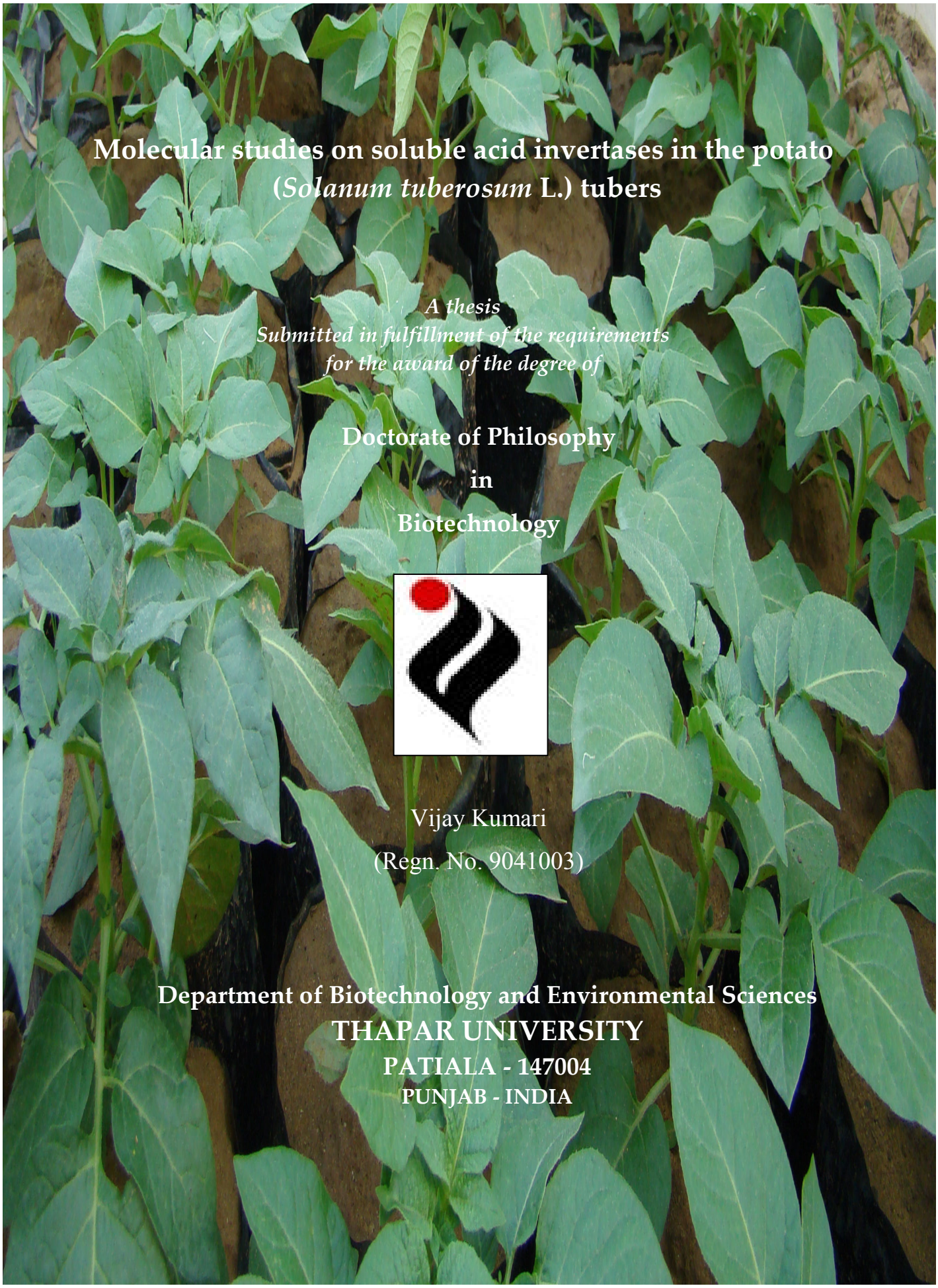

Molecular studies on soluble acid invertases in the potato (*Solanum tuberosum* L.) tubers

A thesis
Submitted in fulfillment of the requirements
for the award of the degree of

Doctorate of Philosophy

in

Biotechnology

Vijay Kumari

(Regn. No. 9041003)

Department of Biotechnology and Environmental Sciences

THAPAR UNIVERSITY

PATIALA - 147004

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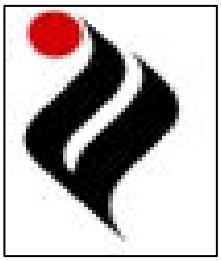

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CERTIFICATE

This is to certify that the thesis entitled, "**Molecular studies on soluble acid invertases in the potato (*Solanum tuberosum* L.) tubers**" which is submitted by Ms. Vijay Kumari, in fulfillment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of the candidate's own independent 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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DECLARATION

I, hereby declare that the work which is being presented in the thesis, **"Molecular studies on soluble acid invertases in the potato (*Solanum tuberosum* L.) tubers "** submitted by me for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is true and original record of my own independent original research work under the supervision of Dr. N. Das, Associate 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.

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(Ms. Vijay Kumari)

Date: Feb 2012

Place: Thapar University
Patiala

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Vijay Kumari

(Ms. Vijay Kumari)

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List of Abbreviations

Name	Abbreviation
ADP	Adenine dinucleotide phosphate
Amp	Ampicillin
APS	Ammonium per sulfate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
CaMV 35S	Cauliflower mosaic virus 35S
CIN	Cytoplasmic invertase
CTAB	Cetyltrimethyl ammonium bromide
CWIN	Cell wall invertase
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
FW	Fresh weight
GA_3	Gibberillic acid
GBSS	Granule-bound starch synthase
Glc	Glucose
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	Milligram
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
min	Minute
mL	Milliliter
mM	Millimolar
MOPS	3-(N-morpholino)-propanesulphonic acid
MS	Murashige and Skoog
NCBI	National Centre for Biotechnology Information
nm	Nanometer
O.D.	Optical density
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
pI	Isoelectric point
pmoles	Picomoles
PMSF	Phenylmethyl sulphonyl fluoride
RNA	Ribonucleic acid
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
STET	Sucrose Tris EDTA Triton X100
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
Tris	Tris-(hydroxymethyl-) aminomethane
TSS	Transcription start site
UDP	Uridine dinucleotide phosphate
UTR	Untranslated region
V	Volt
v/v	Volume per volume
Val	Valine
VIN	Vacuolar invertase
w/v	Weight per volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
YEM	Yeast extract mannitol

Dedicated to my family

Introduction

1. Introduction

1.1. The potato crop: Potato (*Solanum tuberosum* L.) is now regarded as the world's most important non-grain food crop. The potato is a tuber (somatic storage tissue) grown underground on a specialized plant part known as stolon. Therefore, it is a modified stem in a botanical sense. This vegetable crop having high yield potential and high nutritive value is cultivated in many countries. Potato is the most important tuber crop in terms of production, accounting for about 45% of the total world production of all tuber crops (five species together account for almost 90% of total world production-potato, cassava, sweet potato, yams, and taro). The potato tubers are a globally important dietary source of starch, protein, antioxidants and vitamins. Therefore, it plays a major role with regard to global food security. After wheat and rice, potato is the most important food crop, with a world-wide production of around 330 million tons in 2009 (http://www.fao.org). Potatoes are consumed by over one billion people world over; nearly half of them are from the developing countries. The cultivated *Solanum tuberosum* subsp. *tuberosum* is considered to be originated from Andean and Chilean landraces developed by pre-Colombian cultivators. The wild species progenitors of these landraces probably derived from a group of ~20 similar wild species referred to as *S. brevicaule* complex, distributed from central Peru to northern Argentina. The Spanish introduced the potato to Europe in the second half of the 16th century. Subsequently, this crop was introduced to many territories and ports throughout the world by European mariners. Potato was introduced to India from Europe in the beginning of early 17th century. The potential of the potato crop was realized in India soon after independence in 1947. The Central Potato Research Institute (CPRI), Shimla was established in 1949 which took a leading role for the improvement of potato crop through conventional breeding techniques.

Now India ranks third in terms of area of potato cultivation, and it is the second largest country of around 34.39 million tons production of potato in 2009 (http://www.fao.org). China is now the biggest potato producer in the world.

The potato, an herbaceous annual plant can grow up to 20-40 inches (50 to 100 cm) high. The ends of its underground stems or 'stolons', may enlarge greatly to form a few to more than 20 tubers of variable shapes and sizes (Fig. 1). The skin of potato tuber varies in colour from brownish white to deep purple. Its flesh normally ranges in color from white to yellow but it also may be purple. The tubers bear lateral buds (eyes) that grow into new plants when the conditions are favorable for growth.

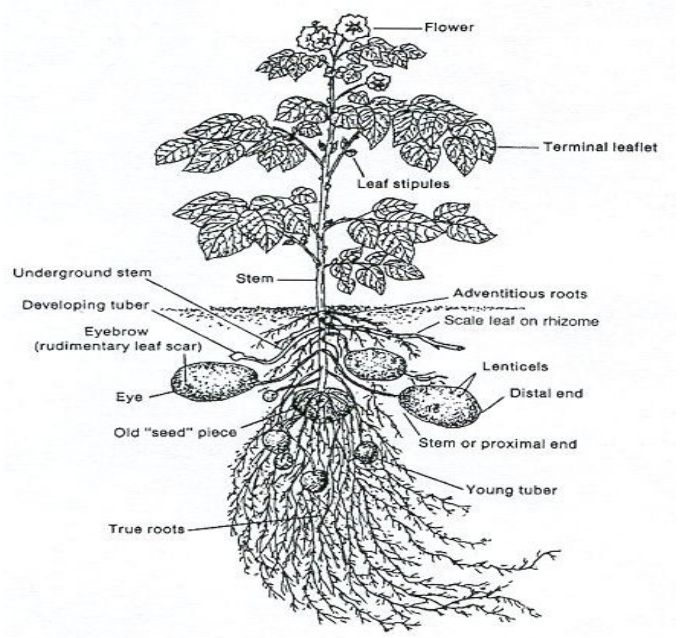


Fig. 1. Schematic view of a potato plant showing various parts (Source: www.google.com)

Leaves are alternate and irregularly pinnate compound. They bear white, pink, red, blue, or purple flowers with yellow stamens. Flowers are pentamerous, actinomorphic, perfect and have sympetalous colored corollas. After flowering, some varieties of potato plants produce small green fruits, each containing up to 300 true seeds also called "true seed" or "botanical

seed". Potato fruit contains large amounts of the toxic alkaloid solanine and is therefore not suitable for consumption.

Ploidy level of potato: The genus *Solanum* consists of approximately 2000 members. Out of this, only about 160 wild and seven cultivated species are able to form tubers. The most common cultivated species of potato i.e., *tuberosum* is a hybrid between the diploid species *S. stentotomum* and the diploid weed *S. sparsipilum* with subsequent chromosome doubling (Ramanna and Hermsen 1979). The potato has a series of ploidy levels, based on a 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 potato cultivars are autotetraploid (2n = 4x = 48) and display a high degree of heterozygosity, and suffer acute inbreeding depression. Moreover, they are susceptible to many harmful pests and pathogens. Because of these attributes, it is relatively difficult to adopt classical breeding approaches for improvement of such tuber crops.

Life cycle of potato: Potato plant has a short life span ranging from 80 to 150 days from planting to maturity depending on the cultivars. Tuber induction, initiation, enlargement, dormancy, and sprouting represent the typical life cycle of a potato plant. The initiation and development of potato in the plant is known as tuberization. A potato plant tuberizes only when environmental conditions are favorable i.e., short-day photoperiod (daylight) and cool night temperature. The potato growing conditions in India are entirely different from those in Europe and North America except some available Indian hills during summer. But this temperate potato production constitutes only about 8-10 % of the total production. In India, therefore, the potato is regarded as a short-duration crop with an average cropping stand of 90-100 days. Usually, potato plant is propagated through tubers (vegetative or asexual

propagation). The tubers meant for propagation are known as “seed tubers” or “seed potatoes”. In addition to tubers, a potato plant can also be propagated through botanical seeds, which are known as True Potato Seeds (TPS). TPS is an alternative means of propagation where production of ‘seed potatoes’ is not feasible. For usual cultivation, TPS is not popularly used; but it is important with respect to potato breeding perspectives.

Potato plants suffer from a variety of viral, bacterial, nematode and fungal diseases which have serious consequences in terms of tuber yield and consumer acceptance. Examples of viral pathogens are potato viruses X and Y. *Streptomyces scabies* and *Erwinia carotovora* represent two bacterial pathogens of tubers that cause serious losses. The most serious losses occur due to fungal pathogens, such as *Phythophthora infestans* that causes potato blight disease. In the areas of molecular biology, biochemistry and biotechnology potato is an attractive system both in terms of basic and applied research. Moreover, it provides vast genetic resources. Currently, the primary focus of applied research is on disease control, starch metabolism and nutritional aspects. Many useful novel proteins/metabolites could be produced in potato tubers (potato tubers being used as ‘factories’ or ’bioreactor’). In recent years, there is increasing demand for processed potato products. Not all potato cultivars are suitable for processing sectors. Because the tuber dry matter content and level of reducing sugars are two important parameters for selecting a potato variety for processing. A related problem is the undesirable ‘cold-induced sweetening’ of potato tubers during storage at low temperature. The major objectives of global potato breeding programs (both conventional and molecular breeding) include optimization of production levels, value addition, and proper storage after harvest and resistance to various biotic and abiotic stresses.

1.2. An overview of sucrose metabolism in plants: The primary function of photosynthesis is to provide energy and carbon sufficient to support maintenance and growth of the photosynthetic tissues and the remaining parts of plant body. Therefore, proper allocation, translocation and partitioning of photoassimilates are basic processes in plants where sucrose plays a very pivotal role. Sucrose is a disaccharide composed of glucose and fructose. The abbreviated name of sucrose is either Glc ($\alpha 1 \leftrightarrow 2\beta$) Fru or Fru ($\beta 2 \leftrightarrow 1\alpha$) Glc. During daylight hours, photoassimilate generated by the photosynthetic reduction cycle (PCR) is temporarily accumulated in the leaf as starch in the chloroplast stroma or sucrose in the mesophyll vacuole. This nonreducing sugar is a major intermediate product of photosynthesis. The conversion of photoassimilate 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 or tissues. The latter process is known as translocation which occurs through the vascular phloem tissue. 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 sucrose, there is an unusual linkage between the anomeric C-1 of glucose and the anomeric C-2 of fructose. Amylases or other common carbohydrate cleaving enzymes can not hydrolyze this bond. Sucrose fails to react with amino acids and proteins nonenzymatically because of the unavailability of the anomeric carbons. Because of these attributes sucrose has been selected as the transport form of carbon during the course of evolution. In many plants sucrose represents the most common form of sugar found in

translocation stream where it is transported from leaves (source) to nonphotosynthetic tissues (sink) such as flower, fruit, seed, and root. Once it has reached these sink tissues, sucrose is degraded into hexoses or their derivatives for various metabolic and biosynthetic processes. In some plants sucrose is found as a storage carbohydrate. For example, in sugarbeets and sugarcane it is stored in the vacoules of specialized storage cells. Being one of the more abundant natural products, sucrose not only plays a vital role in plant growth and development but is also a leading commercial commodity. Moreover, sucrose and hexoses play very important signaling roles in regulating gene expression and plant development (Koch 2004; Rolland et al. 2006; Smeekens et al. 2010).

On the basis of cell fractionation and enzyme localization studies it has been established that sucrose synthesis occurs exclusively in the cytosol of photosynthetic cells; whereas, starch synthesis occurs in the plastids (chloroplast in leaf tissue). These processes are coordinated by a variety of regulatory mechanisms which are again responsive to changes in light level and rate of photosynthesis. The principal pathway for sucrose synthesis in photosynthetic cells involves the following biochemical steps: the enzyme sucrose phosphate synthase catalyzes the conversion of UDP-glucose and fructose-6-phosphate to UDP and sucrose-6-phosphate; sucrose-6-phosphate is hydrolyzed to sucrose and Pi by the enzyme sucrose phosphate phosphatase. It is generally believed that energy provided by sucrose-6-phosphate hydrolysis (~12.5 kJ mol^{-1}) play a role in the high sucrose accumulation typical of sugarcane and other sucrose-storing plants. The other pathway involves the enzyme sucrose synthase which uses UDP-glucose and fructose for conversion to UDP and sucrose with a free energy change is approximately +14 kJ mol^{-1}. Most of the evidence indicates that under normal conditions sucrose synthase operates in the reverse direction to break down sucrose. Sucrose translocated

from the leaf tissue to storage organs such as roots, tuber tissue, and developing seeds is most commonly utilized in starch biosynthesis. Some of the important biochemical steps include the following: sucrose synthase catalyzes the conversion of sucrose and UDP to fructose and UDP-glucose; UDP-glucose reacts with PPi to form UTP and glucose-1-phosphate catalyzed by UDP-glucose pyrophosphorylase; and ADP-glucose pyrophosphorylase catalyzes the conversion of glucose-1-phosphate and ATP to ADP-glucose and PPi. ADP-glucose is then converted to starch by starch synthase. The cleavage reaction of sucrose is catalyzed by two enzymes in higher plants: Sucrose synthase (Sus, EC 2.4.1.13) or invertase (INV, EC 3.2.1.26). Sus degrades sucrose in the presence of UDP into UDP-glucose and fructose, where as the INV hydrolyses sucrose into glucose and fructose. Current understanding on the metabolic aspects indicates that Sus is mainly involved in the biosynthesis of sugar polymers including starch and cellulose and generation of energy (ATP) (Chourey et al. 1998; Ruan and Chourey 2006; Coleman et al. 2009).

Potato tuber-an important sink tissue in plants: Tuber development in potato provides a very good model for studying carbohydrate partitioning between the autotrophic source tissue and nonphotosynthetic sink tissue. Potato tubers are somatic storage tissues. Morphologically, potato tubers are modified stems, expanded radially by a process of cell expansion and limited cell division. During growth of the main potato stem, underground lateral shoots (stolons) characterized by elongated internodes, hooked apical tips, and diageotropic growth development. Upon tuber initiation, cessation of stolon growth coincides with the cessation of mitotic activity in the apical meristem and marked increase in radial expansion and mitotic division of cells in the subapical meristem (Cutter 1978). The transformation of stolons into tubers impacts greatly on the physiology of the entire plant, because the developing tubers

subsequently become the largest sinks (Oparka 1985). Various biochemical activities are known to be involved in the different phases of tuberization. In stolons undergoing extensive growth, the carbohydrate metabolism plays a very important role. Sucrose is delivered to the stolons by apoplastic phloem transport. Acid invertases cleave incoming sucrose into hexoses to provide cells with fuel for respiration, with carbon and energy for the various functions. Cleavage of sucrose into glucose and fructose could greatly increase the osmotic pressure of cells, suggesting a possible function of invertases in cell elongation and plant growth (Gibeaut et al. 1990). With the first visible sign of tuber initiation, there is a switch from predominantly apoplastic unloading of sucrose into stolons towards predominantly symplastic unloading into tubers. Thus, in very early stages of tuber development i.e., during the elongation phase of stolon growth, apoplasmic sucrose unloading predominates. In swelling stolons, a marked decline in invertase activity indicates that a switch from the invertase-sucrolytic pathway to a sucrose synthase-sucrolytic pathway occurs in parallel. A detailed study of the activity of the two potential routes of sucrose degradation unequivocally showed that acid invertase predominates during early stages of tuberization, whereas, sucrose synthase becomes the major sucrolytic activity once starch synthesizing tubers become the major sink for the incoming sucrose (Appeldoorn et al. 1997). During the stolon to tuber transition, sucrose utilization changes from hydrolytic to sucrolytic breakdown (Hajirezaei et al. 2000). In fact, this biochemical event is crucial for subsequent developmentally regulated starch biosynthesis in the potato tubers.

***1.3. Plant invertases-a diverse enzyme family*:** Studies on invertases are gaining importance increasingly both in terms of basic and applied research during the last more than two decades. Invertases are ubiquitous enzymes that irreversibly cleave sucrose into fructose and

glucose. Growing body of evidences suggests that there are several isoenzymes of plant invertases involved in a wide range of regulatory functions in growth and development apart from their major roles in primary carbon metabolism (Sturm 1999; Fotopoulos 2005; Ruan et al. 2010). Several isoforms of invertase exists with different biochemical properties and subcellular locations (Tymowska-Lalanne and Kreis 1998a). The specific functions of the different invertase isoforms are not clear, but they appear to regulate the entry of sucrose into the different utilization pathways. Based on their solubility, subcellular localization, pH optima and isoelectric point, three different types of invertase isoenzymes can be distinguished (summarized in Table 1): i) vacuolar invertase (VIN)-most plant species contain at least two isoforms of vacuolar invertase which accumulate as soluble proteins (soluble acid invertases) in the lumen of this acidic compartment; ii) cell wall invertase (CWIN)-several isoforms of extracellular invertase (cell wall invertases) that are ionically bound to the cell wall have been detected; iii) neutral invertases-also known as cytoplasmic invertases (CIN). Plants have at least two isoforms of cytoplasmic invertase with pH optima for sucrose cleavage in the neutral or slightly alkaline range (Sturm 1999). Neutral and alkaline invertases are less well characterized but, in contrast to the acid invertases, these enzymes appear to be sucrose specific. These three types of invertase isoenzymes, which are encoded by small gene families, are regularly found in plants (Draffehn et al. 2010). Cell wall and vacuolar invertases are N-glycosylated forms with acid pH optima (between pH 4.5 and 5.0) and attack the disaccharide from the Fru residue. Thus CWIN and VIN are β-fructofuranosidases and also hydrolyze other β-Fru-containing oligosaccharides such as raffinose and stachiose. On the contrary, neutral and alkaline invertases (CIN) having pH optima 7.0 to 7.8, appear to be sucrose specific.

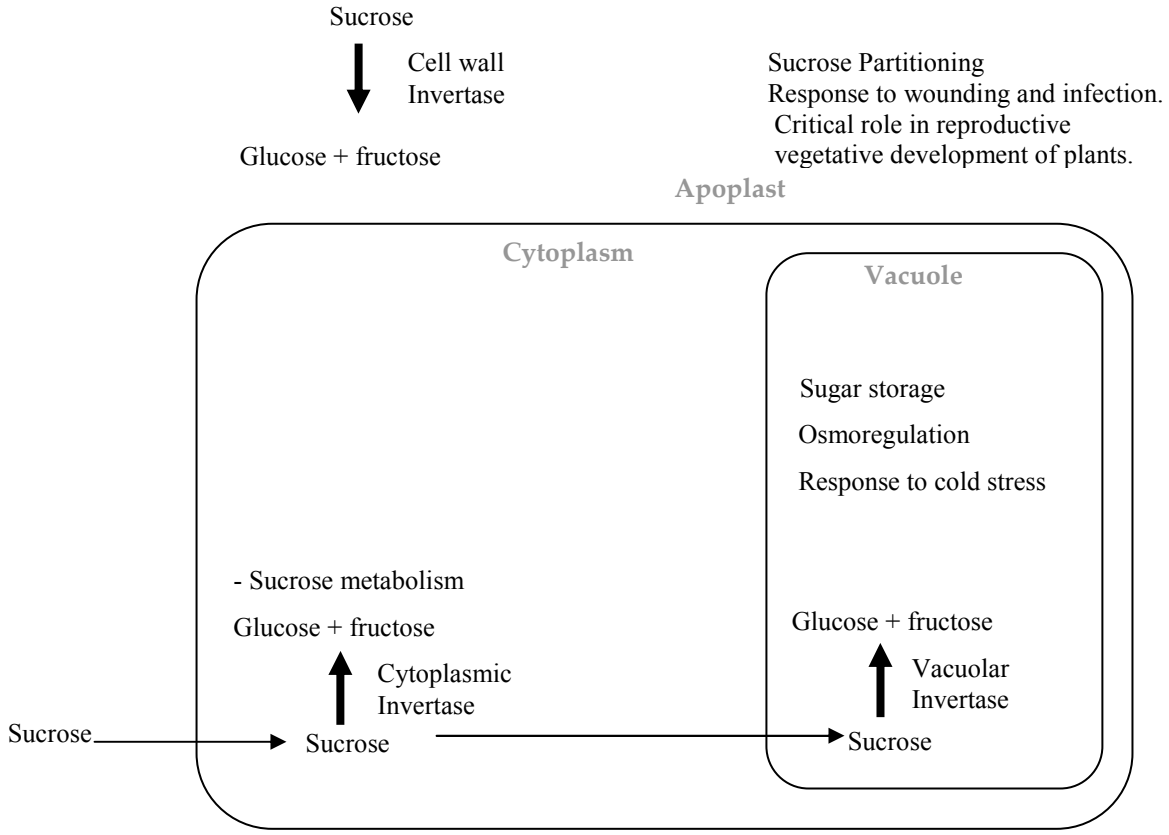
Table 1. Properties of plant invertase isoenzymes

Type of invertase	pH optima	Localization	Solubility	N-Glycosylation	pI
Cell wall (acid)	4.5 - 5.0	Apoplast	Insoluble	+	Basic
Vacuolar (acid)	4.5 - 5.0	Vacuole	Soluble	+	Acidic
Cytoplasmic	7.0 - 7.8	Cytoplasm	Soluble	–	Acidic

***1.4. Role of invertases in plant growth and development*:** Based on the growing body of evidences it is generally believed that CWIN plays crucial role during flower, seed, and fruit development; VIN as a key modulator for hexose accumulation and cell expansion, but the role of CIN in plant development still remains to be elucidated (Sturm 1999; Ruan et al. 2010). The physiological functions of plant invertase isoenzymes are complex and depend upon the kind of tissue and the subcellular location (Sturm and Tang 1999; Tang et al. 1999; Roitsch and Gonza´lez 2004). Biological role of each type of invertase in plants is briefly described here:

Cell wall invertase (CWIN): Long-distance transport of photoassimilates from source leaves into sink organs is driven by differences in osmotic potentials. The important roles of CWIN in regulating phloem unloading and sink strength have been analyzed in transgenic carrot plants with its antisense suppression under the control of the CaMV 35S promoter. This resulted in decreased carbohydrate content in roots and increased leaf-to-root ratio of plants (Tang et al. 1999). Anther-specific repression of CWIN Nin88 negatively affected pollen development, thus causing male sterility in tobacco (Goetz et al. 2001). Similarly, cold- or ABA-induced male sterility in the monocot rice was found to be due to disruption of sugar transport in anthers, caused by repression of the cell wall invertase and monosaccharide transporter gene expression (Oliver et al. 2007). Recently, Zanor et al. (2009) demonstrated that silencing the expression of a CWIN, Lin5, in tomato led to reduced pollen viability and elongation, hence seed number. Based on these experimental evidences, it is now believed

that cell wall invertase plays critical role in reproductive and vegetative development of plants. Subcellular locations and proposed functions of plant invertases are given below:



Schematic view: Plant cell contains different invertases isoforms in the apoplasts, cytoplasm and vacuole. Hexoses generated by the activities of the different enzymes have different functions. This was adapted from Sturm (1999) with some modifications.

