2$, 1.0 mM EDTA, 5.0 mM DTT or 0.2% 2-mercaptoethanol, 0.1% v/v Triton X 100] was added and mixed well and transferred to the microfuge tube. Centrifugation was carried out at 15000 g for 30 sec to 1.0 min and clear supernatant was transferred to fresh microfuge tube and kept at -20ºC for further use.

3.2.23. *Protein estimation by Folin-Lowry method*

Under alkaline conditions, the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue. The following solutions were used: Solution A (Alkaline sodium carbonate solution) – 2% Sodium carbonate in 0.1 N NaOH; Solution B (Copper sulphate-sodium potassium tartrate solution) – 2% of sodium potassium tartrate in 1% copper sulphate; BSA stock (0.2 mg mL^{-1}), Folin-ciocalteu reagent (diluted with water in 1:1). 5.0 mL of alkaline solution (mixture of 50 mL solution A and 1.0 mL of solution B freshly prepared) was added to 1.0 mL of test solution (protein sample and volume made up with water), mixed well and kept at room temperature for 10 min, then 0.5 mL of diluted Folin-ciocalteu reagent was added and mixed immediately. Samples were kept at room temperature for 30 min and absorbance was taken at 750 nm. Standard curve was made using BSA stock.

3.2.24. *SDS-PAGE*

Proteins were separated through SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight). SDS gel electrophoresis of samples that have identical charge per unit mass due to binding of SDS results in fractionation by size. SDS-PAGE gel have two parts one resolving gel and other is stacking gel.

Composition of resolving gel (10.0 mL):

Components	**7%**	**10%**
Water	5.1 mL	4.0 mL
30% Acrylamide/Bis-acrylamide (30% / 0.8% w/v)	2.3 mL	3.4 mL
4X Separating gel buffer (1.5 M Tris-HCl , pH 8.8)	2.5 mL	2.5 mL
10% SDS	100 µL	100 µL
10% APS	50 µL	50 µL
TEMED	5.0 µL	5.0 µL

Composition of stacking gel (5.0 mL):

Components	**4%**
Water	3.0 mL
30% Acrylamide/Bis-acrylamide (30% / 0.8% w/v)	0.8 mL
4X Stacking gel buffer (0.5 M Tris-HCl , pH 6.8)	1.25 mL
10% SDS	25 μL
10% APS	50 μL
TEMED	2.5 μL

The components of the resolving gel was mixed in a test tube and poured in the casting apparatus followed by over layering with water saturated isobutanol. After 30 min when the gel was polymerized, isobutanol was decanted and gel was washed with water. Stacking gel was poured over the resolving gel and comb was placed 1X PAGE running buffer. After polymerization, the comb was removed and the gel was submerged in 1X PAGE running buffer. The protein samples were mixed with equal volume of 2X sample and boiled for 3-5 min. Gel was run at 100 volts for 2 hrs. (1X PAGE running buffer 3.03 g Tris base, 14.4 g glycine, 1.0 g SDS and made up the volume to one litre). Gel was stained in staining solution for 2-3 h. After destaining, the protein bands were visualized and photographed.

3.2.25. *Antigenic peptide and polyclonal antibodies*

The entire deduced amino acid sequence of SPS as isolated in this study (1054-amino acid *KC-SPS1*, Accession No. ACD50895) was examined for the prediction of antigenic peptide using the MIF (Molecular Immunology Foundation) Bioinformatics tool based on the method of Kolaskar and Tongaonkar (1990) (http://immunax.dfci. harvard.edu/ Tools/antigenic.pl). The current website for the same is http://imed.med. ucm.es/Tools/ antigenic.html under the server of Dr. Reche's Immunomedicine Group, Universidad Complutense Madrid. A 17-mer polypeptide SQQRGKKLYIVLISLHG (159-175 of KC-SPS1), having predicted antigenic determinant was synthesized by Biomatrix Technologies, New Delhi, India. This region of the polypeptide did not show any considerable sequence identity with other proteins in the database. Moreover, it showed hydrophilic peaks in the hydropathy plot as generated by the Kyte & Doolittle scale. Polyclonal antibody was raised in the rabbit using the Keyhole Limpet Hemocyanin (KLH)-conjugated peptide immunogen by Bangalore Genei (India) Pvt. Ltd., India.

3.2.26. *Protein blot analysis*

After electrophoresis, the gel was washed in water and a transfer sandwich was made consisting of two blotting papers on spongy support of transfer system saturated with transfer buffer (2.9 g of glycine, 5.8 g of Tris base, 0.37 g of SDS, 200 mL of methanol and volume made to one litre with water). Then PVDF membrane (pre-treated with methanol for 10 min) was placed and then gel was placed over the membrane. The membrane was covered with two blotting papers and second spongy support of transfer system and this transfer sandwich was placed in electro-blotter for transfer of proteins. Electroblotting was carried out at 70 volts for 2 hrs. The blot was semidried and blocked in the blocking buffer for 1 hr SPS protein specific polyclonal antibody (50 μL) was added in 5.0 mL of blocking buffer and blot was incubated at 25ºC overnight with agitation. The blot was washed thrice with PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.2 g KH_2PO_4 and made volume to 1.0 L) and once washed with phosphate free buffer (150 mM NaCl, 50 mM Tris-HCl). Blot was incubated in blocking buffer (10 mL) containing secondary antibody IgG (10 μL) for one hour at 25 ºC. The blot was washed thrice with phosphate free buffer. Then blot was incubated with DAB containing buffer with hydrogen peroxide for 5-10 min and reaction was stopped by washing the blot several times with sterile water and placed in the PBS buffer and photographed.

3.2.27. *SPS assay*

SPS assay was performed as described by Hill et al. (1996). The desalted protein extracts were prepared from the tubers and leaves of different potato cultivars as mentioned above. SPS activity is based on quantifying the fructosyl moiety of sucrose using anthrone reagent. SPS activity was assayed under two different conditions: a) saturating substrates in presence of the activator, Glc 6-P i.e. V_{max} assay, and b) limiting substrates with inorganic phosphate (Pi) as an inhibitor i.e. V_{sel} assay. For V_{max} assay, the reaction mixture: (70 μL) contained 6 mM UDP-Glc, 12 mM Fru 6-P, 36 mM Glc 6-P, 50 mM Mops-NaOH (pH 7.5), 15 mM $MgCl_2$, 1 mM DTT, and protein extract. For V_{sel} assay, the reaction mixture (70 μL) contained 6 mM UDP-Glc, 5 mM Pi, 2 mM Fru 6-P, 10 mM Glc 6-P, protein extract along with the remaining ingredients as used in V_{max} assay. Reactions were run for 15 min at 25ºC and terminated by addition of 70 μL of 30% (w/v) KOH, followed by incubation for 10 min in a boiling water bath. After cooling, 1 ml of 0.14% (w/v) anthrone in 13.8 M H_2SO_4 was added to each reaction mixture, mixed well, and incubated at 40ºC for 20 min before measuring absorbance at 620 nm

Sr. No.	**Chemicals/Biochemicals**	**Stock conc. (mg mL^{-1})**	**Working conc. (μg mL^{-1})**	**Solvent used**
1.	Ethidium bromide	5	0.5-1.0	Sterile water
2.	X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside)	20	20 (30 μL per plate)	N, N-dimethyl-formamide
3.	IPTG (Isopropyl thio-β-D-galactoside)	100	100 (8 μL per plate)	Sterile water
4.	Ampicillin	50	50	Sterile water
5.	Kanamycin	50	50	Sterile water
6.	Rifampicin	15	15	Methanol
7.	Streptomycin	50	50	Sterile water
8.	Cefotaxime	250	250	Sterile water
9.	Gentamycin	40	40	Sterile water

Sr. No.	**Buffers**	**Composition and preparation**
1.	0.5 M Tris-HCl (pH 8.0)	For 100 mL stock, 6.05 g of Tris base was dissolved in 50 mL water and pH was adjusted with 6.0 N HCl and made up volume with water and autoclaved.
2.	0.5 M EDTA (pH 8.0)	For 100 mL stock, 18.6 g of sodium salt of EDTA was dissolved in 50 mL water and pH was adjusted with concentrated NaOH and made up volume with water and then autoclaved.
3.	3.0 M Sodium acetate (pH 5.5)	For 50 mL stock, 12.3 g of Sodium acetate was dissolved in water and pH was adjusted with glacial acetic acid and volume made upto 50 mL
4.	0.5 M Sodium acetate (pH 4.7)	For 50 mL stock, 2.05 g of Sodium acetate was dissolved in water and adjust the pH with glacial acetic acid and final volume 50 mL.
5.	5 M Potassium acetate	49 g of potassium acetate was dissolved in water and made final volume 100 mL and autoclaved.
5.	3 M Potassium acetate (pH4.8)	29.4 g of potassium acetate was dissolved in water and 11.5 mL of glacial acetate was added and made final volume 100 mL and autoclaved.
7.	0.2 M MOPS (pH 7.5)	4.2 g of MOPS was dissolved in water and pH adjusted to 7.5 using NaOH and volume made to 100 mL.
8.	0.5 M $MgCl_2$	10.2 g of $MgCl_2$ was dissolved in sterile water to a final volume of 100 mL
9.	1.0 M Sorbitol	18.2 g of sorbitol was dissolved in sterile water to a final volume of 100 mL
10.	0.5 M KCl	3.73 g of KCl was dissolved in sterile water to a final volume of 100 mL
11.	0.5 M Sucrose	17.1 g of sucrose was dissolved in sterile water to a final volume of 100 mL
12.	*Solutions used for plasmid isolation by alkali lysis method* :	
	Solution I:	Glucose 50 mM; Tris-HCl 25 mM (pH 8.0); EDTA 10 mM (pH 8.0)
	Solution II:	NaOH 0.2 N; SDS 1.0%
	Solution III:	Potassium acetate (3M)
13.	*The buffer for plasmid isolation by boiling method (STET)*	8.0 % (w/v) Sucrose, 0.5 % (w/v) Triton X 100, 50 mM EDTA (pH 8.0), 10 mM Tris-HCl (pH 8.0) Volume was made up by water and autoclaved.

14.	STE Buffer	0.3 M NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0)
15.	Saline EDTA	0.15 M Sodium chloride, 0.1 M EDTA (pH 8.0)
16.	TE Buffer (1X)	10.0 mM Tris-HCl (pH 8.0), 1.0 mM EDTA (pH 8.0) Volume was made up with water and autoclaved.
17.	TBE Buffer (5X)	54 g L^{-1} Tris base, 28 g L^{-1} Boric acid, 3.8 g L^{-1} EDTA The pH of the buffer was set at 8.0 Volume was made up with water and autoclaved.
18.	TAE Buffer (5X)	24.2 g L^{-1} Tris-base, 5.7 mL L^{-1} Glacial acetic acid, 10 mL L^{-1} 0.5M EDTA (pH 8.0). Volume was made up by water and autoclaved.
19.	DNA Gel Loading Buffer (5X)	35 % (w/v) Sucrose or 40% glycerol, 20.0 mM EDTA (pH 8.0), 0.1 % (w/v) Bromophenol blue Volume was made up with sterile water.
20.	DNA extraction buffer	50 mM Tris-HCl pH 8.0, 50 mM EDTA (pH 8.0), 250 mM NaCl, 15% sucrose
21.	REX buffer for RNA extraction	100 mM LiCl, 100 mM Tris-HCl (pH~8.0), 1.0 % SDS, 10 mM EDTA (pH~ 7.3), 0.2% ß- Mercaptoethanol
22.	Protein extraction buffer	50 mM MOPS-NaOH (pH 7.5), 10 mM $MgCl_{2,}$ 1.0 mM EDTA, 5.0 mM DTT or 0.2% β Mercaptoethanol, 0.1%(v/v) Triton X 100
23.	*Formaldehyde gel buffers:*	
	5X Formaldehyde Gel running buffer	0.1 M MOPS, 40 mM sodium acetate, 5 mM EDTA (pH 8.0)
	Formaldehyde Gel loading buffer	50% glycerol, 1 mM EDTA (pH 7.5), 0.25% bromophenol blue
24.	Solutions for Folin Lowry protein estimation:	*Solution I*: Alkaline Sodium carbonate(20 g of sodium carbonate was dissolved in final volume of 0.1 N NaOH) *Solution II*: Copper sulphate-sodium potassium tartrate solution (5g L^{-1} $CuSO4.5H_2O$ was dissolved in 10 g L^{-1} of Sodium potassium tartrate) (Mix solution I 50 mL with 1 mL solution II for use)
25.	*SDS-PAGE buffers:*	
	10 X Electrophoresis buffer	For one litre: 30.3 g Tris Base, 144 g glycine, 10 g SDS
	30% Acrylamide	Acrylamide (29.2) : Bis Acrylamide (0.8)
	4X Separating gel buffer	1.5 M Tris-HCl (pH 8.8)
	4X Stacking gel buffer	0.5 M Tris-HCl (pH 6.8)

	Separating gel overlay solution	Water saturated butanol
	10 % SDS	10 g of SDS was dissolved in 70 mL sterile water and made volume to 100 mL.
	2X Sample buffer (10 mL)	0.5 M Tris-HCl (pH 6.8)- 2.5 mL,10% SDS- 4.0 mL, glycerol- 2.0 mL, β-Mercepta-ethanol-0.8 mL, 0.1% Bromophenol blue-0.3 mL and made the volume with water
	10% APS	10 mg of APS dissolved in 1 mL of sterile water
	TEMED	Commercially available
	Staining solution	0.15% Coomassie Brilliant Blue-R250, 30% Methanol, 10% glacial acetic acid and made final volume with water.
	Destaining solution	30% Methanol, 10% glacial acetic acid and made final volume with water
26.	*Western blot buffers:*	
	Transfer Buffer	2.9 g L^{-1} Glycine, 5.8 g L^{-1} Tris Base, 0.37 g L^{-1} SDS, 200 mL methanol made in sterile water under sterile conditions
	PBS	8.0 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na_2HPO_4, 0.24 g L^{-1} KH_2PO_4 autoclaved after dissolving all the components in water.
	Phosphate removal buffer	150 mM NaCl, 50 mM Tris-HCl

Note: Deionized water was used for all the solutions as mentioned above.

Restriction Enzymes

Various hexacutter and tetracutter restriction enzymes such as ***Eco*RI, *Bam*HI, *ECl*136II, *Sac*I, *Sma*I, *HincII* and *Hind*III** were used in this study. Restriction digestion was carried out in the buffer as supplied by the manufacturers. Depending on enzyme, reaction was carried out at appropriate temperature, and BSA added if required.

Description of some restriction enzymes used in this study:

Restriction enzymes	**Restriction site**	**Reaction temperature**
*Eco*R1	GAATTC	37ºC
*Bam*H1	GGATCC	37ºC
*Hind*III	AAGCTT	37ºC
*ECl136*II	GAGCTC	37ºC
*Sac*1	GAGCTC	37ºC
*Sma*1	CCCGGG	25ºC
*Hinc*II	GTY/RAC	37ºC

Other Enzymes

Apart from restriction enzymes some other enzymes were used as mentioned below: Several reactions were carried out using buffers as supplied by the manufacturers. Depending on enzyme, reaction was carried out at appropriate temperature.

Different enzymes used in the study:

Sr. No.	Various enzymes	Stock conc.	Working conc.	Other relevant details
1.	Lysozyme	10 mg mL^{-1}	300-400 µg mL^{-1}	Sterile water was used for stock preparation
2.	Ribonuclease A	10 mg mL^{-1}	10-20 µg mL^{-1}	Dissolved in 10 mM Tris-HCl (pH 8.0) and 15 mM NaCl buffer, and kept in boiling water bath for 10 min followed by slow cooling to room temperature and stored at -20 ºC for subsequent use.
3.	T4 DNA Ligase	400 U $µL^{-1}$	~ 40 U $µL^{-1}$	Prior to use, the enzyme was diluted using dilution buffer as provided by the manufacturer.
4.	Klenow Fragment of *E. coli* DNA polymerase I	5 U $µL^{-1}$	2 U per 50 µL of reaction volume	Klenow enzyme treatment was carried out in the buffer as supplied by the manufacturer.
5.	*Taq* DNA Polymerase	3U $µL^{-1}$	3 U $µL^{-1}$	----

Various media formulations:

Sr. No.	**Media**	**Composition**
1.	Luria Bertani (LB) Medium	0.5% (w/v) Yeast extract, 1.0% (w/v) Tryptone, 1.0% (w/v) NaCl, 1.5% (w/v) Agar-Agar Volume was made up with water and autoclaved.
2.	YEM Medium	0.4 g L^{-1} Yeast extract, 10.0 g L^{-1} Mannitol, 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.5 g $L^{-1}$$K_2HPO_4$, 0.1 g L^{-1} NaCl, 1.5% Agar-Agar
3.	Regeneration medium	MS medium, Zeatin (2.5 mg L^{-1}), GA_3 (3.0 mg L^{-1}), IAA (0.01 mg L^{-1})
4.	Selective regeneration medium	MS medium, Zeatin (2.5 mg L^{-1}), GA_3 (3.0 mg L^{-1}), IAA (0.01 mg L^{-1}), kanamycin (80 mg L^{-1}) and cefotaxime (250 mg L^{-1})
5.	Selective micropropagation media	MS medium, IAA (0.05 mg L^{-1}), kanamycin (80 mg L^{-1}) and cefotaxime (250 mg L^{-1})

Composition and Stock Preparations for Murashige and Skoog (MS) Basal Medium:

MS Major Salts:

S. No.	MS Major Salts	MS Basal conc. (mg L^{-1})	Amount required for 100X stock (g L^{-1})	Use of stock for 1L medium (mL)
1.	KNO_3	1900.0	190.0	10.0
2.	NH_4NO_3	1650.0	165.0	10.0
3.	$MgSO_4.7H_20$	370.0	37.0	10.0
4.	$CaCl_2.2H_2O$	440.0	44.0	10.0
5.	KH_2PO_4	170.0	17.0	10.0

Note: All the MS major salts stock solutions were prepared separately.

MS Minor Salts:

S. No.	MS Minor Salts	MS Basal conc. (mg L^{-1})	Amount required for 1000X stock (g L^{-1})	Use of stock for 1L medium (mL)
1.	H_3BO_4	6.20	6.20	1.0
2.	$MnSO_4.4H_2O$	22.30	22.30	1.0
3.	$ZnSO_4.7H_2O$	8.60	8.60	1.0
4.	$Na_2MoO_4.2H_2O$	0.25	0.25	1.0
5.	$CuSO_4.5H_2O$	0.025	0.025	1.0
6.	$CoCl_2.6H_2O$	0.025	0.025	1.0
7.	KI	0.83	0.83	1.0

Note: $Na_2MoO_4.2H_2O$ was added first followed by the H_3BO_4 during preparation of minor salts stock solutions.

MS Vitamins:

S. No.	Name of Vitamins	MS Basal Conc. (mg L^{-1})	Amount required for 1000X stock (mg mL^{-1})	Use of stock for 1L medium (mL)
1.	Nicotinic Acid	0.5	0.5	1.0
2.	Pyridoxine HCl	0.5	0.5	1.0
3.	Thiamine HCl	0.1	0.1	1.0
4.	Glycine	2.0	2.0	1.0
5.	Myo-inositol	100.0	100.0	1.0

Note: All the MS vitamins stock solutions were prepared separately.

S. No.	Name of Chemical	MS Basal Conc. (mg L^{-1})	Amount required for 1000X stock (mg mL^{-1})	Use of stock for 1L medium (mL)
1.	$Fe_2EDTA. 2H_2O$ (sodium salt)	30.0	30.0	1.0

Note: Preparation of MS basal medium included major salts, minor salts, vitamins, $Fe_2EDTA.2H_2O$, 3.0% sucrose, 0.7-0.8% agar agar. The pH of the media was set at 5.8 using 0.01N HCl or 0.01N NaOH.

Various phytohormones:

Sr. No.	**Phytohormones**	**Stock conc** (mg mL^{-1})	**Working Conc** (mg L^{-1})	**Details of preparation**
1.	Zeatin	2.5	2.5	Zeatin was dissolved in 0.1 N HCl, heated gently, and made the volume by adding sterile water. The pH was adjusted to ~5.0.
2.	IAA	2.0	0.1	Auxin namely IAA (Indole acetic acid) was dissolved in 0.1 N KOH, stirred gently and made up the volume by adding sterile water.
3.	2,4-D	2.0	2.0	2, 4-D (2, 4-dichlorophenoxy-acetic acid) was dissolved in 0.1 N KOH, stirred gently and made up the volume by adding sterile water.
4.	Gibberellins (GA_3)	3.0	3.0	GA_3 was dissolved in 95% ethanol, stirred gently and made the volume by adding sterile water.

Chapter 4

Results and Discussion

Chapter 4 Results and Discussion

4.1. *First Objective:* Molecular cloning and characterization of cDNA and genomic clones corresponding to SPS in the Indian potato cultivars

Considerable progress had been made on molecular and biochemical studies on SPS during last more than two decades. A number of SPS cDNA/genomic clones have been reported from different plant species including commercially important members of *Solanaceae* family. Research reports and database search revealed that multiple SPS forms are present in the *Solanaceae* family members namely tobacco and tomato as described earlier. It is very likely that autotetraploid heterozygous potato genome contains multiple genes belonging to each SPS family. Different potato cultivars/clones would be quite useful in exploring their rich genetic resources. From potato, only one SPS member has been reported from the cv. Desiree till date. In this study, a full-length cDNA encoding a distinct form of SPS through RT-PCR approach was isolated and characterized from the potato cultivar Kufri Chipsona-1 (a processing variety) using tuber RNA. This constitutes the first report from the Indian potato cultivar. Apart from molecular cloning, sequence analyses and comparison, multiple sequence alignment from three important members of *Solanaceae* family (belonging to the different classes of gene families), and phylogenetic analysis using a relatively large number of full-length amino acid SPS sequences from taxonomically different plant species are presented in the following sections. As a preliminary study, the overall expression pattern of SPS was analyzed in the leaves, freshly-harvested and cold-stored tubers from some of the Indian potato cultivars using semi-quantitative RT-PCR, protein blot analysis, and assaying total extractable activities of SPS enzyme under different conditions are also presented. A few partial SPS genomic clones were also isolated from potato (data not shown). The major focus of this study was on isolation and characterization SPS cDNA clone from potato, and its uses in transgenics as described sequentially in this thesis.

4.1.1. *RNA extraction, RT-PCR, and SPS cDNA cloning*

Total RNA was extracted from freshly harvested tubers of Kufri Chipsona-1 and Kufri Chandramukhi by SDS-Phenol method (Gilman et al. 1987). The intactness of the RNA preparations was checked by normal and formaldehyde agarose gel electrophoreses (data not shown), and the A_{260}/A_{280} ratio was found to be nearly 2.0. Reverse transcription (RT) was performed using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit from Fermentas Life Sciences containing M-MuLV reverse transcriptase and the reverse primer, SPS-3601. Approx. 2.0 μg of total RNA from freshly harvested tuber of Kufri Chipsona-1 was used as template in reverse transcription reaction following the steps according to the manufacturer's instructions. In order to isolate full-length cDNA, PCR was carried out using the RT product as template, the SPS cDNA-specific primers, D20-SPS and SPS-3601, and high fidelity XT-5 PCR System (Bangalore Genei). The size of the RT-PCR product was found to be ~3.6 kb. Similar product could be obtained in other potato cultivars using the same primers. The nucleotide sequence of the cDNA clone specific to Kufri Chipsona-1 (3591 bp, designated SPS-C1) was analyzed by NCBI BLAST tool, and found to encode a distinct form of SPS (Fig. 1); the sequence information was submitted to the NCBI database (GenBank ID: EU684230; protein_id ACD50895).

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agttctctctcgctgtccttatcatttcaccacctccataaatcttgaaacatcttttctactccgttaatctctctagcacacggcgga   90

ggagtgcggcggaggagATGGCGGGAAACGATTGGATTAACAGTTACTTAGAGGCGATACTGGATGTTGGACCAGGGCTAGATGATAAGA  180
                M  A  G  N  D  W  I  N  S  Y  L  E  A  I  L  D  V  G  P  G  L  D  D  K  K

AGTCATCGTTGTTGTTGAGAGAAAGAGGGAGGTTTAGTCCGACGAGGTACTTTGTTGAGGAAGTTATTACTGGATTCGATGAGACTGATT  270
 S  S  L  L  L  R  E  R  G  R  F  S  P  T  R  Y  F  V  E  E  V  I  T  G  F  D  E  T  D  L

TGCATCGTTCGTGGATCCGAGCACAAGCTACTCGGAGTCCGCAGGAGAGGAATACTAGGCTCGAGAATATGTGCTGGAGGATTTGGAATT  360
 H  R  S  W  I  R  A  Q  A  T  R  S  P  Q  E  R  N  T  R  L  E  N  M  C  W  R  I  W  N  L

TGGCTCGCCAGAAAAAGCAGCTTGAGGGAGAGCAAGCTCAGTGGATGGCAAAACGCCGTCAAGAACGTGAAAGAGGTCGCAGAGAAGCAG  450
 A  R  Q  K  K  Q  L  E  G  E  Q  A  Q  W  M  A  K  R  R  Q  E  R  E  R  G  R  R  E  A  V

TTGCTGATATGTCAGAGGATCTATCTGAGGGAGAGAAAGGAGATATAGTCGCTGACATGTCATCTCATGGTGAAAGTACCAGAGGCCGAT  540
 A  D  M  S  E  D  L  S  E  G  E  K  G  D  I  V  A  D  M  S  S  H  G  E  S  T  R  G  R  L

TGCCTAGAATCAGTTCTGTTGAGACAATGGAAGCATGGGTCAGTCAGCAGAGAGGAAAGAAGCTTTATATCGTGCTTATAAGTTTACATG  630
 P  R  I  S  S  V  E  T  M  E  A  W  V  S  Q  Q  R  G  K  K  L  Y  I  V  L  I  S  L  H  G

GTTTAATTCGGGGTGAGAATATGGAGCTTGGACGGGATTCTGATACTGGTGGTCAGGTGAAGTATGTTGTTGAACTTGCGAGGGCCTTAG  720
 L  I  R  G  E  N  M  E  L  G  R  D  S  D  T  G  G  Q  V  K  Y  V  V  E  L  A  R  A  L  G

GGTCGATGCCAGGTGTATATCGGGTTGACTTGCTTACTAGACAAGTATCTTCACCAGAAGTAGATTGGAGCTATGGTGAGCCGACAGAGA  810
 S  M  P  G  V  Y  R  V  D  L  L  T  R  Q  V  S  S  P  E  V  D  W  S  Y  G  E  P  T  E  M

TGCTGACGCCAATAAGTACAGACGGCTTGATGACTGAGATGGGGGAGAGTAGTGGTGCTTATATTATTCGCATTCCTTTTGGACCAAGAG  900
 L  T  P  I  S  T  D  G  L  M  T  E  M  G  E  S  S  G  A  Y  I  I  R  I  P  F  G  P  R  E

AGAAATATATTCCAAAAGAACAGCTATGGCCCTATATTCCCGAATTTGTTGATGGTGCACTTAACCATATTATTCAAATGTCCAAAGTTC  990
 K  Y  I  P  K  E  Q  L  W  P  Y  I  P  E  F  V  D  G  A  L  N  H  I  I  Q  M  S  K  V  L

TTGGGGAGCAAATTGGTAGTGGCTATCCTGTGTGGCCTGTTGCCATACACGGACATTATGCTGATGCTGGCGACTCAGCTGCTCTCCTGT 1080
 G  E  Q  I  G  S  G  Y  P  V  W  P  V  A  I  H  G  H  Y  A  D  A  G  D  S  A  A  L  L  S

CAGGTGCTTTAAATGTACCAATGCTTTTCACTGGTCACTCACTTGGTAGAGATAAGTTGGAGCAACTGTTGCGACAAGGTCGTTTGTCAA 1170
 G  A  L  N  V  P  M  L  F  T  G  H  S  L  G  R  D  K  L  E  Q  L  L  R  Q  G  R  L  S  K

AGGATGAAATAAACTCAACCTACAAGATAATGCGGAGAATAGAGGCTGAAGAATTAACTCTTGATGCTTCCGAAATTGTCATCACTAGTA 1260
 D  E  I  N  S  T  Y  K  I  M  R  R  I  E  A  E  E  L  T  L  D  A  S  E  I  V  I  T  S  T

CAAGACAGGAGATTGACGAGCAATGGCGTTTGTATGATGGGTTTGATCCAATATTAGAGCGTAAGTTACGTGCAAGGATCAAGCGCAATG 1350
 R  Q  E  I  D  E  Q  W  R  L  Y  D  G  F  D  P  I  L  E  R  K  L  R  A  R  I  K  R  N  V

TCAGCTGTTATGGCAGGTTTATGCCTCGTATGGCTGTAATTCCTCCTGGGATGGAGTTCCACCATATTGTGCCACATGAAGGTGACATGG 1440
 S  C  Y  G  R  F  M  P  R  M  A  V  I  P  P  G  M  E  F  H  H  I  V  P  H  E  G  D  M  D

ATGGTGAAACAGAAGGAAGTGAAGATGGGAAGACCCCGGATCCACCTATTTGGGCAGAGATTATGCGCTTCTTTTCTAATCCAAGGAAGC 1530
 G  E  T  E  G  S  E  D  G  K  T  P  D  P  P  I  W  A  E  I  M  R  F  F  S  N  P  R  K  P

CTATGATACTCGCACTTGCTAGGCCTGATCCCAAGAAGAACCTCACTACTTTAGTGAAAGCATTTGGTGAATGTCGTCCATTGAGAGAGC 1620
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 M  I  L  A  L  A  R  P  D  P  K  K  N  L  T  T  L  V  K  A  F  G  E  C  R  P  L  R  E  L

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TGATAGATAAGTATGATCTTTATGGTCAAGTAGCTTATCCTAAACACCACAAGCAGTCAGATGTTCCTGATATCTACCGTCTTGCTGCAA 1800
 I  D  K  Y  D  L  Y  G  Q  V  A  Y  P  K  H  H  K  Q  S  D  V  P  D  I  Y  R  L  A  A  K

AGACTAAGGGTGTTTTTATTAATCCAGCTTTTATTGAGCCTTTTGGACTGACTTTGATTGAGGCAGCAGCTTATGGTCTCCCAATGGTAG 1890
 T  K  G  V  F  I  N  P  A  F  I  E  P  F  G  L  T  L  I  E  A  A  A  Y  G  L  P  M  V  A

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ACTGTAAAACTTATCTATCCCGGATAGCTAGCTGCAAACCAAGGCAACCACGCTGGCTGAGATCCATTGATGATGATGATGAAAATTCAG 2160
 C  K  T  Y  L  S  R  I  A  S  C  K  P  R  Q  P  R  W  L  R  S  I  D  D  D  D  E  N  S  E