Vacuolar invertase (VIN): Currently, vacoular invertase is regarded as a key modulator for hexose accumulation and cell expansion (Ruan et al. 2010) as supported by the following observations. Vacuolar activity increases while hexose accumulates during fruit ripening in the domestic tomato (*Solanum Lycopersicum*) but remains much lower in the sucrose-accumulating fruit from wild tomato species (Klann et al. 1993; Jin et al. 2009). The

suppression of vacuolar invertase expression leads to the conversion of hexose-accumulation to sucrose-storing fruit in the domesticated tomato species (Klann et al. 1996). Similar findings have been reported in other sugar storage sinks such as grape berry (Davies and Robinson 1996) and potato tubers (Greiner et al. 1999). High level of vacuolar invertase activity was observed in a wide range of expanding tissues, including developing potato tubers (Ross et al. 1994), carrot taproot (Tang et al. 1999) and maize ovaries (Zinselmeier et al. 1995b; Andersen et al. 2002). Collectively, these results clearly suggest that vacuolar invertase controls the sugar composition in these plant organs. It is now commonly believed that VIN plays a role in maintaining cell turgor and cell expansion. Tissues undergoing rapid cell expansion usually have a high hexose and low sucrose concentration suggesting an important role for sucrose-metabolizing enzymes (Sebkova et al. 1995).

Neutral/alkaline invertase or cytoplasmic invertase (CIN): Literature suvey reveals that substantial progress has been made in understanding the roles of CWIN and VIN as mentioned above. But much less information is available till to date on the function of CIN because of its instability and low intracellular activity at neutral pH. However, some recent reports suggest that CIN may have potential roles in plant development (Ruan et al. 2003; Roitsch and Gonza´lez 2004; Ruan et al. 2010).

Invertase-mediated sugar signaling: Invertase hydrolyzes sucrose into glucose and fructose and plays a major role in plant development and in responses to various biotic and abiotic stresses (Sturm 1999; Essmann et al. 2008). The main distinctive feature of invertases is the production of glucose instead of UDP-glucose. This aspect is noteworthy when considering many evidences that support signaling role of sugars in plant development. The significance of sugar metabolism is not only in providing substrates for metabolic processes but also in

producing signals perceived by cells (Koch 2004). Sugars in plants are not only nutrients but also important regulators of gene expression. Therefore, in terms of the control of cell differentiation and plant development invertases may be indirectly involved. Both glucose and fructose are implicated in the signaling pathways by which sucrose alerts the plant cell to nutritional requirements or constraints. This may be sensed by plant cells so that metabolism is modified by altering gene expression. Thus invertase action amplifies the sucrose signal by producing two "messenger" molecules for the price of one. Invertase activity needs to be tightly regulated *in vivo* to ensure ordered plant development (Ruan and Chourey 2006).

Some more important advances in the area of plant invertase research during the last few decades are provided in the next 'Review of Literature' section.

Review of Literature

2. Review of Literature

Literature survey reveals that biochemical, molecular and genetic studies on various invertase isoenzymes have been carried out in many plant species including economically important crops such as tomato, carrot, potato and others during the last more than two decades. In other words, substantial progress has been made along with some exciting advancements in this area. The major focus areas of studies in many laboratories include: a) isolation and biochemical characterization of plant invertases; b) molecular cloning, sequence characterization and expression studies; c) sequence analyses and comparison, prediction of structure-function relationship in the invertases; d) identifying a variety of intracellular and extracellular factors that influence invertase gene expression and regulation of enzyme activity at post-translational level through interactions with the endogenous inhibitors; e) understanding the emerging biological roles of invertases during plant growth and development; f) elucidating cross-talk between invertase-mediated sugar signaling and hormonal control of development; g) sugar and invertase mediated responses to various abiotic stresses, and g) potential applications of current knowledge on invertases in plant biotechnology mainly crop improvements through transgenics. For example, transgenic approach is found to be considerably effective for inhibition of cold-induced sweetening (an undesirable phenomenon as it leads to high accumulation of reducing sugars in potato tubers) in the harvested potato tubers during low temperature storage. Apart from the biochemical properties of the invertases, there were some exciting advances in elucidating the three-dimensional (3D) structures based on X-ray crystallography which helped in gaining insights into glycoside hydrolase family 32 (GH32) and 68 (GH68) enzymes with regard to their functional implications. Such studies helped in explaining the reaction mechanism and the

role of the crucial amino acids in substrate binding or stabilization i.e., in overall catalysis. The following sections cover some important aspects of recent progress of plant invertase research based on the published reports. Here the focus is mostly on the soluble acid invertases (vacuolar invertases) along with their implication with respect to low-temperature sweetening in the potato tubers.

***2.1. Enzymatic properties of vacuolar and cell wall invertases*:** Vacuolar and extracellular invertases have been purified from several plant species (Unger et al. 1992). Vacuolar and cell wall invertases share some biochemical properties e.g., they cleave sucrose most efficiently between pH 4.5 and 5.0 and attack the disaccharide from fructose residue. Thus these so called acid invertases (both vacuolar and cell wall invertases) are β-fructofuranosidases and also hydrolyze other β-fructose containing oligosaccharides such as raffinose and stachiose as a substrate but with significantly reduced cleavage efficiency. These acid invertases have low K_m values for Suc, and activity is inhibited by heavy metals such as Hg^{2+} and Ag^{+} suggesting the presence of a sulfhydryl group at the catalytic site. Glc acts as a non-competitive inhibitor and Fru as competitive inhibitor for acid invertases. In most of the cases molecular masses of the mature N-glycosylated polypeptides are between 55 and 70 kD (Sturm 1999).

It was revealed that the N-terminal fivefold β-propeller domain consists of three common conserved motifs in the active sites that accommodate three crucial amino acid residues. More specifically, these are the nucleophile (the aspartate in the WMNDPNG-motif), transition-state stabilizer (the aspartate in the RDP-motif) and the acid/base catalyst (the glutamate in the EC-motif) (Verhaest et al. 2005; Lammens et al. 2009). Apart from plants, acid invertases are also isolated and characterized in yeast and bacteria. Both VIN and CWIN are β-fructo-furanosidases, and share a high degree of overall sequence homology and two conserved

amino acid motifs. Their common features are the presence of the pentapeptide NDPNG (βF-motif) towards the N-terminus of the mature proteins. The above sequence NDPNG can be regarded as a hallmark of plant acid invertases. Another conserved sequence motif i.e., WECXDF occurs towards the C-terminus consisting of conserved Glu and Cys residues with a few neighboring amino acids. Vacuolar and cell wall invertases are glycoproteins (N-linked glycosylation); it has been demonstrated that inhibition of glycosylation results in rapid degradation of vacuolar and cell wall invertase (Pagny et al. 2003). Close inspection of the deduced amino acid sequence also revealed that unlike cell wall acid invertase, a short C-terminal extension is present in vacuolar acid invertase as found through cDNA cloning studies in carrot (Sturm and Chrispeels 1990).

***2.2. Molecular cloning and characterization studies on acid invertases*:** Sturm and Chrispeels (1990) first reported plant cell wall invertase cDNA clone from carrot. Since its isolation a number of cDNA sequences encoding acid invertases from taxonomically different plant species have been deposited in the databases and reported in the literature. Considerable progress has been made on cell wall invertase both at biochemical and molecular level in different plant species, such as carrot, potato, tomato, *Arabidopsis*, pea and others (Unger et al. 1994; Hedley et al. 1993; Ohyama et al. 1998). Likewise, molecular cloning and characterization studies on vacuolar (soluble) invertase were also carried out in many plant species including economically important crops such as potato (Zhou et al. 1994; Zrenner et al. 1996), tomato (Ohyama 1992; Elliot et al. 1993), mung bean (Arai et al. 1992), Capsicum (GenBank Acc. No. U87849), common tobacco (GenBank Acc. No. AJ305044), sweet potato (Huang et al. 2003; Wang et al. 2005), muskmelon (Tian et al. 2009), grape berries (Davies and Robinson 1996), upland cotton (Wang et al. 2010), *Arabidopsis* (Haouazine-Takvorian et

al. 1997), Broccoli (Coupe et al. 2003), maize (Kim et al. 2000), rice (Ji et al. 2005), chicory (Van den Ende et al 2002), *Pachysandra terminalis* (Buxaceae) (Van den Ende et al. 2011), poplar and also some other plant species.

The cDNA cloning studies on plant acid invertases revealed that each isoenzyme is encoded by different gene along with their allelic variants (Sturm 1999; Tymowska-Lalanne and Kreis 1998a). The polypeptides encoded by these genes can be divided into two main classes with different properties. One class of the predicted polypeptides corresponds to CWIN with a basic pI, and the other class of polypeptides represents VIN with an acidic pI. The plant cDNA derived amino acid sequences all belong to a large protein family, and also related to the sequences of invertases from yeast and bacteria. VIN is closely related to CWIN but distally related to CIN as revealed by phylogenetic analyses. Acid invertases are synthesized as pre-proproteins as revealed by the sequence information of the mature polypeptides. The N-terminal domain up to 100 amino acid residues long, most likely consists of a signal peptide and an N-terminal propeptide (Sturm and Chrispeels 1990; Unger et al. 1994) which get cleaved off during transport and protein maturation. The signal peptide is required for entry into the ER lumen for further processing through the secretory pathway, and an N-terminal propeptide, is thought to either play a role in protein folding and stability and/or in the regulation of enzyme activity (Hasilik and Tanner 1987). The function of the putative propeptides is not clear, but in analogy to other "preproenzymes" they may play a role in protein folding, protein targeting (Klionski et al. 1988). In comparison with the sequences of plant CWIN, vacuolar invertases were found to contain short hydrophobic C-terminal extensions which might be involved in the vacuolar targeting of the protein as reviewed in Sturm (1999).

2.3. Invertase and stress responses of plants: Apart from carbohydrate metabolism and other physiological roles during plant development soluble acid invertase plays a very important role in a variety of biotic and abiotic stress responses (Tymowska-Lalanne and Kreis 1998a; Roitsch et al. 2003; Roitsch and Gonza´lez 2004). The accumulation of soluble sugars such as sucrose, glucose and fructose by plants at low, non-freezing temperatures is a widespread and well-established phenomenon. In fact, this is regarded as an adaptive response to cold stress, as sugars have long been known to have an osmoprotective function in plants. It was demonstrated long ago that plant tissues, organs and intact plants showed enhanced resistance after hardening in a medium containing sucrose, a proof of protective activity of sugars. Cold-induced destructive oxidation processes, such as peroxidation of lipids (POL), in the biological membranes, are prevented by the ability of sugars to combine with free radicals. At low temperature along with other proteins, increased synthesis of invertases in plants play very crucial roles since the composition of soluble carbohydrates get changed. Tolerance to chilling was compared in potato cv. Desiree under *in vitro* conditions transformed with a yeast-derived invertase gene under the control of the *B33* class 1 tuber-specific promoter and control plants transformed only with reporter gene. As compared to the control, contents of sucrose increased by 21% and glucose by 13% respectively. The soluble sugars also act as low-molecular weight antioxidants and stabilizers of cell membranes under the hypothermic conditions besides osmoregulatory and cryoprotective functions. The potato plants transformed with the yeast invertase gene acquire a higher tolerance to low temperatures as compared to the control plants, apparently due to the changes in sugar ratio produced by the foreign invertase (Deryabin et al. 2003). The monosaccharides produced as a result of

disaccharide hydrolysis not only provide cryoprotective and osmoregulatory function but also meet the necessary metabolic and energy requirements (Deryabin et al. 2005).

2.4. Cold-induced sweetening in potato tubers: Potato plants are best grown in temperate climate. Therefore, continuous production of tubers is not possible round the year. Rising temperatures cause sprouting of tubers, weight loss and rotting. Such problems can be overcome by storing potatoes between 10^0-12^0C along with use of isopropyl N-chlorophenyl carbamate (CIPC) as sprout suppressant. It is not an acceptable approach because of its toxic effects and other environmental concerns. Therefore, harvested tubers need to be stored for a certain period of time at refrigerated cold stores at 2^0-4^0C which is an alternative to the treatment of dormancy-prolonging chemicals. Storage at such low temperatures for several weeks leads to breakdown of starch into sugars in tubers with consequent accumulation of reducing sugars-a phenomenon known as 'cold-induced sweetening'. This process is also regarded as an adaptive response to cold stress. The level of sugar accumulation during low temperature storage depends on the cultivar genotype and environmental factors. Several enzyme activities are closely related to the phenomenon of low-temperature sweetening. It is commonly believed that the cold-induced hexose accumulation is caused by an imbalance between starch breakdown and glycolytic activity. There are several plausible explanations pertaining to the cold-induced sweetening. (a) The starch is thought to be degraded via mainly phosphorolytic route based on the relative activities of phosphorylase and amylolytic enzymes (overall secondary structure of starch is also known to influence on its degradability). An increase in the activity of one or more starch degrading enzymes may be involved the above process. (b) After starch breakdown, sucrose is formed in cytosol via UDP-glucose pyrophosphorylase and sucrose-phosphate synthase catalyzed reaction. (c) Sucrose is further

hydrolyzed by invertases to reducing sugars i.e. glucose and fructose (Isherwood 1973; Pollock and Rees 1975; Richardson et al. 1990; Zrenner et al. 1996). Zrenner et al. *(*1996) have reported that the activity of acid invertase increases in tubers stored at low temperatures, and the level of activity differs between the potato cultivars. This observation was further substantiated by Matsuura-Endo et al. (2004). They studied on biochemical changes during low temperature storage of the potato tubers from a few Japanese cultivars. The purpose was to measure the changes of sugar content and the available vacuolar acid invertase activity in cold-stored tubers to find possible correlations, if any. When stored at 4^0C, three types of changes were noticed with the progress of time among the potato cultivars: a) increased level of reducing sugars during storage (type-1); b) low levels of reducing sugars throughout the storage period (type-2); and c) increased sucrose, but not reducing sugars (type-3). During storage at 4^0C, the activity of vacuolar acid invertase was increased in the type-1 cultivars, whereas in type-2 and type-3 cultivars, the activities remained low or very low. RT-PCR analysis of acid invertase showed that the transcript of the enzyme accumulated in the tubers stored at 4^0C in type-1 cultivars but not in type-3. These results clearly indicate that the activity of vacuolar acid invertase is related to the type of changes in terms of sugar content during low-temperature storage. Varietal differences are also quite evident in the above study.

Accumulation of reducing sugars negatively affects potato quality: The increase of reducing sugar level in cold-stored tubers severely affects the processing quality of their products such as chips, flakes, fries, curls etc. Because excess reducing sugars react with free amino groups of proteins/amino acids resulting in the formation of a complex of large molecular weight molecules-known as nonenzymatic *Maillard reaction* that results in formation of brown-to black-pigmented products which is not acceptable to consumers (Shallenberger et al. 1959).

Moreover, high temperature processing of the tuber slices results in black/brown-colored, bitter-tasting products. In the United States alone, approximately 15% of potatoes are rejected at processing plants annually due to high levels of reducing sugars (Sowokinos 2004). More problematically, the Maillard reaction also generates acrylamide, a neurotoxin and a potential carcinogen. It is formed from asparagine and reducing sugars via an *N*-glycoside intermediate in a side reaction of the Maillard reaction (Mottram et al. 2002; Stadler et al. 2002). In fried potato products, the amount of acrylamide directly depends on the reducing sugar content of the tubers, and is increased by prior storage of the potatoes at low temperature (Olsson et al. 2004; De Wilde et al. 2005; Williams 2005). In 2002, high levels of acrylamide were reported in carbohydrate rich foods processed at high temperatures. Reducing sugars is one of the major substrates for acrylamide formation in the processed potato products (Goekmen and Palazoglu 2008). One effective way to decrease acrylamide content is to decrease the reducing sugar level in raw tubers (Matsuura-Endo et al. 2006; Muttucumaru et al. 2008).

2.5. Various strategies for inhibition of acid invertase gene function in potato: Cold-induced sugar accumulation was mostly studied in potato tubers. Biochemical studies on the potato tubers stored at low temperatures clearly indicated that acid invertase and its isoforms are the key enzymes causing reducing sugar accumulation. Therefore, the level of reducing sugars can be minimized through inhibition of acid invertase gene functions. Here a few case studies are described briefly.

Zrenner et al. (1996) made a significant contribution in terms of vacuolar invertase cDNA cloning and characterization from potato along with antisense approach for inhibition of invertase gene function. They isolated a cDNA clone, designated as INV-19 (EMBL Acc. No. X70368), encoding vacuolar invertase by screening potato leaf cDNA library using carrot

CWIN cDNA as a probe. The 2063-bp INV-19 contains an ORF of 1905 bp encoding a polypeptide of 635 amino acids (lacking a few amino acids at the N-terminus). At nucleotide level, INV-19 shared 98% identity with other potato cDNA sequences encoding soluble acid invertases (GenBank Accession Nos. AY341425 and L29099). In potato, the complete ORF encoding VIN consists of 639 amino acids. INV-19 cDNA sequence showed 94% sequence identity with tomato vacuolar invertase (GenBank Acc. No. Z12026). INV-19 specific transcript was found in leaves, roots, stems, flower and stolon, but absent in growing and sprouting tubers. They used the potato cv. Desiree for genetic transformation, and generated transgenic potato plants by expressing INV-19 cDNA in antisense orientation under the constitutive CaMV 35S promoter. In the cold-stored transgenic potato tubers soluble acid invertase activity was found to be inhibited. Almost no correlation was found between the total amount of invertase activity and the accumulation of reducing sugars, but there was a striking correlation between the hexose/sucrose ratio and the extractable soluble invertase activity. Based on this study it was concluded that soluble acid invertases does not control the total amount of soluble sugars but determines the hexose-to-sucrose ratio in cold-stored potato tubers. The less accumulation of reducing sugars in the transgenic cold-stored potato tubers is a promising aspect of this study. This observation was further substantiated by Greiner et al. (1999). In this study transgenic potato tubers were developed by expressing a homolog of a tobacco CWIN inhibitor (*Nt-inhh*) under the control of CaMV 35S promoter. The activity of vacuolar acid invertase is reduced by approx. 80%, whereas cell wall invertase activity remained unchanged. Cold-induced hexose accumulation was reduced by up to 75%, without any effect on potato tuber yield. The selective inhibition of vacuolar invertase by *Nt-inhh* did

not affect the tuber number, tuber weight. The processing quality of tubers was greatly improved and the starch quantity and quality remained unaltered.

McKenzie et al. (2005) reported that the tuber glucose:sucrose ratio showed correlation with both basal (with natural invertase inhibitors) and total (after destroying invertase inhibitor) acid invertase activity. There was a positive correlation between basal invertase activity and the glucose:sucrose ratio before the storage period began. At the beginning of storage, destruction of the inhibitor actually led to a reduction in the specific activity of acid invertase. This was possibly due to the non-specific destruction of invertase protein during the removal of the inhibitor. Following five months of cold storage, acid invertase activity was greater when the inhibitor was destroyed in all clones. It clearly indicated that invertase inhibitor activity could be induced by the cold treatment process itself, possibly as a way to minimize the effects of increased invertase activity until a higher temperature condition returns. Together, this report suggests that the invertase inhibitor may play an important role in controlling acid invertase activity in different clones and basal acid invertase activity may be useful to screen potato breeding clones for low glucose forming potential without the need for a significant period of cold treatment.

In order to meet the demands of the processing quality, the activity of invertases should be drastically reduced, which could disturb other metabolic activities occurring in the potato plant. In stead of constitutive expression, an expression vector using Nt-VIF cDNA (*Nicotiana tabacum* vacuolar inhibitor of beta fructosidase, a vacuolar invertase inhibitor from *Nicotiana tabacum*) under the tuber-specific class I patatin promoter was used by Cheng et al. (2006). After storing potato tubers of 14 transgenic lines at 4^0C or 20^0C for 30 days, their activities of vacuolar invertase and reducing sugar content were analysed. The results

clearly showed that there were no significant differences in reducing sugar content between transgenic and untransformed or control tubers stored at 20^0C. However, reducing sugar content of transgenic lines was reduced at 4^0C compared to the control, from 34.0% to 76.8%. The selective inhibition of vacuolar invertase by potato tuber specific promoter class I patatin promoter does not affect tuber number, tuber weight, starch content and other development aspects. Bhaskar et al. (2010) adopted RNAi approach for silencing the vacuolar invertase gene function in potato. They reported that nearly complete silencing of the vacuolar invertase gene function which prevented reducing sugar accumulation in cold-stored tubers. The RNAi potato lines revealed a clear correlation between the vacuolar invetase gene expression and the accumulation of reducing sugars in cold-stored tubers. Thus cold induced sweetening and its associated acrylamide problems can be controlled by suppression of the vacuolar invertase gene by such RNAi-based gene silencing approach.

The potato breeding community made efforts for many decades to develop "cold chippers," cultivars that do not accumulate reducing sugars during cold storage (Mackay et al. 1990; Thill and Peloquin 1995; Xiong et al. 2002). None of the potato cultivars in production can be stored at cold temperatures without undergoing cold induced sweetening. In contrast to *S. tuberosum*, several wild *Solanum* species, including some accessions of *Solanum raphanifolium* (2n = 2x = 24), show excellent potential as cold chippers (Hamernik 1998; McCann et al. 2010). Potato chips processed from tubers of *S. raphanifolium* accessions stored for 3 months at 2^0C showed acceptable color (Hamernik 1998). This cold-chipping phenotype was shown to be heritable by crossing cold-chipping *S. raphanifolium* accessions with other diploid potato clones (Hamernik 1998; Hamernik et al. 2009).

2.6. Our national scenario of potato research: At present food and nutritional security is an important global priority. India is a vast country with large and rapidly growing population. Therefore, potato is regarded as a potential crop to fight hunger and malnutrition. In recent years, there is growing demand for processed potato products in our country. The reason is increased urbanization, rise in per capita income, increase in number of working women and expanding tourist trade. Apart from human consumption as vegetable, we need to promote the development of potato processing sectors. Therefore, there is an urgent need to provide good quality and adequate quantities of raw materials round the year.