AAACAGATTCACCTAGTGATTCCTTGAGAGATATTCATGATATATCTCTGAATTTGAGAGTGTCATTAGATGGGGAAAAGAATGACAATA 2250
 T  D  S  P  S  D  S  L  R  D  I  H  D  I  S  L  N  L  R  V  S  L  D  G  E  K  N  D  N  K

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 E  N  A  D  N  T  L  D  P  E  V  R  R  S  K  L  E  N  A  V  L  S  L  S  K  G  A  L  K  S

GCACATCAAAATCTTGGTCGTCAGACAAGGCAGACCAAAACCCTGGTGCTGGTAAATTCCCAGCGATTAGGAGGATGCGACATATTTTTG 2430
 T  S  K  S  W  S  S  D  K  A  D  Q  N  P  G  A  G  K  F  P  A  I  R  R  M  R  H  I  F  V

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CCATTGGATTTATCCCGGCTACATCTTTCAATATATCAGAAGTACAGTCTTTCCTGCCTTCAGAGGGCATGAATCCTACTGATTTTGATG 2610
 I  G  F  I  P  A  T  S  F  N  I  S  E  V  Q  S  F  L  P  S  E  G  M  N  P  T  D  F  D  A

CTTACATATGCAATAGTGGTGGTGATCTTTATTATTCGTCCTTCCATTCTGAGCAAAATCCTTTTGTAGTTGACTTGTACTATCACTCAC 2700
 Y  I  C  N  S  G  G  D  L  Y  Y  S  S  F  H  S  E  Q  N  P  F  V  V  D  L  Y  Y  H  S  H

ATATTGAGTATCGTTGGGGGGGCGAAGGATTGAGAAAGACTTTGGTGCGTTGGGCCGCCTCTATCATTGATAAGAATGGTGAAAATGGAG 2790
 I  E  Y  R  W  G  G  E  G  L  R  K  T  L  V  R  W  A  A  S  I  I  D  K  N  G  E  N  G  D

ATCACATTGTTGTTGAGGATGAAGACAATTCAGCTGACTACTGCTATACTTTCAAAGTCTGCAAGCCTGGGACGGTTCCTCCATCTAAAG 2880
 H  I  V  V  E  D  E  D  N  S  A  D  Y  C  Y  T  F  K  V  C  K  P  G  T  V  P  P  S  K  E

AGCTTAGAAAAGTAATGCGAATTCAGGCACTTCGTTGTCACGCTGTTTATTGTCAAAATGGGAGTAGGATTAATGTGATCCCTGTACTGG 2970
 L  R  K  V  M  R  I  Q  A  L  R  C  H  A  V  Y  C  Q  N  G  S  R  I  N  V  I  P  V  L  A

CATCTCGGTCCCAAGCACTCAGGTACTTATATCTGCGATGGGGAATGGACTTGTCGAAGTTGGTGGTTTTCGTCGGAGAAAGTGGTGATA 3060
 S  R  S  Q  A  L  R  Y  L  Y  L  R  W  G  M  D  L  S  K  L  V  V  F  V  G  E  S  G  D  T

CCGATTATGAAGGATTAATCGGTGGTCTACGCAAGGCTGTCATAATGAAAGGCCTCTGCACTAATGCAAGCAGCTTAATTCACGGTAATA 3150
 D  Y  E  G  L  I  G  G  L  R  K  A  V  I  M  K  G  L  C  T  N  A  S  S  L  I  H  G  N  R

GGAATTACCCGCTATCTGATGTTTTACCATTCGACAGCCCTAATGTCATCCAAGCGGACGAGGAATGTAGCAGCACCGAAATCCGTTGCT 3240
 N  Y  P  L  S  D  V  L  P  F  D  S  P  N  V  I  Q  A  D  E  E  C  S  S  T  E  I  R  C  L

TACTGGTGAAACTAGCGGTACTCAAAGGAtaatacccttcccccttttgattgtcaaaaacctatatgagctataagactatgccatgaaa 3330
 L  V  K  L  A  V  L  K  G

agaatggccatccatttggcttgtcttttgaagctgttaatacttttcaacagactacaaaatgagatgagtcctttgatcctctttaaa 3420

ggacataaaagctttatgcaagaaccagtgctgtaaagttatagaatttcttttgctatatatgacattcgacagaacctgttccggttc 3510

atcgagaaaaagaaataaatttcaacttataaacatgcctgatcatgtaaattatcatatacatccatcggaaggcattat          3591
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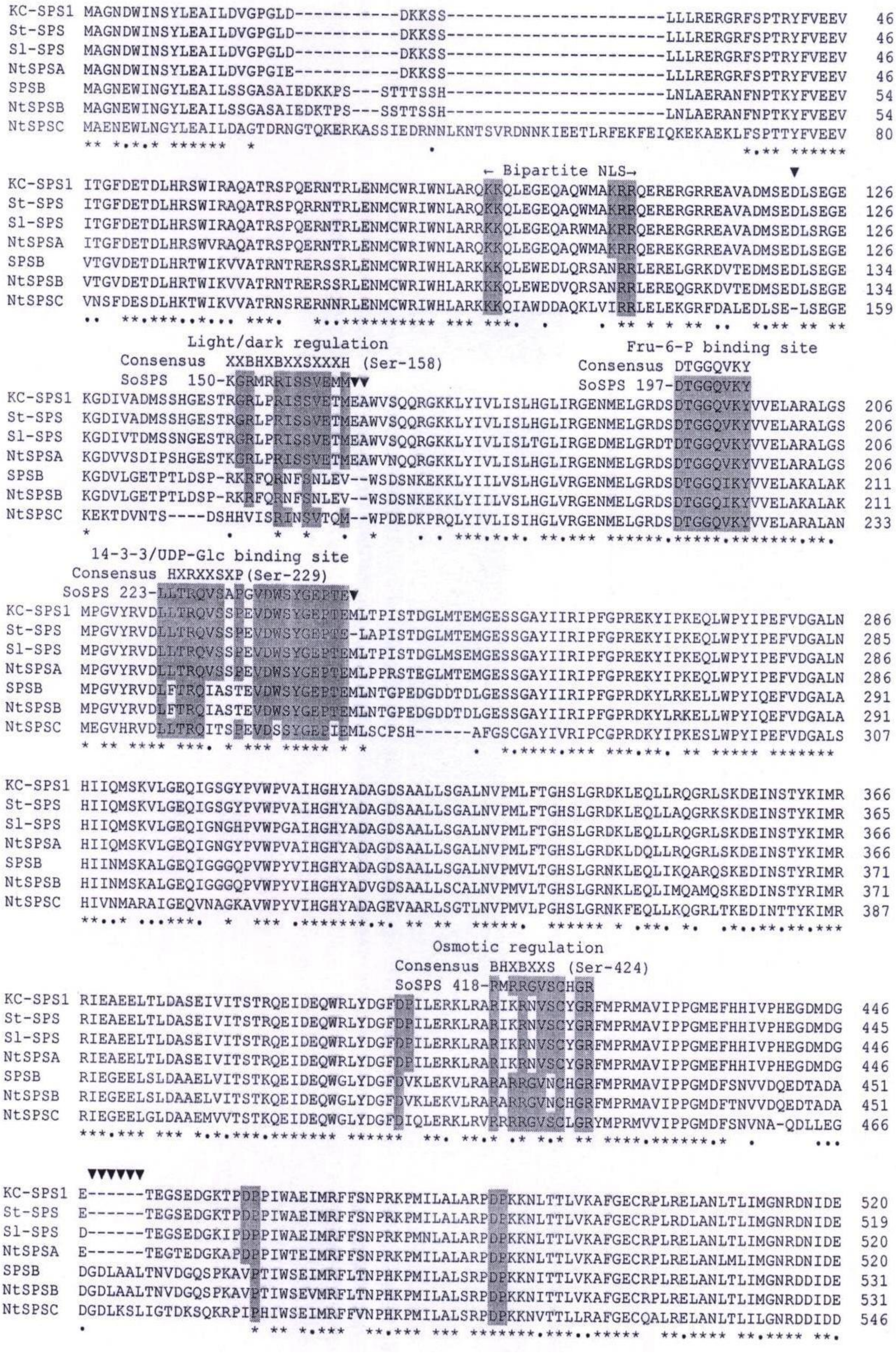
Fig. 1 3591-nt SPS cDNA sequence encoding sucrose-phosphate synthase (SPS) from the potato cultivar Kufri Chipsona-1 showing deduced amino acid sequence (1054-aa). The 5′-UTR and 3′-UTR are shown in *lower cases*.

4.1.2. *Sequence analyses and comparison of different SPS forms*

The 3591-bp SPS-C1 consisted of 107-bp 5'-UTR, 3165-bp ORF (bases 108-3272), and 319-bp 3'-UTR. The predicted protein, designated as KC-SPS1, consisted of 1054 amino acids (Fig. 1). The G/C content of the 5'-UTR, coding region, and 3′-UTR of SPS-C1 were found to be 48%, 43% and 34%, respectively. NCBI BLAST search revealed that SPS-C1 shared 99% sequence identity with SPS-P4, a full-length SPS cDNA clone from Desiree (X73477). The coding region of SPS-C1 showed significant sequence identities with some other plant SPS cDNAs: 96% with muskmelon (DQ364058), 95-96% with tomato (NM_001247910, NM_001246991, AF071786), 93% with tobacco (AF194022); but prominent sequence divergence was noticed in their 5'-UTRs and 3'-UTRs (data not shown). NCBI blastp analysis revealed that KC-SPS1 shared 99% sequence identity with SPS-P4 encoded polypeptide, i.e. St-SPS (Q43845). Moreover, one extra amino acid i.e. a Met residue at position 235 of KC-SPS1 made it a distinct member of SPS A family in potato (Fig. 2). KC-SPS1 was close to some other SPS A family members: 95-97% sequence identity with tomato (AAC24872, NP_001233920, NP_001234839), 97% with muskmelon (ABC96184, ABF47344); and 94% with tobacco (AAF06792). SPS genes in higher plants, particularly in dicots, could be grouped into three distinct families, designated A, B, and C as analyzed earlier by Langenkämper et al. (2002). A total of 43 full-length SPS amino acid sequences (ranging from 1025 to 1240) from a number of different plant species were used in making a phylogenetic tree showing sequence relatedness between them (Fig. 3). Sequence and phylogenetic analyses clearly indicated that KC-SPS1 was a distinct member of SPS A family. Although potato and muskmelon were not close taxonomically; interestingly, the SPS A forms of these plant species showed remarkable sequence identity (refer to protein_ids: ACD50895, ABF47344). However, the sequence identity was considerably decreased if compared with the SPS A family members from other plant species. KC-SPS1 showed only 59% sequence identity with a tomato SPS B form (SPSB, AFD64638) and 53% sequence identity with a tobacco SPS C form (NtSPSC, ABA64520) indicating significant sequence divergence between the families.



```
KC-SPS1  MAGNDWINSYLEAILDVGPGLD-----------DKKSS-----------------------LLLRERGRFSPTRYFVEEV  46
St-SPS   MAGNDWINSYLEAILDVGPGLD-----------DKKSS-----------------------LLLRERGRFSPTRYFVEEV  46
Sl-SPS   MAGNDWINSYLEAILDVGPGLD-----------DKKSS-----------------------LLLRERGRFSPTRYFVEEV  46
NtSPSA   MAGNDWINSYLEAILDVGPGIE-----------DKKSS-----------------------LLLRERGRFSPTRYFVEEV  46
SPSB     MAGNEWINGYLEAILSSGASAIEDKKPS---STTTSSH-----------------------LNLAERANFNPTKYFVEEV  54
NtSPSB   MAGNEWINGYLEAILSSGASAIEDKTPS---SSTTSSH-----------------------LNLAERANFNPTKYFVEEV  54
NtSPSC   MAENEWLNGYLEAILDAGTDRNGTQKERKASSIEDRNNLKNTSVRDNNKIEETLRFEKFEIQKEKAEKLFSPTTYFVEEV  80
         ** *.*.* ******  *                  .                                *.** ******

                                                   ← Bipartite NLS→                ▼
KC-SPS1  ITGFDETDLHRSWIRAQATRSPQERNTRLENMCWRIWNLARQKKQLEGEQAQWMAKRRQERERGRREAVADMSEDLSEGE  126
St-SPS   ITGFDETDLHRSWIRAQATRSPQRRNTRLENMCWRIWNLARQKKQLEGEQAQWMAKRRQERERGRREAVADMSEDLSEGE  126
Sl-SPS   ITGFDETDLHRSWIRAQATRSPQERNTRLENMCWRIWNLARRKKQLEGEQARWMAKRRQERERGRREAVADMSEDLSRGE  126
NtSPSA   ITGFDETDLHRSWVRAQATRSPQERNTRLENMCWRIWNLARQKKQLEGEQAQWMAKRRQEREKGRREAVADMSEDLSEGE  126
SPSB     VTGVDETDLHRTWIKVVATRNTRERSSRLENMCWRIWHLARKKKQLEWEDLQRSANRRLERELGRKDVTEDMSEDLSEGE  134
NtSPSB   VTGVDETDLHRTWIKVVATRNTRERSSRLENMCWRIWHLARKKKQLEWEDVQRSANRRLEREQGRKDVTEDMSEDLSEGE  134
NtSPSC   VNSFDESDLHKTWIKVVATRNSRERNNRLENMCWRIWHLARKKKQIAWDDAQKLVIRRLELEKGRFDALEDLSE-LSEGE  159
         ..  **.***..*... ***.   *..********** *** ***.  .     . ** * * ** ..  *.** ** **

                    Light/dark regulation                         Fru-6-P binding site
             Consensus  XXBHXBXXSXXXH (Ser-158)              Consensus DTGGQVKY
             SoSPS  150-KGRMRRISSVEMM▼▼                      SoSPS 197-DTGGQVKY
KC-SPS1  KGDIVADMSSHGESTRGRLPRISSVETMEAWVSQQRGKKLYIVLISLHGLIRGENMELGRDSDTGGQVKYVVELARALGS  206
St-SPS   KGDIVADMSSHGESTRGRLPRISSVETMEAWVSQQRGKKLYIVLISLHGLIRGENMELGRDSDTGGQVKYVVELARALGS  206
Sl-SPS   KGDIVTDMSSNGESTRGRLPRISSVETMEAWVSQQRGKKLYIVLISLTGLIRGEDMELGRDTDTGGQVKYVVELARALGS  206
NtSPSA   KGDVVSDIPSHGESTKGRLPRISSVETMEAWVNQQRGKKLYIVLISLHGLIRGENMELGRDSDTGGQVKYVVELARALGS  206
SPSB     KGDVLGETPTLDSP-RKRFQRNFSNLEV--WSDSNKEKKLYIILVSLHGLVRGENMELGRDSDTGGQIKYVVELAKALAK  211
NtSPSB   KGDVLGETPTLDSP-RKRFQRNFSNLEV--WSDSNKEKKLYIILVSLHGLVRGENMELGRDSDTGGQIKYVVELAKALAK  211
NtSPSC   KEKTDVNTS----DSHHVISRINSVTQM--WPDEDKPRQLYIVLISIHGLVRGENMELGRDSDTGGQVKYVVELARALAN  233
         *              .    *  *   .  *    . . ***.*.*. **.*** *****.*****.*******.**.

          14-3-3/UDP-Glc binding site
        Consensus HXRXXSXP(Ser-229)
       SoSPS 223-LLTRQVSAPGVDWSYGEPTE▼
KC-SPS1  MPGVYRVDLLTRQVSSPEVDWSYGEPTEMLTPISTDGLMTEMGESSGAYIIRIPFGPREKYIPKEQLWPYIPEFVDGALN  286
St-SPS   MPGVYRVDLLTRQVSSPEVDWSYGEPTE-LAPISTDGLMTEMGESSGAYIIRIPFGPREKYIPKEQLWPYIPEFVDGALN  285
Sl-SPS   MPGVYRVDLLTRQVSSPEVDWSYGEPTEMLTPISTDGLMSEMGESSGAYIIRIPFGPREKYIPKEQLWPYIPEFVDGALN  286
NtSPSA   MPGVYRVDLLTRQVSSPEVDWSYGEPTEMLPPRSTEGLMTEMGESSGAYIIRIPFGPREKYIPKEQLWPYIPEFVDGALN  286
SPSB     MPGVYRVDLFTRQIASTEVDWSYGEPTEMLNTGPEDGDDTDLGESSGAYIIRIPFGPRDKYLRKELLWPYIQEFVDGALA  291
NtSPSB   MPGVYRVDLFTRQIASTEVDWSYGEPTEMLNTGPEDGDDTDLGESSGAYIIRIPFGPRDKYLRKELLWPYIQEFVDGALA  291
NtSPSC   MEGVHRVDLLTRQITSPEVDSSYGEPIEMLSCPSH------AFGSCGAYIVRIPCGPRDKYIPKESLWPYIPEFVDGALS  307
         * ** **** ***. * *** ***** * *          .   *.****.*** ***.**. ** ***** *******

KC-SPS1  HIIQMSKVLGEQIGSGYPVWPVAIHGHYADAGDSAALLSGALNVPMLFTGHSLGRDKLEQLLRQGRLSKDEINSTYKIMR  366
St-SPS   HIIQMSKVLGEQIGSGYPVWPVAIHGHYADAGDSAALLSGALNVPMLFTGHSLGRDKLEQLLAQGRKSKDEINSTYKIMR  365
Sl-SPS   HIIQMSKVLGEQIGNGHPVWPGAIHGHYADAGDSAALLSGALNVPMLFTGHSLGRDKLEQLLRQGRLSKDEINSTYKIMR  366
NtSPSA   HIIQMSKVLGEQIGNGYPVWPVAIHGHYADAGDSAALLSGALNVPMLFTGHSLGRDKLDQLLRQGRLSKDEINSTYKIMR  366
SPSB     HIINMSKALGEQIGGGQPVWPYVIHGHYADAGDSAALLSGALNVPMVLTGHSLGRNKLEQLIKQARQSKEDINSTYRIMR  371
NtSPSB   HIINMSKALGEQIGGGQPVWPYVIHGHYADVGDSAALLSCALNVPMVLTGHSLGRNKLEQLIMQAMQSKEDINSTYRIMR  371
NtSPSC   HIVNMARAIGEQVNAGKAVWPYVIHGHYADAGEVAARLSGTLNVPMVLPGHSLGRNKFEQLLKQGRLTKEDINTTYKIMR  387
         **..* ..***.   *  *** .*******.*. ** **  *****.  ****** * .**. *.  .*..**.**.***

                                              Osmotic regulation
                                          Consensus BHXBXXS (Ser-424)
                                          SoSPS 418-RMRRGVSCHGR
KC-SPS1  RIEAEELTLDASEIVITSTRQEIDEQWRLYDGFDPILERKLRARIKRNVSCYGRFMPRMAVIPPGMEFHHIVPHEGDMDG  446
St-SPS   RIEAEELTLDASEIVITSTRQEIDEQWRLYDGFDPILERKLRARIKRNVSCYGRFMPRMAVIPPGMEFHHIVPHEGDMDG  445
Sl-SPS   RIEAEELTLDASEIVITSTRQEIDEQWRLYDGFDPILERKLRARIKRNVSCYGRFMPRMAVIPPGMEFHHIVPHEGDMDG  446
NtSPSA   RIEAEELTLDASEIVITSTRQEIDEQWRLYDGFDPILERKLRARIKRNVSCYGRFMPRMAVIPPGMEFHHIVPHEGDMDG  446
SPSB     RIEGEELSLDAAELVITSTKQEIDEQWGLYDGFDVKLEKVLRARARRGVNCHGRFMPRMAVIPPGMDFSNVVDQEDTADA  451
NtSPSB   RIEGEELSLDAAELVITSTKQEIDEQWGLYDGFDVKLEKVLRARARRGVNCHGRFMPRMAVIPPGMDFTNVVDQEDTADA  451
NtSPSC   RIEGEELGLDAAEMVVTSTKQEIDEQWGLYDGFDIQLERKLRVRRRRGVSCLGRYMPRMVVIPPGMDFSNVNA-QDLLEG  466
         ***.*** *** *.*.***.******* ******  **. **.* .* *.* ** ****.******.*  .      ...

          ▼▼▼▼▼▼
KC-SPS1  E------TEGSEDGKTPDPPIWAEIMRFFSNPRKPMILALARPDPKKNLTTLVKAFGECRPLRELANLTLIMGNRDNIDE  520
St-SPS   E------TEGSEDGKTPDPPIWAEIMRFFSNPRKPMILALARPDPKKNLTTLVKAFGECRPLRDLANLTLIMGNRDNIDE  519
Sl-SPS   D------TEGSEDGKIPDPPIWAEIMRFFSNPRKPMNLALARPDPKKNLTTLVKAFGECRPLRELANLTLIMGNRDNIDE  520
NtSPSA   E------TEGTEDGKAPDPPIWTEIMRFFSNPRKPMILALARPDPKKNLTTLVKAFGECRPLRELANLMLIMGNRDNIDE  520
SPSB     DGDLAALTNVDGQSPKAVPTIWSEIMRFLTNPHKPMILALSRPDPKKNITTLVKAFGECRPLRELANLTLIMGNRDDIDE  531
NtSPSB   DGDLAALTNVDGQSPKAVPTIWSEVMRFLTNPHKPMILALSRPDPKKNITTLVKAFGECRPLRELANLTLIMGNRDDIDE  531
NtSPSC   DGDLKSLIGTDKSQKRPIPHIWSEIMRFFVNPHKPMILALSRPDPKKNVTTLLRAFGECQALRELANLTLILGNRDDIDD  546
         .                 * ** *.***  **.*** *** *******.***..*****  **.**** **.**** **.
```

Fig. 2

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KC-SPS1 MSSTNSALLLSILKMIDKYDLYGQVAYPKHHKQSDVPDIYRLAAKTKGVFINPAFIEPFGLTLIEAAAYGLPMVATKNGG  600
St-SPS  MSSTNSALLLSILKMIDKYDLYGQVAYPKHHKQSDVPDIYRLAAKTKGVFINPAFIEPFGLTLIEAAAYGLPMVATKNGG  599
Sl-SPS  MSSTNSALLLSILKMIDKYDLYGQVAYPKHHKQSDVPDIYRLAAKTKGVFINPAFIEPFGLTLIEAAAYGLPMVATKNGG  600
NtSPSA  MSSTNSSVLLSILKMIDKYDLYGQVAYPKHHKQADVPDIYRLAAKTKGVFINPAFIEPFGLTLIEAAAYGLPMVATKNGG  600
SPSB    MSAGNASVLTTVLKLVDRYDLYGQVAFPKHHKQSDVPEIYRLAGKTKGVFINPALVEPFGLTLIEASAHGLPMVATKNGG  611
NtSPSB  MSAGNASVLTTVLKLVDRYDLYGQVAFPKHHKQSDVPEIYRLAGKTKGVFINPALVEPFGLTLIEASAHGLPMVATKNGG  611
NtSPSC  MSSSSSAVLTTVIKLIDKYNLYGQVAYPKHHKQPDVPDIYRLAAKTKGVFINPALVEPFGLTLIEAAAYGLPIVATKNGG  626
        **  .  .* ...*..*.* ****** ****** ***.*****.********** .********** * ***.*******

KC-SPS1 PVDIHRVLDNGLLVDPHDQQAIADALLKLVADKQLWAKCRANGLKNIHLFSWPEHCKTYLSRIASCKPRQPRWLRSIDDD  680
St-SPS  PVDIHRVLDNGLLVDPHDQQAIADALLKLVADKQLWAKCRANGLKNIHLFSWPEHCKTYLSRIASCKPRQPRWLRSIDDD  679
Sl-SPS  PVDIHRVLDNGLLVDPHDQQAIADALLKLVADKQLWAKCRANGLKNIHLFSWPEHCKTYLSRIASCKPRQPRWLRPGDDD  680
NtSPSA  PVDIHRVLDNGLLVDPHDQQAIADALLKLVADKHLWAKCRANGLKNIHLFSWPEHCKTYLSRIASCKPRQPRWLRNDDDD  680
SPSB    PVDIHRALNNGLLVDPHDQQAIADALLKLVSEKNLWHECRKNGWKNIHLFSWPEHCRTYLTRIAACRMRHPQWKTDNPSD  691
NtSPSB  PVDIHRALNNGLLVDPHDQQAIADALLKLVSEKNLWHECTKNGWKNIHLFSWPEHCRTYLTRIAACRMRHPQWKTDNPSD  691
NtSPSC  PVDILKALNNGLLIDPHDQKAIADALLKLVADKNLWLECRKNGLKNIHRFSWPEHCRNYLSHVQHCRNRHPANRLEVM--  704
        **** ..* ****.***** ********** .* **  *  ** **** *******..*....  *. * *

KC-SPS1 DENSETDSPSDSLRDIHDISLNLRVSLDGEKNDNKENADNTLDPEVRRSKLENAVLSLSKGALKSTSKSWSSDKADQNPG  760
St-SPS  DENSETDSPSDSLRDIHDISLNLRFSLDGEKNDNKENADNTLDPEVRRSKLENAVLSLSKGALKSTSKSWSSDKADQNPG  759
Sl-SPS  DENSETDSPSDSLRDIHDISLNLRFSLDGEKNDNKENADSTLDPEVRKSKLENAVLSLSKGAPKSTSKSWSSDKADQNPG  760
NtSPSA  DENSETDSPSDSLRDIHDISLNLRFSLDGEKNDNKENADNTLDPEVRKSKLENAVLSWSKGVLKSTPKAWSSDKGDQNSG  760
SPSB    ELAAEESSLNDSLKDVQDMSLR--LSVDGEKTSLNESFDASATADAVQDQVN-RVLSK---MKRPETSKQESEGDKKDNV  765
NtSPSB  ELAAEESSLNDSLKDVQDMSLR--LSVDGEKTSLNESFDASATADAVQDQVN-RVLSK---MKRSETSKQESEGDKKDNV  765
NtSPSC  -KPTLEEPMSESLRDVEDLSLKFSIDVDFKANGE-------LDMARRQQELVEKLSR----------------KANSISK  760
             ..**.*. *.**    .*   .         .        .    .

                Consensus  DXDX(T/V)
                StSPP1  15-DLDYT (Motif I of SPP)
                SoSPS  778-DCDVT
KC-SPS1 AGKFPAIRRMRHIFVIAVDCDASSGLSGSV-----KKIFEAVE-KERAEGSIGFIPATSFNISEVQSFLPSEGMNPTDFD  834
St-SPS  AGKFPAIRRRRHIFVIAVDCDASSGLSGSV-----KKIFEAVE-KERAEGSIGFILATSFNISEVQSFLLSEGMNPTDFD  833
Sl-SPS  AGKFPAIRRRRHIFVIAVDCDASSGLSGSV-----KKIFEAVE-KERSEGSIGFILASSFNISEVQSFLVSEGMSPTDFD  834
NtSPSA  PGKFPAIRRRRHIFVIAVDCDASSGLSESV-----RKIFEAVE-KERAEGSIGFILASSFNISQVQSFLVSEGMKPTDFD  834
SPSB    PSKYPMLRRRRKLIVIALDCYDTNGAPQKKMIQIIQEILKTIKSDPQVARVSGFAISTAMSMSELAAFLISGNIKVTEFD  845
NtSPSB  PSKYPMLRRRRKLIVIALDCYDTNGAPQKKMIQIIQEILKTIKSDPQVARVSGFAISTAMSMSELAAFLKSGNIKVTEFD  845
NtSPSC  PIISYCPGRRQVLYVVATDCYNSKGTPTETLSLTVKNIMQVAG---SRSSQIGLVLSTGLSLDETKEALNSCPTNLEDFD  837
               *   . *.* **  '. *          *                   * .  .  ..     * *   . .**

KC-SPS1 AYICNSGGDLYYSSFHSEQ-NPFVVDLYYHSHIEYRWGGEGLRKTLVRWAASIIDKNGENGDHIVVEDEDNSADYCYTFK  913
St-SPS  AYICNSGGDLYYSSFHSEQ-NPFVVDLYYHSHIEYRWGGEGLRKTLVRWAASIIDKNGENGDHIVVEDEDNSADYCYTFK  912
Sl-SPS  AYICNSGGDLYYSSFHSEQ-NPFVVDLYYHSHIEYRWGGEGLRKTLVRWAASITDKNGENGEHIVVEDEDNSADYCYTFK  913
NtSPSA  AYICNSGGDLYYSSFHSEQ-NPFVVDLYYHSHIEYRWGGEGLRKTLVRWAASIIDKKGENEDHIVVEDEDNSADYCYTFK  913
SPSB    ALICSSGSEVFYPGTSSEEHGKLYPDPDYSSHIEYRWGGDGLRKTIWKLMNTQEGKEEKSVTCAIEEDVKSSNSHCISYL  925
NtSPSB  ALICSSGSEVFYPGTSSEEHGKLYPDPDYSSHIEYRWGGDGLRKTIWKLMNTQEGKEEKSVTCAIEEDVKSSNSHCISYL  925
NtSPSC  ALICSSGSEIYYPW------RDFGLDEDYEAHIEYRWAGENIKSAVMRLGKHE-----EGSEHDIAQCSSACSSRCYSYS  906
        * **.** .. *             .*  *  ******.*. .. . . .                  .       . * .

                                         StSPP1 167-LDTLPQGAGKGQALAYLLKKLKSEGKLPNNTLACGDSGN-DAELFS
                                         SoSPS  948-LNVIPVLASRSQALRYLFMRWGVE--LSNFVVFVGESGDTDYEGLL
KC-SPS1 VCKPGTVPPSKELRKVMRIQALRCHAVYCQNGSRINVIPVLASRSQALRYLYLRWGMD--LSKLVVFVGESGDTDYEGLI  991
St-SPS  VCKPGTVPPSKELRKVMRIQALRCHAVYCQNGSRINVIPVLASRSQALRYLYLRWGMD--LSKLVVFVGESGDTDYEGLI  990
Sl-SPS  VCKPGKVPPAKELRKVMRIQALRCHAVYCQNGSRINMIPVLASRSQALRYLYLRWGMD--LSKLVVFVGESGDTDYEGLI  991
NtSPSA  VRKLGTVPPAKELRKLMRIQALRCHAVYCQNGSRINVIPVLASRSQALRYLYLRWGMD--LSKLVVFVGESGDTDYEGLI  991
SPSB    IKDRSKAKKVDDMRQKLRMRGLRCHLMYCRNSTRMQVVPLLASRSQALRYLFVRWRLN--VANMCVILGETGDTDYEELI 1003
NtSPSB  IKDRSKAKKVDDMRQKLRMRGLRCHLMYCRNSTRMQVVPLLASRSQALRYLFVRWRLN--VANMCVILGETGDTDYEELI 1003
NtSPSC  ITPGAKVPKVNDLRQRLRMRGFRCSVIYTHAASRLNVTPLFASRSQALRYLSVRWGVG--LSSMVVFVGEKGDTDYEGLL  984
        .    .   ..*  .*. . ** ..*.   .*... *. ********** .** . . .  . * .** ****** *.

    212-IPDVYGVMV (Motif III of SPP)
    991-GGVHKTVLL           ▼▼
KC-SPS1 GGLRKAVIMKGLCTNAS-SLIHGNRNYPLSDVLPFDSPNVIQADEECSSTEIRCLLVKLAVLKG 1054
St-SPS  GGLRKAVIMKGLCTNAS-SLIHGNRNYPLSDVLPFDSPNVIQADEECSSTEIRCLLEKLAVLKG 1053
Sl-SPS  GGLRKAVIMKGLCTNAS-SLIHGNRNYPLSDVLPFDSPNVIQADEECSSTEIRSLLEKLAVLKG 1054
NtSPSA  GGLRKAVIMKGLCASAS-SLIHGNSNYPLSDVLPFDSPNVVQSAEECSSTEIRSSLEKLGVLKG 1054
SPSB    SGTHKTLILKGAVEEGSENLLRTSGSYLREDVVPPESPLIIYTGGNETVEEFANALKQVCR--- 1064
NtSPSB  SGTHKTLILKGAVEEGSENLLRTSGSYLREDVVPPESPLITFTSGNETVEEFANALRQVSR--- 1064
NtSPSC  VGLHKTVILKGSVEHASEMLLHNEDSFRTDDVVPQDSTNICVAEGYE-PQDISAALEKLEVM-- 1045
         * .* .*.**    .*  *..   .    **.* .*  .          .    *  .
```

Fig. 2 contd.

Fig. 2 Comparison of the predicted amino acid sequences of seven full-length SPS forms from the *Solanaceae* family. The first four sequences i.e. KC-SPS1 of this study (ACD50895), St-SPS (potato cv. Desiree, Q43845), Sl-SPS (tomato, NP_001234839), NtSPSA (tobacco, AAF06792) belong to SPS A family; the next two, SPSB (tomato, AFD64638) and NtSPSB (tobacco, ABA64521) represent SPS B family; and the last one i.e. NtSPSC (tobacco, ABA64520) is a member of SPS C family. Multiple sequence alignment was based on Clustal Omega tool along with some minor manual adjustments. Dashes indicate gaps that arise during alignment. The *symbol Asterisks* (*) indicate the identical amino acids between all the SPS sequences presented here. The *symbol bullet* '•' denotes identical amino acid residues between the SPS A family members along with conservative substitutions in the other members. The significant regulatory/binding motifs of SPS are shown. For direct comparison, the corresponding motifs of spinach SPS (P31928) along with the consensus sequences (wherever available) are included in the most of the cases in the sequence figure. The identical amino acids within the various SPS motifs are gray highlighted. Likewise, the sequence motifs in SPP-like domain are compared with the Motifs I and III of StSPP1 (potato, ABS52707) by highlighting the identical/conservative amino acids with dark yellow. Some distinguishing sequence features between the SPS families are shown by *downward arrows* (▼). A string of basic (KHHK) and acidic (DDDDE) amino acids are *double overlined*.

Based on the ProtParam tool, the calculated molecular weight (MW) of KC-SPS1 was found to be ~118.5 kDa with a predicted isoelectric point (p*I*) of 5.97. Out of its total 1054 amino acids, 132 were strongly basic (+) (Lys, Arg), 147 were strongly acidic (−) (Asp, Glu), 344 are hydrophobic (Ala, Ile, Leu, Phe, Trp, Val), and 246 were polar (Asn, Cys, Gln, Ser, Thr, Tyr). The instability index (II) of KC-SPS1 was computed as 45.77, which classified the protein as unstable. The amino acid composition data revealed that some of the amino acids such as Arg (7.1%), Asp (6.8%), Ser (8.2%), and Ile (6.5%) occurred more frequently as compared to their average occurrence; whereas, the amino acids, namely Thr (3.7%), Gln (2.9%), Ala (6.5%), Phe (2.7%), and Cys (1.4%) occurred less frequently (Doolittle 1989). The predicted p*I*s of some other SPS A members from potato (Q43845), tomato (NP_001234839), and tobacco (AAF06792) were found to be 6.07, 6.16, and 6.10, respectively with slightly higher instability indices (46.83, 47.79, 47.15). The calculated MW of SPSB and NtSPSC were found to be ~119.5 and ~118.0 kDa with predicted p*I*s of 6.13 and 6.26, respectively. The II of these forms were also classified as unstable.

4.1.3. *Sequence alignment, regulatory/binding motifs and distinguishing sequence features*

Multiple sequence alignment was done using a total of seven SPS members from three economically important crops belonging to the *Solanaceae* family: two from potato cultivars (family A), two from tomato (each from family A and B), and three from tobacco (each from family A, B and C) as shown in Fig. 2. The purpose was to examine sequence similarities and divergences, nature and location of the amino acid substitutions, deletions/insertions within and between the different SPS family members. Apart from three regulatory phosphorylation sites (Ser-158, Ser-229 and Ser-424) involved in light/dark regulation, 14-3-3 protein binding, and osmotic stress activation, other important motifs in SPS include the putative nuclear bipartite signal, Fru-6-P and UDP-Glc binding sites, aspartate-proline (DP) pairs. All these motifs were mostly located in the N-terminal half of KC-SPS1 and other SPS forms in the *Solanaceae* family. Molecular cloning and sequence comparison also revealed that SPS contained a sucrose-phosphatase (SPP)-like domain immediately after linker towards the carboxy-terminus. Only Motif I and Motif III of SPP could be detected in SPP-like domain of KC-SPS1 towards the C-terminus. Apart from an extra amino acid, KC-SPS1 was found to vary at 10 more locations, mostly nonconservative substitutions, if compared with St-SPS: E70R, T237A, R349A, L353K, E504D, V705F, M770R, P810L, P824L, and V1047E. More variations, at 33 locations and mostly nonconservative substitutions, could be noticed if compared with a tomato SPS A form (Sl-SPS, 1054 aa): Q88R, Q98R, E124R, A132T, H137N, H174T, N181D, S188T, T246S, S301N, Y303H, V308G, E447D, T456I, I477N, S676P, I677G, V705F, N720S, R728K, L743P, M770R, A802S, P810L, P824L, N829S, I887T, D895E, T919K, S923A, V950M, C1044S, and V1047E. More sequence divergence was prominent if compared between KC-SPS1 and tobacco NtSPSA (1054 aa): L21I, D22E, I60V, R109K, I130V, A132S, M134I, S135P, R142K, S159N, T237P, I239R, D242E, S301N, E345D, S451T, T456A, A463T, T509M, A527S, L528V, S554A, Q634H, S676N, I677D, V705F, R728K, L738W, A742V, S747P, S749A, A755G, P759S, A761P, M770R, G788E, K791R, P810L, T812S, E818Q, P824V, N829K, N890K, G894E, C915R, P917L, S923A, V929L, T1005A, N1006S, R1015S, I1031V, A1033S, D1034A, C1044S, L1045S, V1047E, A1050G. The majority of these substitutions occurred towards the C-terminus.

Segment-wise comparison between the different SPS family members of the *Solanaceae* family resulted in the following observations. In the N-terminal of all the SPS forms, the first 15 amino acid residues were nearly conserved. Although the region corresponding to 36-670 aa of KC-SPS1 appeared to be conserved in all SPS forms, the regulatory/binding motifs and some of their flanking regions showed significant variations among them. In case of bipartite NLS, all the A members showed KK (11 aa) KRR, whereas KK (12 aa) RR was found in the B and C forms. The light/dark regulation motifs in A and B members were in agreement with the consensus XXBHXBXXSXXXH of Ser-158; whereas in

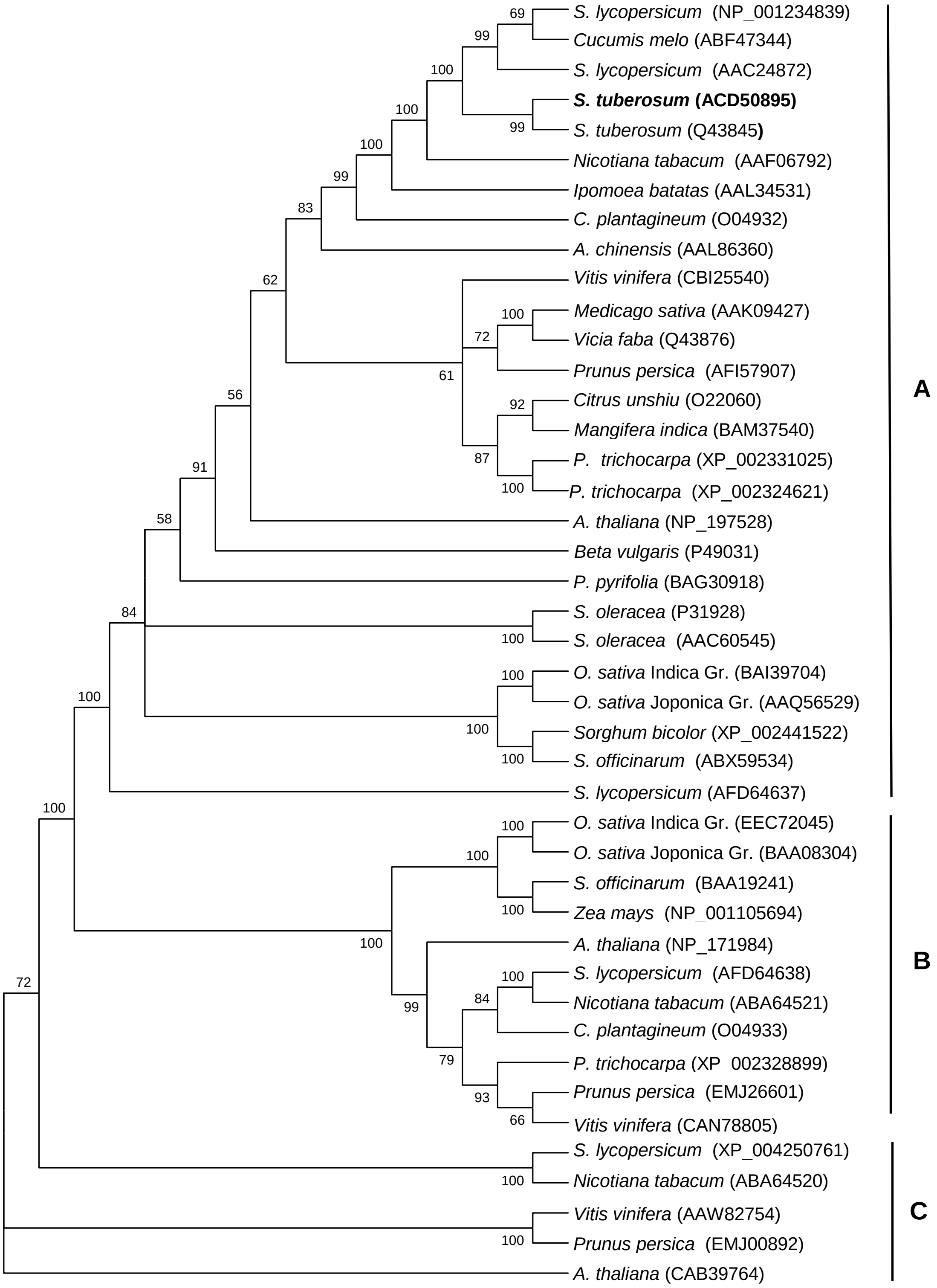

Fig. 3 The phylogenetic tree was generated by the MEGA 5.0 software (Tamura et al. 2011) using the Neighbor-Joining method (Saitou and Nei 1987). **The** bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed**.** The number at each node represented the bootstrap value. The evolutionary analyses involved 43 full-length SPS sequences from the different plant species as available in the published reports and/or databases (the name of the plant species and the GenBank accession numbers are indicated at each branch). KC-SPS1 (1054 amino acids, ACD50895) of this study occupied a distinct position in the phylogenetic tree (shown in bold cases). Representatives of the three SPS gene families (A, B, C) are also indicated.

NtSPSC, deviation could be found since a basic residue was lacking at position -6 relative to the critical phosphoserine. This consensus sequence is critical for recognition by the Ca^{2+}-independent protein kinase, PK_{III}. DTGGQVKY, consensus for the Fru-6-P binding motif, was nearly conserved in all SPS forms. 14-3-3 and UDP-Glc binding sites occur in an overlapping manner. The A family members contained 14-3-3 biding site (HXRXXSXP, consensus for Ser-229); but the B and C family members lacked the Ser-229, even critical Ser was replaced by Thr in NtSPSC. Like Fru-6-P binding motif, the UDP-Glc binding site was nearly conserved in all SPS forms reflecting commonalities in their functions. The A family members followed the consensus sequence, BHXBXXS for osmotic regulation. NtSPSC mostly followed this consensus; interestingly, the presence of 4 consecutive R residues in this motif made it more distinct. But the members of the B family lacked the critical serine residue. Aspartate-proline (DP) dipeptides occurred at 4 places in A family members; whereas only two of them were found in other family members. Plant sucrose-phosphatase (SPP) and haloacid dehalogenase (HAD) superfamily of phosphatases/ hydrolases consisted of three conserved motifs, Motif I, Motif II, and Motif III (Lunn et al. 2000). Motif I is associated with the active site having consensus DXDX(T/V), in which the first D residue forms an acylphosphate intermediate during catalysis (Collet et al. 1998). Only Motifs I and III could be predicted in the SPP-like domain of different SPS family members. Motif I in A members was very close to the consensus as compared to the other family members.

Sequence comparison between the SPS families also revealed more features. For example, the 20-aa segment of KC-SPS1 (16-35), 28-aa segment of SPSB (16-43), and 54-aa segment of NtSPSC (16-69) towards the N-terminal region appeared to be unique for the respective SPS families. The regions between putative bipartite NLS and the light/dark regulation motif showed considerable variations. Thus, the region between light/dark regulation motif and Fru-6-P binding site was mostly conserved except of some minor variation. Likewise, the region between Fru-6-P and 14-3-3/UDP-Glc binding sites was mostly conserved in all the SPS forms. The relatively large segment between 14-3-3/UDP-Glc binding site and the osmotic regulatory motif was highly conserved except in NtSPSC where it was shorter by 6 aa. A segment of ~90 aa corresponding to 675 to 765 of KC-SPS1 is regarded as linker as it connects to C-terminal SPP-like domain. The linker region was found to vary significantly between the family members because of amino acid substitutions and insertions/deletions. Considerable sequence divergence could also be noticed in the regions separating Motifs I and III of SPP-like domain, and the remaining

C-terminal regions. Interestingly, some distinguishing features could be noticed between the SPS families. For example, D residue at position 121 could be noticed only in A and B forms; the dipeptide EA was found only in A forms immediately after light/dark regulation motif. Downstream to the osmotic regulation motif, a segment GDLAAL (between the positions 447 and 448 of KC-SPS1) and its close variant could be found only in B and C forms. Ser residue at position 1009 was found only in the A family; whereas, the corresponding position is occupied by E(N/M) in other family members. A string of 5 acidic aa i.e. DDDDE (678-682) was noticed only in A-family members.

All the important regulatory/binding motifs were well conserved in SPS A forms, and in compliance with the respective consensus sequences. Therefore, it is apparent that A-family SPS activity is most adaptive to environmental conditions because of multiple options for posttranslational modifications (Chen et al. 2005). The SPS A forms showed variations at number of places that included mostly nonconservative substitutions. Such changes might influence their structure-function relationships hence overall performance. Multiple sequence alignment clearly showed segment-wise sequence identities, and also prominent variations between the different SPS family members due to cumulative point mutations, deletions/insertions mostly confined towards N- and C-terminal regions. The variations between the SPS forms were associated with some of the regulatory/binding motifs, and more prominently in their flanking regions. The family-specific unique regions and distinguishing sequence features as predicted in this study could be regarded as identity features which could provide some biochemical clues in establishing the evolutionary relatedness between them.

4.1.4. *Hydropathy plots*

The hydropathy profiles were generated for KC-SPS1, SPSB and NtSPSC with 9 amino acids running window using the ProtScale tool based on the Kyte-Doolittle scale (Fig. 4A-C). The purpose was to compare between the SPS A, B and C family members. For segment-wise analysis and comparison between different SPS members, the positions of the important SPS regulatory/binding motifs were clearly indicated in each hydropathy plot. In KC-SPS1, the motifs corresponding to nuclear bipartite signal, light/dark regulation, 14-3-3/UDP-Glc binding sites and osmotic regulation belonged mostly to the hydrophilic regions; whereas the other motifs namely Fru-6-P binding site, SPP Motif I and SPP Motif III were found in the hydrophobic regions. The regions of SPSB and NtSPSC corresponding to these motif positions showed more or less similar hydropathic

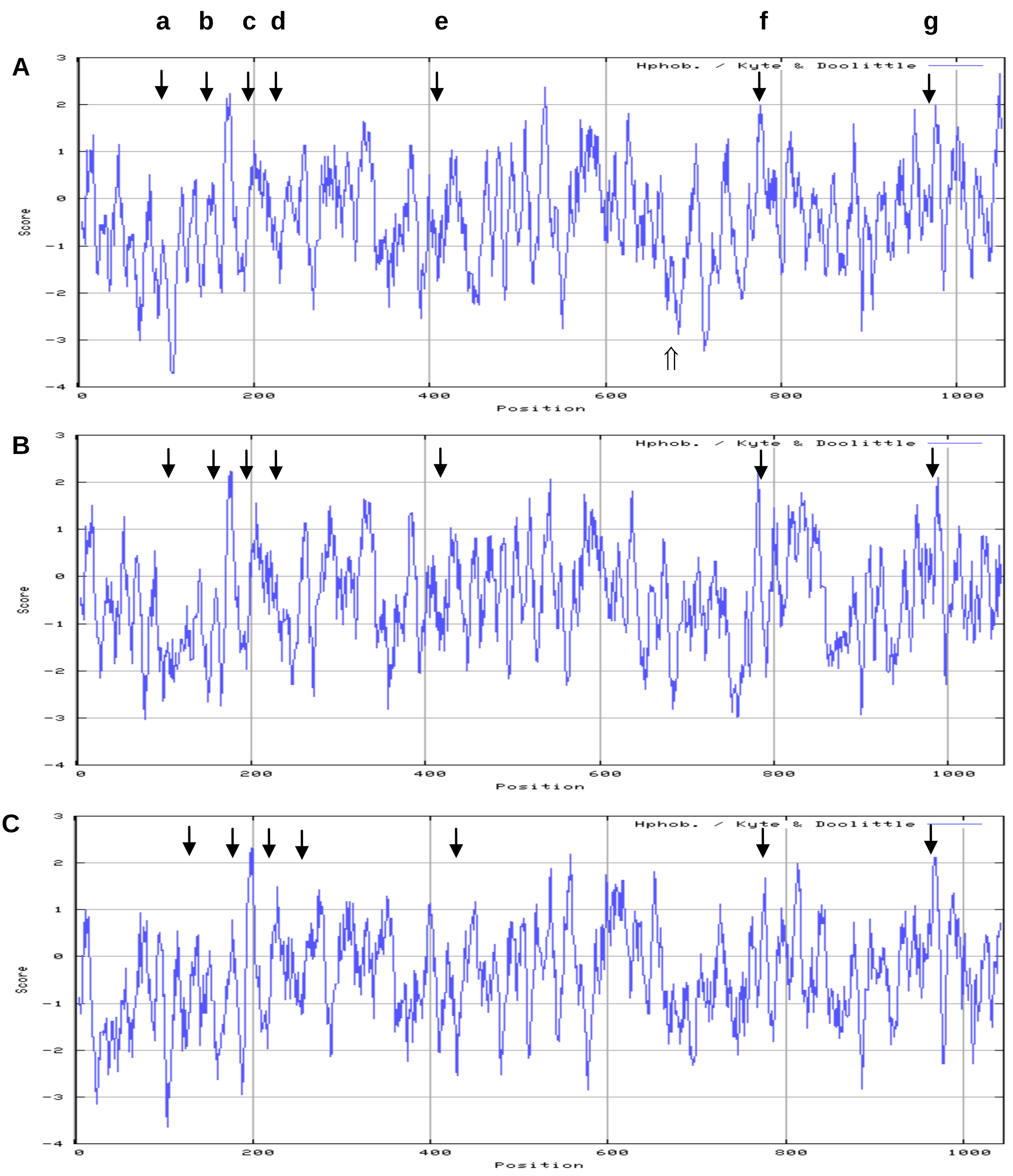

Fig. 4 Hydropathy plots of the predicted amino acid sequences of SPS from *Solanaceae* family based on the Kyte and Doolittle scale. **A** KC-SPS1 from potato, member of SPS A family (1054 aa, ACD50895); **B** SPSB from tomato, member of SPS B family (1064 aa, AFD64638); **C** NtSPSC from tobacco, member of SPS C family (1045aa, ABA64520). Various regulatory/binding motifs of SPS are also shown by downward arrows: **a** nuclear bipartite signal, **b** light/dark regulation, **c** Fru-6-P binding, **d** 14-3-3/UDP-Glc binding sites, **e** Osmotic regulation, **f** SPP Motif I, **g** SPP Motif III. The *upward arrow* '⇑' in **A** indicates the position of a string of acidic amino acids.

characters. But the flanking regions of some of the motifs, for example the N-terminal regions, the regions between 14-3-3/UDP-Glc binding sites and osmotic regulation, the linker regions, the regions between SPP Motif I and Motif III were found to vary significantly if compared between these SPS forms.

4.1.5. *Prediction of secondary structures*

With the help of ProtScale tool, α-helix, β-sheet, β-turn, and random coil in KC-SPS1, SPSB and NtSPSC were also predicted in order to compare their secondary structures. Only segment-wise propensities towards the formation of α-helices of the three SPS forms are shown in Fig. 5A-C. Except nuclear bipartite signal, most of the regulatory/binding motifs appeared to show fewer proclivities towards the formation of α-helices as compared to the other regions.

4.1.6. *SPS expression studies in different potato cultivars*

We used six Indian potato cultivars namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chandramukhi, Kufri Jyoti, Kufri Ashoka, and Kufri Pukhraj in this study. The former two are processing varieties. Kufri Chandramukhi and Kufri Ashoka are early maturing; whereas, the other cultivars are medium maturing. The potato plantlets were hardened and acclimatized and then cultivated in the field for ~12 weeks. The field-grown potato tubers immediately after harvest, referred to as ‘freshly harvested tuber’, were initially stored at room temperature for three weeks, and then transferred to 4ºC and 25ºC for next four weeks, termed as ‘cold-stored’ and ‘warm-stored’ potato tubers, respectively. The tubers and leaves were frozen in liquid nitrogen and stored at -70ºC for further molecular and biochemical studies.

Semi-quantitative RT-PCR: The SPS gene expression pattern was studied in the leaves, warm-stored and cold-stored tubers of the potato cultivars (see ‘Materials and Methods’). By semi-quantitative RT-PCR, ~1.3 kb cDNA could only be amplified using total RNA from the leaves and tubers of each potato cultivar (Fig. 6). The size was in agreement with the transcripts of SPS A forms towards the 5′-end. The transcript levels appeared to be comparable between the leaves and warm-stored tubers (Fig. 6A-B). The transcript levels

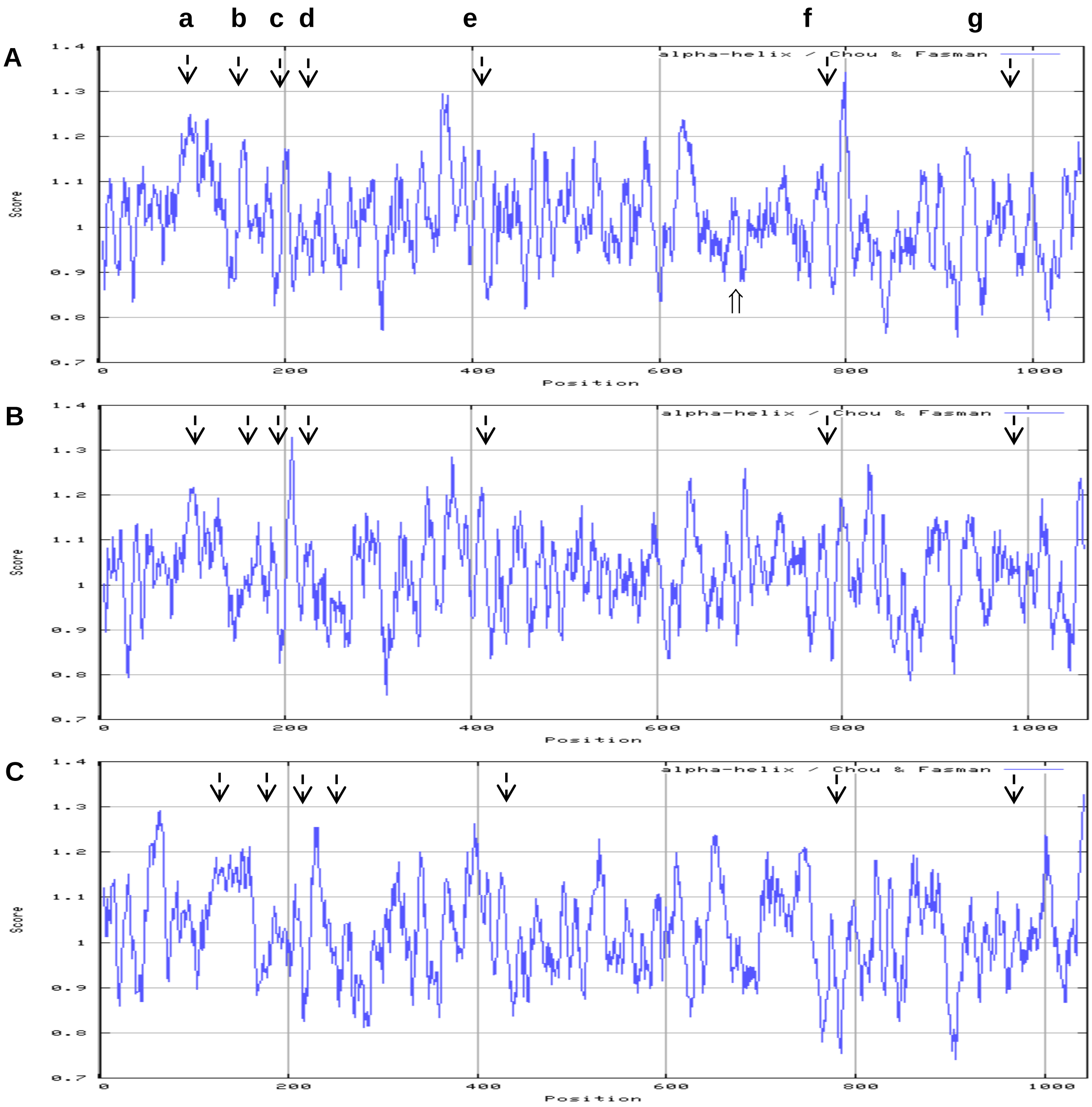

Fig. 5 Prediction of α-helices in the amino acid sequences of SPS from *Solanaceae* family based on the Chou and Fasman scale. **A** KC-SPS1 from potato, member of SPS A family (1054 aa, ACD50895); **B** SPSB from tomato, member of SPS B family (1064 aa, AFD64638); **C** NtSPSC from tobacco, member of SPS C family (1045aa, ABA64520). Various regulatory/binding motifs of SPS are also shown by downward arrows: **a** nuclear bipartite signal, **b** light/dark regulation, **c** Fru-6-P binding, **d** 14-3-3/UDP-Glc binding sites, **e** Osmotic regulation, **f** SPP Motif I, **g** SPP Motif III. *The upward arrow* '⇑' in **A** indicates the position of a string of acidic amino acids.

did not change significantly in the cold-stored tubers of most of the cultivars except Kufri Chipsona-1 and Kufri Jyoti where relatively higher transcript level was noticed (Fig. 6C). The actin expression level in the warm-stored tubers was uniform; whereas, it was nearly uniform in most of the cultivars in the cases of leaves as well as cold-stored tubers.

Protein gel blot analysis of potato cultivars: Protein gel blot analyses were carried out using SPS-specific polyclonal antibody to detect the protein band in the tuber protein extracts from different cultivars. IgG-HRP conjugated secondary antibody and DAB system were used. Protein blot analysis could detect a faint protein band of ~ 120 kDa in both warm- and cold-stored tubers (Fig. 7B, C). Very faint or almost negligible band could be detected in the leaf protein extracts (Fig. 7A).

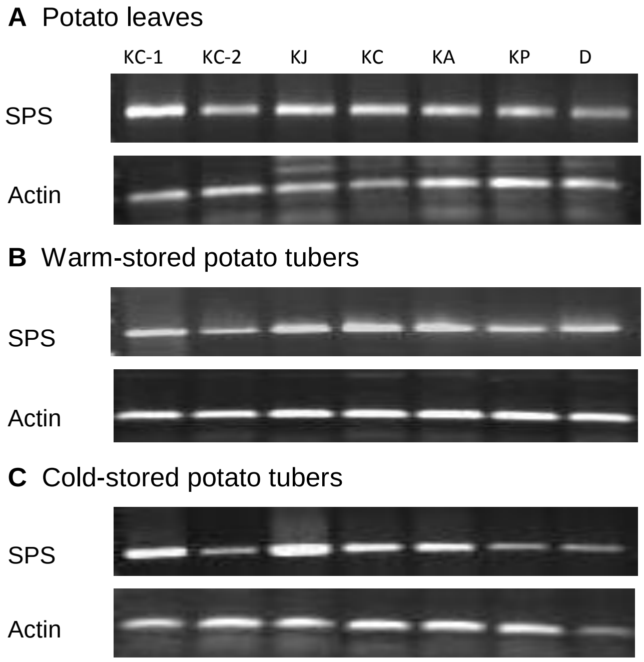

Fig. 6 Semi-quantitative RT-PCR approach for SPS expression analysis in the leaves and tubers from the mature field-grown potato cultivars using the primers D20-SPS and F20-SPS. **A** Potato leaves, **B** Warm-stored potato tubers (transferred to 25^0C for four weeks), **C** Cold-stored potato tubers (transferred to 4ºC for four weeks). Potato cultivars are Kufri Chipsona-1 (KC-1), Kufri Chipsona-2 (KC-2), Kufri Jyoti (KJ), Kufri Chandramukhi (KC), Kufri Ashoka (KA), Kufri Pukhraj (KP), and the cv. Desiree (D). The size of the SPS-specific amplified product was found to be ~1.3 kb in each case. Actin-specific primers were used as control (the size of the amplified product ~0.25 kb).

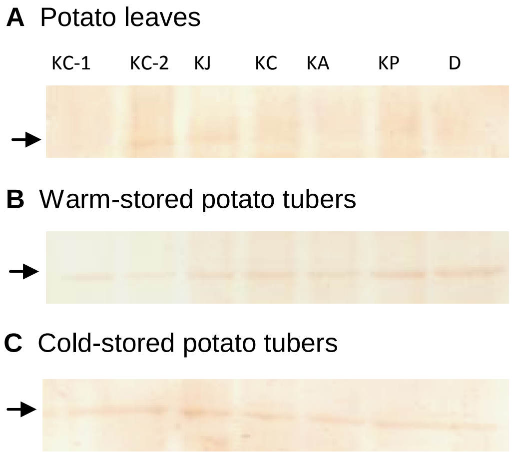

Fig. 7 Protein gel blot analysis using soluble proteins from potato leaves and tubers and polyclonal antibodies raised against SPS-specific peptide antigen (Materials and methods). **A** Potato leaves, **B** Warm-stored potato tubers, **C** Cold-stored potato tubers. Potato cultivars are Kufri Chipsona-1 (KC-1), Kufri Chipsona-2 (KC-2), Kufri Jyoti (KJ), Kufri Chandramukhi (KC), Kufri Ashoka (KA), Kufri Pukhraj (KP), and the cv. Desiree (D). The size of the immuno-detected band was ~ 120 kDa (as shown by arrow).

4.1.7. *SPS activity in potato cultivars*

SPS activity is often measured under two different conditions: a) V_{max} activity i.e. the 'nonselective assay,' in presence of nearly saturating substrate concentrations without inhibitor; b) V_{sel} activity i.e. the 'selective assay,' in presence of limiting substrate concentrations and inhibitor (Pi). Both V_{max} and V_{sel} activities of SPS were determined in the leaves, freshly-harvested, warm- and cold-stored tuber protein extracts. The SPS activity data clearly showed cultivar-specific variations. The values were higher in freshly harvested tubers as compared to leaves in most of the potato cultivars (Table 1). There was no significant change in V_{max} activity between warm- and cold-stored tubers in majority of the cultivars; but V_{sel} activity was found to be 2 to 3-fold higher in the latter for all the cultivars (Table 2). In the cases of Kufri Chipsona-1, Kufri Jyoti, and Kufri Pukhraj, warm- and cold-stored tubers were associated with decrease in V_{max} activity as compared to the freshly harvested tubers; whereas, similar treatments resulted in opposite effects in the other cultivars.