A large number of potato cultivars have been released by CPRI (Central Potato Research Institute, Shimla) and AICPIP (All India Coordinated Potato Improvement Project) by using mostly conventional breeding programs. These cultivars are suitable for different agro-climatic regions in our country. In terms of generation time, they belong to three categories namely early, medium and late-maturing. Although many potato varieties are available in our country but not all of them are suitable for processing industries. For example, the potatoes required for processing need to have 21-23% tuber dry matter and reducing sugars below 150 mg per 100 g fresh weight of tubers. Because the yield, texture and quality of processed potato products largely depend upon the two most important parameters, the tuber dry matter and reducing sugar content. So far processing varieties are concerned; Kufri Chipsona-1, Kufri Chipsona-2 along with a few others are notable medium-maturing varieties. Several biochemical attributes of Chipsona varieties made them suitable for processing sectors. Some other salient features of the existing Indian cultivar varieties include resistance to late blight, ability to give high yields under short days, less duration growing periods suitable for the plains, tolerance to several pathogenic viruses, immunity to wart disease and resistance to

nematodes. All these important attributes in potato clearly reflect the success of our conventional breeding programs. Some laboratories are also involved in improving the nutritive quality of potato tubers (Chakraborty et al. 2000). However, the common problem of cold sweetening still exists in the above varieties. In other words, post harvest storage of this food crop is equally important. Again, the level of sugar accumulation during low temperature storage also depends upon the cultivar genotype as well as environmental factors. Efforts are also being made in identifying potato clones/cultivars that accumulate less reducing sugars. Optimization of potato production in terms of yield and quality needs to be ensured by overcoming several biotic and abiotic stresses. Apart from this, there is an urgent need to develop indigenous varieties that accumulate acceptable levels of hexose and soluble sugars during storage at low temperatures.

2.7. Rationale behind the present study: Potato is a very important member of the *Solanaceae* family that includes several other economically important species such as tomato, eggplant, petunia, pepper and tobacco. The cultivated potato varieties are usually heterozygous, autotetraploid (2n=4x=48), and suffer acute inbreeding depression. Moreover, most cultivars are susceptible to many devastating pests and pathogens. These attributes are the major barriers to potato improvement using classical breeding approaches. Therefore, advanced molecular breeding strategies need to be facilitated in this vegetable crop.

By linkage and association studies quantitative trait loci (QTLs) and quantitative trait alleles (QTAs) have been identified for potato tuber yield and starch content and chip quality (i.e., cold sweetening). All these loci were found to colocalize with three independent potato invertase loci encoding five invertase genes: *Pain-1*(present in chromosome III encodes vacuolar invertase)*, InvGE, InvGF, InvCD141* and *InvCD111*. Functional studies on natural

variants of invertase genes in some potato cultivars and clones were carried out, and a number of cDNA alleles corresponding to each of these genes were cloned and sequenced (Draffehn et al. 2010). Different potato cultivars/clones with their rich genetic resources have become quite attractive systems both in terms of basic and applied aspects of plant invertase research. It was also shown that multiple allelism occurs for potato genes that affect morphological characteristics and various metabolic pathways (van de Wal et al. 2001). High level of natural allelic variation is common in potato invertase genes. Therefore, in potato it is likely that depending on the cultivar genotype, kind of tissues and the subcellular locations, more allelic variants of acid invertases and the corresponding isoforms are involved in sucrose metabolism. Therefore, in-depth understanding of structure and function of different invertase genes, their allelic variants in potato, and various factors that influence their expression in different tissues, would be quite useful. Because such efforts are very essential for adopting advanced molecular breeding strategies.

As mentioned earlier, there are a number of high-yielding Indian potato cultivars including some processing varieties suitable to different agro climatic zones of the Indian subcontinent. These cultivars vary with regard to their overall genetic makeup, crop yield, maturation time, disease resistance, tuber dry matter content, reducing and soluble sugar content, and also extent of cold-induced sweetening. With regard to molecular cloning, characterization and expression studies, there is no report available on various invertase isoenzymes from the Indian potato cultivars till to date. Therefore, we need to know particularly about the allelic variants of the acid invertases in the individual potato cultivars. The correlation between the cultivar-specific cold-induced hexose accumulation and available invertase activity in the tubers is also lacking. At present it is commonly believed that in potato, invertases, together

with other proteins, are involved in the accumulation hexoses and other soluble sugars in the cold-stored potato tubers as reported in the literature. Therefore, various molecular strategies could be adopted for inhibition of acid invertase gene functions in these potato cultivars. Such efforts will be useful in redesigning of potato crops to obtain ‘value-added’ agricultural products; subsequently to benefit the farmers, consumers and the processing sectors. Keeping in view the above points, the objectives were framed for the present study.

Objectives

3. Objectives

- **To study the changes in sugar content and soluble acid invertase activity during low-temperature storage of potato tubers**

- **To carry out genomic/cDNA cloning and characterization studies on soluble acid invertase in the potato cultivar(s)**

- **To make genetic constructs by using soluble acid invertase cDNA/genomic clones in order to repress its gene function in the potato tuber**

- **To generate transgenic potato lines with the above constructs followed by screening for desired ones**

Materials and Methods

4. Materials and Methods

4.1. Materials

4.1.1. Procurement of potato germplasm and other materials: The germplasm of various potato cultivars such as Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Chandra-mukhi (KCM), Kufri Jyoti (KJ), Kufri Ashoka (AS) and Kufri Pukhraj (PR) (for our convenience the names in short form are shown within parenthesis) were procured from Central Potato Research Institute (CPRI), Shimla, India and are routinely maintained in our laboratory on MS basal medium.

The required 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 present 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.

4.1.2. Establishment of potato germplasm: The high-yielding Indian potato cultivars namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chandramukhi, Kufri Jyoti, Kufri Ashoka, and Kufri Pukhraj as used in this study were procured from Central Potato Research Institute (CPRI), Shimla, India. These cultivars vary with regard to their genetic makeup, maturation time and growth in different agro-climatic zones of the Indian subcontinent. The cultivars Kufri Chandramukhi and Kufri Ashoka 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, 70% relative humidity) for four to five weeks on MS basal medium. The aseptically grown micropropagated potato plantlets of the above cultivars were used for genomic DNA isolation. Kufri Chipsona-1 (a processing cultivar) was used in the present study for *Agrobacterium*-mediated genetic transformation.

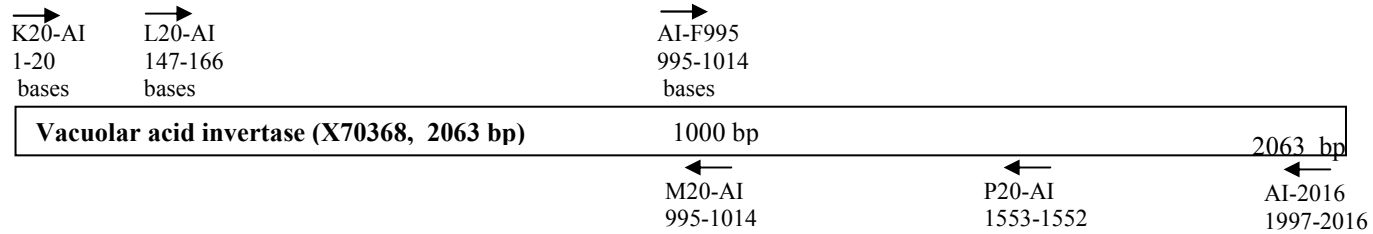
4.1.3. Bacterial strains and plasmids

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, whereas, those transformed with pUC19 plasmid were 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. pUC19 Vector: pUC19 (GenBank Acc. No. X02514) is a commonly used plasmid cloning vector in *E.coli* (Yanisch-Perron et al. 1985). Its size is 2686 bp. It is a high copy number plasmid. It carries a 54-bp multiple cloning site that contains unique sites for a number of different hexanucleotide-specific restriction endonucleases. The binary vector pBI121 (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 Acc. No. AF485783) was used as control; and *GBSSI*-GUS gene fusion was made based on this binary vector.

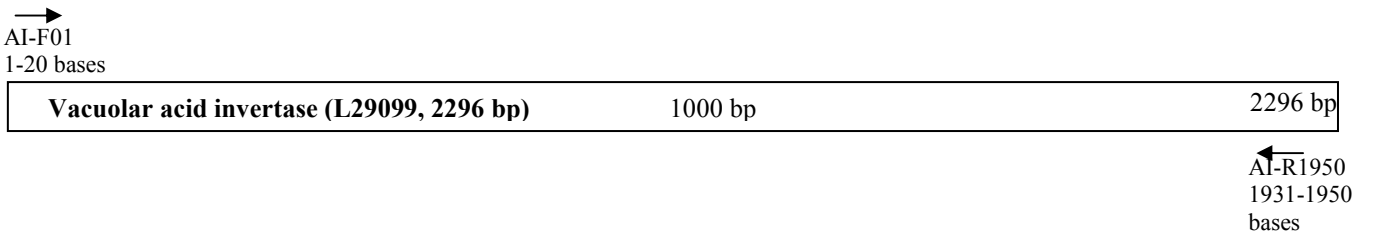
4.1.4. Designing of oligonucleotide primers: The following oligonucleotide primers were designed and the details of the different primer sequences are described below:

Primers based on Acid invertase cDNA sequence: Based on the available potato vacuolar acid invertase cDNA sequence in the database (GenBank Accession No. X70368) corresponding to the potato cultivar Desiree, the following oligonucleotide primers were designed: the forward primer K20-AI, 5′-AGTACCATTCCAGTTATGAC-3′ (corresponding to the bases 1-20); forward primer L20-AI, 5′-CCGATCCTCAACAACCAGTC-3′ (corresponds to the bases147-166); forward primer AI-F995, 5′-GCAAGATCACTATGCTATTG-3′(corresponds to the bases 995-1014); reverse primer M20-AI, 5′-CAATAGCATAGTGATCTTGC-3′ (complementary to the bases 995-1014); reverse primer P20-AI, 5′-ACTGGCGTTAGCT CAGATAG-3′ complementary to the bases 1533-1552) and the other reverse primer AI-2016, 5′-TAAGTAGAGTATAACACTAC-3′ (complementary to the bases 1997-2016). Likewise, based on the vacuolar acid invertase cDNA sequence from potato (GenBank Accession No. L29099) the following oligonucleotide primers were designed: the forward primer AI-F01, 5′-GCACGAGTATGGCCACGCAG-3′ (corresponding to the bases 1-20); the reverse primer AI-R1950, 5′-GAAGAAGATATGGCTTGATG-3′ (complementary to the bases 1931-1950). The locations of the different oligonucleotide primers are schematically shown below:

A



B



GBSSI gene-specific primers: The different primers were designed based on the available granule-bound starch synthase (*GBSSI*) gene sequence in the database (GenBank Accession No. 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 following primers were designed which are specific to constitutive actin gene and used in the present study, also described by Dohmann et al. (2005). 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 No. AF485783) as given below:

CaMV 35S promoter-specific primers: Forward primer AF35-01, 5'-ATTCAAATAG AGGACCTAAC-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'-TGGTCAGTCCCTTATGTTAC-3' corresponding to the bases 5832-5851 and two reverse primers US-R6747, 5'-CAAGTCCGC

ATCTTCATGAC-3' complementary to the bases 6728-6747 and US-R7719, 5'-TTCGAGC TCGGTAGCAATTC-3' complementary to the bases 7700-7719.

nptII gene-specific primers: Forward primer NPT-F1, 5'-GTTTCGCATGATTGAACAAG-3' corresponding to the bases 2831-2850 including initiation codon of Kanamycin resistance ORF and reverse primer NPT-R01, 5'-CGCTCAGAAGAACTCGTCAA-3' complementary to the bases 3616-3635 including stop codon.

pBI121 binary vector specific primers: Forward primer ORB-F01, 5'-TTGGCATGCACAT ACAAATG-3'corresponding to the bases 2364-2383 outside the right border.

4.2. Methods

4.2.1. Genomic DNA isolation from potato cultivars: Different amounts of plant material ranging from 0.1 g to 2.0 g were used for total DNA isolation using a modified method based on the protocol describes by Dellaporta et al. (1983). One of the isolation steps involved potassium acetate to remove carbohydrates and other bulky impurities. Briefly the steps are given here: ~2.0 g of plant material was ground to fine powder in the presence of liquid nitrogen and transferred quickly to a conical flask containing 15 mL of 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 mixed well and incubated at 65^{o}C for 20 min with intermittent gentle shaking. 5.0 mL of 5.0 M potassium acetate solution was added, mixed vigorously and incubated further on ice for 20 min and then centrifuged at 4000g, 4^{o}C for 20 min. The supernatant was filtered through two layers of fine muslin cloth and 0.70 vol of isopropanol was added, 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 crude DNA pellet was washed with ice

cold 70% ethanol, air dried and suspended in 500 µL of TE buffer (10 mM Tris-HCl pH 8.0) and 1.0 mM EDTA (pH 8.0). For further purification of DNA, DNase-free RNase treatment was carried out 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 finally dissolved in 200-250 µL of TE buffer. For 0.1 to 0.2 g plant materials, the same protocol was adopted by scaling down the necessary steps accordingly. The quality and quantity of the potato DNA samples were initially checked spectrophotometrically by measuring the A_{260}/A_{280} ratio and absorbance at 260 nm, respectively.

4.2.2. Agarose gel electrophoresis: Agarose gel electrophoresis was performed using standard methods (Sambrook et al. 1989). 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.

4.2.3. Restriction digestion of DNA: Restriction endonucleases are the enzymes that recognize short DNA sequences and cleave the double-stranded DNA at specific sites within or adjacent to the recognition sequences. Routinely restriction digestion was carried out in a reaction volume of 20 µL for 3-4 hrs at specified temperatures. The main components of a restriction digestion reaction are as follows: 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.

4.2.4. RNA Isolation from potato tissues (Phenol SDS/lithium chloride method): Different methods have been reported in the literature for the isolation of RNA from plant tissues.

However, isolation of good quality of RNA in terms of intactness and quantity as well is relatively difficult from plant tissues containing high polysaccharides, phenolics, nucleases and other storage macromolecules. Therefore depending on the plant materials suitable isolation protocol needs to be followed or modified. Here a simple, cost-effective and efficient method described by Gilman (1987) was used for the isolation of RNA from field-grown potato (*Solanum tuberosum* L.) tubers. Approximately, 2.0 g of plant material were frozen in liquid nitrogen and pulverized to a fine powder and homogenized in 13 mL of RNA extraction buffer [Composition of RNA Extraction Buffer: 100 mM LiCl, 100 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 7.45), 1.0% SDS, 0.2% β-Mercaptoethanol] followed by direct extraction with 4.5 mL phenol/chloroform. Samples of the homogenized slurry were transferred to 30 mL screw-cap centrifuge tubes and centrifuged at 7700g for 30 mins at 4^0C. Again 4.0 mL of chloroform was added to upper aqueous layer. Mixed properly and centrifuged at 7700g for 10 mins at 4^0C. In order to remove DNA impurities, RNA was selectively precipitated from the aqueous phase by adding one-third volume of 8.0 M LiCl under ice-cold condition followed by incubation for at least two hours to overnight. The crude RNA was pelleted down by centrifugation at 10000 g, 4^oC for 15 min. The crude RNA pellet was air dried and suspended in 400 µL of DEPC treated water. For further purification of RNA, solvent extraction was carried out twice using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1), followed by precipitation of RNA using 0.1 vol of 3.0 M sodium acetate (pH 5.5) and 2.5 vol of ethanol and finally dissolved in 200-250 µL of DEPC treated water. The yield of RNA was up to 150 µg g^{-1} of tuber tissue. The same RNA isolation protocol also worked well in case of micropropagated potato plantlets as source materials. The intactness of the total RNA samples was checked by denaturing formaldehyde agarose gels and further

confirmed by RT-PCR (Reverse Transcription followed by the Polymerase Chain Reaction) using different potato gene-specific oligonucleotide primers.

4.2.5. Formaldehyde agarose gel electrophoresis: RNA samples were denatured by treatment with formamide and separated by electrophoresis through agarose gels containing formaldehyde (Sambrook et al. 1989). Preparation of 100 mL of 1.5 % agarose gel containing formaldehyde: 1.5 g of agarose in 62 mL of sterile water was boiled and cooled up to 55ºC. 20 mL of 5X MOPS electrophoresis buffer and 18 mL of deionized formaldehyde was added and the gel was casted in gel casting apparatus and was allowed to set for one hour at room temperature. 1.5% agarose gel is suitable for resolving RNAs in the 0.5-8.0 kb size range. The gel was submerged in the 1X MOPS electrophoresis buffer. For denaturation of RNA samples, 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 and incubated at 65ºC for 15 min. Then 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} until the bromophenol blue was migrated to the end. 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.

4.2.6. 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 quickly chilled on ice.

4.2.7. Polymerase chain reactions: PCR is iterative process, consisting of three cycling parameters, 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 composition for a typical 50 µL PCR reaction was set as follows: 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.

4.2.8. Klenow enzyme treatment: 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 along with 3' recessed termini. As a polishing step here, Klenow treatment served two purposes: firstly, removal of the extra 'A' residue at the 3' ends; secondly, filling up the recessed 3' termini in the amplified DNA products. 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 carried out at 28°C for 40 min and then terminated by incubating at 65°C for 5-7 min.

4.2.9. Purification of DNA fragments: For purification, the following methods were adopted:

Recovery of DNA bands through electro-elution: For this purpose, the dialysis membranes were used which were sterilized and treated to make them free from any contaminating impurities. Sterilized dialysis membranes were prepared as follows:

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 clean microfuge tube and then solvent extraction was done with saturated phenol twice followed by mixture of phenol and chloroform (1:1). Centrifugation was done at 8,000 rpm for 10 min. Upper aqueous layer was transferred to a clean microfuge tube and $1/10^{th}$ volume of 3M sodium acetate (CH_3COONa) was added followed by the addition of 2.5 volumes of ethanol. The tubes were mixed well and kept at -20°C. 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.

Recovery of DNA bands using Qiagen kit: QIAEX II agarose gel extraction protocol was followed for purification of DNA bands. For this, DNA samples were run in two lanes on 0.8 % agarose in 1X TAE buffer along with a control lane in which sample was also loaded. The gel was run for sufficient time and then, the control lane was excised with the help of a clean scalpel and visualized on a UV-transilluminator. The position of DNA bands of interest was marked and then, corresponding bands were excised from sample lanes without UV exposure. The gel slices were weighed separately in 1.5 mL microfuge tube. Following this, three volumes of Buffer QX1 was added to one volume of gel as per the manufacturer's instructions. QIAEX II solution was resuspended by vortexing for 30 seconds and 20 µL of it was added to each sample. In order to solubilize the agarose, the samples were incubated at 50°C for 10 min with intermittent vortexing after every 2 min. This was done to keep QIAEX II in suspension. 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. In the same manner, 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. 7 µL of sterile water and 7 µL of TE buffer were added to the pellet and resuspended by vortexing. It was incubated at 50°C for 10 min and then centrifuged for 30 seconds. The supernatant containing the purified DNA was carefully transferred into a clean microfuge tube. The above two steps were repeated to increase the yield. Lastly, the eluates were combined and 2.5 µL was loaded on 0.8 % agarose gel in order to check the yield.

4.2.10. Ligation reaction: A ligation reaction was set up in order to ligate the insert into 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. It can join blunt as well as the cohesive end termini. The main components of a ligation reaction were as follows: Vector, 0.3-0.5 μg; Insert, 0.5-1.0 μg and T4 DNA Ligase enzyme and buffers were used according to the manufacturer's instructions. 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 at ~15°C for cohesive end ligation and at 21°C for blunt end ligation. The reaction was carried out for 3-5 hrs.

4.2.11. Genetic transformation of E. coli DH5α with plasmid vectors: *E.coli* DH5α was transformed with various DNA samples using the $CaCl_2$ method (Mandel and Higa 1970).

Preparation of competent cells: A single bacterial colony was inoculated in 25 mL of Luria broth and incubated at 125 rpm at 37°C for overnight. A small aliquot of overnight-grown culture was used to re-inoculate 25 mL of fresh Luria broth and then incubated at 37°C with shaking to obtain an O.D. around 0.4-0.6 at 590 nm. The culture was kept on ice to arrest the cell growth. Cell pellet was recovered by centrifuging the cells at 6500 rpm for 10 min. The pellet was resuspended properly in 10 mL of ice-cold 100 mM $CaCl_2$. The cells were recovered by centrifugation at 6500 rpm for 10 min. The pellet was resuspended in 1.0 mL of ice-cold 100 mM $CaCl_2$ and kept in ice for 2½ hrs.

Transformation of plasmid vector: 100 μL of the competent cell suspension was dispensed in sterile microfuge tubes and kept at 0°C. 6-7 μL of ligation mix was added to each tube containing competent cell suspension, mixed well and kept at 4°C for 30 min. Heat shock was performed at 42°C for 2 min to all the tubes, followed by the addition of 1 mL LB and

incubation at 37°C for 1½ hrs. Centrifugation was carried out at 6500 rpm for 6-7 min and 600-800 µL of supernatant was discarded to concentrate the cells. The pellet was resuspended and 100 µL of the above transformed cell suspension was plated on LA medium containing ampicillin (50 µg mL^{-1}) with X-Gal and IPTG. The plates were incubated at 37°C for 16-18 hrs. The transformants were further analyzed on the basis of blue/white colour selection in the case of pUC19 vector.

4.2.12. Plasmid isolation: Plasmid isolation was carried out by the following two methods: *Alkali lysis method:* This method was essentially same as described by Birnboim and Doly (1979) having the following steps in mini scale preparation. Transformant *E. coli* colony was inoculated in 25 mL LB containing either ampicillin or kanamycin (working conc. of 50 µg mL^{-1}). The culture was incubated at 37ºC overnight with shaking at 120 rpm. 1.5 mL overnight grown culture was centrifuged and the supernatant was decanted. 200 µL of Solution I was added followed by addition of 30 µL lysozyme (stock 10 mg mL^{-1}) and mixed well. Then 400 µL of Solution II was added and mixed gently by inverting the tubes. Then 300 µL of Solution III was added and mixed well till curdy white precipitate was formed. The tubes were kept in ice for 30 min by high speed centrifugation for 15 min. The supernatant was transferred to a sterile microfuge tube and 2.0 µL of DNase free RNAse (10 mg mL^{-1}) was added, mixed and incubated at 37ºC for 30 min. Extraction was done with equal volume of phenol and chloroform mixture (1:1) followed by equal volume of chloroform. Upper aqueous layer was transferred to a sterile microfuge tube followed by the addition of equal volume of isopropanol. Kept at 4ºC for 20 min, and centrifuged at 12,000 rpm for 15 min. Supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was air-dried and dissolved in 30 µL of TE buffer and stored at -20ºC.

Boiling method: Plasmid isolation in mini scale was carried out by boiling prep method as described by Holmes and Quigley (1981). In this process bacterial transformant colonies were inoculated aseptically in 4.5 mL LB containing ampicillin in test tubes. The culture was incubated at 37°C/120 rpm for overnight. Cells were harvested from 1.5 mL overnight grown culture in microfuge tubes. The pellet was loosened by vortexing, followed by resuspension in 800 µL of STET buffer. 30 µL of lysozyme was added to the bacterial suspension and mixed well. Each microfuge tube containing cell suspension was kept in boiling water bath for 1.5 min. After cooling down to room temperature high speed centrifugation (12,000 rpm) was carried out for 15 min. After removing the pellet, 2.0 µL of RNase solution was added to the supernatant to remove the contaminating RNA. After incubation at 37°C for 45 min equal volume of phenol: chloroform was added, mixed for 5-7 min and centrifugation was performed at 10,000 rpm for 10 min. To the upper aqueous layer, $1/10^{th}$ volume of 3M sodium acetate (CH_3COONa) and equal volume of isopropanol was added and incubated at 4°C for 45 min for the precipitation of plasmid DNA. Then the tubes were centrifuged at 12,000 rpm for 15 min. The DNA pellet was washed with chilled 70 % ethanol to ensure the removal of excess salts and other impurities. Finally, DNA pellet was air dried at room temperature and dissolved in 20-30 µL of TE buffer.