Table 1 SPS activity [nmol min^{-1}(gFW^{-1})] in the leaves and freshly harvested tubers from different potato cultivars

Potato cultivars	SPS activity [nmol min^{-1}(gFW)$^{-1}$]			
	Leaves		freshly harvested tubers	
	V_{max} ± SD	V_{sel} ± SD	V_{max} ± SD	V_{sel} ± SD
Kufri Chipsona-1	359 ± 19 a	44 ± 3 a	507 ± 53 a	53 ± 7 a
Kufri Chipsona-2	232 ± 13 c	27 ± 2 b	323 ± 42 b	33 ± 5 b
Kufri Jyoti	326 ± 21 a	38 ± 5 ab	493 ± 58 a	47 ± 4 a
Kufri Chandramukhi	237 ± 17 c	30 ± 4 b	330 ± 29 b	37 ± 3 b
Kufri Ashoka	281 ± 12 b	33 ± 4 b	332 ± 24 b	27 ± 3 bc
Kufri Pukhraj	255 ± 15 c	35 ± 5 b	525 ± 57 a	49 ± 4 a
Desiree	285 ± 16 b	29 ± 3 b	247 ± 19 c	23 ± 2 c

Values sharing a common letter within a column are not significant at $P \leq 0.05$

Values are the mean ± SD of n =3 independent tubers/leaves

Table 2 SPS activity [nmol min^{-1}(gFW)$^{-1}$] in the tubers from different potato cultivars stored at 25^{0}C and 4^{0}C for 4 weeks (prior to transfer to the experimental temperatures, the harvested potato tubers were kept at room temperature for three weeks)

Potato cultivars	SPS activity [nmol min^{-1}(gFW)$^{-1}$]			
	tubers stored at 25°C		tubers stored at 4°C	
	V_{max} ± SD	V_{sel} ± SD	V_{max} ± SD	V_{sel} ± SD
Kufri Chipsona-1	387 ± 23 a	33 ± 3 d	443 ± 27 a	113 ± 9 a
Kufri Chipsona-2	396 ± 17 a	34 ± 5 d	432 ± 19 a	101 ± 7 b
Kufri Jyoti	320 ± 12 cd	28 ± 3 d	396 ± 21 a	87 ± 5 c
Kufri Chandramukhi	307 ± 13 cd	37 ± 4 d	343 ± 17 bcd	93 ± 6 bc
Kufri Ashoka	379 ± 16 ab	39 ± 7 d	387 ± 13 a	117 ± 9 a
Kufri Pukhraj	357 ± 17 abc	32 ± 5 d	395 ± 15 a	123 ± 7 a
Desiree	287 ± 11 d	33 ± 6 d	311 ± 14 cd	97 ± 5 b

V_{max} values sharing a common letter are not significant at $P \leq 0.05$

V_{sel} values sharing a common letter are not significant at $P \leq 0.05$

Values are the mean ± SD of n =3 independent tubers

Sequence divergence in the 5′-terminal regions of different SPS family transcripts in the *Solanaceae* family members is contributed significantly by 5′-UTR and a part of N-terminal coding region. The primers, D20-SPS and F20-SPS were used in semi-quantitative RT-PCR analyses. D20-SPS was specific to the 5′-UTR of SPS A form; whereas, F20-SPS referred to the conserved coding region. Therefore, it may be concluded that the ~1.3 kb RT-PCR product was specific to SPS A family transcripts in both photosynthetic and nonphotosynthetic tissues of potato (Fig. 6). There was nearly uniform level of expression in the leaves, freshly-harvested and warm-stored tubers of all the cultivars; some fluctuations in the cold-stored tubers were probably due to genotypic variations between them. The RT-PCR data strengthened the view that SPS A forms were probably involved in housekeeping functions. Occurrence of SPS A forms in various potato tissues does not necessarily mean that the respective SPS genes are under similar transcriptional control. Therefore, expression patterns of the individual members of this SPS gene family need to be studied carefully. By immunodetection technique (Fig. 7), a band corresponding to ~ 120 kDa protein could be detected in both warm- and cold-stored tuber protein extracts. But for leaf protein extracts, the band intensity was either very faint or negligible, suggesting poor extraction and/or low cellular level of SPS that remained to be understood. Protein blot analysis probably indicated the expression of SPS A form; since the 17-mer antigenic polypeptide was mostly specific to SPS A form. V_{max} activities were only indicative of the total extractable SPS activity from different potato tissues (Tables 1, 2). Cultivar-specific variations could be due to genotypic variations and metabolic status of the tissues. V_{sel} activities in the cold-stored tubers were significantly higher as compared to the freshly harvested and warm-stored tubers suggesting the altered kinetic properties of some SPS forms appeared at low temperature as reported earlier (Hill et al. 1996; Reimholz et al. 1997; Deiting et al. 1998). However, individual contribution of various SPS forms towards the overall catalytic activities in different potato tissues still remain an enigma which needs to be addressed through corroborative experimental approaches.

In conclusion, apart from isolation of a cDNA encoding distinct SPS A form, thorough sequence analyses and comparison within and between SPS families, preliminary studies on expression patterns and assay of SPS activities in different tissues as documented in this report will be quite useful for further in-depth studies on SPS in potato and other important members of the *Solanaceae* family.

4.2. *Second Objective:* Making various Ti-plasmid based antisense & sense genetic constructs by using different regions of SPS cDNA/genomic clones under constitutive and tuber-specific promoters

As discussed earlier in the 'Introduction', a number of enzymes are believed to play important biochemical roles during cold-induced sweetening in the potato tubers. During this process, SPS also plays an important role. Since the increased Suc accumulation could be well correlated with increased SPS activity in both photosynthetic nonphotosynthetic tissues, such as potato tuber. Moreover, altered kinetic properties of SPS were thought to be important in controlling the flux of carbons to free sugars under stress situation (i.e. drought and cold). Most importantly, the undesirable cold sweetening process in potatoes was associated with activation of SPS, appearance of a new form of this enzyme, increased SPS activity, stimulation of sucrose synthesis, and the decline of hexose phosphates (Hill et al. 1996; Reimholz et al.1997; Deiting et al. 1998). Reimholz et al. (1997) along with the presence of multiple forms of SPS in the cold-stored potato tubers. Apart from SPS, other SBRPs namely UDP-glucose pyrophosphorylase and sucrose 6-phosphate phosphatase are also involved in cold-induced sweetening. Therefore, inhibition of sucrose biosynthesis could be regarded as more direct approach to address the problem of CIS in potato tubers. As SPS is a major enzyme of sucrose biosynthesis, inhibition of SPS gene function(s) in potato became target of research in many laboratories. Based on a full-length SPS cDNA clone clone i.e. SPS-P4 (GenBank ID: X73477)), and a partial SPS cDNA clone i.e. SPS-P1, Krause et al (1998) made a few antisense and sense genetic constructs under the CaMV 35S promoter; which were subsequently used for genetic transformation of the reference cultivar Desiree. There was some degree of success. For example, ~75% reduction in SPS expression showed a reproducible but non-proportional (10 to 40%) decrease of soluble sugars in cold-stored tubers. With regard to commercially important potato cultivars including Indian varieties, no such reports are available till date. In this study, several molecular approaches, considerably different from the above, were adopted for inhibition of SPS gene function in Indian potato cultivar, Kufri Chipsona-1. Different regions of the SPS cDNA under study were employed in making a series of antisense and sense binary genetic constructs under both constitutive (CaMV 35S) and tuber-specific (GBSSI) promoters. All these constructs were used in *Agrobacterium*-mediated potato transformation. Using the potato cultivar, Kufri Chipsona-1, a number of transgenic potato plants were generated with respect to the individual genetic constructs.

Transgenic potato tubers produced and analyzed at both biochemical and molecular level after storage at different experimental temperatures. All these steps are precisely described in the following sections.

4.2.1. *Binary vectors used in this study*

The binary vector pBI121 was used for making various types of antisense and sense genetic constructs under the constitutive CaMV 35S promoter (Fig. 8A). The other binary vector, namely pAN-GB01, having GBSSI-GUS gene fusion (by replacing the CaMV 35S promoter in pBI121 with the GBSSI promoter) was used in this study (Fig. 8B).

One of the objectives of this work was to use the tuber-specific promoter in making some of the binary genetic constructs. PCR approach using GBSSI allele-specific primers led us to isolate two partial GBSSI alleles from the potato cultivar Kufri Chandramukhi, designated as GKC1 and GKC2, and the sequence information were submitted to the NCBI under the GenBank IDs EU548081 and EU548082, respectively. The GKC1 promoter was found to be a tuber-specific promoter. This promoter was used in making the binary vector, designated as pAN-GB01 having GBSSI-GUS gene fusion, was generated earlier in our laboratory (Bansal et al. 2012).

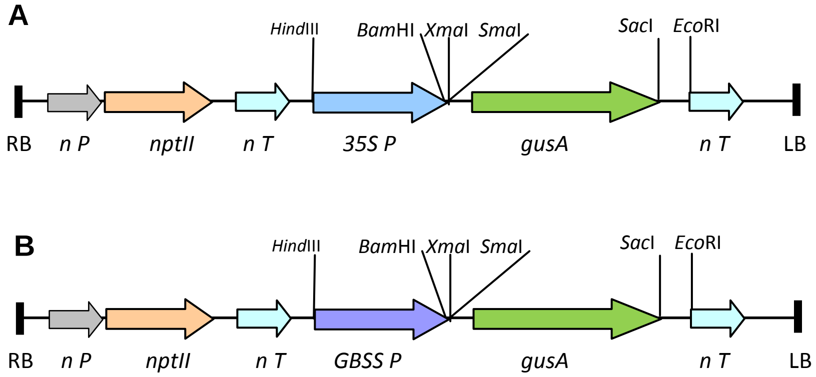

Fig. 8 Schematic view of the T-DNA regions of the binary vectors used for making genetic constructs. *RB* right border; *LB* left border; *n P* the nos promoter; *nT* the nos terminator; *nptII* neomycin phosphotransferase; *gusA* β-glucuronidase; *35S P CaMV 35S* promoter; *GBSS P* the GBSS promoter **A** binary vector pBI121 having the constitutive CaMV 35S promoter upstream of the GUS gene (GenBank ID: AF485783); B The binary vector pAN-GB01, constructed by replacing the CaMV 35S promoter by the ~ 0.75 kb GBSSI promoter isolated from GKC1 allele of cv. Kufri Chandramukhi

4.2.2. *Antisense and sense binary genetic constructs*

For inhibition of SPS gene function, several approaches were adopted. Different regions of the SPS cDNA i.e., SPS-C1 of the study as shown in Fig. 9 were employed in making a total of eight binary genetic constructs. The details are provided below:

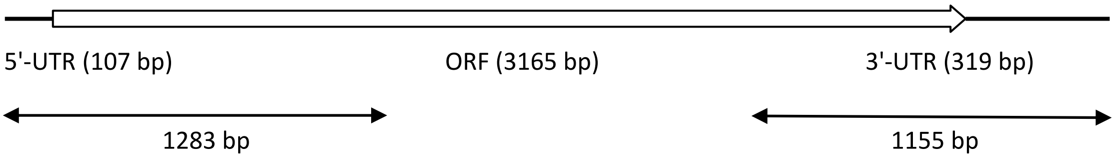

Fig. 9 Schematic view of the SPS cDNA, SPS-C1. The 1283-bp (towards the 5′-terminus) and 1155-bp (towards the 3′-terminus) regions were used in making various genetic constructs

A. List of antisense and sense genetic constructs using 1283-bp towards the 5′ terminus of the SPS cDNA, SPS-C1:

- **i) SA** (antisense construct under the constitutive CaMV 35S promoter)
- **ii) SB** (sense construct under the constitutive CaMV 35S promoter)
- **iii) SC** (antisense construct under the tuber-specific GBSSI promoter)
- **iv) SD** (sense construct under the tuber-specific GBSSI promoter)

B. List of antisense and sense genetic constructs using 1155-bp towards the 3′ terminus of the SPS cDNA, SPS-C1:

- **i) SE** (antisense construct under the constitutive CaMV 35S promoter)
- **ii) SF** (sense construct under the constitutive CaMV 35S promoter)
- **iii) SG** (antisense construct under the tuber-specific GBSSI promoter)
- **iv) SH** (sense construct under the tuber-specific GBSSI promoter)

SA (antisense orientation) and SB (sense orientation) constructs under the CaMV 35S promoter: 1283-bp region was amplified by PCR using SPS-C1 as template and the primer pair, D-20 SPS and F-20 SPS. The amplified product was treated with Klenow enzyme and purified using Qiagen kit. The blunt-ended cDNA fragment was cloned in either orientation into the binary vector pBI121 by replacing the reporter GUS gene (*Sma*I and *Ecl1*36II were used to remove the GUS gene). The antisense (Fig. 10A) and sense (Fig. 10B) orientations were further checked by restriction analyses and PCR.

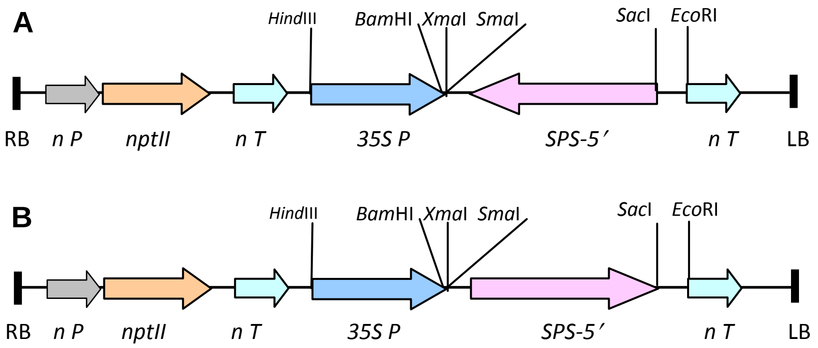

Fig.10 Schematic view of the pBI121-based genetic constructs. **A** SA (the antisense construct under the CaMV 35S promoter); **B** SB (the sense construct under the CaMV 35S promoter)

SC (antisense orientation) and SD (sense orientation) constructs under the GBSSI promoter: Like SA and SB constructs as mentioned above, the 1283-bp region of SPS-C1 was cloned into *Sma*I-*Ecl*136II site of the binary vector pAN-GB01 by replacing the reporter GUS gene. The antisense (Fig. 11A) and sense (Fig. 11B) orientations were further checked by restriction analyses and PCR.

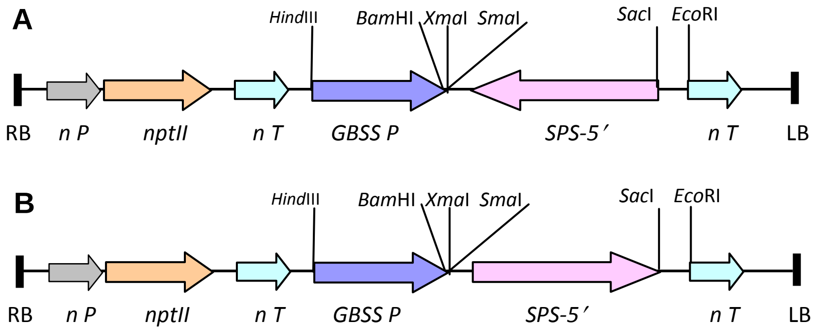

Fig. 11 Schematic view of the pAN-GB01-based genetic constructs. **A** SC (the antisense construct under the GBSSI promoter); **B** SD (the sense construct under the GBSSI promoter)

SE (antisense orientation) and SF (sense orientation) constructs under the CaMV 35S promoter: First, the 1155-bp region towards the 3'-terminus of SPS-C1, was amplified by PCR using the same template as above and the primers, SPS-F2447 and SPS-3601. The PCR-amplified DNA was then cloned into the *Sma*I site of pUC19 in either orientation, and the inserts were released by *Bam*HI and *Ecl136*II digestion. These DNA fragments were then cloned into *Bam*HI-*ECl*136II site in pBI121 by replacing the GUS gene (Fig.

12). The antisense (Fig. 12A) and sense (Fig. 12B) orientations were further checked by restriction analyses and PCR.

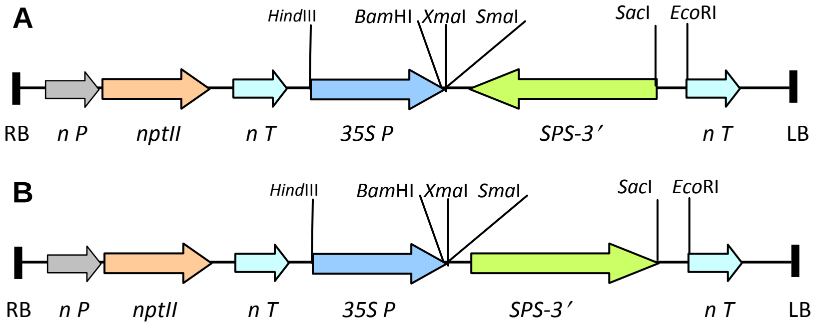

Fig.12 Schematic view of the pBI121-based genetic constructs. **A** SE (the antisense construct under the CaMV 35S promoter); **B** SF (the sense construct under the CaMV 35S promoter)

SG (antisense orientation) and SH (sense orientation) constructs under the GBSSI promoter: For making SG and SH constructs, the same methodology was adopted as mentioned in the above section i.e. for SE and SF constructs. Here the binary vector, pAN-GB01 was used in place of pBI121. The antisense (Fig. 13A) and sense (Fig. 13B) orientations were further checked by restriction analyses and PCR.

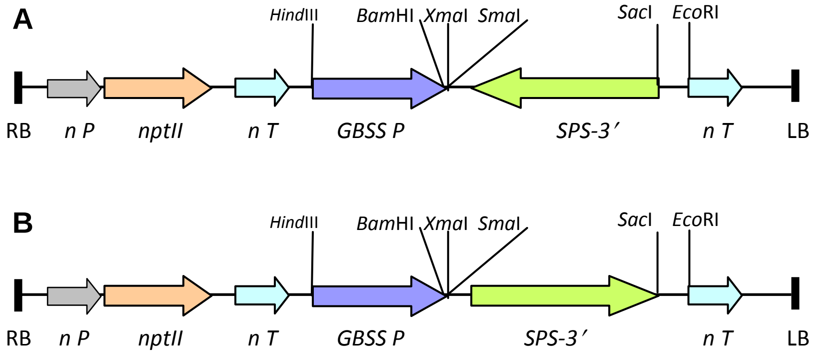

Fig. 13 Schematic view of the pAN-GB01-based genetic constructs. **A** SG (the antisense construct under the GBSSI promoter); **B** SH (the sense construct under the GBSSI promoter)

4.3. *Third Objective: Agrobacterium*-mediated genetic transformation of potato plants with the above genetic constructs followed by screening of the transgenic potato lines

4.3.1. ***Transformation of Agrobacterium tumefaciens strain (LBA4404) with the binary genetic constructs:*** Each of the Ti-plasmid based genetic constructs as mentioned earlier was used to transform *Agrobacterium tumefaciens* strain, LBA4404 by either of the following techniques: electroporation, triparental mating. The individual *Agrobacterium* transformants were selected on selective YEM media containing streptomycin (50 µg/mL) and kanamycin (50 µg/mL). Recombinant plasmid was isolated from each of the *Agrobacterium* transformants. Restriction analyses by appropriate enzymes and PCR were carried out to check the correct orientation of the inserts and intactness of the genetic constructs (data not shown).

4.3.2. ***Generation of transgenic potato plants through Agrobacterium-mediated transformation:*** The internodal stem segments of the micropropagated potato plantlets (cv. Kufri Chipsona-1) were co-cultivated with the individual *Agrobacterium* transformants using the modified protocol as reported by Beaujean et al. (1998), Bansal et al. (2012). The composition of shoot regeneration medium was MS basal containing zeatin 2.5 mg/L, GA_3 3.0 mg/L, IAA 0.01 mg/L along with kanamycin 80 mg/L and cefotaxime 250 mg/L for primary selection of transgenic potato plants. Shoot initiation from the internodal stem segments corresponding to the different genetic constructs are shown in Fig.14. Multiple shoot regeneration with minimum intervening callus phase was noticed during *Agrobacterium*-mediated potato transformation (Fig.15).

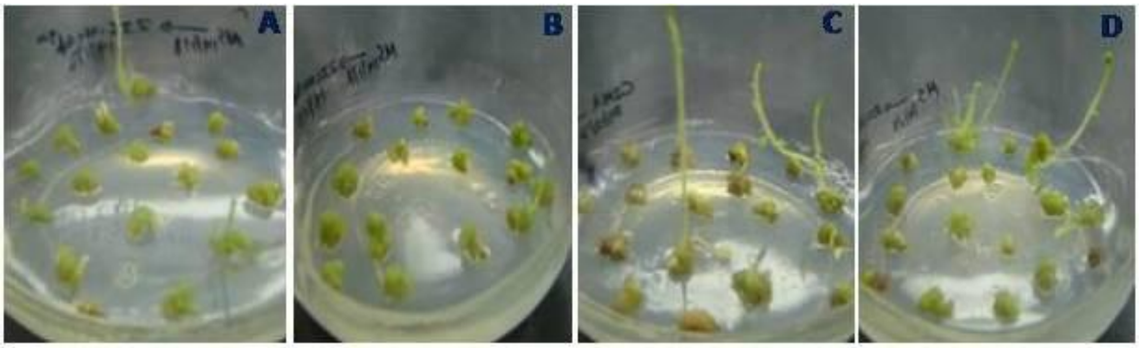

Fig. 14 Shoot **i**nitiation from internodal stem segments of Kufri Chipsona-1 in the selective media corresponding to the different binary genetic constructs. **A** SA construct, **B** SC construct, **C** SF construct, **D** SG construct

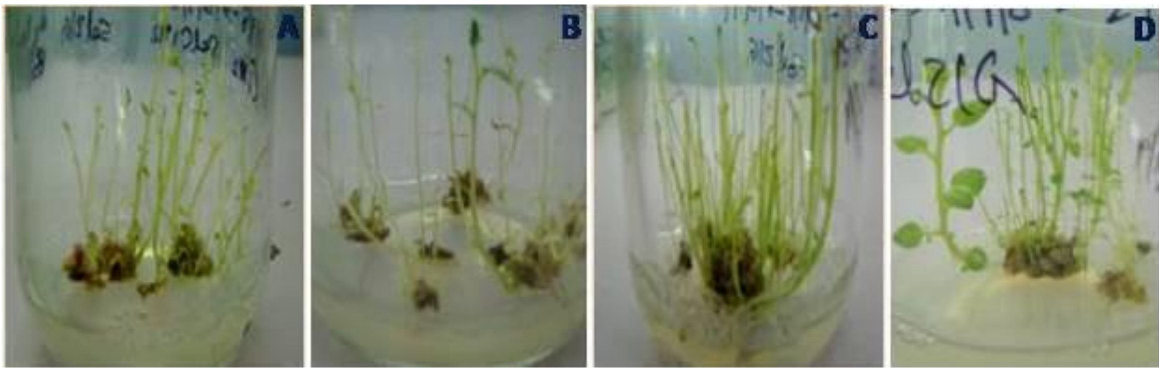

Fig. 15 Multiple shoot regeneration from internodal stem segments of Kufri Chipsona-1 in the selective media corresponding to the different binary genetic constructs with minimum intervening callus phase. **A** SA construct, **B** SB construct, **C** SF construct, D SH construct

Micropropagation of the regenerated shoots on selective medium: The initial regenerated shoots were further transferred to the rooting media to obtain the complete plantlets. In some cases, rooting was delayed from shoot explants.

Checking the presence of transgene(s) in the regenerated potato plantlets: To detect the presence of transgene (s), total genomic DNA was isolated from the transgenic potato plantlets, and PCR was carried out using different combinations of forward and reverse primers corresponding to the CaMV 35S/GBSSI promoters, and the respective SPS cDNA fragments in the individual genetic constructs. PCR amplification occurred in most of regenerated potato plantlets grown in the selective medium indicating the stable integration of transgene(s) into the potato genome (data not shown). Some of the selected micropropagated transgenic potato plantlets are shown below (Fig. 16A-D).

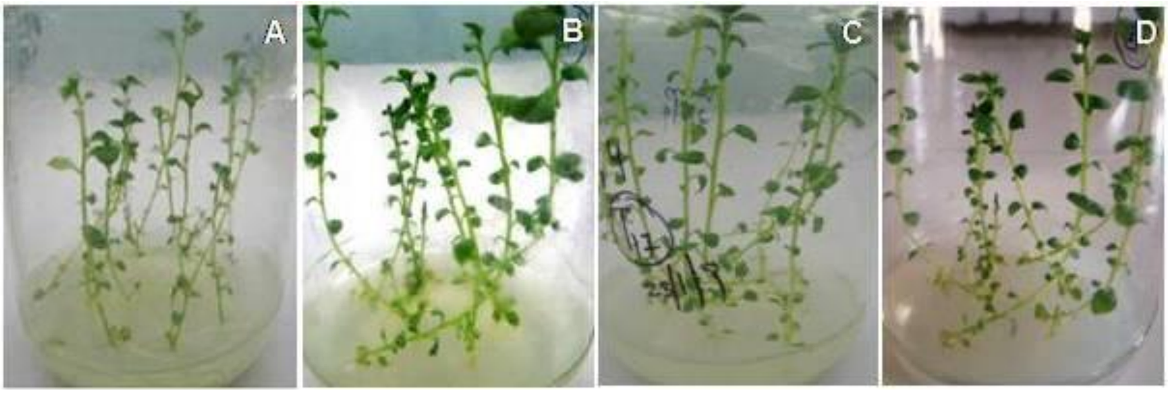

Fig. 16 Micropropagated transgenic potato plantlets corresponding to different binary genetic constructs. **A** SA-1, **B** SC-3, **C** SF-7, **D** SH-6

4.3.3. *Production and screening of the transgenic potato mini tubers*

Production of potato mini tubers under field conditions: The untransformed (control), and 25–30 transgenic potato plants corresponding to each genetic construct were hardened and acclimatized, and then grown in the restricted experimental plots under field conditions; and mini tubers were harvested after 90 to 100 days. In terms of overall vegetative growth of the different transgenic potato plants and various phenotypic attributes of the mini tubers namely size, shape and yield per plant were not distinguishable from the control plants and the tubers. For control see Fig. 17 and a representative transgenic line is shown in Fig. 18 (others are not shown).

Fig. 17 Different phases of cultivation (from micropropagation to the harvest of mini tubers) of the control potato, Kufri Chipsona-1 under field conditions. **A** micropropagated potato plantlets, **B** hardening and acclimatization, **C** 3-wk old potato plants in polybags, **D** gowing transgenic plants (~8-wk old), **E** ~10-wk old plant showing developing tubers, **F** harvested mature mini tubers

Detection of transgene (s) in the potato mini tubers by PCR: Immediately after harvest, total genomic DNA was also isolated from both transgenic and control potato tubers. The presence of transgene was checked by PCR using these DNA preparations as templates and different primer pairs as described previously. The PCR-amplified DNA products corresponding to most of the transgenic potato plants under study were analyzed by agarose gel electrophoresis; some of them are shown in Fig. 19 and Fig. 20. The sizes of

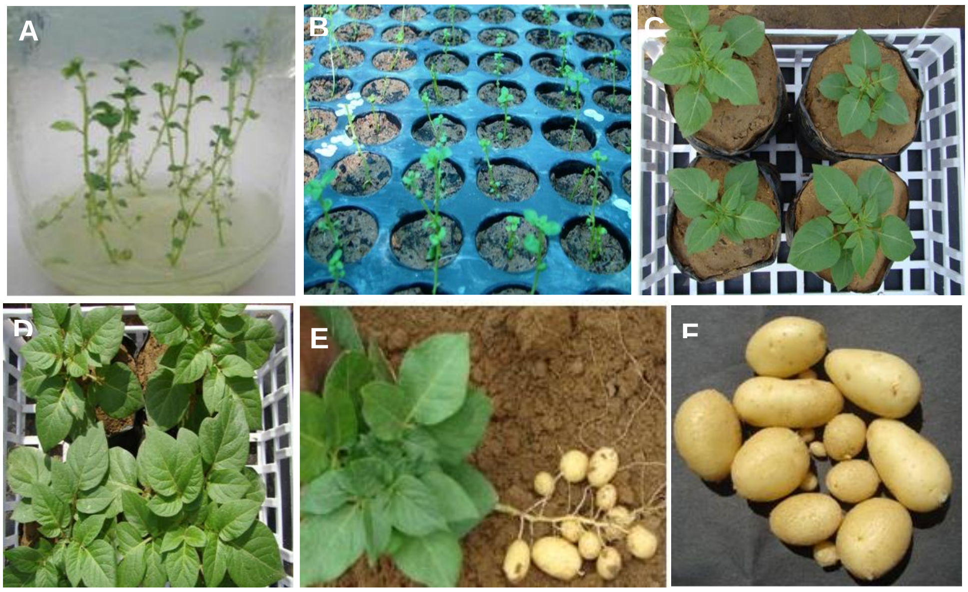

Fig. 18 Different phases of cultivation (from micropropagation to the harvest of mini tubers) of transgenic plant SA-1. **A** micropropagated potato plantlets, **B** hardening and acclimatization, **C** ~4-wk old plants in polybags, **D** ~8-wk old growing transgenic plants, E ~10-wk old plant showing developing tubers, **F** harvested mature mini tubers

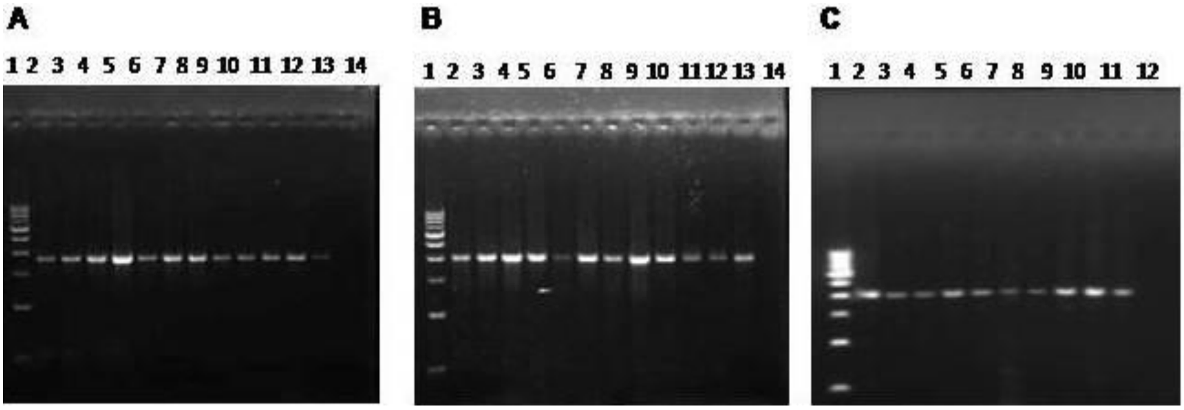

Fig. 19 PCR analyses using tuber genomic DNA as template corresponding to transgenic potato plants with 5' region of SPS-C1. **A** ~1.9 kb fragment amplified using AF-3501 forward primer (from CaMV 35S promoter region) and D-20 SPS reverse primer; **B** ~2.0 kb fragment amplified using GB1-F01 forward primer (from GBSSI promoter region) and D-20 SPS reverse primer; **C** ~2.0 kb fragment amplified using GB1-F01 forward primer and F-20 SPS reverse primer. Lane1 0.5 kb DNA ladder in each figure ; **A** Lanes 2 to 13 different transgenic lines SA-1, SA-2, SA-3, SA-7, SA-8, SA-11, SA-16, SA-17, SA-18, SA-19, SA-21, SA-23 and Lane 14 KC-1 as control; **B** Lanes 2 to 13 different transgenic lines SC-1, SC-2, SC-3, SC-4, SC-5, SC-6, SC-7, SC-8, SC-9, SC-10, SC-13, SC-15 and Lane 14 KC-1 as control ; **C** Lanes 2 to 11 different transgenic lines SD-1, SD-2, SD-3, SD-4, SD-6, SD-7, SD-8, SD-9, SD-11, SD-12 and Lane 12 KC-1 as control

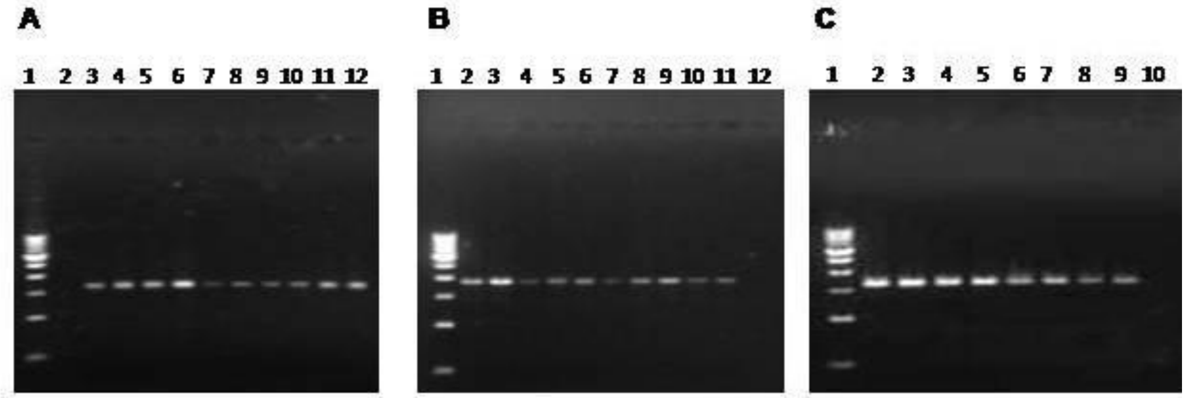

Fig. 20 PCR analyses using tuber genomic DNA as template corresponding to transgenic potato plants with 3' region of SPS-C1. **A** ~1.65 kb fragment amplified using AF-3501 forward primer (from CaMV 35S promoter region) and F-2447 SPS reverse primer; **B** ~1.9 kb fragment amplified using GB1-F01 forward primer (from GBSSI promoter region) and F-2447 SPS reverse primer; **C** ~1.65 kb fragment amplified using AF-3501 forward primer and SPS-3601, SPS reverse primer. Lane1 0.5 kb DNA ladder in each figure ; **A** Lane 1 KC-1 as control and Lanes 3 to 12 different transgenic lines SE-1, SE-2, SA-3, SE-4, SE-5, SE-8, SE-9, SE-10, SE-14, SE-15; **B** Lanes 2 to 11 different transgenic lines SG-1, SG-2, SG-3, SG-4, SG-5, SG-6, SG-7, SG-8, SG-9, SG-10 and Lane 12 KC-1 as control ; **C** Lanes 2 to 9 different transgenic lines SF-1, SF-2, SF-3, SF-4, SF-6, SF-7, SF-11, SF-14, SF-15, SF-19 and Lane 10 KC-1 as control

the amplified DNA products corresponding to the individual transgenic potato plants appeared to be consistent and as expected. PCR data clearly indicated the presence of transgene (s) in the tubers of the transgenic potato plants as detected previously in the respective potato plantlets.

4.4. *Fourth Objective:* Biochemical studies on sucrose-phosphate synthase (SPS) activity and the level of sugar accumulation in the transgenic potato tubers at various experimental temperatures

4.4.1. *Storage of potato mini tubers at different experimental temperatures*

Storage of potato tubers at low temperatures leads to the accumulation of soluble sugars i.e., both reducing sugars and sucrose. The extent of sugar accumulation depends on the cultivar genotype. One of the major objectives of this study was to inhibit SPS gene function in potato through antisense and sense expression of different regions of SPS cDNA under the influence of both constitutive and tuber-specific promoters. Apart from screening of the transgenic potato lines by PCR approach as described above, primary screening of the tubers of 25–30 transgenic potato plants corresponding to each genetic construct was done based on the reduced accumulation of sucrose and reducing sugars in the cold-stored transgenic mini tubers. The tubers of around 10 transgenic plants were selected which showed significantly reduced sucrose and reducing sugar contents upon storage at 4ºC for 4 weeks as compared to the control tubers. To obtain sufficient amounts of both control and transgenic mini tubers for further detailed analyses, four to six replicates of each preselected potato transformants were transferred to the restricted experimental plots for production of mini tubers under field conditions.

To analyze the phenomenon of cold-induced sweetening, potato mini tubers were harvested from different transgenic along with control potato plants grown under field conditions. Immediately after harvest, both the transgenic and control potato tubers were stored at room temperature for three weeks referred to as ‘freshly harvested tuber (FH)’, and then transferred to 4^0C and 25^0C for the next four weeks, termed as ‘cold-stored (CS)’ and ‘warm-stored (WS)’ potato tubers, respectively. The storage of the mini tubers at these temperatures were also continued up to eight weeks. All these tubers stored at different temperatures for varying time periods were sliced and frozen in liquid nitrogen, and then kept at -70^0C for further molecular and biochemical studies. As biochemical studies, contents of reducing sugars, sucrose and total sugars along with extractable SPS activities were measured; whereas SPS expression studies were based on semi-quantitative RT-PCR and protein blot analyses. Although, the sugar and SPS activity data for 4 and 8 weeks of storage at different experimental temperatures were more or less comparable, only the data for 4 weeks of storage were mostly presented in the following Results sections. The data for 8 weeks of storage were presented in Annexure-I which could be useful for comparison with 4-wk storage data.

4.4.2. *Determination of soluble sugars*

a) Soluble sugar contents in the untransformed (control) potato tubers

The potato cultivar, Kufri Chipsona-1 was used as a control in the study. For comparison with the transgenic tubers, sugar data of the control tubers after storage at different experimental temperatures are provided here. In the FH tubers, the sucrose and reducing sugar contents were 4.52 ± 0.59 mg g^{-1} FW and 0.69 ± 0.24 mg g^{-1} FW, respectively. In the CS tubers, the sucrose and reducing sugar contents were 7.83 ± 0.53 mg g^{-1} FW and 3.84 ± 0.08 mg g^{-1} FW, respectively; whereas, the respective values for the WS tubers were 2.02 ± 0.33 mg g^{-1} FW and 1.22 ± 0.05 mg g^{-1} FW. The data clearly indicated that sucrose level was increased significantly in the cold-stored tubers. Storage at low temperature led to nearly 6-fold increase of reducing sugar as compared to freshly harvested tubers. Therefore, cold-induced sweetening appeared to be quite prominent even in the processing potato cultivar.

b) Soluble sugar contents in the transgenic potato tubers

In this study, a series antisense and sense binary genetic constructs as mentioned earlier were used in generating transgenic potato plants. For screening of the transgenic potato lines, sucrose and reducing sugar contents were determined in the potato tubers stored at different temperatures for varying periods of time. For this purpose, tubers from around 25 transgenic potato lines corresponding to each binary genetic construct were analyzed. Again from each genetic construct, sugar data of the tubers from around ten potato lines showing significantly ($P<0.05$) lower accumulation of soluble sugars during cold storage are analyzed in the following sections.

SA construct-based transgenic tubers: In the FH tubers, sucrose contents were found ranging from 2.18 ± 0.56 to 8.82 ± 1.01 mg g^{-1} FW; the reducing sugar contents varied between 0.21 ± 0.18 and 1.61 ± 0.39 mg g^{-1} FW. In the CS tubers, sucrose contents were found in the range of 1.13 ± 0.29 to 2.45 ± 0.62 mg g^{-1} FW; whereas the values of reducing sugar contents were between 0.55 ± 0.30 to 3.03 ± 0.84 mg g^{-1} FW. In the WS tubers, the ranges of the sucrose and reducing sugar contents were found to be 1.22 ± 0.29–5.21 ± 0.51 mg g^{-1} FW and 0.05 ± 0.01–0.78±0.09 mg g^{-1} FW, respectively. In some of the cold-stored transgenic tubers corresponding to the lines, namely SA-7, SA-8, SA-11 and SA-16, the sucrose and reducing sugar contents were reduced significantly as compared to the control. For sugar data see Fig. 21 and Tables 11-13 (Annexure-I).

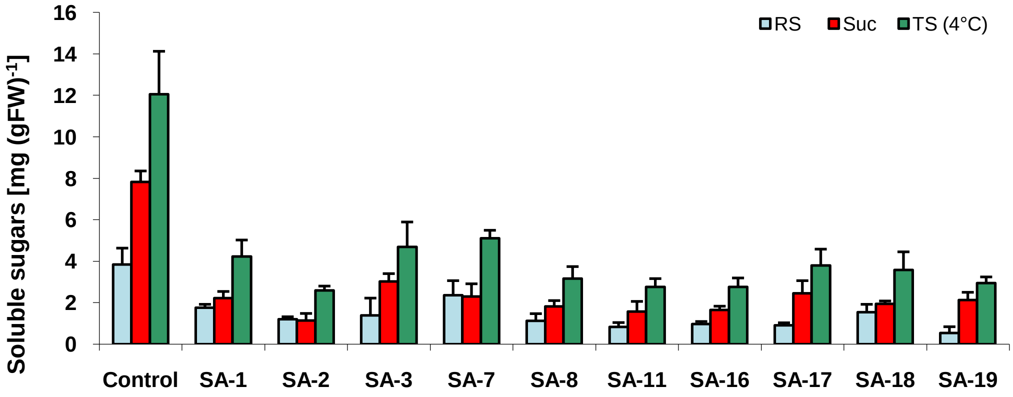

Fig. 21 Soluble sugar contents (reducing sugar, sucrose and total sugars) of SA construct-based 'cold-stored' transgenic tubers. Control refers to cv. Kufri Chipsona-1 (untransformed). The results are mean ± SD of 4 individual tubers.

SB construct-based transgenic tubers: In the FH tubers, sucrose contents were found ranging from 1.47 ± 0.38 to 4.87 ± 0.31 mg g^{-1} FW; the reducing sugar contents varied between 0.36 ± 0.08 and 1.46 ± 0.67 mg g^{-1} FW. In the CS tubers, sucrose contents were found in the range of 1.14 ± 0.29 to 4.04 ± 0.24 mg g^{-1} FW; whereas the values of reducing sugar contents were between 0.16 ± 0.01 to 1.64 ± 0.21mg g^{-1} FW. In the WS tubers, the ranges of the sucrose and reducing sugar contents were found to be 0.71 ± 0.38 to 2.28 ± 0.23 mg g^{-1} FW and 0.30 ± 0.05 –1.71 ± 0.26 mg g^{-1} FW, respectively. In some of the cold-stored transgenic tubers corresponding to the lines, namely SB-2, SB-3, SB-4, SB-6 and SB-8, the sucrose and reducing sugar contents were reduced significantly as compared to the control. For sugar data see Fig.22 and Tables 14-16 (Annexure-I).

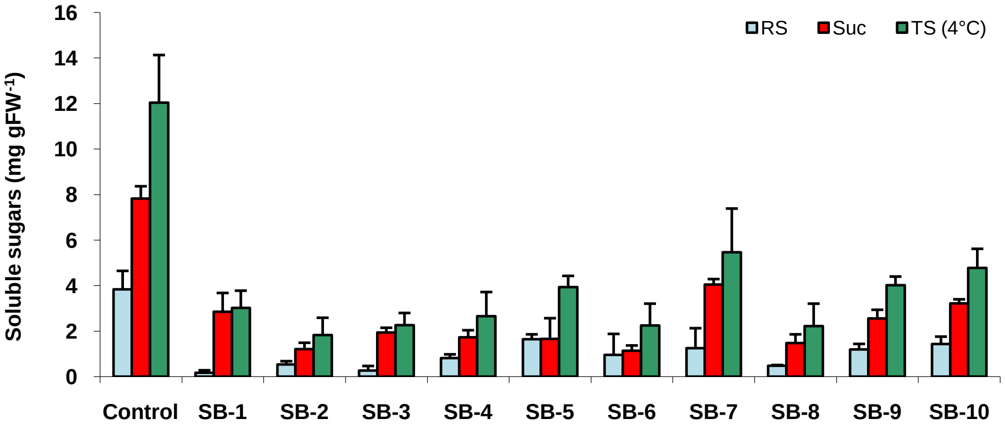

Fig. 22 Soluble sugar contents (reducing sugar, sucrose and total sugars) of SB construct-based 'cold-stored' transgenic tubers. Control refers to cv. Kufri Chipsona-1 (untransformed). The results are mean ± SD of 4 individual tubers.

SC construct-based transgenic tubers: In the FH tubers, sucrose contents were found ranging from 1.77 ± 0.63 to 3.93 ± 0.71 mg g^{-1} FW; the reducing sugar contents varied between 0.35 ± 0.14 and 1.65 ± 0.31 mg g^{-1} FW. In the CS tubers, sucrose contents were found in the range of 0.73 ± 0.09 to 2.56 ± 0.33 mg g^{-1} FW; whereas the values of reducing sugar contents were between 0.59 ± 0.28 to 2.47 ± 0.48mg g^{-1} FW. In the WS tubers, the ranges of the sucrose and reducing sugar contents were found to be 1.31 ± 0.39 to 2.48 ± 0.52 mg g^{-1} FW and 0.11 ± 0.02 –1.40 ± 0.22mg g^{-1} FW, respectively. In some of the cold-stored transgenic tubers corresponding to the lines, namely SC-6, SC-7, SC-8 and SC-10, the sucrose and reducing sugar contents were reduced significantly as compared to the control. For sugar data see Fig. 23 and Tables 17-19 (Annexure-I).

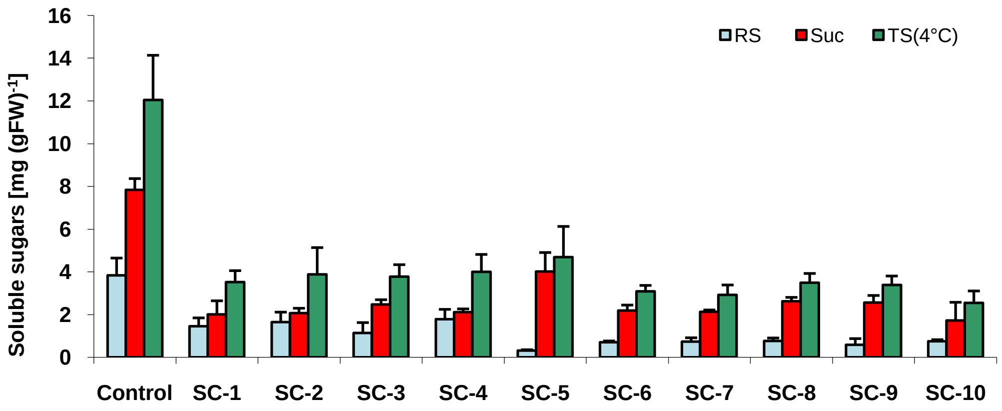

Fig. 23 Soluble sugar contents (reducing sugar, sucrose and total sugars) of SC construct-based 'cold-stored' transgenic tubers. Control refers to cv. Kufri Chipsona-1 (untransformed). The results are mean ± SD of 4 individual tubers.

SD construct-based transgenic tubers: In the FH tubers, sucrose contents were found ranging from 3.05 ± 0.32 to 6.72 ± 1.60 mg g^{-1} FW; the reducing sugar contents varied between 0.14 ± 0.08 and 1.34 ± 0.10 mg g^{-1} FW. In the CS tubers, sucrose contents were found in the range of 0.34 ± 0.21 to 3.11 ± 0.30 mg g^{-1} FW; whereas the values of reducing sugar contents were between 0.09 ± 0.02 to 1.63 ± 0.32mg g^{-1} FW. In the WS tubers, the ranges of the sucrose and reducing sugar contents were found to be 1.31 ± 0.38 to 2.89 ± 0.26 mg g^{-1} FW and 0.06 ± 0.03 –0.82 ± 0.21mg g^{-1} FW, respectively. In some of the cold-stored transgenic tubers corresponding to the lines, namely SD-1, SD-4, SD-7, SD-9, SD-11 and SD-12, the sucrose and reducing sugar contents were reduced significantly as compared to the control. For sugar data see Fig. 24 and Tables 20-22 (Annexure-I).

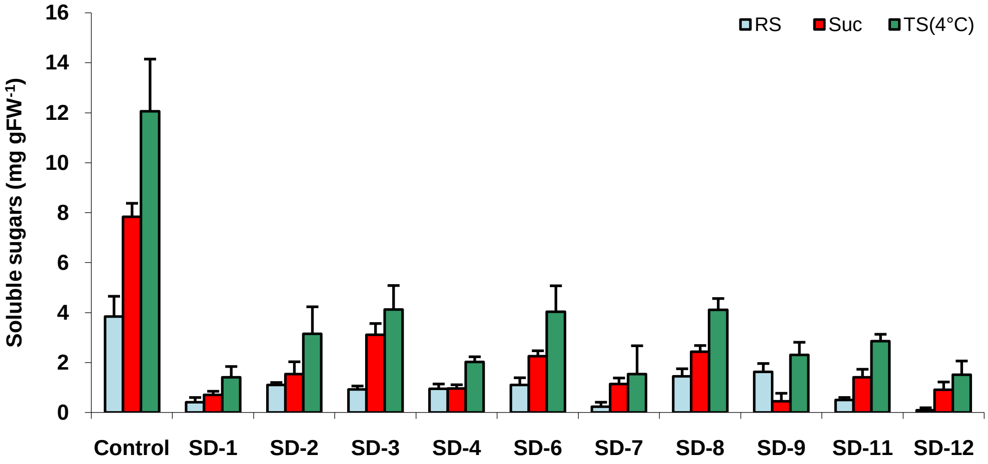

Fig. 24 Soluble sugar contents (reducing sugar, sucrose and total sugars) of SD construct-based 'cold-stored' transgenic tubers. Control refers to cv. Kufri Chipsona-1 (untransformed). The results are mean ± SD of 4 individual tubers.

SE construct-based transgenic tubers: In the FH tubers, sucrose contents were found ranging from 4.25 ± 0.28 to 8.01 ± 1.52 mg g^{-1} FW; the reducing sugar contents varied between 0.33 ± 0.14 and 1.23 ± 0.16 mg g^{-1} FW. In the CS tubers, sucrose contents were found in the range of 1.41 ± 0.15 to 3.52 ± 0.31 mg g^{-1} FW; whereas the values of reducing sugar contents were between 0.29 ± 0.02 to 2.02 ± 0.25mg g^{-1} FW. In the WS tubers, the ranges of the sucrose and reducing sugar contents were found to be 1.13 ± 0.21 to 2.50 ± 0.42 mg g^{-1} FW and 0.05 ± 0.02–0.31 ± 0.09 mg g^{-1} FW, respectively. In some of the cold-stored transgenic tubers corresponding to the lines, namely SE-3, SE-8, SE-9, SE-10, SE-14 and SE-15 the sucrose and reducing sugar contents were reduced significantly as compared to the control. For sugar data see Fig. 25 and Tables 23-25 (Annexure-I).

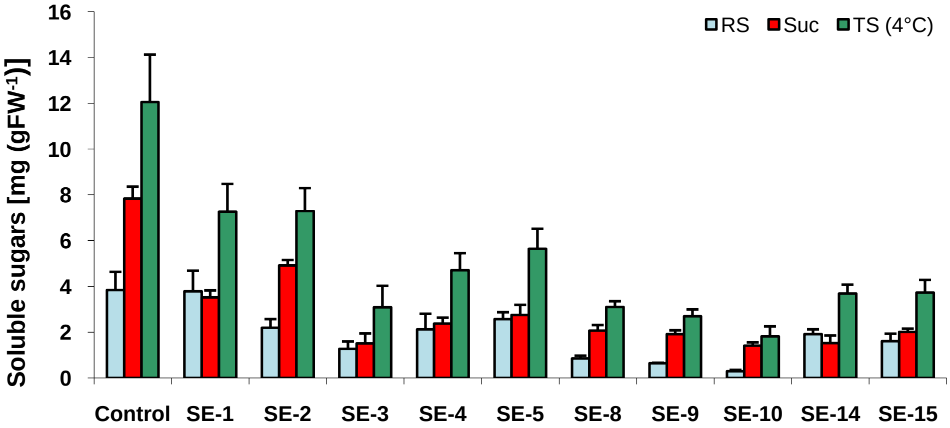

Fig. 25 Soluble sugar contents (reducing sugar, sucrose and total sugars) of SE construct-based 'cold-stored' transgenic tubers. Control refers to cv. Kufri Chipsona-1 (untransformed). The results are mean ± SD of 4 individual tubers.

SF construct-based transgenic tubers: In the FH tubers, sucrose contents were found ranging from 3.63 ± 0.38 to 5.96 ± 0.23 mg g^{-1} FW; the reducing sugar contents varied between 0.07 ± 0.03 and 0.76 ± 0.22 mg g^{-1} FW. In the CS tubers, sucrose contents were found in the range of 2.33 ± 0.137 to 4.87 ± 0.33 mg g^{-1} FW; whereas the values of reducing sugar contents were between 0.73 ± 0.37 to 2.50 ± 0.33 mg g^{-1} FW. In the WS tubers, the ranges of the sucrose and reducing sugar contents were found to be 1.14 ± 0.22 to 2.96 ± 0.26 mg g^{-1} FW and 0.07 ± 0.03–0.76 ± 0.22 mg g^{-1} FW, respectively. In some of the cold-stored transgenic tubers corresponding to the lines, namely SF-7, SF-14 and SF-19, the sucrose and reducing sugar contents were reduced significantly as compared to the control. For sugar data see Fig. 26 and Tables 26-28 (Annexure-I).

SG construct-based transgenic tubers: In the FH tubers, sucrose contents were found ranging from 1.18 ± 0.21 to 3.69 ± 0.43 mg g^{-1} FW; the reducing sugar contents varied between 0.12 ± 0.07 and 2.33 ± 0.79 mg g^{-1} FW. In the CS tubers, sucrose contents were found in the range of 2.07 ± 0.20 to 7.48 ± 1.21 mg g^{-1} FW; whereas the values of reducing sugar contents were between 0.34 ± 0.16 to 1.69 ± 0.41mg g^{-1} FW. In the WS tubers, the ranges of the sucrose and reducing sugar contents were found to be 0.98 ± 0.16 to 2.17 ± 0.19 mg g^{-1} FW and 0.05 ± 0.04–0.12 ± 0.06 mg g^{-1} FW, respectively. In some of the cold-stored

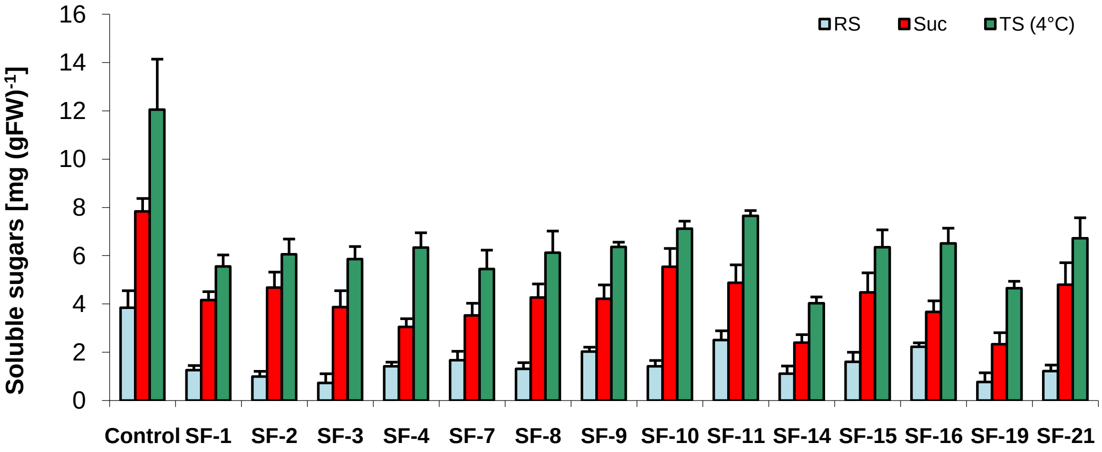

Fig. 26 Soluble sugar contents (reducing sugar, sucrose and total sugars) of SF construct-based 'cold-stored' transgenic tubers. Control refers to cv. Kufri Chipsona-1 (untransformed). The results are mean ± SD of 4 individual tubers.

transgenic tubers corresponding to the lines, namely SG-3, SG-4, SG-5, SG-6 and SG-10the sucrose and reducing sugar contents were reduced significantly as compared to the control. For sugar data see Fig. 27 and Tables 29-31 (Annexure-I).

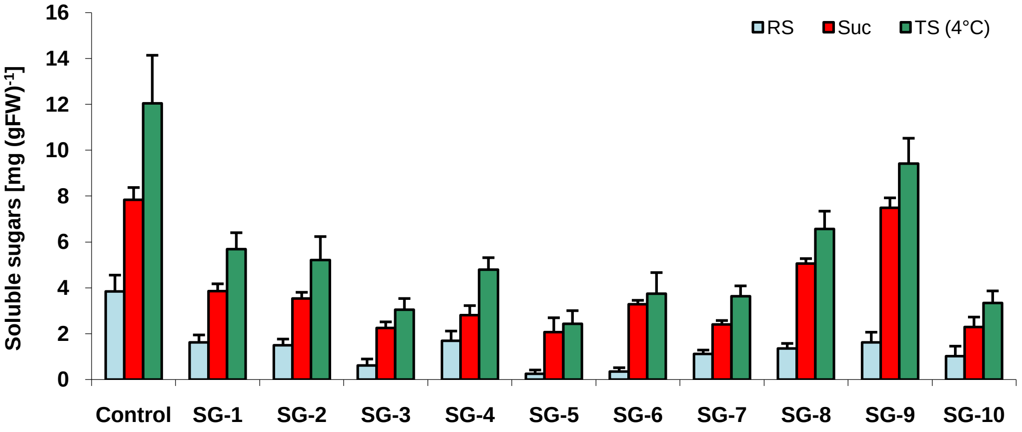

Fig. 27 Soluble sugar contents (reducing sugar, sucrose and total sugars) of SG construct-based 'cold-stored' transgenic tubers. Control refers to cv. Kufri Chipsona-1 (untransformed). The results are mean ± SD of 4 individual tubers.

SH construct-based transgenic tubers: In the FH tubers, sucrose contents were found ranging from 3.69 ± 0.28 to 6.64 ± 1.34 mg g^{-1} FW; the reducing sugar contents varied between 0.26 ± 0.06 and 0.62 ± 0.21 mg g^{-1} FW. In the CS tubers, sucrose contents were found in the range of 1.69 ± 0.41 to 3.75 ± 0.28 mg g^{-1} FW; whereas the values of reducing sugar contents were between 0.44 ± 0.06 to 2.66 ± 0.37 mg g^{-1} FW. In the WS tubers, the ranges of the sucrose and reducing sugar contents were found to be 1.02 ± 0.09 to 2.91 ± 0.18 mg g^{-1} FW and 0.12 ± 0.04–0.57 ± 0.25mg g^{-1} FW, respectively. In some of the cold-stored transgenic tubers corresponding to the lines, namely SH-6, SH-12, SH-16, SH-18 and SE-21, the sucrose and reducing sugar contents were reduced significantly as compared to the control. For sugar data see Fig. 28 and Tables 31-34 (Annexure-I).

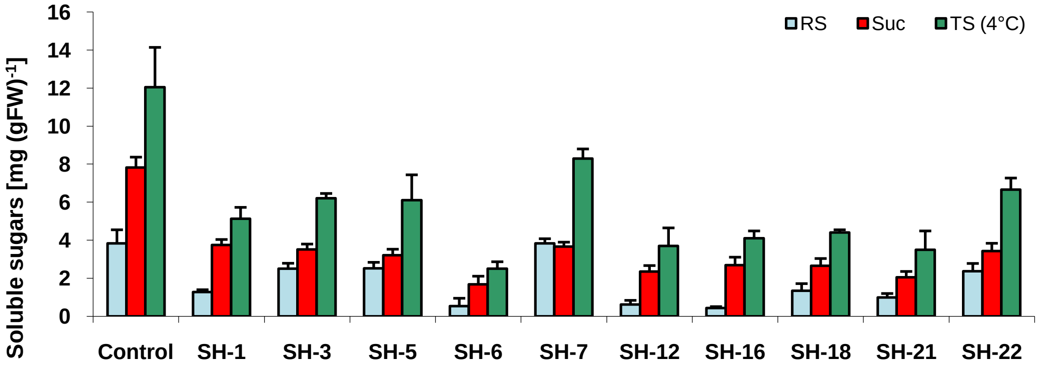

Fig. 28 Soluble sugar contents (reducing sugar, sucrose and total sugars) of SH construct-based 'cold-stored' transgenic tubers. Control refers to cv. Kufri Chipsona-1 (untransformed). The results are mean ± SD of 4 individual tubers.

4.4.3. *SPS assay in transgenic potato mini tubers*

As discussed in the previous section, major screening of the transgenic potato lines corresponding to the individual genetic constructs was based on the considerable lower accumulation of soluble sugars, reducing sugar and sucrose contents in particular, in the cold-stored tubers as compared to the control. This exercise helped in identifying some promising transgenic potato lines corresponding to each genetic construct of the study as described earlier. One of the major objectives was to inhibit SPS gene function through transgenics. Therefore, extractable SPS activities were determined in both control and transgenic mini tubers after storage at different experimental temperatures.

SPS activity is regulated by posttranslational modifications via phosphorylation, and allosteric control by the metabolic effectors, such as Glucose-6-phosphate (Glc-6-P) (activator) and inorganic phosphate (Pi) (inhibitor) (Huber and Huber 1996). Keeping in view, SPS activity was assayed under two different conditions: using a) saturating substrates in presence of the activator, Glc 6-P i.e. $V_{\max}$ assay; and b) limiting substrates with inorganic phosphate (Pi) as an inhibitor i.e. V_{sel} assay. All these assays were carried out according to the protocol described by Hill et al. (1996).

a) SPS assay in the control mini tubers

The maximum SPS activities i.e., $V_{\max}$ in the control tubers were found to be 507±53 (FH), 393±13 (WS) and 429±13 (CS) nmol $\text{min}^{-1}(\text{gFW})^{-1}$; whereas, V_{sel} activities were 53±7 (FH), 37±7 (WS) and 123±7 (CS) nmol $\text{min}^{-1}(\text{gFW})^{-1}$. The data clearly indicated that V_{sel} value of SPS was significantly higher in the CS tubers as compared to the WS tubers.

b) SPS assay in the transgenic mini tubers

Extractable SPS activities were also determined in some of the selected transgenic mini tubers corresponding to different genetic constructs after storage at different experimental temperatures, and presented in the Tables 3–10. Comparison with the control tubers clearly showed substantial inhibition of SPS activity in the transgenic tubers.

SA construct-based transgenic tubers: The maximum SPS activities ($V_{\max}$) in cold-stored transgenic potato tubers were in the range of 97 ± 8 to 121 ± 4 nmol $\text{min}^{-1}(\text{gFW})^{-1}$ whereas V_{sel} values were in the range of 30 ± 2 to 53 ± 2 nmol $\text{min}^{-1}(\text{gFW})^{-1}$. In warm-stored tubers it was 95 ± 2 to 123 ± 3 nmol $\text{min}^{-1}(\text{gFW})^{-1}$ and for V_{sel} values range was 27 ± 2 to 51 ± 3 nmol $\text{min}^{-1}(\text{gFW})^{-1}$. The SPS activities data are presented in Table 3.

Table 3 SPS activities [nmol $\text{min}^{-1}(\text{gFW})^{-1}$] in the tubers from SA-based transgenic potato lines and control tubers. Potato tubers were stored at 25ºC and 4ºC for 4 weeks

Transgenic potato lines	SPS activity [nmol $\text{min}^{-1}(\text{gFW})^{-1}$]			
	tubers stored at 25ºC		tubers stored at 4ºC	
	V_{max} ± SD	V_{sel} ± SD	V_{max} ± SD	V_{sel} ± SD
SA-1	97 ± 2 ef	27 ± 2 c	107 ± 9 def	33 ± 3 c
SA-3	115 ± 2 de	51 ± 3 bc	119 ± 8 d	53 ± 2 bc
SA-7	123 ± 3 d	33 ± 3 c	97 ± 8 ef	30 ± 2 c
SA-8	95 ± 2 ef	31 ± 4 c	121 ± 4 d	35 ± 4 c
Control	393 ± 13 a	37 ± 7 c	429 ± 13 a	123 ± 7 a

V_{max} values sharing a common letter are not significant at $P \leq 0.05$
V_{sel} values sharing a common letter are not significant at $P \leq 0.05$