4.2.13. Sequence analyses: The nucleotide sequences of the cDNAs were analyzed by NCBI Blast tools. The deduced 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). In order to calculate the theoretical molecular weight, isoelectric point (pI), and amino acid composition of the predicted amino acid sequences of vacuolar invertase 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. Likewise, the different ProtScale tools of ExPASy were used for prediction of hydrophobic character (Kyte and Doolittle 1982), 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.bluetractorsoftware.co.uk/). Isochore plots were generated by another EMBL-EBI sequence analysis tool (http://www.ebi.ac.uk/Tools/ emboss/cpgplot/). For multiple sequence alignment the ClustalW2 tool, an EMBL-EBI sequence analysis tool with its default parameters (http://www.ebi.ac.uk/Tools/) was used. Both the ClustalW2 and the MultAlin software (Corpet 1988) (http://www.multalin. toulouse.inra.fr/multalin/); tools were used in predicting the consensus sequence. In order to generate phylogenetic tree, multiple sequence alignment was done first by the MultAlin software, followed by the neighbor-joining method using MEGA 5.0 software (Saitou and Nei 1987; Tamura et al. 2011). For the above purpose, a total of 45 predicted vacuolar invertase sequences covering 27 plant species from different taxonomic groups were used: *Solanum tuberosum* cultivars (ACC93584, ACC93585, ABF18956, AAQ17074, ADM47340, AAA50305, CAA49831); *Solanum lycopersicum* cultivars (NP_001234843, NP_001234618, BAA01954, CAA78060, CAA78061); *Capsicum annum* (AAB48484); *Nicotiana tabacum* (CAC83577); *Cucumis melo* (ABX55832); *Ipomoea batatas* (AAK71505, AAK71504, AAD01606); *Oryza sativa* Japonica Gr. (AAK72492, AAD10239); *Oryza sativa* Indica Gr. (CAH67112); *Coffea canephora* (ABI17894); *Daucus carota* (CAA53097, CAA53098, CAA53099, CAA47636); *Glycine max* (XP_003533514); *Citrus sinensis* (BAF34363, AAL27709); *Gossypium hirsutum* (ACQ82802); *Ricinus communis* (XP_002510944); *Pachysandra terminalis* (CBM41476); *Sorghum bicolor* (XP_002446857); *Vitis vinifera*

(AAB47172); *Cichorium intybus* (CAD12104); *Arabidopsis lyrata* (XP_002888009); *Arabidopsis thaliana* (NP_564798); *Brassica oleracea* (AAG36943, AAG36942); *Pyrus pyrifolia* (BAF35859); *Populus trichocarpa* (XP_002303519); *Vigna radiata* (BAA01107); *Phaseolus vulgaris* (AAB68679); *Vicia faba* (CAA89992) and *Pisum sativum* (AAM52062).

4.2.14. Genetic transformation of Agrobacterium strain:

Electroporation: *Agrobacterium tumefaciens* strain LBA4404 was grown in 20 mL of YEM broth for overnight at 28ºC/160 rpm. The overnight grown bacterial culture was transferred to prechilled 30 mL oak ridge centrifuge tube and the bacterial cells were pelleted by centrifugation at 6500 rpm for 10 min at 4°C. The cell pellet was washed thrice with ice cold 10% glycerol (10-15 mL). Finally, the cell pellet was 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. To the aliquot of electrocompetent cells (20 µL) in ice cold microfuge tube, plasmid DNA (1.0-3.0 µg) was added and mixed well. The cuvette was placed in the chamber slide, the slide was pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber and pulse was made. 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 selection of transformed cells, the cells were concentrated (centrifuged at 6000 rpm for 10 min) and then 100 µL was spread on YEM (rif^{15} + kan^{50}) plates.

Transformation by triparental mating: In triparental mating, the donor strain (*E. coli* harboring Ti plasmid with gene of interest) was mated with conjugal 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). After mating, *A. tumefaciens* strains harboring the engineered plant transformation vector (Ti plasmid with gene of interest) were selected by growth in the presence of antibiotics for which resistance is provided by genetic markers unique to those recipient *Agrobacterium* and Ti plasmid vector (Ti plasmid with gene of interest). The steps involved were: 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. A single colony of each freshly grown strain was patched separately close to each other on Luria agar plates. The three patches were mixed with sterile loop and the plates were incubated at 28ºC for 24 hrs. The small portion of triparental patch was picked with the help of loop and serially diluted in 0.9% saline and 100 μL of it was spread on Luria agar containing antibiotics rifampicin (15 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) and incubated at 28ºC. Single colonies of transformed *Agrobacterium* were streaked on YEM medium containing rifampicin (15 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) for further use.

4.2.15. Agrobacterium mediated co-cultivation: The well characterized transformed *Agrobacterium* strain (corresponding to individual genetic constructs) was used for co-cultivation. 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 of five to six weeks old potato plantlets (Kufri Chipsona-1), grown in MS medium, were co-cultivated with diluted culture for 10 min, blot the internodal stem segments on sterile filter paper, placed horizontally on MS basal medium and 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 initial 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. Around ten independent transgenic potato lines corresponding to each binary genetic construct were screened by PCR approach using different sets of gene-specific primer pairs.

4.2.16. Production of mini tubers, and storage at different experimental temperatures: After proper hardening and acclimatization, the selected tissue culture raised transgenic and nontransgenic potato plantlets (including control and other cultivars) were grown in polybags for mini tuber production in the restricted experimental plots. Mini tubers were harvested from the cultivars as well as from transgenic lines corresponding to each genetic construct after three and half months of cultivation in the field (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 (25^0C and 4^0C).

4.2.17. Semi-quantitative RT-PCR: Semi-quantitative RT-PCR was carried out to know the vacuolar invertase expression pattern in the freshly harvested and cold-stored tubers from the seven field-grown potato cultivars. 2.0 μg of total RNA (free from DNA impurities) from each potato sample was used for reverse transcription in a reaction volume of 20 μL using

oligo $(dT)_{18}$ primer and the 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 vacuolar invertase cDNA specific forward and reverse primers, K20-AI and M20-AI, and 1.0 unit of *Taq* DNA polymerase (Bangalore Genei). After initial denaturation at 94ºC for 1 min 30 s, the thermal cycling parameters during PCR were: denaturation at 94ºC for 1 min, annealing at 55ºC for 2 min, polymerization at 72ºC for 2 min for 30 cycles followed by final extension at 72ºC for 5 min. As a control, the primers FW-ACT2 (forward primer) and RV-ACT2 (reverse primer) specific to the housekeeping actin gene were used to amplify ~250 bp fragment using the same 3.0 µL individual RT mixture as template. The invertase and actin specific RT-PCR products were resolved in 0.8% and 1.2% agarose gel electrophoresis, respectively**.**

4.2.18. Extraction of soluble sugars: Total soluble sugars were extracted from the freshly harvested tubers and tubers stored at different temperatures (4ºC and 25ºC) for four and eight weeks. The potato tuber slice (~ 200 mg) was crushed thoroughly using mortar and pestle to make fine paste, 1.0 mL of 80% alcohol was added, mixed well and then transferred the slurry into microfuge tube and rinsed with 1.0 mL of 80% alcohol and transferred to the same tube. Then incubated in water bath at 80ºC for 45 min, centrifuged at 10000 rpm for 10 min and the supernatant containing soluble extract was collected in a beaker. The pellet was re-extracted with 80% alcohol and the supernatant obtained was collected in the same beaker. The supernatant kept at 65ºC for nearly two hours to ensure complete evaporation of solvent. The residual matter in the beaker was thoroughly solubilized in a total volume of 1.5 mL sterile water and the soluble extract was transferred to a clean and fresh microfuge tube. 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).

4.2.19. Estimation of reducing sugars by Nelson-Somogyi's Method: The reducing sugars when heated with alkaline copper tartrate reduce the copper from Cu^{2+} to cuprous oxide form. 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.

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

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

100 µL and 200 µL aliquots of each soluble sugar extract were taken in clean test tubes. Standards prepared by taking different aliquots of D-glucose (stock 0.1 mg mL^{-1}) so that the tubes contained the following amounts 10, 20, 40, 60, 80, 100, 120, 140,150 µg along with blank. 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.

4.2.20. Estimation 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.

4.2.21. Protein extraction from potato tubers: 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.

4.2.22. 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. 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) and 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 O.D. was taken at 750 nm. Standard curve was made using BSA stock (0.2 mg mL^{-1}) 20 µg, 40 µg, 60 µg, 80 µg and 100 µg.

4.2.23. SDS PAGE: SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), is a technique used to separate proteins 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 SDS-PAGE gel casting apparatus was arranged and all the components of resolving gel were mixed well in a test tube and gel was poured into the apparatus. A layer of water saturated isobutanol was also poured over the gel immediately. Gel was allowed for polymerization for minimum 30 min. After polymerization, isobutanol was decanted and gel was washed with water. Stacking gel was poured over the resolving gel and comb was placed. Gel was allowed for polymerization for 30 min. Equal volume of 2X sample buffer was added to the protein samples and boiled for 3-5 min, cooled to room temperature and loaded in the

gel. Gel electrophoresis was carried out at 100 volts for 2 hrs in 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 removed from the apparatus, stained in staining solution for one hour followed by destaining, protein bands were visualized and photographed.

4.2.24. Antigenic peptide and polyclonal antibody: The entire deduced amino acid sequence of vacuolar invertase as isolated in this study (639-amino acid *KC-VIN2,* Accession No. ACC93585) 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 16-mer polypeptide NGPLYHKGWYHLFYQY, corresponding to the amino acids 123-138 of *KC-VIN2* (see Fig. 7), 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.

4.2.25. 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 vacuolar invertase 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.

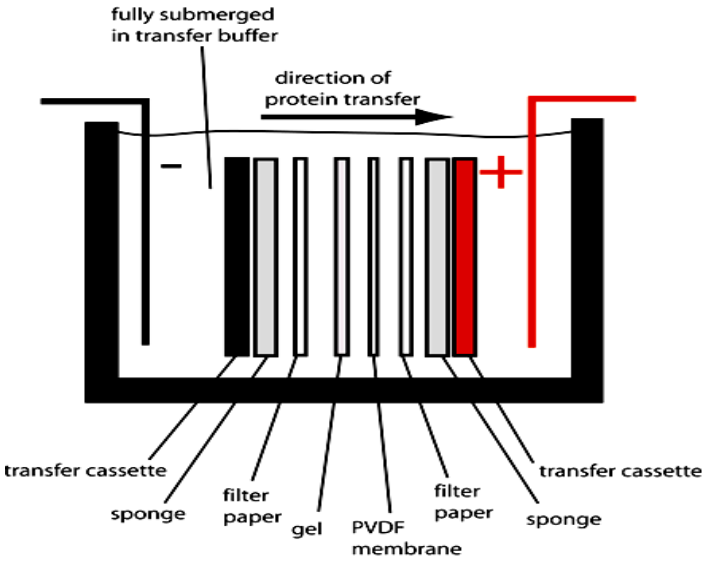


Fig. 2. Schematic view of setting of protein transfer sandwich for electroblotting

4.2.26 Assay of vacuolar acid invertase: The determination of vacuolar invertase activity in the freshly harvested and cold-stored potato tubers was carried out as per the protocol described by Greiner et al. (1999). Approximately 500 mg of tuber sample was ground to fine powder and homogenized in 1.0 mL of extraction buffer [30 mM MOPS (3-(N-morpholino)-propanesulphonic acid), 250 mM sorbitol, 10 mM $MgCl_2$, 10 mM KCl, 1 mM phenylmethyl sulphonyl fluoride (PMSF)], followed by centrifugation for 10 min (6000g, 4^0C). Crude extracts of potato tubers usually contain a small protein that has an inhibitory effect on soluble acid invertase activity (Pressey 1967; Bracho and Whitaker 1990). However, Ross and Davies (1992) have pointed out that techniques such as rapid vortexing and foaming of extracts, which were developed to destroy the inhibitor, are severe and that a possible loss of invertase could occur. In this study, the enzyme activity was assayed without destroying the inhibitor effect. From each supernatant an aliquot of 20 µL was added to 200 µL reaction buffer containing 30 mM sodium acetate (pH 4.7) and 30 mM sucrose, followed by incubation at 30^0C for 1 hr. The reaction was then stopped by the addition of 1.0 mL alkaline copper tartrate solution and the liberated hexoses were assayed by Nelson-Somogyi's method as described by Sadasivam and Manickam (1987) with proper controls and D-glucose as the standard. Here one unit of vacuolar invertase activity refers to the amount of enzyme which liberates 1.0 nmol of hexose min^{-1} per mg of tuber FW.

M&M: Appendix-I

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

M&M: Appendix-II

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
27.	*Soluble invertase assay buffer*	
	Extraction buffer	30 mM 3-(N-morpholino) - propanesulphonic acid (MOPS), 250 mM sorbitol, 10 mM $MgCl_2$, 10 mM KCl, 1 mM phenylmethyl sulphonyl fluoride (PMSF) made in sterile water under sterile conditions
	Reaction buffer	30 mM sodium acetate (pH 4.7) and 30 mM sucrose

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.

Description of Different Enzymes:

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.

M&M: Appendix-VII

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.

Results and Discussion

5. Results and Discussion

5.1. To study the changes in sugar content and soluble acid invertase activity during low-temperature storage of potato tubers

5.1.1. Biochemical and invertase expression studies in the tubers of different potato cultivars: Some of the high-yielding Indian potato cultivars namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chandramukhi, Kufri Jyoti, Kufri Ashoka, and Kufri Pukhraj along with Desiree as a reference cultivar were used in the present study. These cultivars vary with regard to their genetic makeup, maturation time, disease resistance, and growth features. They are suitable to the different agro-climatic zones of the Indian subcontinent. Efforts were made to measure the level of reducing and total sugar content in the tubers after storage at different experimental temperatures for varying time periods. For this purpose, these cultivars were grown in the field and mini tubers were harvested, kept for three weeks at room temperature and finally shifted to various experimental temperatures, such as 25^0C, 4^0C for a period of four and eight weeks. Tissue slices from intact tubers were frozen in liquid nitrogen and stored at -70^0C for further biochemical and molecular studies. As a preliminary study, the expression pattern of vacuolar invertase gene (s) was also analyzed in the freshly harvested and cold-stored tubers from different cultivars using semi-quantitative RT-PCR, and further substantiated by protein blot analysis and by assaying extractable vacuolar invertase enzyme activity. The details are described in the following sections:

*Reducing sugar content of control (non-transgenic) potato tubers***:** Total soluble sugars were extracted from weighed amount of frozen potato tuber slices. The level of reducing sugars (in mg g^{-1} of tuber fresh weight) was estimated by Nelson-Somogy's method by using glucose as standard (Table 2 and Fig. 3). In the case of freshly harvested tubers from different cultivars, the reducing sugar level was found to be in the range of 0.55-1.80 mg g^{-1} FW. The reducing sugar level at 25°C for four weeks was found to be in the range of

0.95-1.95 mg g^{-1} FW. But the storage of tubers at 4ºC for four weeks led to the accumulation of reducing sugars, and was found to be in the range of 3.92-8.50 mg g^{-1} FW; whereas storage for eight weeks led to more accumulation of reducing sugar i.e., 12.55-24.73 mg g^{-1} FW. The reducing sugar level was found to be high in Kufri Ashoka (24.73 mg g^{-1} FW), whereas, the value was low for Kufri Chipsona-1 (13.55 mg g^{-1} FW). Around 20-fold increase of the level of reducing sugars was noted for most of the above cultivars if stored for eight weeks at 4ºC. Varietal differences were noted among the cultivars with regard to sugar accumulation. The common pattern was that the level of reducing sugars was markedly increased usually after four weeks of storage at low temperature.

Table 2. Reducing sugar contents in the freshly harvested and stored tubers at different temperatures from the potato cultivars

Potato Cultivars	Reducing sugars (mg g^{-1} FW)			
	Freshly harvested	25ºC (one month)	4ºC (one month)	4ºC (two months)
Kufri Chipsona-1	0.60 ± 0.08d	1.24 ± 0.04^{c}	3.92 ± 0.35^{d}	13.55 ± 1.16^{de}
Kufri Chipsona-2	0.75 ± 0.10d^{a}	1.03 ± 0.02^{d}	5.65 ± 0.45^{c}	14.70 ± 0.66^{d}
Kufri Jyoti	1.26 ± 0.09^{c}	1.56 ± 0.08^{b}	5.90 ± 0.53^{bc}	19.05 ± 0.33^{b}
Kufri Chandramukhi	1.34 ± 0.12^{bc}	1.84 ± 0.02^{b}	6.25 ± 0.55^{bc}	15.04 ± 0.21^{d}
Kufri Pukhraj	1.60 ± 0.15^{ab}	1.95 ± 0.07^{a}	6.73 ± 0.47^{bc}	17.15 ± 1.03^{c}
Kufri Ashoka	1.80 ± 0.13^{a}	1.57 ± 0.12^{b}	8.50 ± 0.65^{a}	24.73 ± 0.28^{a}
Desiree	0.55 ± 0.09^{d}	0.95 ± 0.04^{d}	7.18 ± 0.57^{ab}	12.55 ± 0.03^{e}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

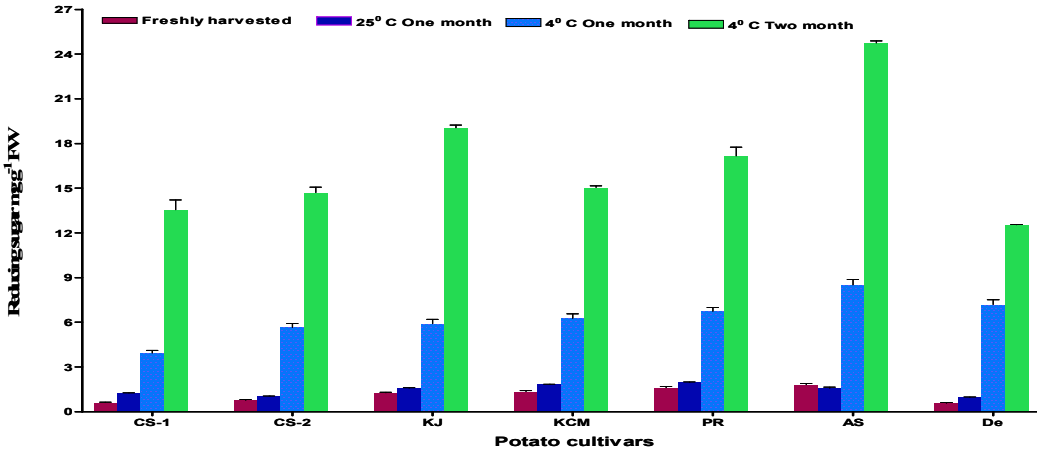


Fig. 3. Reducing sugar contents of potato tubers from different potato cultivars after storage at different temperatures

Total soluble sugar contents of potato tubers: The level of total soluble sugar was also measured in the tubers from different potato cultivars by anthrone method using sucrose as standard (Table 3 and Fig. 4). The total sugar content of freshly harvested tubers was found to be in the range of 1.85-4.85 mg g^{-1} FW. Freshly harvested tubers of Kufri Chipsona-1 had minimum soluble sugars i.e. 1.85 mg g^{-1} FW and maximum found in Kufri Ashoka i.e., 4.85 mg g^{-1} FW. The total sugar level at 25°C was found to be low, in the range of 2.45-6.93 mg g^{-1} tuber fresh weight. But the total sugar content in the tubers was markedly increased in all the cultivars after storage at 4ºC. It was found to be in the range of 10.51-16.36 mg g^{-1} FW after four weeks at 4ºC i.e., maximum in Kufri Ashoka (16.36 mg g^{-1} FW) and minimum in Kufri Chipsona-1 (10.51 mg g^{-1} FW), and considerably increased further after storage for eight weeks which was found to be in the range of 17.83-33.35 mg g^{-1} FW. The level of total sugars was increased around 10-fold for most of the above cultivars if stored for eight weeks at 4ºC. Varietal differences were also there between the cultivars with regard to total sugar accumulation. The common pattern was that the level of total sugars was increased after four weeks of storage at low temperature.

Table 3. Total soluble sugar contents in the freshly harvested and stored tubers at different temperatures from the potato cultivars

Potato Cultivars	Total sugars (mg g^{-1} FW)			
	Freshly harvested	25ºC (One month)	4ºC (One month)	4ºC (Two months)
Kufri Chipsona-1	1.85 ± 0.13^{d}	2.45 ± 0.17^{e}	10.51 ± 0.35^{d}	17.83 ± 0.85^{d}
Kufri Chipsona-2	2.73 ± 0.18^{c}	3.61 ± 0.23^{d}	12.58 ± 0.37^{c}	22.47 ± 0.71^{c}
Kufri Jyoti	3.10 ± 0.23^{c}	4.85 ± 0.27^{c}	15.43 ± 0.43^{a}	23.43 ± 0.83^{bc}
Kufri Chandramukhi	3.84 ± 0.27^{b}	5.73 ± 0.10^{b}	13.98 ± 0.33^{b}	22.52 ± 0.67^{c}
Kufri Pukhraj	2.98 ± 0.21^{c}	5.85 ± 0.17^{b}	15.93 ± 0.45^{a}	25.59 ± 0.78^{b}
Kufri Ashoka	4.85 ± 0.32^{a}	6.93 ± 0.2^{a}	16.36 ± 0.51^{a}	33.35 ± 0.93^{a}
Desiree	3.25 ± 0.25^{bc}	4.95 ± 0.23^{c}	13.34 ± 0.34^{bc}	19.35 ± 0.77^{d}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

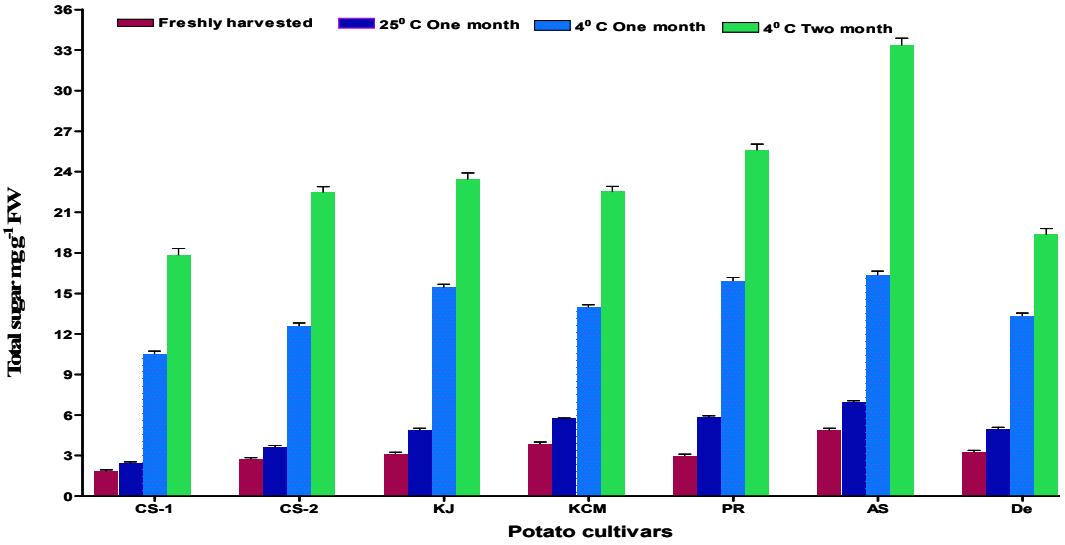


Fig. 4. Total soluble sugar contents of potato tubers from different potato cultivars after storage at different temperatures

5.1.2. Semi-quantitative RT-PCR: Semi-quantitative RT-PCR approach was carried out in order to study the vacuolar invertase gene expression at the level of transcription, both in the freshly harvested and cold-stored tubers from different potato cultivars. By semi-quantitative RT-PCR, ~1.0 kb cDNA corresponding to the 5′-end of the transcript (specific to the primer pair K20-AI and M20-AI) was amplified using total RNA from the tubers. The vacuolar invertase expression was found to be low in the freshly harvested tubers (Fig. 5A), but the level of transcripts was considerably increased in the cold-stored tubers (Fig. 5B) in all the cultivars. In the cold-stored tubers of Kufri Chipsona-1, Kufri Chandramukhi, Kufri Chipsona-2, and Kufri Jyoti relatively more accumulation of transcripts was noticed as compared to the remaining cultivars.