Values are the mean ± SD of n =3 independent tubers

SB construct-based transgenic tubers: The maximum SPS activities (V_{max}) in cold-stored transgenic potato tubers were in the range of 101 ± 3 to 206 ± 6 nmol $\text{min}^{-1}(\text{gFW})^{-1}$ whereas V_{sel} values were in the range of 43 ± 3 to 57±4 nmol $\text{min}^{-1}(\text{gFW})^{-1}$. In warm-stored tubers V_{max} was in the range of 91 ± 2 to 167 ± 2 nmol $\text{min}^{-1}(\text{gFW})^{-1}$ and for V_{sel} values range was 39 ± 2 to 55 ± 3 nmol $\text{min}^{-1}(\text{gFW})^{-1}$. The SPS activities data are presented in Table 4

Table 4 SPS activities [nmol $\text{min}^{-1}(\text{gFW})^{-1}$] in the tubers from SB-based transgenic potato lines and control tubers Potato tubers were stored at 25ºC and 4ºC for 4 weeks

Transgenic potato lines	SPS activity [nmol $\text{min}^{-1}(\text{gFW})^{-1}$]			
	tubers stored at 25ºC		tubers stored at 4ºC	
	V_{max} ± SD	V_{sel} ± SD	V_{max} ± SD	V_{sel} ± SD
SB-3	127 ± 5 d	39 ± 2 c	157 ± 11 c	43 ± 3 c
SB-6	119 ± 2 d	55 ± 3 bc	159 ± 7 c	51 ± 3 bc
SB-7	167 ± 2 c	51 ± 4 bc	206 ± 6 b	57 ± 4 bc
SB-8	91 ± 2 ef	47 ± 2 c	101 ± 6 ef	53 ± 3 bc
Control	393 ± 13 a	37 ± 7 c	429 ± 13 a	123 ± 7 a

V_{max} values sharing a common letter are not significant at $P \leq 0.05$
V_{sel} values sharing a common letter are not significant at $P \leq 0.05$
Values are the mean ± SD of n =3 independent tubers

SC construct-based transgenic tubers: The maximum SPS activities (V_{max}) in cold-stored transgenic potato tubers were in the range of 100 ± 7 to 163 ± 7 nmol $\text{min}^{-1}(\text{gFW})^{-1}$ whereas V_{sel} values were in the range of 38 ± 3 to 48 ± 3 nmol $\text{min}^{-1}(\text{gFW})^{-1}$. In warm-stored tubers V_{max} was in the range of 85±5 to 106±7 and for V_{sel} values range was 27 ± 2 to 43 ± 4 nmol min^{-1}(gFW)$^{-1}$. The SPS activities data are presented in Table 5.

Table 5 SPS activities [nmol min^{-1}(gFW)$^{-1}$] in the tubers from SC-based transgenic potato lines and control tubers Potato tubers were stored at 25ºC and 4ºC for 4 weeks

Transgenic potato lines	SPS activity [nmol min^{-1}(gFW)$^{-1}$]			
	tubers stored at 25ºC		tubers stored at 4ºC	
	V_{max} ± SD	V_{sel} ± SD	V_{max} ± SD	V_{sel} ± SD
SC-1	94 ± 2 ef	27 ± 2 c	107 ± 5 def	38 ± 3 c
SC-3	82 ± 5 f	31 ± 3 c	100 ± 7 ef	39 ± 4 c
SC-9	97 ± 3 ef	43 ± 3 c	104 ± 6 def	48 ± 3 bc
SC-10	106 ± 7 def	43 ± 4 c	163 ± 7 c	40 ± 3 c
Control	393 ± 13 a	37 ± 7 c	429 ± 13 a	123 ± 7 a

V_{max} values sharing a common letter are not significant at $P \leq 0.05$
V_{sel} values sharing a common letter are not significant at $P \leq 0.05$
Values are the mean ± SD of n =3 independent tubers

SD construct-based transgenic tubers: The maximum SPS activities (V_{max}) in cold-stored transgenic potato tubers were in the range of 111 ± 13 to 161 ± 11 nmol min^{-1}(gFW)$^{-1}$ whereas V_{sel} values were in the range of 55 ± 7 to 67 ± 2 nmol min^{-1}(gFW)$^{-1}$. In warm-stored tubers V_{max} was in the range of 77 ± 8 to 120 ± 5 nmol min^{-1}(gFW)$^{-1}$ and for V_{sel} values range was 37 ± 2 to 53 ± 3 nmol min^{-1}(gFW)$^{-1}$. The SPS activities data are presented in Table 6.

Table 6 SPS activities [nmol min^{-1}(gFW)$^{-1}$] in the tubers from SD-based transgenic potato lines and control tubers Potato tubers were stored at 25ºC and 4ºC for 4 weeks

Transgenic potato lines	SPS activity [nmol min^{-1}(gFW)$^{-1}$]			
	tubers stored at 25ºC		tubers stored at 4ºC	
	V_{max} ± SD	V_{sel} ± SD	V_{max} ± SD	V_{sel} ± SD
SD-1	77 ± 8 f	37 ± 2 c	111 ± 13 de	55 ± 7 bc
SD-2	120 ± 5 d	53 ± 3 bc	161 ± 11 c	67 ± 9 b
SD-7	95 ± 5 ef	47 ± 3 bc	125 ± 9 d	61 ± 5 b
SD-12	106 ± 7 def	49 ± 3 bc	129 ± 10 d	67 ± 2 b
Control	393 ± 13 a	37 ± 7 c	429 ± 13 a	123 ± 7 a

V_{max} values sharing a common letter are not significant at $P \leq 0.05$
V_{sel} values sharing a common letter are not significant at $P \leq 0.05$
Values are the mean ± SD of n =3 independent tubers

SE construct-based transgenic tubers: The maximum SPS activities (V_{max}) in cold-stored transgenic potato tubers were in the range of 145 ± 7 to 286 ± 11 nmol min^{-1}(gFW)$^{-1}$ whereas V_{sel} values were in the range of 49 ± 3 to 87 ±5 nmol min^{-1}(gFW)$^{-1}$. In warm-stored tubers $V_{\max}$ was in the range of 115 ± 8 to 166 ± 7 nmol $\text{min}^{-1}(\text{gFW})^{-1}$ and for V_{sel} values range was 31 ± 2 to 59 ± 3 nmol $\text{min}^{-1}(\text{gFW})^{-1}$. The SPS activities data are presented in Table 7.

Table 7 SPS activities [nmol $\text{min}^{-1}(\text{gFW})^{-1}$] in the tubers from SE-based transgenic potato lines and control tubers Potato tubers were stored at 25ºC and 4ºC for 4 weeks

Transgenic potato lines	SPS activity [nmol $\text{min}^{-1}(\text{gFW})^{-1}$]			
	tubers stored at 25ºC		tubers stored at 4ºC	
	$V_{\max}$ ± SD	V_{sel} ± SD	$V_{\max}$ ± SD	V_{sel} ± SD
SE-8	166 ± 7 cd	59 ± 3 cd	286 ± 11 b	87 ± 5 b
SE-9	146 ± 5 de	31 ± 2 de	164 ± 9 cd	49 ± 3 cd
SE-10	115 ± 8 ef	33 ± 3 de	145 ± 7 de	51 ± 4 cd
SE-15	153 ± 5 de	47 ± 2 cd	183 ± 13 cd	67 ± 5 bc
Control	393 ± 13 a	37 ± 7 de	429 ± 13 a	123 ± 7 a

$V_{\max}$ values sharing a common letter are not significant at $P \leq 0.05$
V_{sel} values sharing a common letter are not significant at $P \leq 0.05$
Values are the mean ± SD of n =3 independent tubers

SF construct-based transgenic tubers: The maximum SPS activities ($V_{\max}$) in cold-stored transgenic potato tubers were in the range of 184 ± 9 to 225 ± 13 nmol $\text{min}^{-1}(\text{gFW})^{-1}$ whereas V_{sel} value were in the range of 53 ± 5 to 67 ± 3 nmol $\text{min}^{-1}(\text{gFW})^{-1}$. In warm-stored tubers $V_{\max}$ was in the range of 101 ± 5 to 207 ± 4 nmol $\text{min}^{-1}(\text{gFW})^{-1}$ and for V_{sel} values range was 21 ± 4 to 53 ± 3 nmol $\text{min}^{-1}(\text{gFW})^{-1}$. The SPS activities data are presented in Table 8.

Table 8 SPS activities [nmol $\text{min}^{-1}(\text{gFW})^{-1}$] in the tubers from SF-based transgenic potato lines and control tubers Potato tubers were stored at 25ºC and 4ºC for 4 weeks

Transgenic potato lines	SPS activity [nmol $\text{min}^{-1}(\text{gFW})^{-1}$]			
	tubers stored at 25ºC		tubers stored at 4ºC	
	$V_{\max}$ ± SD	V_{sel} ± SD	$V_{\max}$ ± SD	V_{sel} ± SD
SF-17	189 ± 5 cd	49 ± 3 cd	225 ± 13 c	57 ± 4 cd
SF-19	153 ± 3 de	35 ± 2 de	208 ± 11 c	53 ± 5 cd
SF-11	101 ± 5 ef	21 ± 4 e	184 ± 9 cd	67 ± 3 bc
SF-15	207 ± 4 c	53 ± 3 cd	215 ± 7 c	65 ± 4 bc
Control	393 ± 13 a	37 ± 7 de	429 ± 13 a	123 ± 7 a

$V_{\max}$ values sharing a common letter are not significant at $P \leq 0.05$
V_{sel} values sharing a common letter are not significant at $P \leq 0.05$
Values are the mean ± SD of n =3 independent tubers

SG construct-based transgenic tubers: The maximum SPS activities ($V_{\max}$) in cold-stored transgenic potato tubers were in the range of 112 ± 7 to 222 ± 13 nmol $\text{min}^{-1}(\text{gFW})^{-1}$ whereas V_{sel} values were in the range of 45 ± 3 to 65 ± 5 nmol $\text{min}^{-1}(\text{gFW})^{-1}$. In warm-stored tubers V_{max} was in the range of 91 ± 7 to 197 ± 7 nmol $min^{-1}(gFW)^{-1}$ and for V_{sel} values range was 35 ± 2 to 49 ± 6 nmol $min^{-1}(gFW)^{-1}$. The SPS activities data are presented in Table 9.

Table 9 SPS activities [nmol $min^{-1}(gFW)^{-1}$] in the tubers from SG-based transgenic potato lines and control tubers Potato tubers were stored at 25ºC and 4ºC for 4 weeks

Transgenic potato lines	SPS activity [nmol $min^{-1}(gFW)^{-1}$]			
	tubers stored at 25ºC		tubers stored at 4ºC	
	V_{max} ± SD	V_{sel} ± SD	V_{max} ± SD	V_{sel} ± SD
SG-1	176 ± 5 cd	49 ± 6 cd	222 ± 13 c	46 ± 3 cd
SG-2	197 ± 7 cd	41 ± 5 de	151 ± 9 de	65 ± 5 bc
SG-4	98 ± 4 ef	37 ± 3 de	116 ± 11 ef	45 ± 3 cd
SG-7	91 ± 7 ef	35 ± 2 de	112 ± 7 f	48 ± 4 cd
Control	393 ± 13 a	37 ± 7 de	429 ± 13 a	123 ± 7 a

V_{max} values sharing a common letter are not significant at $P \leq 0.05$
V_{sel} values sharing a common letter are not significant at $P \leq 0.05$
Values are the mean ± SD of n =3 independent tubers

SH construct-based transgenic tubers: The maximum SPS activities (V_{max}) in cold-stored transgenic potato tubers were in the range of 135 ± 13 to 194 ± 9 nmol $min^{-1}(gFW)^{-1}$ whereas V_{sel} values were in the range of 51 ± 3 to 77 ± 5 nmol $min^{-1}(gFW)^{-1}$. In warm-stored tubers V_{max} was in the range of 87 ± 4 to 137 ± 5 nmol $min^{-1}(gFW)^{-1}$ and for V_{sel} values range was 33 ± 2 to 53 ± 3 nmol $min^{-1}(gFW)^{-1}$. The SPS activities data are presented in Table 10.

Table 10 SPS activities [nmol $min^{-1}(gFW)^{-1}$] in the tubers from SH-based transgenic potato lines and control tubers Potato tubers were stored at 25ºC and 4ºC for 4 weeks

Transgenic potato lines	SPS activity [nmol $min^{-1}(gFW)^{-1}$]			
	tubers stored at 25ºC		tubers stored at 4ºC	
	V_{max} ± SD	V_{sel} ± SD	V_{max} ± SD	V_{sel} ± SD
SH-6	87 ± 4 ef	53 ± 3 cd	135 ± 13 de	67 ± 5 bc
SH-12	137 ± 5 de	39 ± 2 de	185 ± 11 cd	51 ± 3 cd
SH-16	116 ± 3 ef	51 ± 3 cd	194 ± 9 cd	65 ± 4 bc
SH-21	103 ± 5 ef	33 ± 2 de	192 ± 11 cd	77 ± 5 b
Control	393 ± 13 a	37 ± 7 de	429 ± 13 a	123 ± 7 a

V_{max} values sharing a common letter are not significant at $P \leq 0.05$
V_{sel} values sharing a common letter are not significant at $P \leq 0.05$
Values are the mean ± SD of n =3 independent tubers

4.4.4. *Expression studies of transgenic lines through semi-quantitative RT-PCR*

To investigate whether the observed SPS activities during cold storage reflected accordingly the level of steady-state mRNA, total RNA was isolated from control and transgenic tubers SPS. SPS transcript levels were analyzed in some of the selected transgenic tubers through semi-quantitative RT-PCR. The data as shown in Fig. 29 indicated very low accumulation of SPS transcript in some of the cold-stored transgenic tubers as compared to the control suggesting inhibition of SPS gene function probably at transcriptional level and/or rapid turnover of SPS mRNA. For internal control, actin gene-specific primers were used.

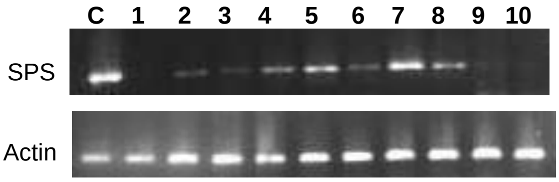

Fig. 29 Semi-quantitative RT-PCR for SPS expression analysis in the transgenic tubers using the primers D20-SPS and F20-SPS. The size of the SPS-specific amplified product was found to be ~1.3 kb in each case. Actin-specific primers were used as control (the size of the amplified product ~0.25 kb). Lane C, KC-1 as control and Lanes 1 to 10 refer to different transgenic lines namely SA-1, SA-8, SC-1, SC-3, SE-10, SG-2, SF-15, SH-21, SD-7, SB-8, respectively.

4.4.5. *Protein gel blot analyses*

Protein gel blot analyses were carried out to know the expression patterns of SPS in the protein extracts of transgenic potato tubers using KC-SPS1 specific polyclonal antibodies and IgG-HRP conjugated secondary antibody using DAB system. In the control tuber extract, a protein band of ~120 kDa was detected as expected; whereas, in the cases of transgenic tuber extracts, the immuno-detected band was found to be very faint or negligible suggesting significant inhibition of SPS gene expression in the cold-stored tubers (Fig. 30A-C).

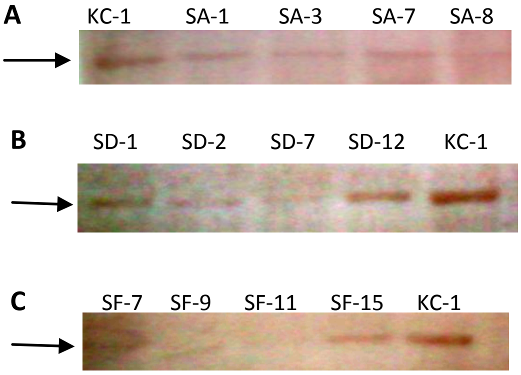

Fig. **30** Protein blot analyses. Lane KC-1 in each panel refers to cold-stored control potato tuber. Other lanes in **A**, **B** and **C** correspond to different cold-stored transgenic potato tubers as specified previously.

4.4.6. *Discussion on transgenics*

Cold-induced sweetening negatively affects the processing attributes of the potato tubers during cold storage. Increased sucrose accumulation in the cold-stored tubers is usually correlated with higher SPS activities as evident in the published reports. SPS along with other proteins/enzymes such as acid invertase, SPP are involved in hexogenesis, and considered to play important roles for this undesirable phenomenon. In order to overcome the problem of cold-induced sweetening in the potato tubers, transgenic approaches were adopted by some laboratories to inhibit the above-mentioned enzymes. Earlier, antisense and sense inhibition of SPS gene function was achieved in the reference cultivar Desiree with varying degree of success (for details, see section 2.6). With regard to the Indian potato cultivars no such efforts were made. In this context, this study is relevant because of the following reasons: i) a number of antisense/sense binary genetic constructs were made under both constitutive and tuber-specific promoters, ii) instead of using full-length SPS cDNA of the study, partial cDNAs (towards both 5′- and 3′- termini) were employed in the above construct, and importantly iii) a commercially important Indian processing potato cultivar namely Kufri Chipsona-1 was used in *Agrobacterium*-mediated genetic transformation. Although a number of transgenic potato lines were generated with regard to each binary genetic construct, cold-stored tubers from some of the lines showed considerably reduced accumulation of both sucrose and reducing sugars as compared with control. It was apparent that both antisense and sense constructs remained effective.

A number of transgenic tubers were identified which showed significant ($P<0.05$) lower accumulation of sucrose and reducing sugars during storage at 4^0C for 4 weeks as compared to the control tubers. Although the ranges of both sucrose and reducing data were mentioned earlier, the sugar data of some promising transgenic potato lines are highlighted here: Sucrose level (cold-stored transgenic tubers): SA-16 (1.65 ± 0.19), SA-11 (1.58 ± 0.49), SB-6 (1.14 ± 0.22), SB-3 (1.94 ± 0.20), SC-1 (2.01 ± 0.63), SC-10 (1.73 ± 0.84), SD-1 (0.71 ± 0.13), SD-11 (1.41 ± 0.31), SE-3 (1.51 ± 0.44), SE-10 (1.41 ± 0.15), SF-14 (2.39 ± 0.31), SF-19 (2.33 ± 0.37), SG-3 (2.25 ± 0.27), SG-5 (2.07 ± 0.20), SH-6 (1.69 ± 0.41) and SH-21 (2.06 ± 0.29); reducing sugar level in the corresponding tubers were 0.97 ± 0.13, 0.84 ± 0.21, 0.95 ± 0.20, 0.56 ± 0.14, 1.46 ± 0.38, 0.75 ± 0.07, 0.41 ± 0.18, 0.50 ± 0.11, 1.27 ± 0.33, 0.29 ± 0.15, 1.10 ± 0.65, 0.77 ± 0.11, 0.61 ± 0.25, 0.56 ± 0.16, 0.53 ± 0.41 and 0.99 ± 0.02, respectively. All these numerical values have the common unit i.e., ‘mg g^{-1} FW’. Some transgenic tubers almost behaved like control tubers (data not shown). This could be due to inefficient antisense/sense expression.

SPS activity is regulated by posttranslational modifications via phosphorylation, and allosteric control by the metabolic effectors, such as glucose-6-phosphate (Glc-6-P) (activator) and inorganic phosphate (Pi) (inhibitor) (Huber and Huber 1996). Therefore, SPS activity was measured under two different conditions, a) saturating substrates (V_{max}) and b) limiting substrates with inhibitor, Pi (V_{sel}). In warm-stored untransformed (control) tubers, V_{max} activity was 393 ± 13 nmol $\mathrm{min}^{-1}(\mathrm{gFW})^{-1}$ and its value was 429 ± 13 nmol $\mathrm{min}^{-1}(\mathrm{gFW})^{-1}$ in cold-stored tubers. As reported in earlier studies (Hill et al. 1996; Krause et al. 1998), V_{max} activity did not change significantly with temperature difference whereas the V_{sel} activity was approximately 3-fold increased in cold-stored tubers (123 ± 7 nmol $\mathrm{min}^{-1}(\mathrm{gFW})^{-1}$) as compared to warm-stored tubers (37±3 nmol $\mathrm{min}^{-1}(\mathrm{gFW})^{-1}$). SPS activity was increased 3-4 fold even in the presence of limiting substrates due to alteration in the kinetic properties of SPS. The V_{max} values of SPS in the cold-stored transgenic tubers corresponding to the SA, SB, SC and SD genetic constructs were found to be in the range of 97 ± 8 to 206 ± 6 nmol min^{-1} $(\mathrm{gFW})^{-1}$, and the V_{max} values were in the range of 87 ± 4 to 207 ± 4 nmol min^{-1} $(\mathrm{gFW})^{-1}$ for SE, SF, SG and SH genetic constructs. The V_{max} values in these cold-stored transgenic tubers clearly indicated significant inhibition of SPS activity, and the V_{sel} activities were also reduced in these tubers as compared to control.

Potato contains multiple SPS forms, SPS-1a (125 kDa), SPS-1b (127 kDa), SPS-2 (135 kDa) & SPS-3 (145 kDa), detected in immunoblots using polyclonal antibodies generated against SPS peptide from amino acid sequence, protein_id Q43845, from cv. Desiree (Reimholz et al. 1997). The cold-induced change in the kinetic properties of SPS was accompanied by the appearance of a new form of SPS with a slightly higher apparent molecular weight, and by an increase of the SPS transcript. In warm-stored tubers SPS-1a is the major form, however cold conditions selectively increased the expression of SPS-1b. Antisense and sense transgenic lines generated by using SPS cDNA (SPS-P4, X73477) showed reduced level of both SPS-1a and SPS-1b (Krause et al. 1998). In fact, both these forms could be the products of the same gene or highly homologous genes. However, they belong to the same SPS family, i.e. SPS A family. In this study, antibodies were raised against a synthetic peptide from SPS-C1 (ACD50895) detected a band ~120 kDa, which was close to SPS-1a and SPS-1b. Protein blot analysis detected either very faint/negligible band indicating significantly lower accumulation of SPS in the transformed tubers. Semi-quantitative RT-PCR data clearly indicated the inhibition of SPS A form at the level of transcription. Therefore, the data suggested that decreased SPS activity in the transformed tubers could be due to inhibition of SPS gene function both at the levels of transcription and translation.

Based on sugar, SPS expression and activity data, the following observations were made:

The level of soluble sugars (both sucrose and reducing sugars) was reduced by 50–80% in both antisense/sense transgenic tubers as compared to control in most of the cases. In cold-stored tubers of the promising transformed lines, the level of SPS expression was significantly reduced as supported by semi-quantitative RT-PCR and protein blot analyses. In the cold-stored SA construct-based transformed tubers, 70–80% decreased SPS activity resulted in 15–30% sucrose content when compared with control. Likewise, 50–75% decreased activity resulted in 15–50% sucrose content corresponding to SB construct. 60–75% decreased activity led to 10–28% sucrose content for SC construct, and 60–75% reduced SPS activity resulted in 5–40% sucrose content for SD construct. In cold-stored tubers of SE construct-based transformed lines 35–65% decreased SPS activity resulted in 20–45% sucrose content; 50–60% reduced SPS activity resulted in 30–60% sucrose content for SF construct. In case of SG construct, 50–75% reduced SPS activity resulted in 25–95% sucrose content; in SH construct, 55–70% decreased activity resulted in 20–45% sucrose content. Results indicate that there was varying degree of SPS inhibition in different transformed tubers. The underlying molecular mechanisms need to be understood categorically. The significant decrease of sucrose content in the cold-stored transformed tubers was also accompanied with concomitant decrease of the level of reducing sugar content -a common trend noted in most of the promising transformed tubers. Therefore, it is very likely that cold-induced accumulation of sucrose at least partially explain the extent of soluble sugar (both reducing and nonreducing) accumulation during cold storage of potato tubers. Antisense expression under the tuber-specific GBSSI promoter appeared to inhibit the SPS gene function slightly more effectively as compared to the CaMV 35S promoter. Moreover, data clearly suggested that the different regions of SPS cDNA could be effective in inhibiting the respective gene function. However, further in-depth studies are required for understanding the precise molecular mechanisms of such inhibition.

In conclusion, the entire study covering molecular cloning of a cDNA to generation of transgenic potato plant represent both basic and applied aspects of research on SPS, particularly in the *Solanaceae* family. With respect to the Indian potato cultivar, this study is quite important and relevant. This is an important step forward for improving a processing potato variety in our country.

Summary of the thesis work

- Based on RT-PCR approach, a cDNA encoding a distinct form of full-length SPS was isolated and characterized using the tuber RNA from the potato cultivar Kufri Chipsona-1 (a processing variety). The nucleotide sequence of the cDNA clone (3591 bp, designated as SPS-C1) was analyzed by NCBI BLAST tool, and the predicted SPS was found to represent SPS A form. The sequence information was submitted to the NCBI database (GenBank ID: EU684230; protein_id ACD50895). This constitutes the first report from the Indian potato cultivar. The deduced amino acid sequences were analyzed, and compared with their homologs from different plant species.
- Multiple sequence alignment was carried out using the SPS sequences from the *Solanaceae* family members belonging to the different SPS gene families. The purpose was to examine carefully the sequence similarities and the variations in the regulatory/ binding motifs between them.
- Segment-wise hydrophobic characters and secondary structures were predicted and compared between the different SPS forms in the *Solanaceae* family.
- For understanding both sequence/evolutionary relatedness, full-length SPS sequences from a number of taxonomically different plant species were used for generating a phylogenetic tree.
- As a preliminary study, the overall expression patterns of SPS was analyzed in the freshly-harvested and cold-stored tubers from some of the Indian potato cultivars using semi-quantitative RT-PCR, protein blot analyses, and assaying total extractable SPS activities (V_{max} and V_{sel}) under different reaction conditions.
- In order to inhibit SPS gene function, a series of antisense and sense binary genetic constructs were made. For this purpose, different regions of the SPS cDNA as isolated in this study were cloned under both the constitutive and tuber-specific promoters as described below.
- A 1283-bp fragment towards the 5′-terminus and 1155-bp fragment towards the 3′-terminus of the full-length SPS cDNA i.e., SPS-C1 were first cloned in pUC19 vector. For directional cloning, the individual cloned inserts in pUC19 were isolated by restriction digestion(s), and placed under the constitutive CaMV 35S promoter in pBI121 and the tuber-specific GBSSI promoter in pAN-GB01 by replacing the existing

GUS gene. The orientation and intactness of the inserts in individual genetic constructs were further checked through restriction digestion, and PCR using specific primers.

- A total of eight binary genetic constructs were made. Genetic constructs based on SPS cDNA towards 5'-end designated as SA, SB (antisense and sense under the CaMV 35S promoter); SC, SD (antisense and sense under the GBSS1 promoter). Likewise, based on SPS cDNA towards 3'-end designated as SE, SF (antisense and sense under the CaMV 35S promoter); SG, SH (antisense and sense under the GBSS1 promoter).
- *Agrobacterium tumefaciens* strain, LBA4404 was transformed with the individual genetic constructs through the following procedures: electroporation and/or triparental mating. The respective *Agrobacterium* transformants were selected in a medium containing streptomycin (50 µg/mL) and kanamycin (50 µg/mL).
- The internodal stem segments of the micropropagated potato plantlets (cv. Kufri Chipsona-1) were co-cultivated with the individual *Agrobacterium* transformants using the protocol as described by Beaujean et al. (1998) and Bansal et al. (2012).
- The regenerated shoots were further transferred to the rooting media to obtain complete plantlets. The kanamycin resistant plantlets with regard to each genetic construct were further screened by using various molecular techniques.
- For initial screening, total DNA was isolated from the transgenic potato plantlets, and PCR was carried out using different combinations of primers corresponding to the CaMV 35S/GBSSI promoter and SPS cDNA fragment as present in the respective genetic construct. PCR amplification occurred in most of the cases which indicated the stable integration of transgene(s) into the potato genome.
- The transgenic potato plantlets after hardening and acclimatization were grown in polybags in the restricted experimental plots for mini tuber production. Mature potato tubers were harvested after 90–100 d. Phenotypically, the overall plant morphology, tuber size and yield were not distinguishable if compared between the transgenic and control plants. The potato tubers immediately after harvest, referred to as ‘freshly-harvested tubers’, were initially stored at room temperature for three weeks, and then transferred to 4^0C and 25^0C for the next four weeks, termed as ‘cold-stored’ and ‘warm-stored’ potato tubers, respectively.

- PCR approach was adopted for checking the presence, intactness, and orientation of the transgene (s) in the transformed mini tubers. For this purpose, promoter-specific forward and gene-specific reverse primers were used during PCR analyses. As expected, in most of the cases, the presence of transgene was detected by this approach.
- For primary screening for the desired phenotypes (i.e. low sugar accumulation during cold storage), soluble sugars (both reducing and nonreducing sugars) were extracted from 'freshly-harvested (FH)', 'cold-stored (CS)' and 'warm-stored (WS)' transgenic and control tubers. 'mg g^{-1} FW' (FW means fresh weight) was used as unit in calculation of sugar contents.
- The potato cultivar, Kufri Chipsona-1 was used as a control in the study. *S*ugar data of the control tubers after storage at different experimental temperatures were determined for comparison with the transformed tubers. In the FH tubers, the sucrose and reducing sugar contents were 4.52 ± 0.59 mg g^{-1} FW and 0.69 ± 0.24 mg g^{-1} FW, respectively. In the CS tubers, the sucrose and reducing sugar contents were 7.83 ± 0.53 mg g^{-1} FW and 3.84 ± 0.08 mg g^{-1} FW, respectively; whereas, the respective values for the WS tubers were 2.02 ± 0.33 mg g^{-1} FW and 1.22 ± 0.05 mg g^{-1} FW. The data clearly indicated that sucrose level was increased significantly in the cold-stored tubers. Storage at low temperature led to nearly 6-fold increase of reducing sugar as compared to freshly harvested tubers. Therefore, cold-induced sweetening appeared to be quite prominent even in the processing potato cultivar.
- A number of transgenic tubers were identified which showed significant ($P<0.05$) lower accumulation of sucrose and reducing sugars during storage at 4^0C for 4 weeks as compared to the control tubers. Although the ranges of both sucrose and reducing data were mentioned earlier, the sugar data of some promising transgenic potato lines are highlighted here: Sucrose level (cold-stored transgenic tubers): SA-16 (1.65 ± 0.19), SA-11 (1.58 ± 0.49), SB-6 (1.14 ± 0.22), SB-3 (1.94 ± 0.20), SC-1 (2.01 ± 0.63), SC-10 (1.73 ± 0.84), SD-1 (0.71 ± 0.13), SD-11 (1.41 ± 0.31), SE-3 (1.51 ± 0.44), SE-10 (1.41 ± 0.15), SF-14 (2.39 ± 0.31), SF-19 (2.33 ± 0.37), SG-3 (2.25 ± 0.27), SG-5 (2.07 ± 0.20), SH-6 (1.69 ± 0.41) and SH-21 (2.06 ± 0.29); reducing sugar level in the corresponding tubers were 0.97 ± 0.13, 0.84 ± 0.21, 0.95 ± 0.20, 0.56 ± 0.14, 1.46 ± 0.38, 0.75 ± 0.07, 0.41 ± 0.18, 0.50 ± 0.11, 1.27 ± 0.33, 0.29 ± 0.15, 1.10 ± 0.65, 0.77 ± 0.11, 0.61 ± 0.25, 0.56 ± 0.16, 0.53 ± 0.41 and 0.99 ± 0.02, respectively.

- SPS activity was measured under two different conditions, a) saturating substrates (V_{max}) and b) limiting substrates with inhibitor, Pi (V_{sel}). In warm-stored untransformed (control) tubers, V_{max} activity was 393 ± 13 nmol $min^{-1}(gFW)^{-1}$ and its value was 429 ± 13 nmol $min^{-1}(gFW)^{-1}$ in cold-stored tubers. V_{max} activity did not change significantly with temperature difference whereas the V_{sel} activity was approximately 3-fold increased in cold-stored tubers (123 ± 7 nmol $min^{-1}(gFW)^{-1}$) as compared to warm-stored tubers (37±3 nmol $min^{-1}(gFW)^{-1}$). SPS activity was increased 3-4 fold even in the presence of limiting substrates due to alteration in the kinetic properties of SPS. The V_{max} values of SPS in the cold-stored transgenic tubers corresponding to the SA, SB, SC and SD genetic constructs were found to be in the range of 97 ± 8 to 206 ± 6 nmol min^{-1} $(gFW)^{-1}$, and the V_{max} values were in the range of 87 ± 4 to 207 ± 4 nmol min^{-1} $(gFW)^{-1}$ for SE, SF, SG and SH genetic constructs. The V_{max} values in these cold-stored transgenic tubers clearly indicated significant inhibition of SPS activity, and the V_{sel} activities were also reduced in these tubers as compared to control.

- SPS transcript levels were analyzed in some of the selected transgenic tubers through semi-quantitative RT-PCR. Semi-quantitative RT-PCR data revealed very low accumulation SPS transcript in some of the cold-stored transgenic tubers as compared to the control indicating inhibition of SPS gene function probably at transcriptional level and/or rapid turnover of SPS mRNA. For internal control, actin gene-specific primers were used.
- Protein gel blot analyses were carried out to know the expression patterns of SPS in the protein extracts of transgenic potato tubers using KC-SPS1 specific polyclonal antibody and and IgG-HRP conjugated secondary antibody using DAB system. In the control tuber extract a protein band of ~120 kDa was detected as expected; whereas, in the cases of transgenic tuber extracts, the immuno-detected band was found to be very faint or negligible suggesting significant inhibition of SPS gene expression in the cold-stored tubers.

- This thesis work dealt with both basic and applied aspects of research on SPS, particularly in the *Solanaceae* family. Moreover, it is relevant with respect to the Indian potato cultivars. For improvement of the potato crop, various molecular/transgenic approaches as adopted in the study would be quite useful.

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Annexure-I

Table 11 Sucrose (mg g^{-1} FW) in SA-based transgenic lines

Transgenic Lines of SA	Sucrose (mg g^{-1} FW)				
	Freshly harvested	25°C one month	25°C two months	4°C one month	4°C two months
SA-1	2.18 ± 1.11 de	2.06 ± 0.23 b	1.96 ± 0.36 ab	2.22 ± 0.33 b	0.31 ± 0.01 d
SA2	1.60 ± 0.71 e	1.23 ± 0.18 b	2.40 ± 0.77 ab	1.14 ± 0.35 b	0.71 ± 0.11 bcd
SA-3	6.28 ± 0.82 bc	1.28 ± 0.53 b	2.12 ± 0.68 ab	3.03 ± 0.84 b	1.71 ± 0.22 bc
SA-7	8.76 ± 1.49 ab	1.96 ± 0.44 b	4.60 ± 0.68 ab	2.31 ± 0.61 b	0.56 ± 0.06 bcd
SA-8	7.79 ± 0.48 ab	1.22 ± 0.29 b	1.84 ± 0.48 ab	1.82 ± 0.35 b	1.88 ± 0.05 b
SA-11	5.28 ± 0.82 bcd	2.06 ± 0.39 b	5.06 ± 1.08 a	1.58 ± 0.49 b	0.22 ± 0.02 cd
SA-16	8.82 ± 1.01 ab	2.03 ± 0.46 b	4.84 ± 0.91 ab	1.65 ± 0.19 b	1.15 ± 0.22 bcd
SA-17	8.01 ± 1.16 ab	2.39 ± 0.77 b	4.46 ± 0.86 ab	2.45 ± 0.62 b	1.59 ± 0.51 bcd
SA-18	6.03 ± 0.61 bc	1.33 ± 0.60 b	2.43 ± 0.74 ab	1.95 ± 0.14 b	1.18 ± 0.07 bcd
SA-19	10.79± 1.54 a	5.21 ± 0.51 a	3.90 ± 1.23 ab	2.13 ± 0.38 b	1.48 ± 0.38 bcd
Control	4.52 ± 0.59 cde	2.02 ± 0.33 b	3.41 ± 0.28 ab	7.83 ± 0.53 a	11.81 ±1.16 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n=4 independent tubers

Table 12 Reducing sugars (mg g^{-1} FW) in SA-based transgenic lines

Transgenic Lines of SA	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25°C one month	25°C two months	4°C one month	4°C two months
SA-1	1.61 ± 0.39 ab	0.78 ± 0.09 b	0.08 ± 0.07 b	1.77 ± 0.16 cd	1.52 ± 0.4 cde
SA-2	0.23 ± 0.15 c	0.37 ± 0.29 c	0.07 ± 0.06 b	1.21 ± 0.12 def	2.48 ± 0.39 c
SA-3	2.14 ± 0.81a	0.10 ± 0.12 cd	0.09 ± 0.07 b	1.39 ± 0.38 de	0.09 ± 0.04 f
SA-7	0.23 ± 0.12 c	0.12 ± 0.04 cd	0.10 ± 0.05 b	2.37 ± 0.70 bc	0.98 ± 0.11 def
SA-8	0.33 ± 0.16 c	0.14 ± 0.09 cd	0.07 ± 0.06 b	1.13 ± 0.29 def	4.04 ±1.53 b
SA-11	0.66 ± 0.52 bc	0.11 ± 0.05 cd	0.18 ± 0.11 b	0.84 ± 0.21 ef	4.78 ± 296 b
SA-16	0.73 ± 0.48 bc	0.03 ± 0.01 d	0.04 ± 0.04 b	0.97 ± 0.13 ef	0.38 ± 0.13 ef
SA-17	0.34 ± 0.22 c	0.22 ± 0.12 cd	0.06 ± 0.06 b	0.92 ± 0.12 ef	1.15 ± 0.52 def
SA-18	0.21 ± 0.18 c	0.05 ± 0.04 d	0.05 ± 0.03 b	1.54 ± 0.39 de	2.21 ± 0.22 cd
SA-19	0.52 ± 0.43 c	0.05 ± 0.01 d	0.09 ± 0.03 b	0.55 ± 0.30 f	1.42 ± 0.32 def
Control	0.69 ± 0.24 bc	1.22 ± 0.05 a	1.59 ± 0.09 a	3.84 ± 0.08 a	6.97 ± 1.56 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 13 Total soluble sugars (mg g^{-1} FW) in SA-based lines

Transgenic Lines of SA	Total sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SA-1	4.58 ± 0.81 bc	3.01± 0.61 bc	2.29 ± 0.72 c	4.23 ± 0.80 b	1.56 ± 0.30 b
SA-2	2.33 ± 0.86 c	1.73 ± 0.51 cd	3.02 ± 0.5 bc	2.60 ± 0.21 b	3.27 ± 0.47 b
SA-3	9.01 ± 4.52 ab	1.76 ± 0.72 cd	2.70 ± 1.07 c	4.70 ± 3.11 b	1.96 ± 0.20 b
SA-7	10.04 ± 1.78 ab	2.4 ± 0.38 bcd	5.19 ± 0.99 ab	5.11 ± 0.39 b	1.59 ± 0.21 b
SA-8	8.81 ± 2.28 ab	1.57 ± 0.09 d	2.25 ± 0.54 c	3.16 ± 0.59 b	5.96 ± 3.15 b
SA-11	6.52 ± .86 bc	2.45 ± 0.28 bcd	6.01 ± 1.42 a	2.77 ± 0.40 b	5.02 ± 1.36 b
SA-16	10.27 ± 2.92 ab	2.39 ± 0.24 bcd	5.53 ± 1.64 a	2.76 ± 0.44 b	1.69 ± 0.88 b
SA-17	8.47 ± 1.51 ab	3.16 ± 0.05 b	5.14 ± 0.54 ab	3.80 ± 0.79 b	3.11 ± 1.61 b
SA-18	6.67 ± 3.05 bc	1.81 ± 0.08 cd	3.01 ± 0.57 bc	3.59 ± 0.87 b	3.44 ± 0.53 b
SA-19	12.4 ± 3.12 a	5.63 ± 0.60 a	4.95 ± 0.66 ab	2.95 ± 0.30 b	3.17 ± 0.45 b
Control	5.29 ± 0.33 bc	3.48 ± 1.07 b	4.20 ± 0.52 abc	12.05 ± 2.08 a	18.86 ± 7.17 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 14 Sucrose (mg g^{-1} FW) in SB-based transgenic lines

Transgenic Lines of SB	Sucrose (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SB-1	3.55 ± 0.46 cde	1.04 ± 0.22 bc	5.16 ± 0.38 de	2.85± 0.82 bc	1.97 ± 0.14 bc
SB-2	3.92 ± 0.12 cde	0.43 ± 0.27 c	6.13 ± 1.34 cd	1.20 ± 0.28 cd	2.04 ± 0.36 bc
SB-3	3.33 ± 0.29 de	0.31 ± 0.26 c	10.9 ± 1.56 b	1.94 ± 0.20 cd	2.14 ± 0.18 bc
SB-4	4.87 ± 0.31 abc	0.71 ± 0.38 c	4.31 ± 0.39 de	1.73 ± 0.30 cd	1.05 ± 0.12 c
SB-5	2.60 ± 0.52 ef	0.66 ± 0.34 c	2.75 ± 0.35 e	1.65 ± 0.91 cd	2.18 ± 0.24 bc
SB-6	3.60 ± 0.38 cde	1.11 ± 0.17 bc	8.21 ± 1.45 bc	1.14 ± 0.22 cd	3.23 ± 0.28 b
SB-7	6.09 ± 1.28 a	1.41 ± 0.61 bc	19.86± 2.06 a	4.04 ± 0.24 b	2.92 ± 0.22 b
SB-8	4.12 ± 1.32 bcd	2.28 ± 0.23 ab	5.78 ± 0.31 cd	1.47 ± 0.38 cd	3.01 ± 0.23 b
SB-9	5.04 ± 0.39 ab	3.29 ± 0.21 a	5.51 ± 0.28 cde	2.56 ± 0.37 bc	1.83 ± 0.23 bc
SB-10	1.47 ± 0.38 f	3.07 ± 0.25 a	8.23 ± 0.27 bc	3.22 ± 0.17 b	3.47 ± 0.34 b
Control	4.52 ± 0.59 bcd	2.02 ± 0.33 ab	3.41 ± 0.28 de	7.83 ± 0.53 a	11.06±1.17 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 15 Reducing sugars (mg g^{-1} FW) in SB-based transgenic lines

Transgenic Lines of SB	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SB-1	4.46 ± 2.91 a	0.40 ± 0.08 e	0.71 ± 0.14 b	0.83 ± 0.01 ef	6.70 ± 0.43 a
SB-2	0.83 ± 0.49 d	2.16 ± 0.23 a	0.19 ± 0.16 c	0.53 ± 0.14 f	5.82 ± 0.24 ab
SB-3	0.90 ± 0.85 d	1.46 ± 0.07 bc	0.19 ± 0.08 c	0.56 ± 0.14 f	2.19 ± 0.35 cd
SB-4	2.85 ± 1.36 b	1.53 ± 0.34 bc	0.15 ± 0.12 c	0.81 ± 0.16 ef	2.74 ± 0.13 c
SB-5	0.36 ± 0.08 d	0.34 ± 0.16 e	0.05 ± 0.04 c	1.64 ± 0.21 b	0.97 ± 0.13 de
SB-6	1.42 ± 0.42 c	1.71 ± 0.26 b	0.21 ± 0.19 c	0.95 ± 0.92 ef	5.19 ±1.13 b
SB-7	1.46 ± 0.67 c	0.50 ± 0.17 e	0.14 ± 0.16 c	1.25 ±0.87 def	0.16 ± 0.05 e
SB-8	1.31 ± 0.25 c	0.30 ± 0.05 e	0.16 ± 0.17 c	0.48 ± 0.02 f	2.79 ± 0.04 c
SB-9	0.84 ± 0.60 d	0.94 ± 0.07 d	0.14 ± 0.14 c	1.19 ± 0.24 def	1.59 ±1.19 cde
SB-10	1.14 ±1.12 c	0.34 ± 0.09 e	0.26 ± 0.19 c	1.43 ± 0.32 cdef	0.93 ± 0.40 de
Control	0.69 ± 0.24 b	1.22 ± 0.05 cd	1.59 ± 0.09 a	3.84 ± 0.07 a	6.97 ±1.56 a

Values sharing a common letter within the column are not significant at P ≤ 0.05. Values are the mean ± SD of n =4 independent tubers

Table 16 Total soluble sugars (mg g^{-1} FW) in SB-based transgenic lines

Transgenic Lines of Dh2-5	Total soluble sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SB-1	8.34 ± 0.25 a	1.60 ± 0.54 cd	6.15 ± 0.69 cd	3.02 ± 0.75 bcd	8.77 ± 0.27 b
SB-2	4.84 ± 0.84 abc	2.78 ± 0.33 bc	6.57 ± 0.26 cd	1.83 ± 0.75 d	8.12 ± 0.58 b
SB-3	4.44 ± 2.04 bc	1.95 ± 0.18 cd	11.49 ±1.01 b	2.26 ± 0.53 cd	4.46 ± 0.84 b
SB-4	7.94 ±1.35 ab	2.51 ± 0.64 bcd	4.74 ± 0.34 de	2.66 ± 1.05 cd	3.85 ± 0.26 b
SB-5	3.33 ± 0.10 c	1.25 ± 0.15 d	3.05 ± 0.28 e	3.93 ± 0.49 bcd	3.33 ± 0.14 b
SB-6	5.29 ± 0.66 abc	2.94 ±0.63 bc	8.74 ± 2.49 bc	2.25 ± 1.95 bcd	8.63 ± 0.98 b
SB-7	7.75 ± 1.04 ab	2.35 ± 0.65 bcd	21.46 ± 2.07 a	5.46 ± 2.92 b	3.24 ± 0.20 b
SB-8	5.66 ±1.28 abc	2.75 ± 0.14 bc	6.17 ± 0.69 cd	2.22 ± 0.98 bcd	5.97 ± 0.52 b
SB-9	6.16 ± 1.66 abc	4.38 ± 0.81 a	5.85 ± 0.92 cde	4.02 ± 0.37 bcd	3.59 ± 2.26 b
SB-10	2.88 ± 2.70 c	3.59 ± 0.24 ab	8.69 ± 1.90 bc	4.78 ± 0.83 bc	4.65 ± 0.50 b
Control	5.29 ± 1.33 abc	3.48 ± 1.11 ab	4.20 ± 0.52 de	12.05 ±2.08 a	18.86± 7.10 a

Values sharing a common letter within the column are not significant at P ≤ 0.05. Values are the mean ± SD of n =4 independent tubers

Table 17 Sucrose (mg g^{-1} FW) in SC- based transgenic lines

Transgenic Lines of SC	Sucrose (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SC-1	3.93 ± 0.71 bc	1.41 ± 0.23 a	0.65 ± 0.21 cd	2.01 ± 0.63bcd	3.02 ± 0.59 c
SC-2	2.66 ± 1.01 c	2.48 ± 0.52 a	2.03 ± 0.40 b	2.06 ± 0.23 bcd	4.27 ± 0.67 c
SC-3	6.64 ± 0.73 a	1.34 ± 0.17 a	0.48 ± 0.06 cd	2.47 ± 0.22 bc	2.85 ± 0.41 c
SC-4	3.56 ± 0.62 bc	2.23 ± 0.28 a	2.23 ± 0.19 b	2.11 ± 0.15 bc	4.28 ± 0.68 c
SC-5	2.77 ± 0.91 c	1.57 ± 0.51 a	0.77 ± 0.29 cd	4.01 ± 0.89 b	5.25 ± 0.74 c
SC-6	2.41 ± 0.74 c	2.30 ± 0.41 a	0.72 ± 0.08 cd	2.19 ± 0.25 bc	5.14 ± 0.48 c
SC-7	3.76 ± 0.61 bc	1.31 ± 0.39 a	0.35 ± 0.07 d	2.12 ± 0.18 ef	7.93 ± 1.15 b
SC-8	1.77 ± 0.63 c	1.53 ± 0.56 a	1.01 ± 0.35 c	2.62 ± 0.13 bc	2.88 ± 0.68 c
SC-9	3.65 ± 0.47 bc	1.64 ±0.11 a	0.89 ± 0.11 cd	2.56 ± 0.33 bc	4.22 ± 0.63 c
SC-10	2.41 ± 0.58 c	1.69 ± 0.33 a	0.36 ± 0.11 d	1.73 ± 0.84 cd	4.10 ± 0.63 c
Control	4.52 ± 0.59 b	2.02 ± 0.33 a	3.41 ± 0.28 a	7.83 ± 0.53 a	11.06 ±1.17 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 18 Reducing sugars (mg g^{-1} FW) in SC-based transgenic lines

Transgenic Lines of SC	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SC-1	0.67 ± 0.68 ab	0.21 ± 0.04 bc	0.11 ± 0.06 b	1.46 ± 0.38 bc	1.41 ± 0.06 cd
SC-2	0.38 ± 0.40 ab	0.17 ± 0.05 bc	0.09 ± 0.08 b	1.65 ± 0.46 b	2.10 ± 0.20 cd
SC-3	0.99 ± 0.86 ab	0.08 ± 0.06 bc	0.14 ± 0.05 b	1.14 ± 0.48 bc	1.53 ± 0.33 cd
SC-4	0.42 ± 0.47 ab	1.40 ± 0.22 a	0.09 ± 0.04 b	1.78 ± 0.46 b	1.36 ± 0.32 cd
SC-5	1.65 ± 1.62 ab	0.11 ± 0.20 b	0.15 ± 0.03 b	0.31 ± 0.04 c	4.31 ± 0.39 b
SC-6	0.09 ± 0.10 ab	0.07 ± 0.02 bc	0.14 ± 0.04 b	0.71 ± 0.05 c	3.22 ± 0.40 bc
SC-7	0.58 ± 0.25 ab	0.09 ± 0.04 bc	0.22 ± 0.08 b	0.73 ± 0.09 c	7.06 ± 2.01 a
SC-8	0.03 ± 0.08 b	0.03 ± 0.02 c	0.11 ± 0.05 b	0.77 ± 0.18 c	0.26 ± 0.17 d
SC-9	2.25 ± 2.06 a	0.24 ± 0.04 b	0.17 ± 0.05 b	0.59 ± 0.28 c	0.91 ± 0.27 d
SC-10	0.35 ± 0.14 ab	0.13 ± 0.05 bc	0.21 ± 0.02 b	0.75 ± 0.07 c	1.64 ± 0.34 cd
Control	0.69 ± 0.24 ab	1.22 ± 0.05 a	1.59 ± 0.09 a	3.84 ± 1.20 a	6.97 ± 1.56 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 19 Total soluble sugars (mg g^{-1} FW) in SC-based transgenic lines

Transgenic Lines of SC	Total soluble sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SC-1	5.13 ± 2.98 ab	1.78 ± 0.36 c	0.91 ± 0.50 c	3.52 ± 0.53 b	4.79 ± 0.64 c