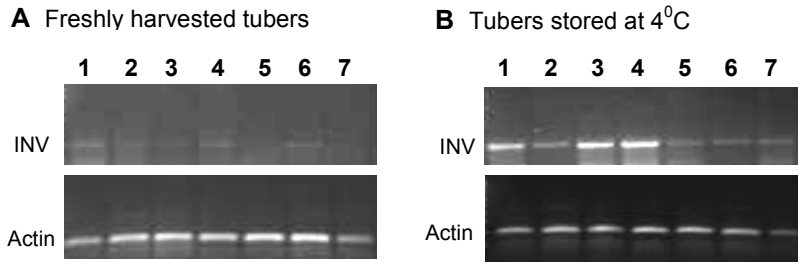


Fig. 5. Semi-quantitative RT-PCR approaches for vacuolar invertase expression analysis in the tubers harvested from the mature field-grown potato cultivars using the primers K20-AI and M20-AI. **A** Freshly harvested tubers from the different potato cultivars; Lanes 1-7 correspond to the potato cultivars Kufri Chipsona-1, Kufri Chandramukhi, Kufri Chipsona-2, Kufri Jyoti, Kufri Ashoka, Kufri Pukhraj, and the cv. Desiree, respectively; **B** Potato tubers stored at 4^0C for four weeks; Lanes 1-7 correspond to the potato cultivars in the same order as mentioned in **A**; the size of the vacuolar invertase-specific amplified product was found to be ~1.0 kb in each case. Actin-specific primers were used as control (the size of the amplified product ~0.25 kb).

5.1.3. Protein gel blot analysis of potato cultivars: Protein gel blot analyses were carried out using vacuolar invertase-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. In all the tuber extracts during low temperature storage, a band of ~ 65 kDa, an expected size of vacuolar invertase, was detected; whereas, in the case of freshly harvested potato tubers the immuno-detected band was found to be very faint or negligible suggesting very less expression of vacuolar invertase gene (data not shown).

5.1.4. Assay of soluble acid invertase in the potato cultivars: Vacuolar invertase activity in the freshly harvested and cold-stored potato tubers was measured using the protocol as described by Greiner et al. (1999). Here one unit of vacuolar invertase activity refers to the amount of enzyme which liberates 1.0 nmol of hexose min^{-1} per mg of tuber FW. In the case of freshly harvested tubers total extractable activities of vacuolar invertase in the different potato cultivars were found to be negligible, ranging from 0.048 ± 0.001 nmol $(min.mg)^{-1}$ (Kufri Chipsona-1) to 0.089 ± 0.002 nmol $(min.mg)^{-1}$ (Desiree); whereas, in the cold-stored

tubers vacuolar invertase activity was increased significantly, ranging from 0.935 ± 0.034 nmol $(min.mg)^{-1}$ (Desiree) to 3.411 ± 0.028 nmol $(min.mg)^{-1}$ (Kufri Jyoti) as shown in the Table 4.

Table 4. Vacuolar invertase activities in the freshly harvested and cold-stored tubers (at 4^0C for four weeks) of different potato cultivars

Potato cultivars	Vacuolar invertase activity [nmol $(min.mg)^{-1}$]	
	freshly harvested	4^0C (one month)
Kufri Chipsona-1	0.048 ± 0.001^{d}	1.586 ± 0.096^{b}
Kufri Chipsona-2	0.055 ± 0.001^{cd}	3.317 ± 0.072^{a}
Kufri Jyoti	$0.051 \pm .001^{cd}$	3.411 ± 0.028^{a}
Kufri Chandramukhi	$0.064 \pm .001^{bc}$	1.265 ± 0.016^{b}
Kufri Pukhraj	$0.076 \pm .013^{ab}$	1.018 ± 0.002^{c}
Kufri Ashoka	0.058 ± 0.001^{cd}	0.951 ± 0.023^{d}
Desiree	0.089 ± 0.002^{a}	0.935 ± 0.034^{d}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

The measured vacuolar invertase activities were reasonably consistent with that of semi-quantitative RT-PCR and protein blot analyses. The increased accumulation of reducing sugars in the cold-stored tubers in different potato cultivars may be partly explained by the higher soluble acid invertase activity.

Matsuura-Endo et al. (2004) worked on some Japanese potato cultivars. They observed mainly three types of changes in the potato tubers stored at 4^0C: a) increased levels of reducing sugars during storage i.e. type-1; b) almost similar pattern as in type-1, but relatively lower levels of reducing sugars throughout storage i.e. type-2; and c) increased levels of sucrose, but not reducing sugars i.e. type-3. They also showed that during storage at 4^0C the vacuolar invertase activity increased only in the type-1; but in the type-2 and type-3 cultivars, invertase levels remained very low. Our data clearly indicated that the potato cultivars namely

Kufri Jyoti, Kufri Chipsona-2, Kufri Chipsona-1, and Kufri Chandramukhi belonged to type-1. Probably, the remaining potato cultivars also belong to the same category.

The data as generated from, semi-quantitative RT-PCR, protein blot analysis and vacuolar invertase assay referred to only preliminary gene expression analyses. Further molecular and biochemical studies are required to know the expression pattern of the individual vacuolar invertase isoforms in the potato cultivars at various stages of their growth and development. Currently, many potato varieties are available in India but most of them are not suitable for processing due to the tuber dry matter and reducing sugar content. So far the processing varieties are concerned, Kufri Chipsona-1 and Kufri Chipsona-2 are notable medium-maturing varieties developed through conventional breeding program by CPRI, Shimla. Several biochemical attributes of Chipsona varieties made them suitable for processing sectors. The results of this study clearly indicated that the undesirable 'cold-induced sweetening' is a major problem during post harvest storage even in the varieties bred for processing attributes. Potato being a cool season crop, and in a country like India cannot ensure adequate supply of good quality potatoes including processing ones round the year. Hence, most of the harvested tubers need to be stored for a considerable time period at low temperature. The results clearly suggested that vacuolar invertase could play a crucial role in accumulation of reducing sugars in the cold-stored potato tubers. Molecular cloning and characterization of cDNAs encoding vacuolar invertase from a potato cultivar, and various molecular approaches for inhibition of invertase gene function are described in the following sections. With regard to the Indian potato cultivars no such reports are available in the literature till to date. Our main focus is on improving the potato cultivars suitable to our agro-climatic conditions.

5.2. To carry out cDNA/genomic cloning and characterization studies on soluble acid invertase in the potato cultivar(s)

In this section, molecular cloning strategies, characterization and sequence analyses of two cDNAs (one nearly full-length and other one is full-length) encoding vacuolar invertase isoforms from the potato cultivar Kufri Chipsona-1 (a processing variety) are described. The deduced amino acid sequences were compared with other homologs from different plant species as available till to date. The vacuolar invertase sequences between the *Solanaceae* family members were compared to examine the presence of any distinguishing sequence features (signature-type sequences). Segment-wise hydrophobic characters were compared between the plant species. Vacuolar invertase sequences from a large number of taxonomically different plant species were used in predicting the consensus sequence, and for generating the phylogenetic tree. This study provided some information not documented in the earlier reports. Attempts were also made for the isolation and characterization of partial genomic clones encoding vacuolar invertase from potato. Molecular cloning and characterization of cDNAs encoding vacuolar invertase are described below:

5.2.1. RNA isolation, RT-PCR, and vacuolar acid invertase cDNA cloning: Total RNA was isolated from five to six-week old aseptically grown plantlets of the potato cultivars, Kufri Chipsona-1 and Kufri Chandramukhi as mentioned earlier. 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. The RNA samples were used as templates in the reverse transcription (RT) reactions using the potato vacuolar invertase-specific reverse primers, AI-2016 and AI-R1950, respectively. The RT products corresponding to each cultivar were used to carry out PCR using two sets of the primer pairs: K20-AI and AI-2016; AI-F01 and AI-R1950, respectively. The size of the amplified product

was around 2.0 kb in each case (Fig. 6A, B). All these RT-PCR products cloned into the *Sma*I site of pUC19 vector which showed similar restriction patterns for a few restriction enzymes such as *Eco*RI, *Bam*HI, and *Hind*III (data not shown). Here, only the cDNA clones corresponding to Kufri Chipsona-1, designated as *AI-01* (2013 bp; the primer pair used, K20-AI and AI-2016), and *AI-02* (1945 bp; the primer pair used, AI-F01 and AI-R1950), were further characterized by sequencing in both directions by Bangalore Genei, Bangalore. The nucleotide sequences were analyzed by NCBI BLAST tool, and found to encode vacuolar acid invertase. The sequence information of *AI-01* and *AI-02* were submitted in NCBI database under GenBank Accession Nos. EU622806 and EU622807, respectively.

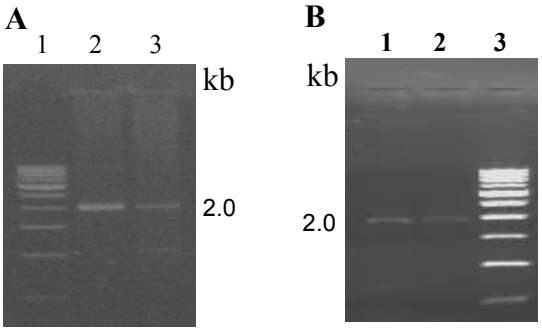


Fig. 6. RT-PCR amplification products (~2.0 kb) using total RNA from the micropropagated plantlets of different potato cultivars, and the vacuolar invertase cDNA specific primers. **A** The primers used K20-AI and AI-2016; Lane 1 0.5 kb DNA ladder; lanes 2 and 3 correspond to total RNA from the cultivars Kufri Chipsona-1 and Kufri Chandramukhi, respectively, **B** The primers used AI-F01 and AI-R1950; lanes 1 and 2 correspond to total RNA from the cultivars Kufri Chipsona-1 and Kufri Chandramukhi, respectively. Lane 3 0.5 kb DNA ladder

5.2.2. Sequence analyses of vacuolar invertase cDNAs: As shown in Fig. 7, the 2013-bp *AI-01* cDNA truncated at the 5′ terminus, contained 1910-bp ORF (bases 1-1910), the corresponding predicted protein consisted of 635 amino acids, designated as *KC-VIN1*; the 1945-bp *AI-02* cDNA contained a complete 1920-bp ORF (bases 4-1923) encoding 639-amino acid protein, designated as *KC-VIN2*. *KC-VIN1* lacks 4 amino acid residues at the

N-terminus. NCBI BLAST search at nucleotide level revealed that *AI-01* and *AI-02* share 99% sequence identity (showing variations at 13 places confined towards 5′ and 3′ regions). It was further revealed that *AI-02* shares 99% sequence identity with vacuolar invertase cDNA sequences from the potato cultivars, namely May Queen (DQ478950), Russet Burbank (L29099), unknown cultivar (AY341425), and it was 98% for the potato cv. Desiree (X70368). In the BLAST output data, few gaps were noted only in the cases of Russet Burbank and Desiree indicating more divergence. In case of tomato, *AI-02* showed 96% sequence identity with the following cultivars, such as Castlemart (M81081), Trujillo accession LA 722 (Z12026), UC82B (Z12025). For other members of *Solanaceae* family, *AI-02* shares 89% and 87% sequence identity with Capsicum (U87849) and tobacco cv. SNN (AJ305044), respectively. Sequence divergence was more prominent for tomato cultivars,

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AI-02     AGTATGGCCACGCAGTACCCCTCAAGTTATGACCCGGAAAACTCCGCCTCCCATTACACATTCCTCCCGGATCAACCTGA   80
AI-01                  ......AT..C.....................................................C..   67
KC-VIN2      M  A  T  Q  Y  P  S  S  Y  D  P  E  N  S  A  S  H  Y  T  F  L  P  D  Q  P  D    26
KC-VIN1                  Y  H  S  S  Y  D  P  E  N  S  A  S  H  Y  T  F  L  P  D  Q  P  D    22

AI-02     TTCCGGCTACCGGAAGTCCCTTAAAATCATCTCCGGCATTTTCCTCTCCTCTTTCCTTCTGCTTTCTGTAGCCTTCTTTC   160
AI-01     .......C.....................................................T.....................   147
KC-VIN2    S  G  Y  R  K  S  L  K  I  I  S  G  I  F  L  S  S  F  L  L  L  S  V  A  F  F  P    52
KC-VIN1    S  G  H  R  K  S  L  K  I  I  S  G  I  F  L  S  S  F  L  L  L  S  V  A  F  F  P    48

AI-02     CGATCCTCAACAACCAGTCACCGGACTTGCAGAGTAACTCCCGTTCGCCGGCGCCGCCGTCAAGAGGTGTTTCTCAGGGA   240
AI-01     ................................................................................   227
KC-VIN2      I  L  N  N  Q  S  P  D  L  Q  S  N  S  R  S  P  A  P  P  S  R  G  V  S  Q  G     79
KC-VIN1      I  L  N  N  Q  S  P  D  L  Q  S  N  S  R  S  P  A  P  P  S  R  G  V  S  Q  G     75

AI-02     GTCTCCGATAAGACTTTTCGAGATGTCGTCAATGCTAGTCACGTTCCTTATGCGTGGTCCAATGCTATGCTTAGCTGGCA   320
AI-01     ...........................................T....................................   307
KC-VIN2   V  S  D  K  T  F  R  D  V  V  N  A  S  H  V  P  Y  A  W  S  N  A  M  L  S  W  Q   106
KC-VIN1   V  S  D  K  T  F  R  D  V  V  N  A  S  H  V  S  Y  A  W  S  N  A  M  L  S  W  Q   102

AI-02     AAGATCTGCTTACCATTTTCAACCTCAAAAAAATTGGATGAACGATCCTAATGGTCCATTGTACCACAAGGGATGGTATC   400
AI-01     ....A...........................................................................   387
KC-VIN2    R  S  A  Y  H  F  Q  P  Q  K  N  W  M  N  D  P  N  G  P  L  Y  H  K  G  W  Y  H   133
KC-VIN1    R  T  A  Y  H  F  Q  P  Q  K  N  W  M  N  D  P  N  G  P  L  Y  H  K  G  W  Y  H   129

AI-02     ATCTTTTTTATCAATACAATCCAGATTCAGCTATTTGGGGAAATATCACATGGGGCCATGCCGTATCCAAGGACTTGATC   480
AI-01     ................................................................................   467
KC-VIN2      L  F  Y  Q  Y  N  P  D  S  A  I  W  G  N  I  T  W  G  H  A  V  S  K  D  L  I    159
KC-VIN1      L  F  Y  Q  Y  N  P  D  S  A  I  W  G  N  I  T  W  G  H  A  V  S  K  D  L  I    155
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AI-02     CACTGGCTCTACTTGCCTTTTGCCATGGTTCCTGATCAATGGTACGATATTAACGGTGTCTGGACTGGGTCCGCTACCAT   560
AI-01     ..............................................................................   547
KC-VIN2   H  W  L  Y  L  P  F  A  M  V  P  D  Q  W  Y  D  I  N  G  V  W  T  G  S  A  T  I   186
KC-VIN1   H  W  L  Y  L  P  F  A  M  V  P  D  Q  W  Y  D  I  N  G  V  W  T  G  S  A  T  I   182

AI-02     CCTACCCGATGGTCAGATCATGATGCTTTATACCGGTGACACTGATGATTATGTGCAAGTGCAAAATCTTGCGTACCCCA   640
AI-01     ..............................................................................   627
KC-VIN2    L  P  D  G  Q  I  M  M  L  Y  T  G  D  T  D  D  Y  V  Q  V  Q  N  L  A  Y  P  T   212
KC-VIN1    L  P  D  G  Q  I  M  M  L  Y  T  G  D  T  D  D  Y  V  Q  V  Q  N  L  A  Y  P  T   208

AI-02     CCAACTTATCTGATCCTCTCCTTCTAGACTGGGTCAAGTACAAAGGCAACCCGGTTCTGGTTCCTCCACCCGGCATTGGT   720
AI-01     ..............................................................................   707
KC-VIN2     N  L  S  D  P  L  L  L  D  W  V  K  Y  K  G  N  P  V  L  V  P  P  P  G  I  G    239
KC-VIN1     N  L  S  D  P  L  L  L  D  W  V  K  Y  K  G  N  P  V  L  V  P  P  P  G  I  G    235

AI-02     GTCAAGGACTTTAGAGACCCGACCACTGCTTGGACCGGACCCCAAAATGGGCAATGGCTCTTAACAATCGGGTCTAAGAT   800
AI-01     ..............................................................................   787
KC-VIN2   V  K  D  F  R  D  P  T  T  A  W  T  G  P  Q  N  G  Q  W  L  L  T  I  G  S  K  I   266
KC-VIN1   V  K  D  F  R  D  P  T  T  A  W  T  G  P  Q  N  G  Q  W  L  L  T  I  G  S  K  I   262

AI-02     TGGTAAAACGGGTATTGCACTTGTTTATGAAACTTCCAACTTCACAAGCTTTAAGCTATTGGATGAAGTGCTGCATGCGG   880
AI-01     ..............................................................................   867
KC-VIN2    G  K  T  G  I  A  L  V  Y  E  T  S  N  F  T  S  F  K  L  L  D  E  V  L  H  A  V   292
KC-VIN1    G  K  T  G  I  A  L  V  Y  E  T  S  N  F  T  S  F  K  L  L  D  E  V  L  H  A  V   288

AI-02     TTCCGGGTACGGGTATGTGGGAGTGTGTGGACTTTTACCCGGTATCGACTGAAAAAACAAACGGGTTGGACACATCATAT   960
AI-01     ..............................................................................   947
KC-VIN2     P  G  T  G  M  W  E  C  V  D  F  Y  P  V  S  T  E  K  T  N  G  L  D  T  S  Y    319
KC-VIN1     P  G  T  G  M  W  E  C  V  D  F  Y  P  V  S  T  E  K  T  N  G  L  D  T  S  Y    315

AI-02     AACGGCCCGGGTGTAAAGCATGTGTTAAAAGCAAGTTTAGATGACAATAAGCAAGATCACTATGCTATTGGGACGTATGA  1040
AI-01     ..............................................................................  1027
KC-VIN2   N  G  P  G  V  K  H  V  L  K  A  S  L  D  D  N  K  Q  D  H  Y  A  I  G  T  Y  D   346
KC-VIN1   N  G  P  G  V  K  H  V  L  K  A  S  L  D  D  N  K  Q  D  H  Y  A  I  G  T  Y  D   342

AI-02     CTTGACAAAGAACAAATGGACACCCGATAAGCCGGAATTGGATTGTGGAATTGGGTTGAAGCTGGATTATGGGAAATATT  1120
AI-01     ..............................................................................  1107
KC-VIN2    L  T  K  N  K  W  T  P  D  K  P  E  L  D  C  G  I  G  L  K  L  D  Y  G  K  Y  Y   372
KC-VIN1    L  T  K  N  K  W  T  P  D  K  P  E  L  D  C  G  I  G  L  K  L  D  Y  G  K  Y  Y   368

AI-02     ATGCATCAAAGACATTTTATGACCCGAAGAAACAACGAAGAGTACTGTGGGGATGGATTGGGGAAACTGATAGTGAATCT  1200
AI-01     ..............................................................................  1187
KC-VIN2     A  S  K  T  F  Y  D  P  K  K  Q  R  R  V  L  W  G  W  I  G  E  T  D  S  E  S    399
KC-VIN1     A  S  K  T  F  Y  D  P  K  K  Q  R  R  V  L  W  G  W  I  G  E  T  D  S  E  S    395

AI-02     GCTGACCTGCAGAAGGGATGGGCATCTGTACAGAGTATTCCAAGGACAGTGCTTTACGACAAGAAGACAGGGACACATCT  1280
AI-01     ..............................................................................  1267
KC-VIN2   A  D  L  Q  K  G  W  A  S  V  Q  S  I  P  R  T  V  L  Y  D  K  K  T  G  T  H  L   426
KC-VIN1   A  D  L  Q  K  G  W  A  S  V  Q  S  I  P  R  T  V  L  Y  D  K  K  T  G  T  H  L   422

AI-02     ACTTCAGTGGCCAGTTGAAGAAATTGAAAGCTTAAGAGCGGGTGATCCTATTGTTAAGCAAGTCAATCTTCAACCAGGTT  1360
AI-01     ..............................................................................  1347
KC-VIN2    L  Q  W  P  V  E  E  I  E  S  L  R  A  G  D  P  I  V  K  Q  V  N  L  Q  P  G  S   452
KC-VIN1    L  Q  W  P  V  E  E  I  E  S  L  R  A  G  D  P  I  V  K  Q  V  N  L  Q  P  G  S   448

AI-02     CAATTGAGCTACTCCATGTTGACTCAGCTGCAGAGTTGGATATAGAAGCCTCATTTGAAGTGGACAAAGTCGCGCTCCAG  1440
AI-01     ..............................................................................  1427
KC-VIN2     I  E  L  L  H  V  D  S  A  A  E  L  D  I  E  A  S  F  E  V  D  K  V  A  L  Q    479
KC-VIN1     I  E  L  L  H  V  D  S  A  A  E  L  D  I  E  A  S  F  E  V  D  K  V  A  L  Q    475
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AI-02     GGAATAATTGAAGCAGATCATGTAGGTTTCAGCTGCTCTACTAGTGGAGGTGCTGCTAGCAGAGGCATTTTGGGACCATT   1520
AI-01     ..........................................................................................   1507
KC-VIN2   G  I  I  E  A  D  H  V  G  F  S  C  S  T  S  G  G  A  A  S  R  G  I  L  G  P  F    506
KC-VIN1   G  I  I  E  A  D  H  V  G  F  S  C  S  T  S  G  G  A  A  S  R  G  I  L  G  P  F    502

AI-02     TGGTGTCGTTGTAATTGCTGATCAAACGCTATCTGAGCTAACGCCAGTTTACTTCTTCATTTCTAAAGGAGCTGATGGTC   1600
AI-01     ..........................................................................................   1587
KC-VIN2    G  V  V  V  I  A  D  Q  T  L  S  E  L  T  P  V  Y  F  F  I  S  K  G  A  D  G  R    532
KC-VIN1    G  V  V  V  I  A  D  Q  T  L  S  E  L  T  P  V  Y  F  F  I  S  K  G  A  D  G  R    528

AI-02     GAGCTGAGACTCACTTCTGTGCTGATCAAACTAGATCCTCAGAGGCTCCGGGAGTTGCTAAACGAGTTTATGGTAGTTCA   1680
AI-01     ........G.................................................................................   1667
KC-VIN2     A  E  T  H  F  C  A  D  Q  T  R  S  S  E  A  P  G  V  A  K  R  V  Y  G  S  S      559
KC-VIN1     A  E  A  H  F  C  A  D  Q  T  R  S  S  E  A  P  G  V  A  K  R  V  Y  G  S  S      555

AI-02     GTACCCGTGTTGGACGGTGAAAAACGTTCGATGAGATTATTGGTGGACCACTCAATTGTGGAGAGCTTTGCTCAAGGAGG   1760
AI-01     ........................A.................................................................   1747
KC-VIN2   V  P  V  L  D  G  E  K  R  S  M  R  L  L  V  D  H  S  I  V  E  S  F  A  Q  G  G    586
KC-VIN1   V  P  V  L  D  G  E  K  H  S  M  R  L  L  V  D  H  S  I  V  E  S  F  A  Q  G  G    582

AI-02     AAGAACAGTCATAACATCGCGAATTTACCCAACAAAGGCAGTGAATGGAGCAGCACGACTCTTCGTTTTCAACAATGCCA   1840
AI-01     ..........................................................G.......................   1827
KC-VIN2    R  T  V  I  T  S  R  I  Y  P  T  K  A  V  N  G  A  A  R  L  F  V  F  N  N  A  T    612
KC-VIN1    R  T  V  I  T  S  R  I  Y  P  T  K  A  V  N  G  A  A  R  L  V  V  F  N  N  A  T    608

AI-02     CAGGGGCTAGCGTGACTGCCTCCGTCAAGATTTGGTCACTTGAGTCGGCTAATATTCGGTCCTTCCCCTTGCAAGACTTG   1920
AI-01     ..........................................................AA......................   1907
KC-VIN2     G  A  S  V  T  A  S  V  K  I  W  S  L  E  S  A  N  I  R  S  F  P  L  Q  D  L      639
KC-VIN1     G  A  S  V  T  A  S  V  K  I  W  S  L  E  S  A  N  I  Q  S  F  P  L  Q  D  L      635

AI-02     TAATTCATCAAGCCATATCTTCTTC                                                            1945
AI-01     ........................ATTCTTTTTTTCATTTGAAGGTTATTTCACCGATGTCCCATCAAGAAAGGGAAGA   1987

AI-01     GAGGGAGAATATGTAGTGTTATACTC                                                           2013
```

Fig. 7. Comparison of the nucleotide sequences of the potato vacuolar invertase cDNAs, *AI-02* and *AI-01* (GenBank Acc. Nos. EU622807 & EU622806, respectively)), and *KC-VIN2* and *KC-VIN1* represent the corresponding deduced amino acid sequences, respectively. Dots in the nucleotide sequences indicate identical nucleotides. In the deduced amino acid sequences, the changes in amino acids are highlighted.

capsicum and tobacco as revealed by presence of gaps in the BLAST search output data. *AI-01* also showed more or less similar sequence identity if compared with the above sequences. The sequence divergence was found to be more prominent for the plant species other than *Solanaceae* family members (data not shown). In these cases, the query coverage length during NCBI BLAST search was considerably reduced; approx. two-third of the coding region of *AI-01* or *AI-02* showed sequence identity in the range of 70-80%. Therefore, none of the vacuolar invertase cDNA clones as isolated and characterized in this study, was found to be identical with the other cDNA sequences reported to date. *AI-01* and *AI-02*

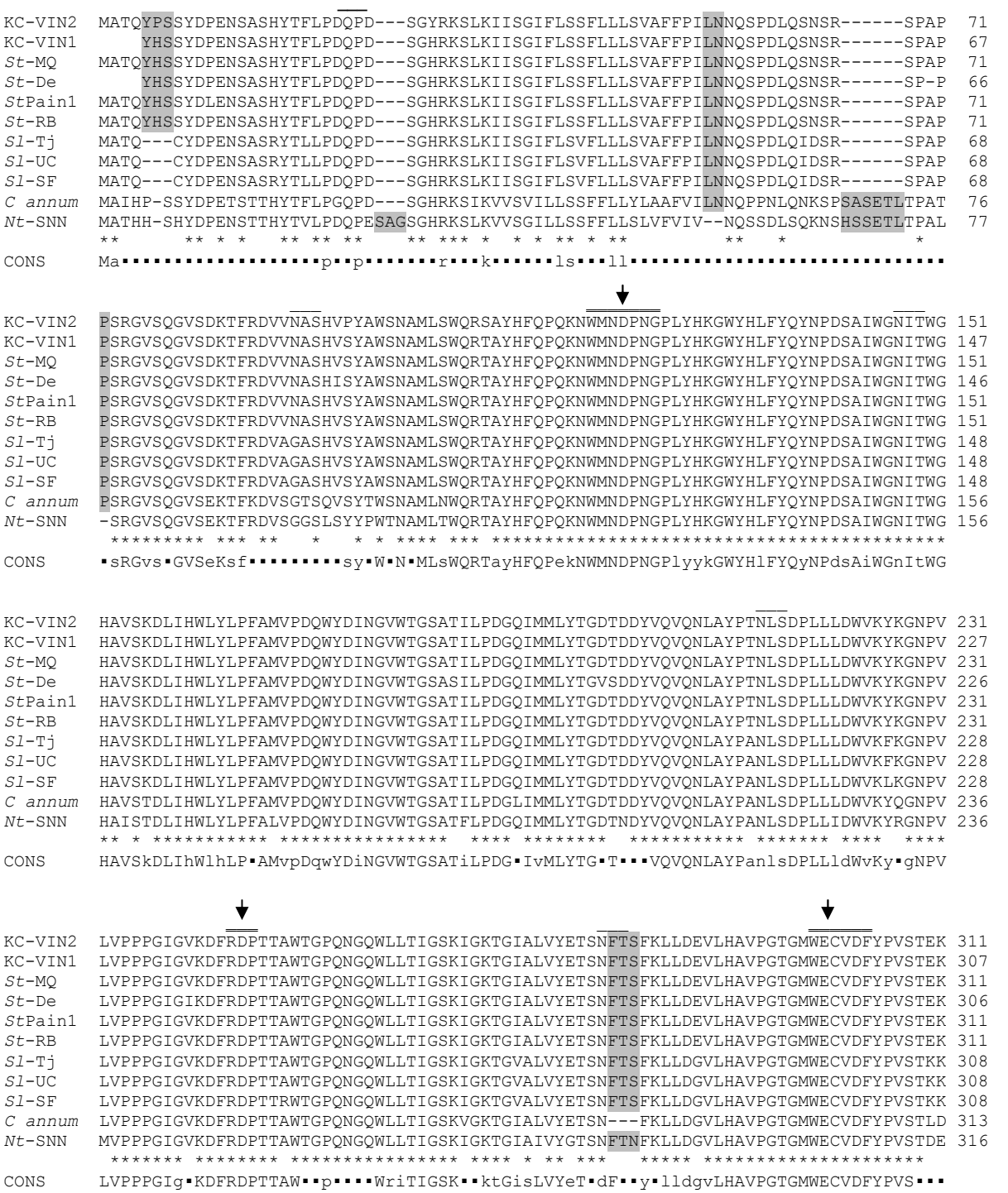
represent two different cDNA alleles from the potato cultivar Kufri Chipsona-1. The overall G+C content of the coding region and 3′ UTR in either cDNA allele were found to be ~46% and ~35%, respectively. However, the G+C content analyses by isochore plot using EBI tools revealed that vacuolar invertase coding regions in potato and other plant species showed considerable variations in different segments; moreover, the overall pattern was not similar between different plant species (data not shown).

NCBI protein-protein BLAST search (blastp) revealed that the 639-amino acid *KC-VIN2* and the 635-amino acid *KC-VIN1* (truncated at N-terminus) share 99% sequence identity. Based on the ProtParam tool (http://web.expasy.org/cgi-bin/protparam), the calculated molecular weight of either of the predicted proteins, *KC-VIN2* and *KC-VIN1*, was found to be nearly 70.0 kDa with a predicted pI of 5.69. However, once 100 amino acid residues are excluded from the N-terminal region, the molecular weight becomes ~60.0 kD with a predicted pI of 5.57 indicating approximate values for the mature proteins. Out of a total 639 amino acids of *KC-VIN2*, 54 are strongly basic (+) (K, R), 66 are strongly acidic (–) (D, E), 221 are hydrophobic (A, I, L, F, W, V), and 183 are polar (N, C, Q, S, T, Y). For the entire predicted protein the instability index (II) was computed as 41.11, which classified the protein as unstable; but the value was computed as 35.81 for predicted mature protein and classified it as stable one. The amino acid composition data also revealed that some of the amino acids such as Asp (6.9%), Pro (6.4%), Ser (9.2%), Trp (3.0%), Tyr (4.5%), and Val (7.4%) occurred more frequently as compared to their average occurrence; whereas, the amino acids, namely Arg (3.1%), Cys (0.6%), Glu (3.4%), Met (1.3%) occurred less frequently (Doolittle 1989). Similar amino acid composition data were obtained in case of *KC-VIN1* (data not shown). BLAST search (blastp) also revealed that *KC-VIN2* shared 97-99% identity with the corresponding sequences from other potato cultivars (CAA49831, AAA50305, ABF18956, AAQ17074); and 94-95%, 87% and 84% for the tomato cultivars (NP_001234843, CAA78061, CAA78060), muskmelon (ABX55832), capsicum (AAB48484), and tobacco

(CAC83577), respectively. The sequence identity was found to be considerably decreased for other plants (data not shown).

***5.2.3. Multiple sequence alignment, distinguishing sequence features and phylogenetic tree*:** In order to examine sequence similarities, nature and location of the amino acid substitutions in the vacuolar invertases, multiple sequence alignment was done using clustalW2, an EBI sequence analysis tool. For this purpose, a total of eleven homologs were chosen from four economically important members of the *Solanaceae* family: six from potato cultivars (*KC-VIN2* and *KC-VIN1* of this study, and the remaining four from other potato cultivars), three from tomato cultivars, one from capsicum, and one from tobacco (Fig.8). Nearly 100-amino acid N-terminal regions of these sequences appeared to be more variable; however, some small segments were found to be conserved in this region. But most of the conserved segments of varying lengths were found in the remaining major parts of the vacuolar invertase homologs. As shown in Fig.8, *KC-VIN2* and *KC-VIN1* were found to vary at 8 locations: P6H, Y29H, P95S, S108T, T536A, R568H, F607V, and R632Q. Out of this, a total of 6 positions represent nonconservative substitutions. All these substitutions are confined to ~100-amino acid N- and C-terminal regions only. Potato vacuolar invertase sequences were found to differ significantly with that of tomato. For example, in case of *KC-VIN2*, apart from the insertion of a 3-amino acid segment i.e.,YPS near N-terminus, it also showed variations at 29 positions if compared with *Sl*-Tj or *Sl*-UC: H17R, F20L, Y29H, S43V, S64I, N65D, V89A, N90G, P95S, S108T, T213A, Y226F, I271V, E288G, E310K, T348G, K356N, K366R, Q384E, A439V, I443T, N448D, H458R, V509I, F525Y, A552G, R554Q, R568H, and R632Q (Fig.8). All these substitutions (mostly nonconservative) were found to occur throughout the entire sequence. More sequence divergence was noticed if compared with the other members of the *Solanaceae* family, such as capsicum, tobacco. Some other important sequence features are also shown in Fig. 8 such as three well-conserved motifs such as, the WMNDPNG-motif (also known as β-fructosidase motif), the RDP-motif (transition-state stabilizer), and the WECVDF-motif i.e., EC-motif or catalytic site. Besides

these motifs, their flanking regions were also found to be well-conserved. All these important motifs occur in a span of nearly 190 amino acid residues. The β-fructosidase motif and the RDP-motif are separated by ~120 amino acid residues,



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KC-VIN2  TNGLDTSYNGPGVKHVLKASLDDNKQDHYAIGTYDLTKNKWTPDKPELDCGIGLKLDYGKYYASKTFYDPKKQRRVLWGW 391
KC-VIN1  TNGLDTSYNGPGVKHVLKASLDDNKQDHYAIGTYDLTKNKWTPDKPELDCGIGLKLDYGKYYASKTFYDPKKQRRVLWGW 387
St-MQ    TNGLDTSYNGPGVKHVLKASLDDNKQDHYAIGTYDLTKNKWTPDNPELDCGIGLKLDYGKYYASKTFYDPKKQRRVLWGW 391
St-De    TNGLDTSYNGPGVKHVLKASLDDNKQDHYAIGTYDLTKNKWTPDNPELDCGIGLKLDYGKYYASKTFYDPKKQRRVLWGW 386
StPain1  TNGLDTSYNGPGVKHVLKASLDDNKQDHYAIGTYDLTKNKWTPDNPELDCGIGLKLDYGKYYASKTFYDPKKQRRVLWGW 391
St-RB    TNGLDTSYNGPGVKHVLKASLDDNKQDHYAIGTYDLTKNKCTPDNPELDCGIGLKLDYGKYYASKTFYDPKKQRRVLWGW 391
Sl-Tj    TNGLDTSYNGPGVKHVLKASLDDNKQDHYAIGTYDLGKNKWTPDNPELDCGIGLRLDYGKYYASKTFYDPKKERRVLWGW 388
Sl-UC    TNGLDTSYNGPGVKHVLKASLDDNKQDHYAIGTYDLGKNKWTPDNPELDCGIGLRLDYGKYYASKTFYDPKKERRVLWGW 388
Sl-SF    TNGLETSYNGPGVKHVLKASLDDNKQDHYAIGTYDLGKNKWTPDNPELDCGIGLRLDYGKYYASKTFYDPKKERRVLWGW 388
C annum  ANGLDTSYNGPGIKHVLKASLDDNKQDHYVIGTYDPVKNKFSPDNPDLDCGIGLRLDYGRYYASKTFYDPKKQRRVLWGW 393
Nt-SNN   ANGLDTSYNGPGIKHVLKASLDDDKHDYYAIGTYDPVKNKWTPDNPQLDVGIGLRLDYGKYYASKTFYDPKEQRRILWGW 396
          *** ******* ********** * * * *****  ***  ** * ** **** **** ***********  ** ****
CONS     ▪NGLDTS▪NGPgVKHVlKASLDDdk▪D▪YAiGTYd▪▪▪▪kwtPDnpe▪DvGIGLr▪DYGKYYASKTFYDq▪k▪RRvlWGW
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KC-VIN2  IGETDSESADLQKGWASVQSIPRTVLYDKKTGTHLLQWPVEEIESLRAGDPIVKQVNLQPGSIELLHVDSAAELDIEASF 471
KC-VIN1  IGETDSESADLQKGWASVQSIPRTVLYDKKTGTHLLQWPVEEIESLRAGDPIVKQVNLQPGSIELLHVDSAAELDIEASF 467
St-MQ    IGETDSESADLQKGWASVQSIPRTVLYDKKTGTHLLQWPVEEIESLRAGDPIVKQVNLQPGSIELLHVDSAAELDIEASF 471
St-De    IGETDSESADLQKGWASVQSIPRTVLYDKKTGTHLLQWPVEEIESLRVGDPIVKQVNLQPGSIELLHVDSAAELDIEASF 466
StPain1  IGETDSESADLQKGWASVQSIPRTVLYDKKTGTHLLQWPVEEIESLRAGDPIVKQVNLQPGSIELLHVDSAAELDIEASF 471
St-RB    IGETDSESADLQKGWASVQSIPRTVLYDKKTGTHLLQWPVEEIESLRGGDPIVKQVNLQPGSIELLHVDSAAELDIEASF 471
Sl-Tj    IGETDSESADLQKGWASVQSIPRTVLYDKKTGTHLLQWPVEEIESLRVGDPTVKQVDLQPGSIELLRVDSAAELDIEASF 468
Sl-UC    IGETDSESADLQKGWASVQSIPRTVLYDKKTGTHLLQWPVEEIESLRVGDPTVKQVDLQPGSIELLRVDSAAELDIEASF 468
Sl-SF    IGETDSESADLQKGWASVQSIPRTVLYDKKTGTHLLQWPVEEIESLRVGDPTVKQVDLQPGSIELLRVDSAAELDIEASF 468
C annum  IGETDSESADLQKGWASVQSIPRTVLFDKKTGTHLLQWPVAEIESLRSGDPKVKEVNLQPGSIELLHVDSAAQFDIEASF 473
Nt-SNN   IGETDSEAADLLKGWASVQSIPRTVLYDKETRTHVLQWPVKEIESLRIGDPLVKRVNLQPGSIELVHVDSAAQLDVEASF 476
         ******* *** ************** ** * ** ***** ****** *** ** * ********  *****  * ****
CONS     IGEtDsE▪aDl▪KGWASvQ▪IPRtV▪▪DkkTgthllqWPVeEvesLR▪▪▪▪▪▪▪▪v▪▪▪pGs▪▪▪l▪v▪▪atqLDI▪AsF
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KC-VIN2  EVDKVALQGIIEADHVGFSCSTSGGAASRGILGPFGVVVIADQTLSELTPVYFFISKGADGRAETHFCADQTRSSEAPGV 551
KC-VIN1  EVDKVALQGIIEADHVGFSCSTSGGAASRGILGPFGVVVIADQTLSELTPVYFFISKGADGRAEAHFCADQTRSSEAPGV 547
St-MQ    EVDKVALQGIIEADHVGFSCSTSGGAASRGILGPFGVVVIADQTLSELTPVYFFISKGADGRAETHFCADQTRSSEAPGV 551
St-De    EVDKVALQGIIEADHVGFSCSTSGGAASRGILGPFGVVVIADQKLSELTPVYFYISKGADGRAETHFCADQTRSSEAPGV 546
StPain1  EVDKVALQGIIEADHVGFSCSTSGGAASRGILGPFGVVVIADQTLSELTPVYFYISKGADGRAETHFCADQTRSSEAPGV 551
St-RB    EVDKVALQGIIEADHVGFSCSTSGGAASRGILGPFGVVVIADQKLSDVTPVYFYISKGADGRAETHFCADQTRSSEAPGV 551
Sl-Tj    EVDKVALQGIIEADHVGFSCSTSGGAASRGILGPFGVIVIADQTLSELTPVYFYISKGADGRAETHFCADQTRSSEAPGV 548
Sl-UC    EVDKVALQGIIEADHVGFSCSTSGGAASRGILGPFGVIVIADQTLSELTPVYFYISKGADGRAETHFCADQTRSSEAPGV 548
Sl-SF    EVDKVALQGIIEADHVGFSCSTSGGAASRGILGPFGVIVIADQTLSDVTPVYFYISKGADGRAETHFCADQTRSSEAPGV 548
C annum  EVDRVTLEGIIEAD-VGYNCSTSGGAASRGILGPFGVVVIADQTLSELTPVYFYISRGADGRAEAHFCADQTRSSEAPGV 552
Nt-SNN   EVDKAALEGTIEAD-VGFNCSTSGGAAKRGILGPFGVVVIADQTLSELTPVYFYIAKGPDGRAETYFCADETRSSEAPGV 555
         ***   * * **** **  ******** ********* ***** **  ***** *  * *****  **** *********
CONS     EvD▪▪al▪▪▪▪e▪▪▪▪▪f▪C▪tsgGa▪▪RG▪LGPFG▪▪V▪AD▪▪LSElTPPYFy▪▪KG▪dg▪▪▪t▪FCaDqtRSs▪A▪▪v
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KC-VIN2  AKRVYGSSVPVLDGEKRSMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNATGASVTASVKIWSLESANI 631
KC-VIN1  AKRVYGSSVPVLDGEKHSMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLVVFNNATGASVTASVKIWSLESANI 627
St-MQ    AKRVYGSSVPVLDGEKHSMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNATGASVTASVKIWSLESANI 631
St-De    AKQVYGSSVPVLDGEKHSMRLLEDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNATGASVTASVKIWSLESANI 626
StPain1  AKQVYGSSVPVLDGEKHSMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNATGSSVTASVKIWSLESANI 631
St-RB    AKQVYGSSVPVLDGEKHSMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNRTGASVTASVKIWSLESANI 631
Sl-Tj    GKQVYGSSVPVLDGEKHSMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNATGASVTASVKIWSLESANI 628
Sl-UC    GKQVYGSSVPVLDGEKHSMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNATGASVTASVKIWSLESANI 628
Sl-SF    GKQVYGSSVPVLDGEKHSMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNATGASVTASVKIWSLESVNI 628
C annum  AKQVYGSSVPVLDGEKHRMRLLVDHSIVESFAQGGRTVITSRIYPTKAVNGAARLFVFNNATGAIVTASLKIWSLESADI 632
Nt-SNN   AKQVYGSSVPVLDDEQHSMRLLVDHSIVESFAQGGRTVITSRIYPTKAINGAARLFVFNNATRASVTASLKIWSLESADI 635
          * ********** *   **** ************************* ****** **** *   **** *******  *
CONS     ▪K▪vyGs▪VPVL▪GEk▪smR▪LVDHSIVEsFaQgGRT▪ITSRiYPTkaiyGAAr▪FlFNNAt▪▪▪VtASlkiW▪▪▪sa▪i
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KC-VIN2  RSFPLQDL 639
KC-VIN1  QSFPLQDL 635
St-MQ    RSFPLQDL 639
St-De    RSFPLQDL 634
StPain1  QSFPLQDL 639
St-RB    RSFPLQDL 639
Sl-Tj    QSFPLQDL 636
Sl-UC    QSFPLQDL 636
Sl-SF    QSFPLQDL 636
C annum  RSFPLQKL 640
Nt-SNN   RSFPLDQL 643
          ****  *
CONS     r▪fp▪▪▪▪
```

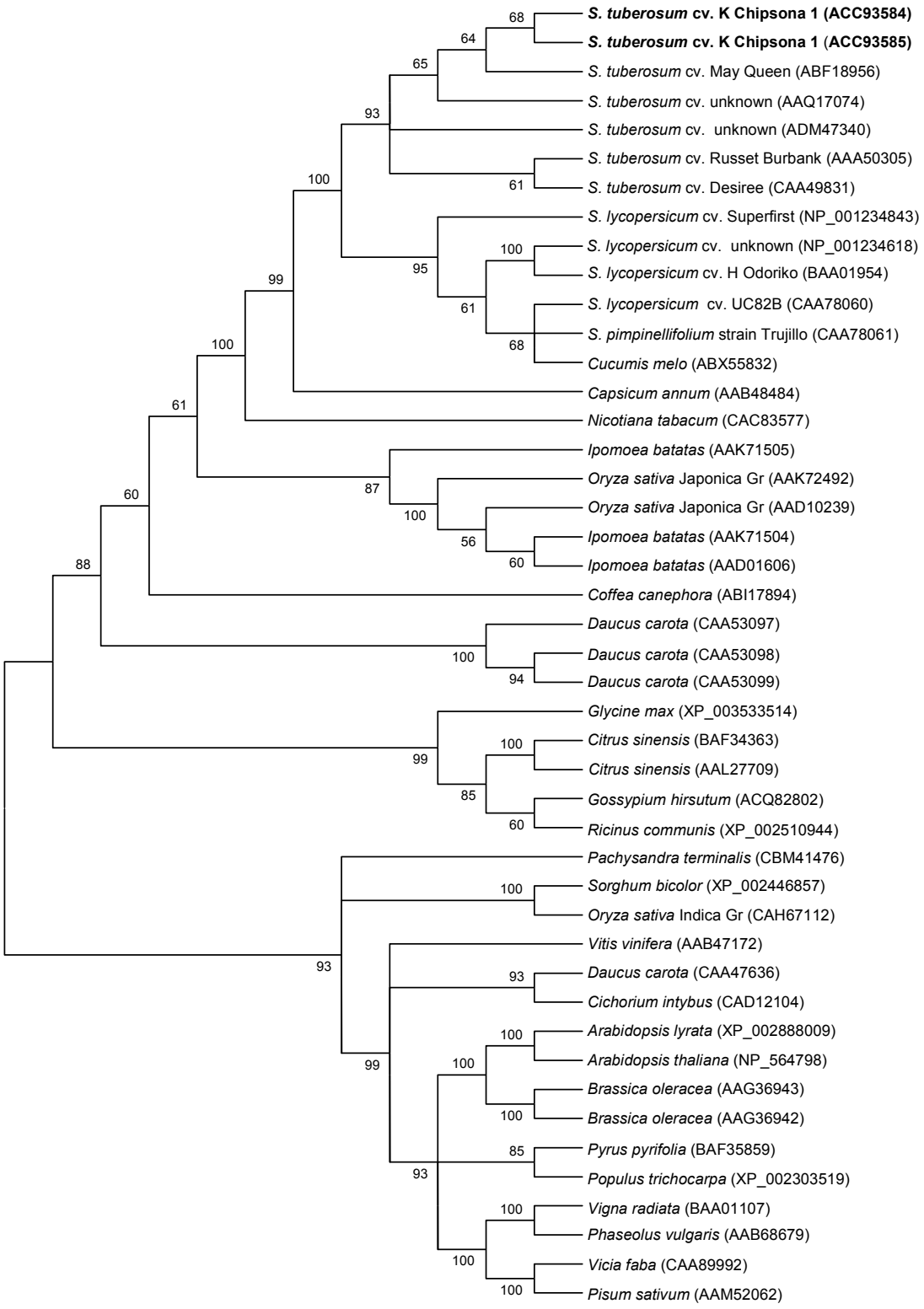
Fig. 8. Comparison of the predicted amino acid sequences of eleven soluble vacuolar invertase homologs from the *Solanaceae* family: *KC-VIN2* (ACC93585) and *KC-VIN1* (ACC93584) from the potato cv. Kufri Chipsona-1), *St*-MQ (ABF18956), *St*-De (CAA49831), *St*Pain1 (ADM47340) and *St*-RB (AAA50305) are from other potato cultivars; *Sl*-Tj (CAA78061), *Sl*-UC (CAA78060), and *Sl*-SF (NP_001234843) are from the tomato cultivars Trujillo, UC82B and SuperFirst, respectively; *C annum* (AAB48484) from Capsicum; *Nt*-SNN (CAC83577) from the tobacco cv. SNN. This multiple sequence alignment is based on ClustalW2 tool along with minor manual adjustments. Dashes indicate gaps that arise during alignment. Asterisks indicate the conserved amino acids between the sequences only from the *Solanaceae* family members. Plant species-specific distinguishing sequence features (signature type) as revealed in this study are highlighted. Six potential N-linked glycosylation sites (Asn-X-Ser/Thr) are single overlined in *KC-VIN2* sequence; likewise, well-conserved WMNDPNG-motif (also known as β-fructosidase motif), RDP-motif (transition-state stabilizer), WECVDF-motif i.e., EC-motif or catalytic site are double overlined (the aspartate in the WMNDPNG-motif, the aspartate in the RDP-motif, and the glutamate in the EC-motif are shown by the downward arrows). For consensus sequence (shown as CONS), a total of 45 vacuolar invertase sequences from 27 taxonomically different plant species were examined with the help of both ClustalW2 and Multalin tools; single letter code in upper case is used for the amino acids conserved in most of the plant species (more than 90%), whereas the amino acids conserved in more than 50% plant species are shown by the respective lower cases; '▪' is used for the variable amino acids.

whereas the catalytic site occurs close to the RDP-motif, separated by ~50 amino acid residues. Similar pattern is also found in the other plant vacuolar invertases. In the earlier reports, it was shown that the aspartate in the WMNDPNG-motif, another aspartate in the RDP-motif, and the glutamate in the EC-motif are indispensable for the functionality of acid invertases. i.e. for substrate binding and catalysis. These three amino acid residues also referred to as 'the catalytic triad'. It is now believed that the aspartate residue in RDP-motif is not directly involved in the catalytic mechanism and probably acts as a transition-state stabilizer. Apart from glutamate, a conserved cysteine residue is also present in the EC-motif. But the precise role of this polar cysteine residue during catalysis is yet to be understood. It is likely that apart from these conserved motifs and their sequences, the distance between these motifs and the amino acid sequences therein may also be crucial for overall effect on the functionality of acid invertases. Six potential N-linked glycosylation sites (Asn-X-Ser/Thr) were found in *KC-VIN2*, *KC-VIN1* and the other potato sequences. In tomato only five such sites were found. Only

three N-linked glycosylation sites were found to be common between the *Solanaceae* family members as examined in this study.