SC-2	3.45 ± 0.09 b	3.02 ± 0.39 abc	2.15 ± 0.58 b	3.88 ± 1.25 b	6.85 ± 2.69 c
SC-3	8.15 ± 3.67 a	1.55 ± 0.12 c	0.77 ± 0.12 c	3.77 ± 0.56 b	4.67 ± 0.80 c
SC-4	4.43 ± 1.58 ab	3.83 ± 1.03 a	2.46 ± 0.33 b	4.00 ± 0.81 b	6.13 ± 1.84 c
SC-5	5.06 ± 2.53 ab	2.04 ± 0.28 bc	1.13 ± 0.32 c	4.69 ± 1.43 b	10.09 ± 2.06 bc
SC-6	3.03 ± 0.72 b	2.66 ± 0.27 abc	0.92 ± 0.17 c	3.08 ± 0.28 b	8.70 ± 0.21 c
SC-7	4.78 ± 0.26 ab	1.67 ± 0.43 c	0.62 ± 0.15 c	2.92 ± 0.46 b	15.81± 2.70 ab
SC-8	2.25 ± 0.08 b	1.96 ± 0.92 bc	1.15 ± 0.13 c	3.49 ± 0.43 b	3.63 ± 0.57 c
SC-9	6.24 ± 2.16 ab	1.96 ± 0.72 bc	1.14 ± 0.13 c	3.39 ±0.41 b	5.58 ± 1.07 c
SC-10	3.17 ± 0.30 b	2.06 ± 0.41 bc	0.65 ± 0.37 c	2.54 ± 0.56 b	6.19 ± 1.57 c
Control	5.29 ± 1.33 ab	3.48 ± 1.07 ab	4.20 ± 0.52 a	12.05 ± 2.08 a	18.93 ± 7.17 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 20 Sucrose (mg g^{-1} FW) in SD-based transgenic lines

Transgenic Lines of SD	Sucrose (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SD-1	9.24 ± 1.86 a	3.34 ± 1.30 a	3.37 ± 0.36 de	0.71 ± 0.13 c	0.68 ± 0.36 c
SD-2	6.72 ± 1.60 b	2.41 ± 0.57 ab	2.38 ± 0.37 e	1.54 ± 0.48 c	4.52 ± 0.45 b
SD-3	3.05 ± 0.32 e	2.44 ± 0.44 ab	3.47 ± 0.68 de	3.11 ± 0.33 b	0.11 ± 0.01 c
SD-4	3.76 ± 0.39 e	1.31 ± 0.38 b	3.36 ± 0.29 de	0.96 ± 0.14 c	1.03 ± 0.19 c
SD-6	7.55 ± 1.68 ab	2.89 ± 0.26 a	2.41 ±1.44 e	2.25 ± 0.21 c	0.25 ± 0.18 c
SD-7	3.24 ± 0.35 e	3.09 ± 0.42 a	19.12 ±1.02 a	1.14 ± 0.23 c	1.33 ± 0.46 c
SD-8	6.11 ± 0.31 bc	2.01 ± 0.48 ab	6.94 ± 0.19 b	2.43 ± 0.24 c	0.33 ± 0.18 c
SD-9	5.82 ± 0.68 bcd	1.97 ± 0.45 ab	6.02 ± 1.18 bc	0.45 ± 0.31 c	1.22 ± 0.26 c
SD-11	4.23 ± 0.53 cde	2.37 ± 0.39 ab	4.23 ± 1.20 cde	1.41 ± 0.31 c	1.57 ± 0.48 c
SD-12	3.86 ± 0.29 de	2.56 ± 0.57 ab	4.53 ± 0.47 cd	0.91 ± 0.30 b	0.53 ± 0.32 c
Control	4.52 ± 0.59 cde	2.02 ± 0.33 ab	3.41 ± 0.28 de	7.83 ± 0.53 a	11.06±1.17 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 21 Reducing sugars (mg g^{-1} FW) in SD-based transgenic lines

Transgenic Lines of SD	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SD-1	0.42 ± 0.20 ab	0.02 ± 0.02 c	0.05 ± 0.03 d	0.41 ± 0.18 cd	2.60 ± 0.41 cd
SD-2	1.10 ± 1.13 ab	0.58 ± 0.28 b	0.35 ± 0.14 bc	1.10 ± 0.28 b	0.25 ± 0.15 e
SD-3	0.36 ± 0.15 ab	0.03 ± 0.02 c	0.13 ± 0.09 cd	0.93 ± 0.12 c	5.19 ± 0.76 ab
SD-4	0.38 ± 0.35 ab	0.82 ± 0.21 b	0.35 ± 0.10 bc	0.95 ± 0.18 c	0.75 ±0.18 de
SD-6	1.21 ±1.03 ab	0.77 ± 0.06 b	0.66 ± 0.28 b	1.11 ± 0.27 b	4.63 ±1.16 ab
SD-7	0.17 ± 0.15 ab	0.06 ± 0.03 c	0.10 ± 0.09 cd	0.23 ± 0.17 cd	0.32 ± 0.21 e
SD-8	0.23 ± 0.19 ab	0.05 ± 0.01 c	0.09 ± 0.03 cd	1.45 ± 0.29 b	4.99 ±1.43 ab
SD-9	1.34 ± 0.10 a	0.04 ± 0.01 c	0.06 ± 0.04 d	1.63 ± 0.32 b	1.45 ± 0.09 de
SD-11	0.14 ± 0.08 b	0.04 ± 0.03 c	0.04 ± 0.02 d	0.50 ± 0.11 ef	2.26 ± 0.29 bc
SD-12	0.08 ± 0.04 b	0.66 ± 0.05 b	0.13 ± 0.08 cd	0.09 ± 0.02 d	3.72 ± 0.83 bc
Control	0.69 ± 0.24 ab	1.22 ± 0.06 a	1.59 ± 0.09 a	3.84 ± 0.07 a	6.97 ±1.56 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 22 Total soluble sugars (mg g^{-1} FW) in SD-based transgenic lines

Transgenic Lines of SD	Total soluble sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SD-1	10.28 ± 2.24 a	3.60 ± 0.64 a	3.68 ± 0.61 b	1.41 ± 0.42 c	3.54 ± 0.44 b
SD-2	8.25 ± 2.64 ab	3.40 ± 1.33 a	3.00 ± 0.79 b	3.15 ± 1.07 bc	5.09 ± 0.59 b
SD-3	3.64 ± 0.55 e	2.79 ± 0.17 a	3.87 ± 0.55 b	4.13 ± 0.94 b	5.30 ± 0.30 b
SD-4	4.42 ± 0.66 cde	2.40 ± 0.06 a	3.88 ± 0.63 b	2.02 ± 0.20 c	1.92 ± 0.93 b
SD-6	9.19 ± 2.46 ab	3.85 ± 2.15 a	3.28 ± 0.77 b	4.04 ± 1.02 b	5.01 ± 2.50 b
SD-7	3.66 ± 0.85 de	3.45 ± 0.41 a	20.24 ± 3.08 a	1.54 ± 1.12 c	1.98 ± 0.73 b
SD-8	6.57 ± 2.53 bcde	2.20 ± 0.12 a	7.18 ± 2.76 b	4.11 ± 0.44 b	5.45 ± 0.72 b
SD-9	7.65 ± 1.12 bcd	2.16 ± 0.72 a	6.22 ± 3.40 b	2.31 ± 0.49 bc	2.86 ± 0.59 b
SD-11	4.75 ± 1.46 cde	2.69 ± 1.03 a	4.40 ± 0.69 b	2.86 ± 0.26 bc	4.18 ± 0.60 b
SD-12	4.15 ± 0.57 de	3.54 ± 0.88 a	5.00 ± 0.68 b	1.51 ± 0.54 c	4.48 ± 0.44 b
Control	5.29 ± 1.33 bcde	3.67 ± 1.07 a	4.20 ± 0.52 b	12.05 ± 2.08 a	18.86 ±7.17 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 23 Sucrose (mg g^{-1} FW) in SE-based transgenic lines

Transgenic Lines of SE	Sucrose (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SE-1	7.01 ± 1.76 bcd	0.81 ± 0.23 d	1.91 ± 0.23 e	3.52 ± 0.31 b	2.27 ± 0.21 ef
SE-2	5.41 ± 0.49 ed	2.12 ± 0.19abcd	2.07 ± 0.38 de	4.91 ± 0.38 b	3.21 ± 0.25 de
SE-3	5.51 ± 0.54 cde	1.25 ± 0.28 bcd	4.63 ± 1.56 ab	1.51 ± 0.44 cd	0.13 ± 0.03 f
SE-4	4.25 ± 0.28 e	1.58 ± 0.49 bcd	5.27 ± 0.48 a	2.38 ± 0.26 bcd	1.61 ± 0.43 ef
SE-5	7.12 ± 1.65 bcd	1.13 ± 0.21cd	4.55 ± 1.61 ab	2.75 ± 0.45 bc	0.65 ± 0.22 f
SE-8	8.01 ± 1.52 b	2.24 ± 0.29 abc	3.88 ± 0.49 abc	2.07 ± 0.25 cd	7.41 ± 0.38 b
SE-9	6.14 ± 1.42 bcd	2.50 ± 0.42 ab	3.78 ± 1.38 abcd	1.92 ± 0.17 cd	5.76 ± 0.44 bc
SE-10	10.15 ± 2.85 a	1.58 ± 0.42 bcd	3.25 ± 0.31bcde	1.41 ± 0.15 d	5.08 ± 0.45 bcd
SE-14	6.09 ± 0.46 bcd	1.51 ± 0.39 bcd	2.29 ± 0.34 cde	1.53 ± 0.33 cd	3.78 ± 0.61 cde
SE-15	7.46 ± 0.58 bc	3.01 ± 0.16 a	4.66 ± 0.61 ab	2.01 ± 0.15 bcd	10.78 ±1.39 a
Control	4.52 ± 0.59 e	2.02 ± 0.33 abcd	3.41 ± 0.28 bcde	7.83 ± 0.53 a	11.02 ±1.12 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 24 Reducing sugars (mg g^{-1} FW) in SE-based transgenic lines

Transgenic Lines of SE	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SE-1	0.11 ± 0.05 b	0.05 ± 0.03 c	0.38 ± 0.08 bc	3.79 ± 0.90 b	1.72 ± 0.19 de
SE-2	1.23 ± 0.16 b	0.28 ± 0.06 b	0.48 ± 0.08 b	2.20 ± 0.38 bc	1.81 ± 0.13 de
SE-3	0.49 ± 0.12 b	0.31 ± 0.09 b	0.38 ± 0.15 bc	1.27 ± 0.33 def	3.82 ±1.62 bc
SE-4	4.28 ± 3.33 a	0.08 ± 0.04 c	0.33 ± 0.1 bc	2.13 ± 0.68 cd	6.09 ± 0.61 ab
SE-5	0.97 ± 0.42 b	0.28 ± 0.07 b	0.21 ± 0.04 c	2.57 ± 0.31 bc	3.69 ±1.28 cd
SE-8	0.52 ± 0.03 b	0.03 ± 0.02 c	0.49 ± 0.09 b	0.85 ± 0.13 efg	1.54 ± 0.47 de
SE-9	0.45 ± 0.21 b	0.05 ± 0.05 c	0.42 ± 0.13 bc	0.65 ± 0.17 fg	2.58 ± 0.55 cde
SE-10	0.33 ± 0.14 b	0.03 ± 0.02 c	0.34 ± 0.06 bc	0.29 ± 0.15 g	2.19 ± 0.2 cde
SE-14	0.52 ± 0.19 b	0.05 ± 0.02 c	0.50 ± 0.06 bc	1.92 ± 0.33 cd	2.33 ± 0.55 cde
SE-15	0.51 ± 0.07 b	0.04 ± 0.04 c	0.44 ± 0.04 b	1.61 ± 0.15 de	1.64 ± 0.68 de
Control	0.69 ± 0.24 b	1.22 ± 0.06 a	1.59 ± 0.09 a	3.84 ± 0.07 a	6.79 ± 1.56 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 25 Total sugars (mg g^{-1} FW) in SE-based transgenic lines

Transgenic Lines of SE	Total soluble sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SE-1	7.24 ±1.68 ab	1.03 ± 0.11 c	2.45 ± 0.18 e	7.26 ± 2.57 b	4.14 ± 0.66 c
SE-2	6.93 ± 0.54 ab	2.54 ± 0.41 abc	2.82 ± 0.04 de	7.29 ± 2.00 b	5.20 ± 0.63 c
SE-3	6.38 ± 2.12 ab	1.76 ± 0.90 bc	5.41 ± 0.76 ab	3.09 ± 0.94 cd	3.97 ± 1.35 c
SE-4	8.74 ± 3.91 ab	2.01 ± 0.94 abc	5.94 ± 0.86 a	4.70 ± 0.76 bcd	8.00 ± 0.55 bc
SE-5	8.55 ±1.83 ab	1.56 ± 0.37 bc	5.20 ±1.09 ab	5.64 ± 0.88 bc	4.50 ± 0.59 c
SE-8	8.90 ± 2.00 ab	2.48 ± 0.71 abc	4.72 ± 1.11 abc	3.10 ± 0.26 cd	9.22 ± 0.86 bc
SE-9	6.89 ± 1.33 ab	2.85 ± 0.31 ab	4.47 ± 0.77 abcd	2.70 ± 0.30 cd	8.66 ± 3.16 bc
SE-10	11.09± 3.30 a	1.91 ± 0.20 abc	3.82 ± 0.88 bcde	1.81 ± 0.45 d	7.59 ±1.52 bc
SE-14	6.94 ± 1.36 ab	1.83 ± 0.29 bc	3.03 ± 0.56 cde	3.69 ± 0.39 cd	6.54 ± 2.12 c
SE-15	8.39 ±1.07 ab	3.16 ± 0.92 ab	5.53 ± 0.92 ab	3.73 ± 0.12 cd	13.41 ±1.27 ab
Control	5.29 ± 1.33 b	3.48 ±1.11 a	4.20 ± 0.52 abc	12.05± 2.08 a	18.61 ±6.79 a

Values sharing a common letter within the column are not significant at P ≤ 0.05. Values are the mean ± SD of n =4 independent tubers

Table 26 Sucrose (mg g^{-1} FW) in SF-based transgenic lines

Transgenic Lines of SF	Sucrose (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SF-1	4.47 ± 0.34 de	2.05 ± 0.23 bcde	2.27 ± 0.37 abcd	4.16 ± 0.34 b	6.63 ± 0.48 b
SF-2	5.99 ± 1.27 a	2.96 ± 0.26 ab	2.54 ± 0.48 abc	4.67 ± 0.64 b	6.93 ± 1.49 b
SF-3	5.41 ± 0.31abcd	1.47 ± 0.25 cde	1.34 ± 0.43 cd	3.87 ± 0.67 bc	8.01 ± 0.51 b
SF-4	4.59 ± 1.37 cde	2.36 ± 0.34 abcd	1.34 ± 0.19 cd	3.05 ± 0.50 a	6.79 ± 1.64 b
SF-7	3.63 ± 0.38 e	1.20 ± 0.24 de	1.12 ± 0.16 d	3.52 ± 0.37 b	5.47 ± 1.22 b
SF-8	4.42 ± 1.26 de	2.50 ± 0.28 abc	2.91 ± 0.16 ab	4.27± 0.55 a	6.03 ± 0.55 b
SF-9	3.92 ± 0.25 e	2.40 ± 0.38 abc	2.51 ± 0.29 abc	4.21 ± 0.57 b	7.01 ± 1.54 b
SF-10	4.63 ± 1.45bcde	3.34 ± 0.34 a	2.24 ± 0.31 abc	5.53 ± 0.76 ab	6.56 ± 0.35 b
SF-11	5.44 ± 0.39abcd	1.14 ± 0.22 e	2.01 ± 0.21 bcd	4.87 ± 0.38 b	6.25 ± 0.79 b
SF-14	4.51 ± 0.26 cde	1.44 ± 0.19 cde	1.51 ± 0.38 cd	2.39 ± 0.31 bcd	4.97 ±1.01 b
SF-15	5.84 ± 1.25 abc	2.13 ± 0.16 bcde	2.60 ± 0.43 abc	4.47 ± 0.39 b	6.14 ± 2.69 b
SF-16	5.66 ± 0.19 abc	2.95 ± 0.18 ab	3.02 ± 0.22 ab	3.67 ± 0.45 bcd	5.40 ± 0.40 b
SF-19	5.96 ± 0.23 a	2.45 ± 0.49 abc	2.48 ± 0.48 abc	2.33 ± 0.37 bcd	6.72 ± 0.58 b
SF-21	4.50 ± 0.38 cde	2.94 ± 0.26 ab	2.36 ± 0.32abcd	4.80 ± 0.90 b	6.07 ± 1.71 b
Control	4.52 ± 0.59 cde	2.02 ± 0.33 bcde	3.41 ± 0.28 a	7.83 ± 0.53 a	11.06±1.17 a

Values sharing a common letter within the column are not significant at P ≤ 0.05. Values are the mean ± SD of n =4 independent tubers

Table 27 Reducing sugars (mg g^{-1} FW) in SF-based transgenic lines

Transgenic Lines of SF	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25°C one month	25°C two months	4°C one month	4°C two months
SF-1	0.39 ± 0.02 abc	0.09 ± 0.01bc	0.11 ± 0.03 de	1.26 ± 0.18bc	1.25 ± 0.18 cd
SF-2	0.02 ± 0.02 c	0.02 ± 0.01c	0.26 ± 0.02 cd	0.99 ± 0.21 d	1.29 ± 0.27 cd
SF-3	0.28 ± 0.07 bc	0.04 ± 0.04 bc	0.56 ± 0.02 b	0.73 ± 0.37 d	2.20 ± 0.14 c
SF-4	0.62 ± 0.31 ab	0.32 ± 0.07 bc	0.63 ± 0.01 b	1.42 ± 0.16bc	1.96 ± 0.11 c
SF-7	0.39 ± 0.18 abc	0.08 ± 0.05 bc	0.19 ± 0.11 cde	1.66 ± 0.81 bc	1.45 ± 0.31 cd
SF-8	0.04 ± 0.02 c	0.11 ± 0.07 bc	0.09 ± 0.02 de	1.31 ± 0.25bc	2.01 ± 0.16 c
SF-9	0.76 ± 0.22 a	0.29 ± 0.12 bc	0.23 ± 0.01 cd	2.02 ± 0.18 bc	2.17± 0.23 c
SF-10	0.41 ± 0.03 abc	0.26 ± 0.26 bc	0.09 ± 0.02 de	1.42 ± 0.23 bc	1.58 ± 0.25 cd
SF-11	0.08 ± 0.06 c	0.05 ± 0.01 bc	0.28 ± 0.01 c	2.50 ± 0.33 b	2.55 ± 0.35 bc
SF-14	0.45 ± 0.06 abc	0.07 ± 0.05 bc	0.09 ± 0.05 de	1.10 ± 0.65 bc	1.53 ± 0.31 cd
SF-15	0.09 ± 0.01 c	0.08 ± 0.05 bc	0.19 ± 0.01 cde	1.60 ± 1.30 bc	0.32 ± 0.15 d
SF-16	0.05 ± 0.01 c	0.42 ± 0.16 b	0.05 ± 0.01 e	2.22 ± 0.16 b	3.80 ± 0.28 b
SF-19	0.07 ± 0.03 c	0.08 ± 0.01 bc	0.13 ± 0.05 cde	0.77 ±0.55c	2.04 ± 0.21 c
SF-21	0.02 ± 0.01 c	0.10 ± 0.04 bc	0.16 ± 0.07 cde	1.21 ± 0.25 bc	2.51 ± 0.28 bc
Control	0.69 ± 0.24 ab	1.22 ± 0.05 a	1.59 ± 0.09 a	3.84 ± 0.07 a	6.97 ±1.56 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 28 Total soluble sugars (mg g^{-1} FW) in SF-based transgenic lines

Transgenic Lines of SF	Total soluble sugars (mg g^{-1} FW)				
	Freshly harvested	25°C one month	25°C two months	4°C one month	4°C two months
SF-1	5.10 ± 0.82 a	2.31 ± 0.56 ab	2.65 ± 0.71 bc	5.55 ± 0.47 bc	8.01 ± 1.42 bcd
SF-2	6.21 ± 1.48 a	3.17 ± 0.23 ab	3.15 ± 0.18 ab	6.05 ± 0.63 bc	8.42 ± 1.31 bcd
SF-3	5.91 ± 1.91 a	1.69 ± 0.44 ab	2.21 ± 0.14 bcd	5.85 ± 0.52 bc	10.31 ± 2.3 b
SF-4	5.48 ± 0.63 a	2.92 ± 0.38 ab	2.11 ± 0.13 bcd	6.33 ± 0.61 b	8.83 ± 0.63 bcd
SF-7	4.30 ± 1.83 b	1.45 ± 0.49 b	1.43 ± 0.14 d	5.45 ± 0.77 e	7.15 ± 0.35 cd
SF-8	4.65 ± 0.65 a	2.81 ± 0.54 ab	3.12 ± 0.50 ab	6.12 ± 0.89 bc	8.15 ± 1.71 bcd
SF-9	4.86 ± 1.88 a	2.96 ±1.20 ab	2.95 ± 0.73 bc	6.36 ± 1.19 de	9.35 ± 1.23 bc
SF-10	5.36 ± 1.13 a	3.84 ± 0.59 a	2.55 ± 0.82 bcd	7.11 ± 0.31 cd	8.32 ± 1.41 bcd
SF-11	5.75 ± 0.69 a	1.35 ± 0.35 b	2.45 ± 0.79 bcd	7.65 ± 0.21 bc	9.05 ± 1.69 bcd
SF-14	5.15 ± 2.12 a	1.65 ± 0.63 b	1.87 ± 0.31 cd	4.02 ± 0.26 b	6.72 ± 0.63 d
SF-15	6.11 ± 2.68 a	2.33 ± 0.91bab	3.10 ± 0.37 ab	6.35 ± 0.71 de	6.57 ± 0.31 d
SF-16	5.85 ± 2.47 a	3.53 ± 0.56 a	3.23 ± 0.42 ab	6.50 ± 0.63 bc	9.40 ± 0.96 bc
SF-19	6.20 ± 1.97 a	2.88 ± 0.86 ab	2.96 ± 0.71 bc	4.65 ±0.28 bc	8.91 ± 0.37 bcd
SF-21	4.80 ± 1.17 a	3.23 ± 0.31 ab	2.75 ± 0.63 bc	6.72 ± 0.84 bc	8.78 ±1.24 bcd
Control	5.29 ± 1.33 a	3.48 ± 1.11 a	4.20 ± 0.55 a	12.05 ± 2.08 a	18.86 ± 7.17 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 29 Sucrose (mg g^{-1} FW) in SG-based transgenic lines

Transgenic Lines of SG	Sucrose (mg g^{-1} FW)				
	Freshly harvested	25°C one month	25°C two months	4°C one month	4°C two months
SG-1	1.18 ± 0.21 c	1.05 ± 0.29 bcd	1.62 ± 0.31 bcd	3.85 ± 0.17 c	0.38 ± 0.11 b
SG-2	0.57 ± 0.22 c	0.38 ± 0.23 d	2.38 ± 0.12 abc	3.53 ± 0.91 cd	0.28 ± 0.09 b
SG-3	0.74 ± 0.31 c	0.64 ± 0.44 cd	1.59 ± 0.34 bcd	2.25 ± 0.27 cde	0.25 ± 0.06 b
SG-4	3.25 ± 0.19 b	0.56 ± 0.19 cd	1.14 ± 0.23 d	2.80 ± .029 cde	0.15 ± 0.03 b
SG-5	1.20 ± 0.22 c	0.41 ± 0.27 d	1.54 ± 0.34 bcd	2.07 ± 0.20 e	0.05 ± 0.03 b
SG-6	2.95 ± 0.25 b	1.55 ± 0.35 abc	1.44 ± 0.39 cd	3.28 ± 0.61 c	0.33 ± 0.17 b
SG-7	3.29 ± 0.24 b	0.19 ± 0.09 d	1.12 ± 0.19 d	2.41 ± 0.16 de	1.16 ± 0.09 b
SG-8	3.69 ± 0.43 ab	0.90 ± 0.19 cd	0.60 ± 0.24 d	5.05 ± 0.23 b	0.38 ± 0.11 b
SG-9	2.90 ± 0.21 b	0.98 ± 0.16 bcd	1.39 ± 0.27 cd	7.48 ± 1.21 a	0.01 ± 0.00 b
SG-10	3.50 ± 0.38 ab	2.17 ± 0.19 a	2.63 ± 0.44 ab	2.29 ± 0.43 b	0.04 ± 0.03 b
Control	4.2 ± 0.59 a	2.02 ± 0.33 ab	3.41 ± 0.28 a	7.83 ± 0.53 a	11.06±1.17 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 30 Reducing sugars (mg g^{-1} FW) in SG-based transgenic lines

Transgenic Lines of SG	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25°C one month	25°C two months	4°C one month	4°C two months
SG-1	2.33 ± 0.79 ab	0.06 ± 0.05 b	0.03 ± 0.01 c	1.62 ± 0.31 bc	4.28 ±1.70 b
SG-2	0.12 ± 0.17 b	0.07 ± 0.04 b	0.04 ± 0.01c	1.49 ± 0.26 bc	1.67 ± 0.21 cde
SG-3	0.13 ± 0.06 b	0.05 ± 0.04 b	0.02 ± 0.01 c	0.61 ± 0.25 cde	1.84 ± 0.68 cde
SG-4	0.27 ± 0.29 b	0.12 ± 0.06 b	0.01 ± 0.01 c	1.69 ± 0.41 b	0.81 ± 0.17 e
SG-5	0.17 ± 0.08 b	0.04 ± 0.02 b	0.04 ± 0.03 c	0.24 ± 0.16 e	1.14 ± 0.31 de
SG-6	4.06 ± 2.42 a	0.04 ± 0.03 b	0.04 ± 0.02 c	0.34 ± 0.16 de	2.26 ± 0.51 cde
SG-7	0.25 ± 0.24 b	0.08 ± 0.11 b	0.01 ± 0.01 c	1.11 ± 0.16 bcde	3.39 ± 0.84 bc
SG-8	0.08 ± 0.04 b	0.06 ± 0.02 b	0.09 ± 0.07 c	1.35 ± 0.21 bcd	1.70 ± 0.30 cde
SG-9	1.38 ±1.41 b	0.10 ± 0.05 b	0.03 ± 0.02 c	1.62 ± 0.43 bc	2.86 ± 0.41 bcd
SG-10	1.47 ± 0.95 b	0.08 ± 0.07 b	0.22 ± 0.08 b	1.02 ± 0.42 bcde	1.61 ± 0.08 cde
Control	0.69 ± 0.24 b	1.22 ± 0.05 a	1.59 ± 0.09 a	3.84 ± 0.70 a	6.97 ±1.56 a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =4 independent tubers

Table 31 Total soluble sugars (mg g^{-1} FW) in SG-based transgenic lines

Transgenic Lines of SG	Total soluble sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SG-1	3.66 ± 0.47 bc	1.32 ± 0.27 bcd	1.86 ± 0.23 cd	5.69 ± 0.70 cd	4.73 ±1.57 b
SG-2	0.85 ± 0.28 d	0.61 ± 0.16 d	2.51 ±1.17 bc	5.21 ± 1.01 cde	2.02 ± 0.11 b
SG-3	1.09 ± 0.13 d	1.00 ± 0.11 cd	1.85 ± 0.31 cd	3.04 ± 0.48 de	2.13 ± 0.88 b
SG-4	3.66 ± 0.41 bc	0.82 ± 0.14 cd	1.32 ± 0.07 cd	4.79 ± 0.51 cde	0.98 ± 0.20 b
SG-5	1.53 ± 0.69 cd	0.63 ± 0.19 d	1.83 ± 0.84 cd	2.43 ± 0.56 e	1.22 ± 0.30 b
SG-6	7.19 ± 0.83 a	1.84 ± 0.28 bc	1.70 ± 0.40 d	3.74 ± 0.91 de	2.72 ± 0.59 b
SG-7	3.71 ±1.38 bc	0.34 ± 0.26 d	1.27 ± 0.15 d	3.63 ±0.44 cde	4.62 ± 1.44 b
SG-8	4.08 ± 0.95 b	1.10 ± 0.74 cd	0.86 ± 0.12 d	6.56 ±0.77 bc	2.16 ± 0.38 b
SG-9	4.43 ± 2.03 b	1.20 ± 0.33 cd	1.62 ± 0.12 cd	9.41± 1.10 ab	2.87 ± 0.43 b
SG-10	5.24 ± 0.75 ab	2.39 ± 0.26 ab	3.17 ± 0.21 ab	3.34 ±0.51 de	1.67 ± 0.24 b
Control	5.29 ± 1.33 ab	3.48 ± 1.11 a	4.20 ± 0.52 a	12.05± 2.08 a	18.86 ± 7.10 a

Values sharing a common letter within the column are not significant at P ≤ 0.05. Values are the mean ± SD of n =4 independent tubers

Table 32 Sucrose (mg g^{-1} FW) in SH-based transgenic lines

Transgenic Lines of SH	Sucrose (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SH-1	4.54 ± 0.34 bc	1.94 ± 0.28ab	2.35 ± 0.31 bcd	3.75 ± 0.28 b	3.22 ± 0.55 d
SH-3	6.58 ± 1.40 a	0.61 ± 0.25 e	2.04 ± 0.13 d	3.51 ± 0.28 b	5.14 ± 0.31 bc
SH-5	7.53 ± 1.29 a	2.91 ± 0.18 a	2.31 ± 0.28 bcd	3.21 ± 0.31 bc	6.01 ± 0.03 bb
SH-6	3.69 ± 0.28 c	1.25 ± 0.31 cde	2.23 ± 0.18 cd	1.69 ± 0.41 d	4.31 ± 0.79 cd
SH-7	6.64 ± 1.34 a	1.67 ± 0.38 bcd	2.41 ± 0.29 abcd	3.66 ± 0.23 b	7.24 ± 1.31 b
SH-12	5.37 ± 0.24 b	2.45 ± 0.22 ab	3.10 ± 0.42 abc	2.35 ± 0.31 d	7.14 ± 1.56 b
SH-16	4.33 ± 0.36 bc	1.20 ± 0.14 cde	2.01 ± 0.16 d	2.69 ± 0.41 c	6.05 ± 1.32 b
SH-18	7.33 ± 1.39 a	2.04 ± 0.21 abc	3.32 ± 0.28 ab	2.66 ± 0.36 c	6.18 ± 1.41 b
SH-21	4.59 ± 0.33 bc	1.02 ± 0.09 de	2.09 ± 0.18 cd	2.06 ± 0.29 cd	7.66 ± 0.92 b
SH-22	5.23 ± 0.31 b	1.69 ± 0.26 bcd	2.91 ± 0.15 abcd	3.44 ± 0.39 b	5.76 ± 0.31 bc
Control	4.52 ± 0.59 bc	2.02 ± 0.33 abc	3.41 ± 0.29 a	7.83 ± 0.53 a	11.06 ±1.17 a

Values sharing a common letter within the column are not significant at P ≤ 0.05. Values are the mean ± SD of n =4 independent tubers

Table 33 Reducing sugars (mg g^{-1} FW) in SH-based transgenic lines

Transgenic Lines of SH	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SH-1	0.27 ± 0.01 c	0.09 ± 0.01 c	0.08 ± 0.01 c	1.28 ± 0.11bc	2.50 ± 0.34 bc
SH-3	0.43 ± 0.04 abc	0.59 ± 0.17 b	0.08 ± 0.07 c	2.50 ± 0.28 b	2.39 ± 0.38 bc
SH-5	0.38 ± 0.07 bc	0.08 ± 0.01 c	0.54 ± 0.26 b	2.52 ± 0.31b	1.42 ± 0.31d
SH-6	0.53 ± 0.04 abc	0.33 ± 0.02 bc	0.01 ± 0.00 c	0.53 ± 0.41 c	2.08 ± 0.33 bc
SH-7	0.27 ± 0.01 c	0.12 ± 0.04 c	0.14 ± 0.06 c	3.84 ± 0.23 a	3.31 ± 0.23 b
SH-12	0.62 ± 0.21 ab	0.57 ± 0.25 b	0.05 ± 0.01 c	0.62 ± 0.21 c	3.52 ± 0.31 b
SH-16	0.26 ± 0.06 c	0.13 ± 0.02 c	0.09 ± 0.08 c	0.44 ± 0.06 c	2.63 ± 0.46 bc
SH-18	0.51 ± 0.02 abc	0.32 ± 0.14 bc	0.04 ± 0.01 c	2.66 ± 0.37 b	1.60 ± 0.39 cd
SH-21	0.31 ± 0.02 c	0.08 ± 0.05 c	0.03 ± 0.04 c	0.99 ± 0.20 c	0.54 ± 0.19 e
SH-22	0.43 ± 0.04 abc	0.29 ± 0.03 bc	0.16 ± 0.02 c	2.38 ± 0.39 b	1.98 ± 0.26 bc
Control	0.69 ± 0.24 a	1.22 ± 0.05 a	1.59 ± 0.09 a	3.84 ± 0.07 a	6.97 ± 1.56 a

Values sharing a common letter within the column are not significant at P ≤ 0.05. Values are the mean ± SD of n =4 independent tubers

Table 34 Total soluble sugars (mg g^{-1} FW) in SH-based transgenic lines

Transgenic Lines of SH	Total soluble sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
SH-1	5.05 ± 0.77 cd	2.17 ± 0.91 cd	2.65 ± 0.21 def	5.03 ± 0.59 c	5.71 ± 0.29 c
SH-3	7.30 ± 0.36 ab	1.38 ± 0.16 ef	2.22 ± 0.16 f	6.20 ± 0.25 bc	7.82 ± 0.49 bc
SH-5	8.12 ± 0.98 a	3.12 ± 0.16 ab	3.05 ± 0.62 bcde	6.11± 1.32 bc	7.65 ±1.12 bc
SH-6	6.72 ± 0.23d	1.81 ± 0.23 de	2.37 ± 0.35 ef	2.51 ± 0.35 de	6.61 ± 0.53 c
SH-7	7.15 ± 1.70 ab	2.07 ± 0.93 cd	2.75 ± 0.21 cdef	8.30 ± 0.49 b	10.71 ±0.68 b
SH-12	6.17 ± 0.85 abc	3.18 ± 1.01 a	3.45 ± 0.71 bc	3.70 ± 0.94 d	10.89 ±1.31 b
SH-16	5.10 ± 1.06 c	1.43 ± 0.28 ef	2.21 ± 0.12 f	4.10 ±0.38 cd	9.01 ± 1.41 b
SH-18	8.06 ± 1.23 a	2.51 ± 0.24 bc	3.57 ± 0.45 ab	4.10 ± 0.14 cd	8.06 ± 0.71 bc
SH-21	7.08 ± 0.82 ab	1.17 ± 0.72 f	2.25 ± 0.21 f	3.50 ± 0.98 d	8.34 ± 2.67 bc
SH-22	5.88 ± 0.28 c	2.17 ± 0.63 cd	3.17 ± 0.56 bcd	6.67 ±0.59 bc	7.93 ± 0.37 bc
Control	5.29 ± 1.33 cd	3.48 ± 1.11 a	4.21 ± 0.52 a	12.05 ±2.08 a	18.86± 7.1 a

Values sharing a common letter within the column are not significant at P ≤ 0.05. Values are the mean ± SD of n =4 independent tubers

Publications in the peer-reviewed journals

1. **Taneja D**, Das N (2014) Molecular cloning, sequence analyses, and expression studies of sucrose-phosphate synthase in the potato (Solanum tuberosum L.) cultivars. Acta Physiologiae Plantarum DOI: 10.1007/s11738-014-1604-y (Published online on 14 June 2014)
2. Bansal A, Kumari V, **Taneja D**, Sayal R, Das N (2012) Molecular cloning and characterization of granule-bound starch synthase I (GBSSI) alleles from Indian potato cultivars, and sequence analysis for detection of *cis*-regulatory motifs. Plant Cell Tissue & Organ Culture (PCTOC) 109: 247-261
3. Kumari V, Bansal A, Aminedi R, **Taneja D**, Das N (2012) Simplified extraction of good quality genomic DNA from a variety of plant materials. African Journal of Biotechnology 11 (24): 6420- 6427
4. **Taneja D**, Das N (2014) Antisense and sense repression of sucrose-phosphate synthase in a potato (*Solanum tuberosum* L.) cultivar, and their effects on sugar accumulation in transgenic tubers at low temperature (manuscript under preparation, to be communicated)