In some families of homologous proteins, certain segments of a protein sequence may be found in the organisms of one taxonomic group but not in other groups; these segments can be referred to as signature sequences for the group in which they are found. Multiple sequence alignment between the *Solanaceae* family members as shown in Fig. 8 also revealed a few such types of distinguishing sequence features (signature type sequences) in the vacuolar invertases. Here the well-conserved segments and amino acid residues inserted in the conserved regions were only considered, such as a 3-amino acid residue, Y(P/H)S in potato near the amino terminus. Likewise, if we further proceed towards C-terminus a few more could be found: a 3-residue, SAG found only in tobacco. Moreover, some segments including single amino acid insertions were found to occur in more than one member of the *Solanaceae* family, such as a 2-residue, LN in potato, tomato, and capsicum; a 6-residue, (S/H)(A/S)SETL in capsicum and tobacco; a single Pro residue in potato, tomato, and capsicum; a 3-residue, FT(S/N) in potato, tomato and tobacco; a single His residue in potato and tomato. Biochemical roles of such distinguishing sequence features remain to be elucidated. At least they are conferring some sort of identity features on the vacuolar invertases in different plant species.

Both ClustalW2 and *Multalin* tools were used to predict the consensus sequence of vacuolar invertases across the different plant species as shown in Fig. 8. For this purpose a total of 45 vacuolar invertase sequences from 27 taxonomically different plant species were used. A number of segments of varying lengths including single amino acid residues were found to be conserved, and mostly they are confined to the predicted mature proteins. A phylogenetic tree (Fig. 9) was generated using the same sequences in order to have an idea of evolutionary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***S. tuberosum* cv. K Chipsona 1 (ACC93584)**
***S. tuberosum* cv. K Chipsona 1 (ACC93585)**
S. tuberosum cv. May Queen (ABF18956)
S. tuberosum cv. unknown (AAQ17074)
S. tuberosum cv. unknown (ADM47340)
S. tuberosum cv. Russet Burbank (AAA50305)
S. tuberosum cv. Desiree (CAA49831)
S. lycopersicum cv. Superfirst (NP_001234843)
S. lycopersicum cv. unknown (NP_001234618)
S. lycopersicum cv. H Odoriko (BAA01954)
S. lycopersicum cv. UC82B (CAA78060)
S. pimpinellifolium strain Trujillo (CAA78061)
Cucumis melo (ABX55832)
Capsicum annum (AAB48484)
Nicotiana tabacum (CAC83577)
Ipomoea batatas (AAK71505)
Oryza sativa Japonica Gr (AAK72492)
Oryza sativa Japonica Gr (AAD10239)
Ipomoea batatas (AAK71504)
Ipomoea batatas (AAD01606)
Coffea canephora (ABI17894)
Daucus carota (CAA53097)
Daucus carota (CAA53098)
Daucus carota (CAA53099)
Glycine max (XP_003533514)
Citrus sinensis (BAF34363)
Citrus sinensis (AAL27709)
Gossypium hirsutum (ACQ82802)
Ricinus communis (XP_002510944)
Pachysandra terminalis (CBM41476)
Sorghum bicolor (XP_002446857)
Oryza sativa Indica Gr (CAH67112)
Vitis vinifera (AAB47172)
Daucus carota (CAA47636)
Cichorium intybus (CAD12104)
Arabidopsis lyrata (XP_002888009)
Arabidopsis thaliana (NP_564798)
Brassica oleracea (AAG36943)
Brassica oleracea (AAG36942)
Pyrus pyrifolia (BAF35859)
Populus trichocarpa (XP_002303519)
Vigna radiata (BAA01107)
Phaseolus vulgaris (AAB68679)
Vicia faba (CAA89992)
Pisum sativum (AAM52062)

Fig. 9

Fig. 9. The phylogenetic tree was generated by the MEGA 5.0 software using the Neighbor-Joining method. This represents a bootstrap consensus tree. The analysis involved 45 vacuolar acid invertase 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). The number at each node represented the bootstrap value, with 1000 replicates. The predicted amino acid sequences of *KC-VIN1* (ACC93584) and *KC-VIN2* (ACC93585) of this study appeared to occupy distinct positions in the phylogenetic tree (shown in bold cases).

relatedness between the different plant species. For this purpose, multiple sequence alignment was done by the MultAlin software, followed by generation of the phylogenetic tree using MEGA 5.0 software by the Neighbor-Joining method (with bootstrap consensus). The vacuolar invertase sequences from different plant species are divided into many distinct groups according to their sequence relatedness. *KC-VIN1* and *KC-VIN2*, as isolated in this study, occupied distinct branches in the phylogenetic tree.

5.2.4. Hydropathy plot, prediction of the secondary structures: For comparison, the hydropathy profiles were generated for the vacuolar invertases from six different plant species, namely potato, sweet potato, oilseed crop *Brassica*, orange, poplar, and rice with 9 amino acids running window using the ProtScale tool based on the Kyte-Doolittle scale, as shown in Fig. 10A-F, respectively. The positions of the WMNDPNG-motif, the RDP-motif, and the WECVDF-motif in each hydropathy plot were clearly indicated for segment-wise comparison between the invertases. As revealed in each plot, some common features were found such as the first two motifs belong to the hydrophilic regions, whereas the catalytic EC-motif appeared on the midpoint of the scale. However, close inspection of the flanking regions of the individual motifs appeared to show variations in terms of their hydropathic characters. In the ~100-amino acid N-terminal regions some segments were found to show similar but others showed variable hydropathic characters. Segment-wise analyses of ~300-amino acid C-terminal regions also showed similar trends.

With the help of ProtScale tool, α-helix (Chou & Fasman scale), β-sheet (Chou & Fasman scale), β-turn (Chou & Fasman scale), and random coil (Deleage & Roux scale) in *KC-VIN2* were predicted (Fig. 11 A-D). Small segments containing the individual motif of the catalytic triad appeared to show fewer propensities towards the formation of regular secondary structures

such as α-helix or β-sheet; however, after the EC-motif, presence of some α-helices and β-sheet structures can be predicted towards the C-terminal regions.

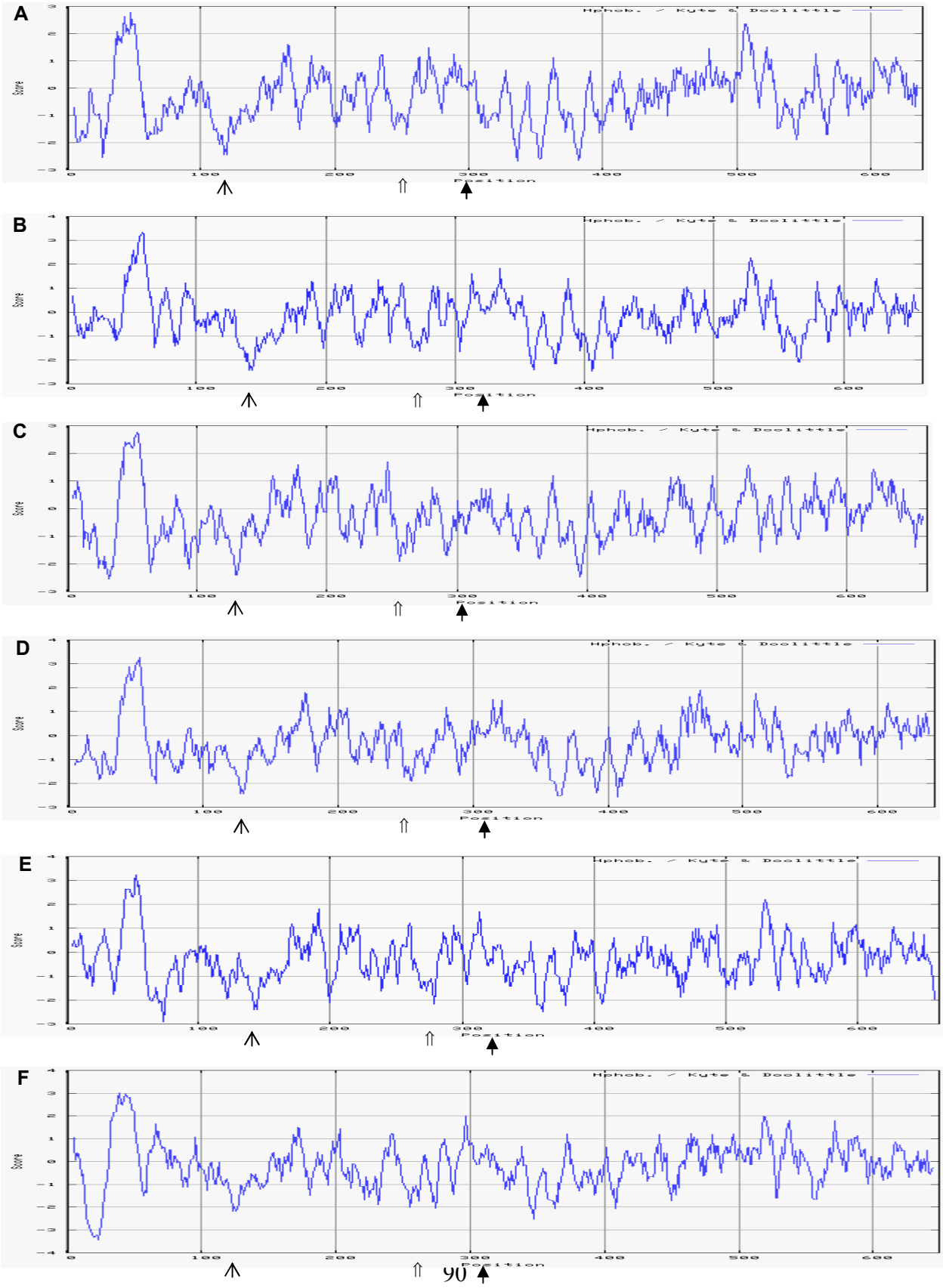

Fig. 10

Fig. 10. Hydropathy plots of the deduced amino acid sequences of vacuolar invertases from plants belonging to different taxonomic groups based on the Kyte and Doolittle scale. **A** Potato (cv. Kufri Chipsona-1, *KC-VIN2*, ACC93585), **B** Sweet potato (cv. Tainong 57, AAK71505), **C** *Brassica oleracea* (cv. Shogun, AAG36943), **D** Orange (cv. Washington, BAF34363), **E** *Populus trichocarpa* (XP_002303519), **F** *Oryza sativa* Indica Gr. (CAH67112). The beginning of the WMNDPNG-motif, the RDP-motif, and the WECVDF-motif in each hydropathy plot are indicated by the following upward arrows⇑, ⇑, and ⇑, respectively

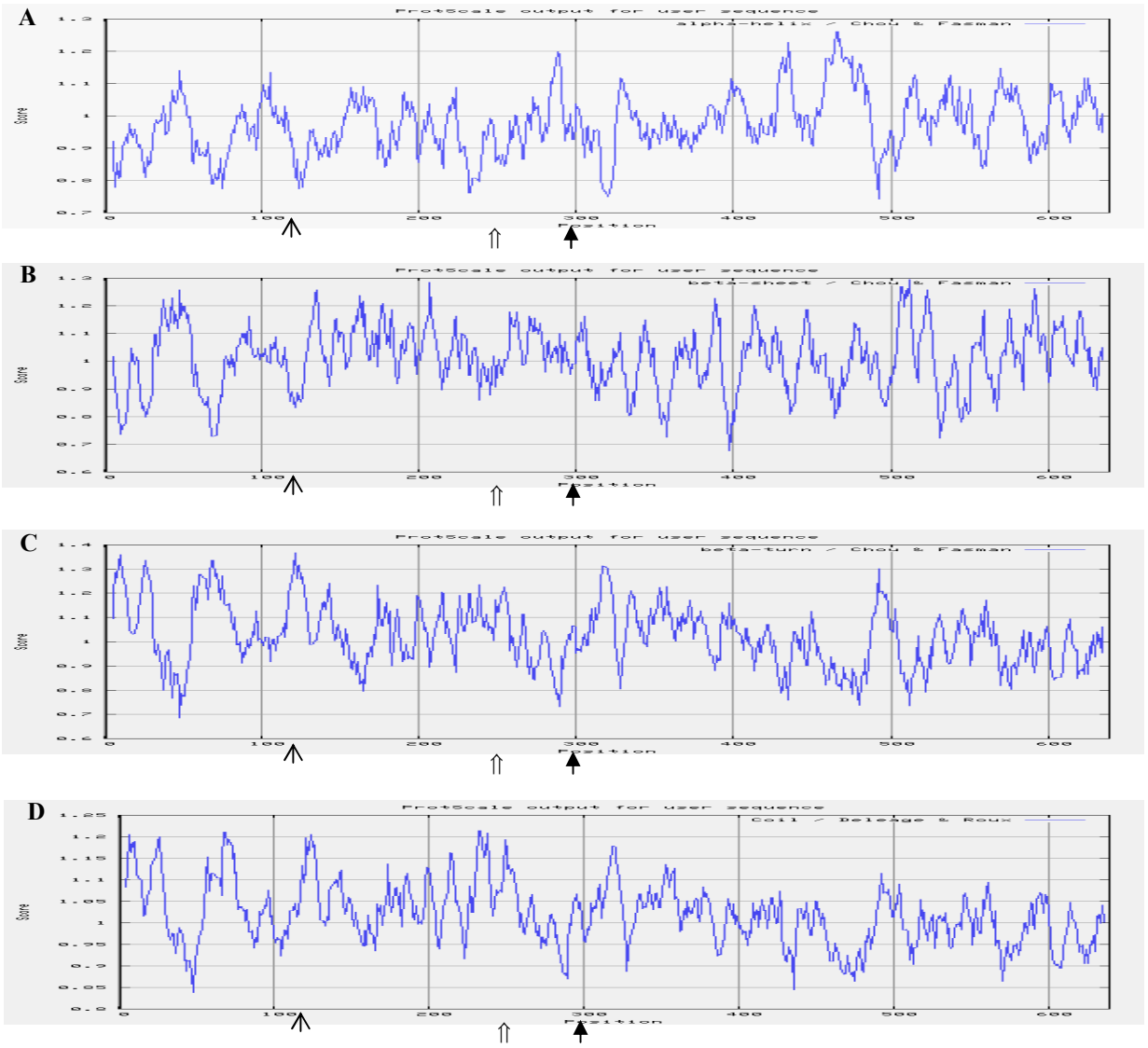

Fig. 11. Secondary structures of the deduced amino acid sequences of vacuolar invertases from *KC-VIN2* based on the Chou & Fasman scale. **A** α-helix (Chou & Fasman scale), **B** β-sheet (Chou & Fasman scale), **C** β-turn (Chou & Fasman scale) and **D** random coil (Deleage & Roux scale). The beginning of the WMNDPNG-motif, the RDP-motif, and the WECVDF-motif in each plot are indicated by the following upward arrows⇑, ⇑, and ⇑, respectively

Two cDNAs encoding vacuolar invertase variants from one commercially important Indian potato cultivar, Kufri Chipsona-1 are isolated and characterized in this study. The genetic resources of other potato cultivars can be further explored to obtain more such allelic variants. In addition to the molecular cloning of the cDNAs from potato, the other interest was on very close inspection of the available vacuolar invertase sequences between the plant species of different taxonomic groups for further analyses and comparison. Sequence alignment between the four members of the *Solanaceae* family (namely potato, tomato, capsicum and tobacco) clearly revealed the sequence relatedness between them along with nature and location of the variations in their primary sequences. Most of the amino acid changes were found to be nonconservative. Therefore, it is likely that such changes may influence in the structure-function relationships and the overall functionalities of the individual vacuolar invertases within and between the plant species. The individual vacuolar invertase forms need to be further studied through biochemical characterization and analysis on their 3-D structures. Sequence alignment of the vacuolar invertase from the *Solanaceae* family members revealed the presence of some distinguishing sequence features (signature type of sequences) not documented in the earlier reports. They may provide some biochemical clues in establishing the evolutionary relatedness at different taxonomic levels.

Hydrophobic characters were predicted and compared in some of the plant species. Based on the large number of available plant invertase sequences, a phylogenetic tree was generated to see the sequence relatedness between them. Moreover, a consensus sequence was predicted presenting variable, moderately conserved and highly conserved amino acid residues and segments. This data would be helpful to carry out site-directed mutagenesis in identifying the crucial amino acids involved in the functionality of vacuolar invertase. Apart from biological roles, invertase is also commercially important enzyme. The data as presented in this report would be useful in protein engineering and other biotechnological applications.

5.2.5. Isolation and characterization of partial genomic clones from potato: Potato genomic DNA was isolated using a simple method based on the protocol described by Dellaporta et al. (1983). One of the steps involved potassium acetate to remove carbohydrates and other bulk impurities. The quality and quantity of the potato DNA samples were initially checked spectrophotometrically by measuring the A_{260}/A_{280} ratio and absorbance at 260 nm, respectively. The A_{260}/A_{280} ratio appeared to be 1.70 to 1.90 for most of the DNA preparations. The genomic DNA from Kufri Chipsona-1 was used as a template for PCR. For the K20-AI and M20-AI primer pair, the size of the amplified DNA band was approx. 2.5 kb and for the K20-AI and P20-AI primer pair, the size of the amplified band was found to be ~ 3.2 kb (Fig. 12).

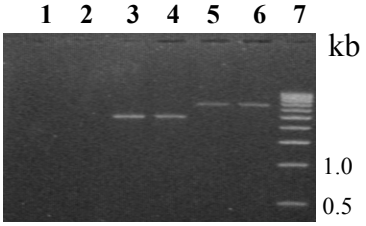

Fig. 12. PCR-amplified genomic DNA products using invertase-specific primers. Lane 1 control PCR with K20-AI & M20-AI primers (no template); Lane 2 control PCR with K20-AI & P20-AI primers (no template); Lanes 3 & 4 CS-1 DNA and KCM DNA as template using K20-AI & M20-AI primer pair; Lanes 5 & 6 CS-1 DNA and KCM DNA as templates using K20-AI & P20-AI primer pair; Lane 7 0.5 kb DNA ladder

The amplified genomic DNA products were cloned into pUC19 vector for further characterization by sequencing. The larger sizes were due to the presence of introns (s). The genomic clones were isolated because of the following reasons: i) to detect the presence of introns (s) in the vacuolar invertase genes; ii) in making antisense and sense binary genetic constructs partial genomic clones could be used in place of the corresponding cDNA and/or its fragments.

In this study, different fragments corresponding to the 5′ and 3′ regions of the cDNAs, *AI-01* and *AI-02,* were only used in making several antisense and sense Ti plasmid-based binary genetic constructs as described categorically in the next section.

5.3. To make genetic constructs by using soluble acid invertase cDNA/genomic clones in order to repress its gene function in the potato tuber

Soluble acid invertases along with other proteins/enzymes in potato tubers are considered to play important roles in cold-induced sweetening. Particularly, invertase gene functions are implicated in hexose (reducing sugars) accumulation in the cold-stored tubers. Therefore, inhibition of acid invertase gene functions in potato became the target for researchers, and different molecular strategies were adopted by many laboratories. Zrenner et al. (1996) adopted antisense approach using full-length vacuolar invertase cDNA under the constitutive CaMV 35S promoter; for genetic transformation they used the potato cv. Desiree (not a regular cultivar, mostly used as a reference). Greiner et al. (1999) expressed tobacco invertase inhibitor in potato tuber (cv. Desiree was used for transformation) under the CaMV 35S promoter. Bhaskar et al. (2010) adopted RNAi approach for silencing vacuolar invertase gene functions in potato (*Solanum tuberosum* 'Katahdin').

In this study, several molecular approaches, considerably different from the above, were adopted for inhibition of vacuolar invertase gene function in one of the Indian potato cultivars. A number of antisense and sense binary genetic constructs were made using both constitutive and tuber-specific promoters for potato transformation. Different regions of the vacuolar invertase cDNAs, *AI-01* and *AI-02* were used in making the genetic constructs to see the effects. Kufri Chipsona-1 (a commercially important Indian potato cultivar) was used in *Agrobacterium*-mediated genetic transformation.

***5.3.1. Types of binary vectors used in this study*:** The binary vector pBI121 (Fig. 13A) was used for making various types of antisense and sense genetic constructs under the constitutive

CaMV 35S promoter. 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. 13B). The relevant details are given below:

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 Accession Nos. 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 in our laboratory (Bansal et al. 2012).

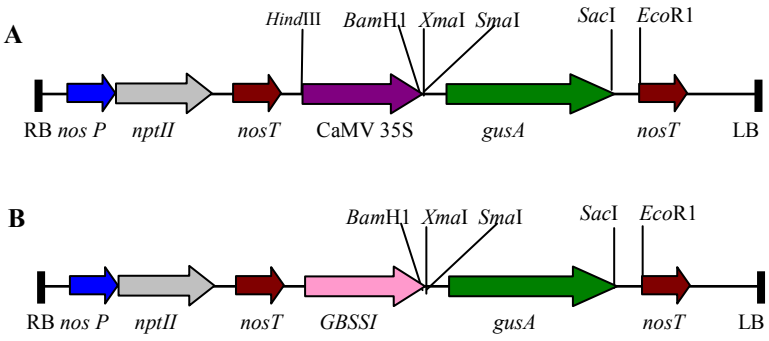

Fig. 13. Schematic view of the binary vectors used for making genetic constructs. **A** binary vector pBI121 having the constitutive CaMV 35S promoter upstream of the GUS gene (GenBank Acc. No. AF485783); **B** The binary vector pAN-GB01, constructed by replacing the CaMV 35S promoter by the ~ 0.75 kb *GBSSI* promoter isolated from GKC1 allele of cv. Kufri Chandramukhi

5.3.2. Construction of the antisense and sense binary genetic constructs: For inhibition of vacuolar invertase gene function, several strategic approaches were adopted. The constitutive and tuber-specific promoters were used for making a series of antisense and sense binary

genetic constructs which were used for the generation of the transgenic potato lines. For this purpose, different regions of the vacuolar invertase cDNAs, *AI-01* and *AI-02*, were employed in making the binary genetic constructs. Kufri Chipsona-1 (a commercially important potato cultivar) was used during *Agrobacterium*-mediated genetic transformation. In this study, a total of eight binary genetic constructs were made as listed below with necessary details:

A. List of antisense and sense genetic constructs using ~1.40 kb towards the 5′ terminus of the vacuolar invertase cDNA, *AI-02:*

i) VA (antisense construct under the constitutive *CaMV 35S* promoter)

ii) VB (sense construct under the constitutive *CaMV 35S* promoter)

iii) VC (antisense construct under the tuber-specific *GBSSI* promoter)

iv) VD (sense construct under the tuber-specific *GBSSI* promoter)

B. List of antisense and sense genetic constructs using ~1.0 kb towards the 3′ terminus of the vacuolar invertase cDNA, *AI-01:*

i) VE (antisense construct under the constitutive *CaMV 35S* promoter)

ii) VF (sense construct under the constitutive *CaMV 35S* promoter)

iii) VG (antisense construct under the tuber-specific *GBSSI* promoter)

iv) VH (sense construct under the tuber-specific *GBSSI* promoter)

VA, the antisense binary construct under the CaMV 35S promoter: The full-length *AI-02* cDNA clone in the required orientation in pUC19 vector was digested with *Hinc*II in order to release the 0.64 kb fragment towards 3′ terminus followed by self circularization of the recombinant pUC19 which consisted of ~1.4 kb acid invertase specific cDNA towards the 5′ terminus. This fragment was released by digestion with *Hinc*II and *Sac*I. Finally, this

cDNA fragment was cloned into the binary vector pBI121 by replacing the reporter GUS gene (*Sma*I and *Sac*I were used to remove the GUS gene) (Fig. 14A).

VB, the sense binary construct under the CaMV 35S promoter: The full-length cDNA clone *AI-02* in other orientation in pUC19 vector was digested with *Bam*HI and *HincII* to release ~1.4 kb cDNA insert towards its 5′ terminus. This fragment was then cloned into *Ecl*136II and *Bam*HI sites of pBI121 by replacing the reporter GUS gene (Fig.14B).

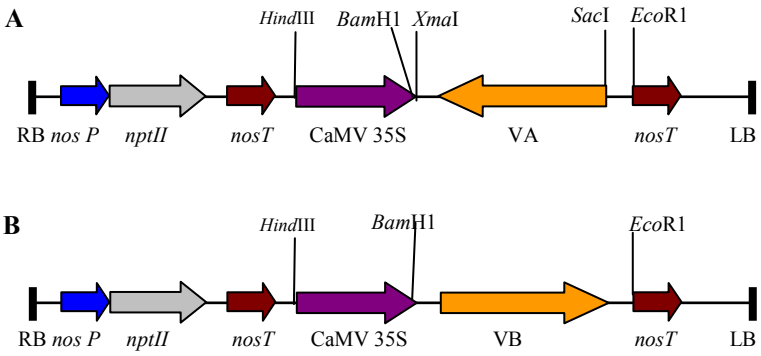

Fig.14. Schematic view of pBI121-based genetic constructs. **A** VA (the antisense construct under the CaMV 35S promoter); **B** VB (the sense construct under the CaMV 35S promoter)

VC, the antisense binary construct under the GBSSI promoter: As mentioned in the VA construct, the ~1.4 kb *Hinc*II-*Sac*I fragment of *AI-02* was cloned into *Sma*I-*Sac*I site of the binary vector pAN-GB01 by replacing the reporter GUS gene (Fig. 15A)

VD, the sense binary construct under the GBSSI promoter: Same approach was adopted as in the VB construct. The binary vector pAN-GB01 was used in place of pBI121 (Fig. 15B).

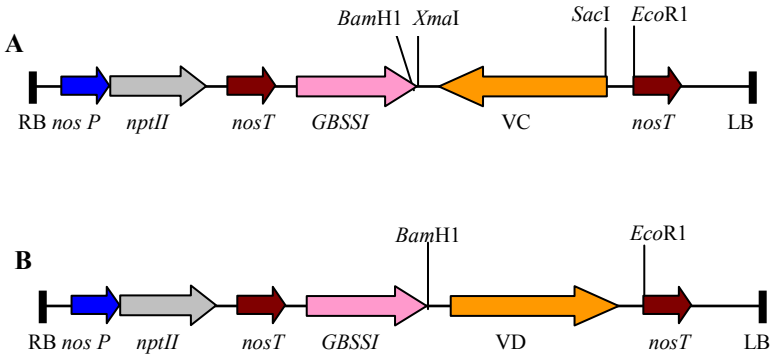

Fig. 15. Schematic view of the pAN-GB01-based genetic constructs. **A** VC (the antisense construct under the *GBSSI* promoter); **B** VD (the sense construct under the *GBSSI* promoter)

VE and VG, the antisense binary genetic constructs under the CaMV 35S promoter and the GBSSI promoter, respectively: The *AI-01* cDNA clone in the required orientation in pUC19 vector was digested with *Bam*HI and *ECl*136II in order to release ~1.0 kb fragment towards the 3′ terminus. This cDNA fragment of *AI-01* was cloned into *Bam*HI-*ECl*136II site in pBI121 by replacing the GUS gene (Fig. 16A), Likewise, the same cDNA fragment was cloned in pAN-GB01 (Fig. 16B)

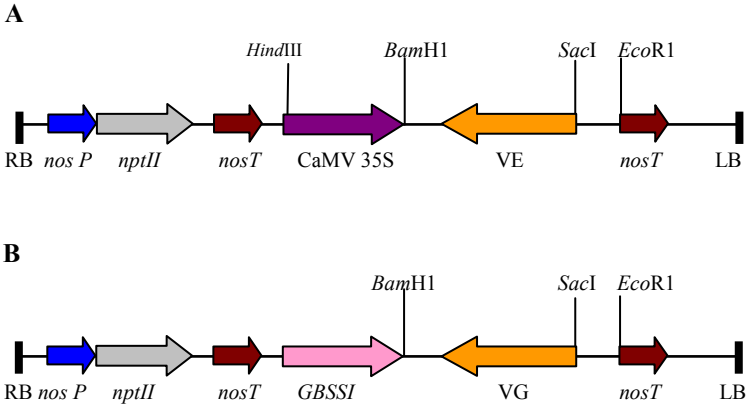

Fig. 16. Schematic view of the antisense genetic constructs. **A** VE, pBI121-based antisense construct under the CaMV 35S promoter; **B** VG, pAN-GB01-based antisense construct under the *GBSSI* promoter

VF and VH, the sense binary genetic constructs under the CaMV 35S promoter and the GBSSI promoter, respectively: The *AI-01* cDNA clone was digested with *Bam*HI, and then treated with Klenow enzyme for filling of the 3′ ends; further digested with *Ecl*136II to release ~1.0 kb fragment towards its 3′ terminus. This cDNA fragment was then cloned into the *Sma*I-*ECl136*II site in the binary vectors pBI121 and pAN-GB01 by replacing the reporter GUS gene (Fig. 17A, B). Here, the respective sense genetic constructs were obtained through screening by restriction analyses and PCR.

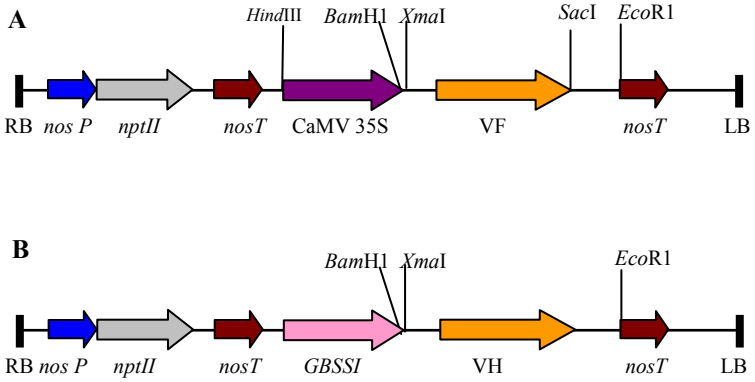

Fig. 17. Schematic view of the sense genetic constructs. **A** VF, pBI121-based sense construct under the CaMV 35S promoter; **B** VH, pAN-GB01-based sense construct under the *GBSSI* promoter

Prior to further use, each of the vacuolar invertase cDNA based antisense and sense binary genetic constructs as mentioned above was checked carefully by restriction analyses and/or PCR to see the intactness, and orientation of the inserts with respect to the constitutive or tuber-specific promoter in either of the binary vectors.

5.4. To generate transgenic potato lines with the above constructs followed by screening for desired ones

5.4.1. 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 rifampcin (15 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).

5.4.2. Agrobacterium mediated co-cultivation and generation of transgenic potato plants: 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). 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 lines. Multiple shoot regeneration with minimum intervening callus phase and subsequently healthy plantlets from the regenerated shoots were noticed for each construct (Fig. 18 & Fig. 19).

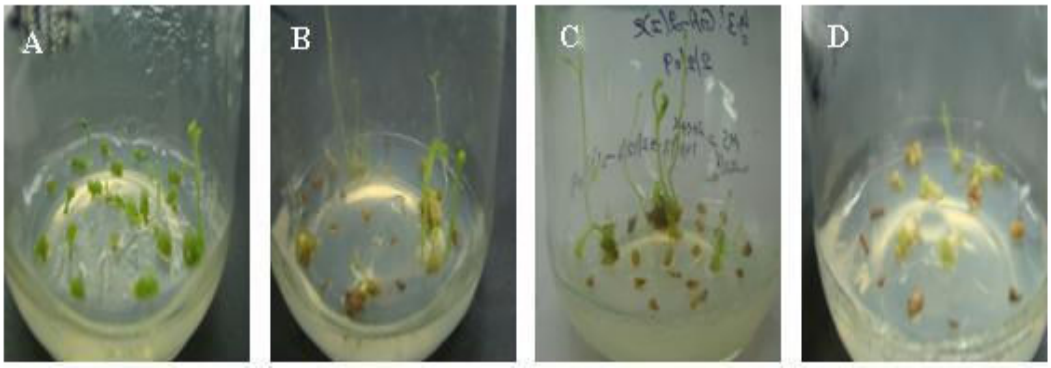

Fig. 18. The schematic view of regeneration of shoots from internodal stem (CS-1) segments with minimal intervening callus phase after co-cultivation on selective media. **A** shoot regeneration corresponding to VA binary construct; **B** shoot regeneration corresponding to VB binary construct; **C** shoot regeneration corresponding to VC binary construct; **D** shoot regeneration corresponding to VD binary construct

Fig. 19. The schematic view of regeneration of shoots from internodal stem (CS-1) segments with minimal intervening callus phase after co-cultivation on selective media. **A** shoot regeneration corresponding to VE binary construct; **B** shoot regeneration corresponding to VF binary construct; **C** shoot regeneration corresponding to VG binary construct; **D** shoot regeneration corresponding to VH binary construct

5.4.3. 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 a number of cases, rooting was delayed from shoot explants. Subsequently complete plantlets were produced from the regenerated shoots. The transgenic potato plantlets corresponding to each genetic construct are shown in the Figs. 20 & 21.

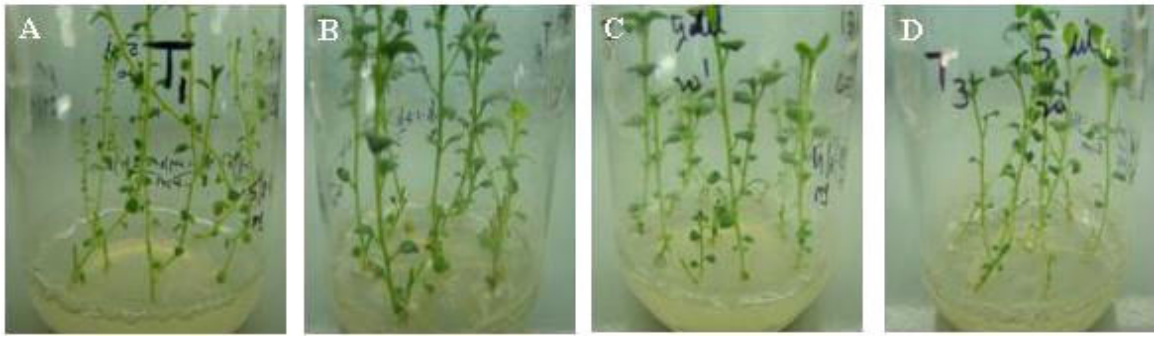

Fig. 20. The schematic view of micropropagated plantlets from regenerated shoots of transgenic potato lines on selective media. **A** VA binary construct based micropropagated plantlets; **B** VB binary construct based micropropagated plantlets; **C** VC binary construct based micropropagated plantlets; **D** VD binary construct based micropropagated plantlets

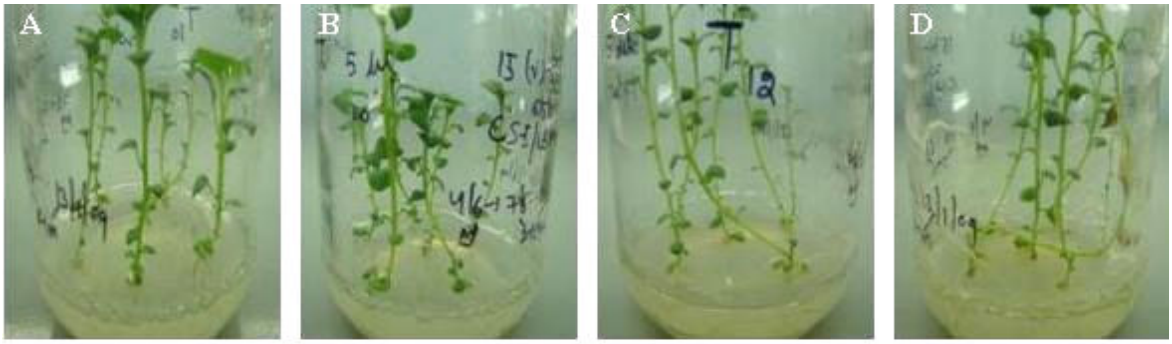

Fig. 21. The schematic view of micropropagated plantlets from the regenerated shoots of transgenic potato lines on selective media. **A** VE binary construct based micropropagated plantlets; **B** VF binary construct based micropropagated plantlets; **C** VG binary construct based micropropagated plantlets; **D** VH binary construct based micropropagated plantlets

PCR approach to check the presence of transgenes: Total genomic DNA was isolated from the plantlets growing in the selective media corresponding to the independent regenerated lines. The presence of the transferred DNA into potato system was checked by PCR using specific oligonucleotide primer pairs (the primers were specific to the CaMV 35S promoter, the *GBSSI* promoter, *AI-01* and *AI-02* cDNAs). A number of regenerated plantlets corresponding to each of the binary genetic constructs were found to show positive response in terms of PCR amplification indicating stable integration of the transgene (s) into potato genome (data not shown). Based on this screening procedure, nearly ten independent transgenic potato lines were selected for each binary genetic construct.

5.4.4. Production of mini tubers from transgenic and control potato plants: After hardening and acclimatization, the transgenic potato plantlets were grown in polybags for mini tuber production in the restricted experimental plot. Similarly, control potato plants were grown. Growth features of the plants were noted. No marked variation was noticed with regard to morphology in comparison to the control plants. Mature potato mini tubers were harvested. The visual examination of the tubers revealed no visible changes with regard to tuber morphology, size and yield as compared to the control tubers. The cultivation process of transgenic potato lines along with control is shown in Fig. 22. These mini tubers were used for various biochemical & molecular studies.

Fig. 22. The schematic view of different phases of potato cultivation i.e. from hardening to harvesting of different transgenic lines. **A** hardening and acclimatization in pro-trays in potting mix of soil and coco-peat; **B** transferred to polybags filled with soil; **C** 35-45 days old growing transgenic lines; **D** two months old growing transgenic lines; **E** and **F** harvesting of mini tubers; **G** & **H** washed and air dried mini tubers from transgenic lines; **I** control (nontransgenic) tubers

5.4.5. Molecular and biochemical characterization of transgenic potato tubers

*PCR analyses of the transgenic potato tubers***:** The total genomic DNA was isolated from the transgenic mini tubers for checking the presence and intactness of transgene using different primer pairs. The first set of primer pairs includes different promoter-specific forward primers and vacuolar invertase cDNA-specific reverse primers to detect the presence of transgene in potato tubers. The second set of primer pairs includes *npt*II specific forward primer and cDNA-specific reverse primers to identify the intactness of the only T-DNA cassette transferred in potato tubers. The third set of primer pairs are specific to outside of right border of T-DNA in the vector along with cDNA-specific reverse primers. The purpose of this last set of primers was to see any bacterial DNA contamination in the genomic DNA isolated from transgenic potato tubers. PCR was carried out using the potato genomic DNA as template corresponding to the different transgenic using different primer pairs, and the amplified DNA products were analyzed by agarose gel electrophoresis as shown in Figures 23-30. All these data indicate the presence of transgene (s) in a number of potato lines corresponding to the individual binary genetic constructs.

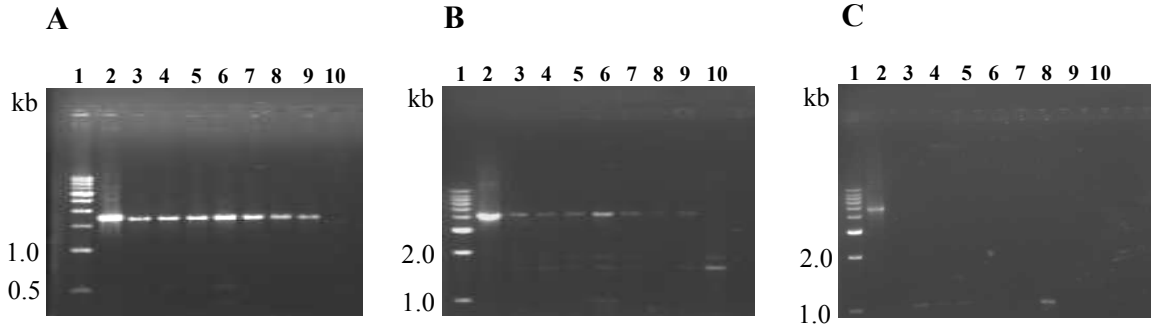

Fig. 23. PCR analyses of **VA** binary construct-based transgenic potato lines using tuber DNA as template. **A** 1.8 kb fragment amplified by using AF-3501 forward primer (from CaMV 35S promoter region) and L20-AI reverse primer; **B** ~4.0 kb fragment amplified by using NPT-F1 forward primer (from *npt*II promoter region) and L20-AI reverse primer; **C** ~4.9 kb fragment amplified by using ORB-F01 forward primer (from out side right border region) and L20-AI reverse primer (no DNA band amplified for the transgenic lines).
In each figure, lanes 3 to 9 correspond to different transgenic lines,VA-2, VA-5, VA-6, VA-10, VA-28, VA-31 and VA-32; lane 10 Kufri chipsona-1 (CS-1) as control; Lane 1 in **A** represent 0.5 kb DNA ladder, whereas in **B** and **C,** lane 1 represents 1.0 kb DNA ladder; lane 2 in each figure, VA binary construct as template

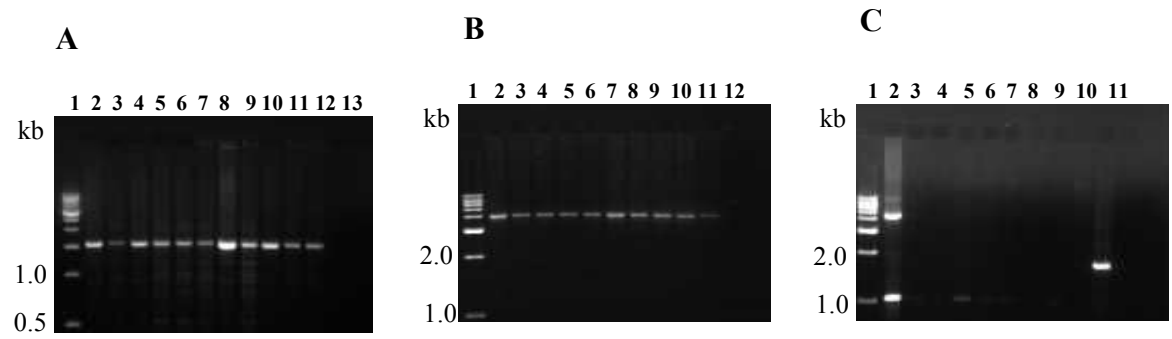

Fig. 24. PCR analyses of VB binary construct-based transgenic potato lines using tuber DNA as template. **A** ~1.5 kb fragment amplified by using AF-3501 forward primer (from CaMV 35S promoter region) and M20-AI reverse primer; **B** ~4.0 kb fragment amplified by using NPT-F1 forward primer (from *npt*II promoter region) and M20-AI reverse primer; **C** ~4.5 kb fragment amplified by using ORB-F01 forward primer (from out side right border region) and M20-AI reverse primer (no DNA band amplified for the transgenic lines).
Lane 1 0.5 kb DNA ladder in **A** and 1.0 kb DNA ladder in **B** & **C**; lane 2 in each figure, VB binary construct as template; **A** lanes 3 to 12 different transgenic lines of VB-1, VB-2, VB-3, VB-5, VB-6, VB-8, VB-9, VB-10, VB-12 and VB-14; **B** lanes 3 to 11 different transgenic lines of VB-1, VB-2, VB-3, VB-5, VB-6, VB-8, VB-9, VB-10 and VB-12; **C** lanes 3 to 10 different transgenic lines of VB-1, VB-2, VB-3, VB-5, VB-6, VB-8, VB-10 and VB-12; lane 13 in **A**, lane 12 in **B**, lane 11 in **C** Kufri chipsona-1 (CS-1) as control

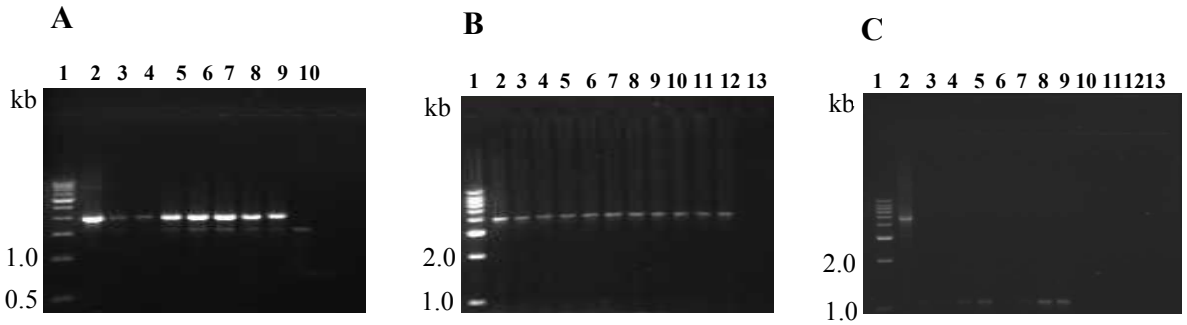

Fig. 25. PCR analyses of VC binary construct-based transgenic potato lines using tuber DNA as template. **A** ~2.0 kb fragment amplified by using GB1-F01 forward primer (from *GBSSI* promoter region) and L20-AI reverse primer; **B** ~4.0 kb fragment amplified by using NPT-F1 forward primer (from *npt*II promoter region) and L20-AI reverse primer; **C** ~4.5 kb fragment amplified by using ORB-F01 forward primer (from out side right border region) and L20-AI reverse primer (no DNA band amplified for the transgenic lines).
Lane1 0.5 kb DNA ladder in **A** and 1.0 kb DNA ladder in **B** & **C**; lane 2 in each figure, VC binary construct as template; **A** lanes 3 to 9 different transgenic lines of VC-12, VC-24, VC-26, VC-28, VC-31, VC-31and VC-36; **B** and **C** lanes 3 to 12 different transgenic lines of VC-12, VC-23, VC-24, VC-25, VC-26, VC-28, VC-31, VC-33, VC-35 and VC-36; lane 10 in **A**, lane 13 in **B** and **C** Kufri chipsona-1 (CS-1) as control

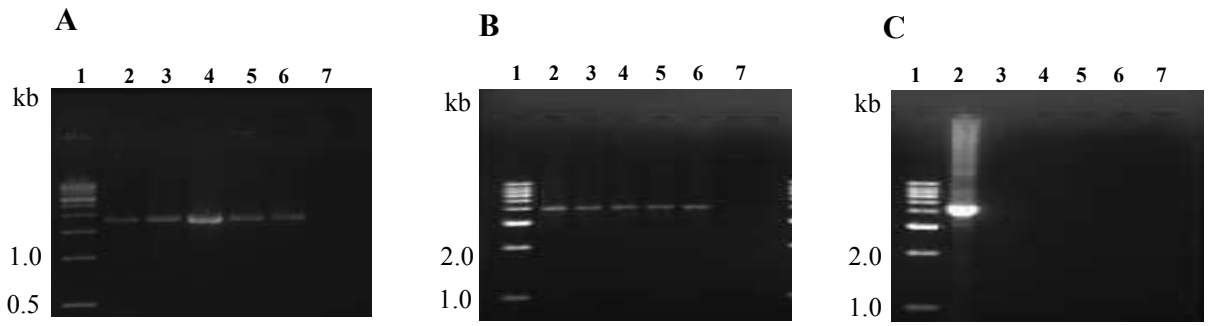

Fig. 26. PCR analyses of VD binary construct-based transgenic potato lines using tuber DNA as template. **A** ~1.8 kb fragment amplified using GB1-F01 forward primer (from *GBSSI* promoter region) and M20-AI reverse primer; **B** ~4.0 kb fragment amplified using NPT-F1 forward primer (from *npt*II promoter region) and M20-AI reverse primer; **C** ~4.5 kb fragment amplified using ORB-F01 forward primer (from out side right border region) and M20-AI reverse primer (no DNA band amplified for the transgenic lines).
Lane1 0.5 kb DNA ladder in **A** and 1.0 kb DNA ladder in **B** & **C**; lane 2 in each figure, VD binary construct as template; lanes 3 to 6 different transgenic lines of VD-1, VD-2, VD-3, and VD-4; lane 7 Kufri chipsona-1 (CS-1) as control

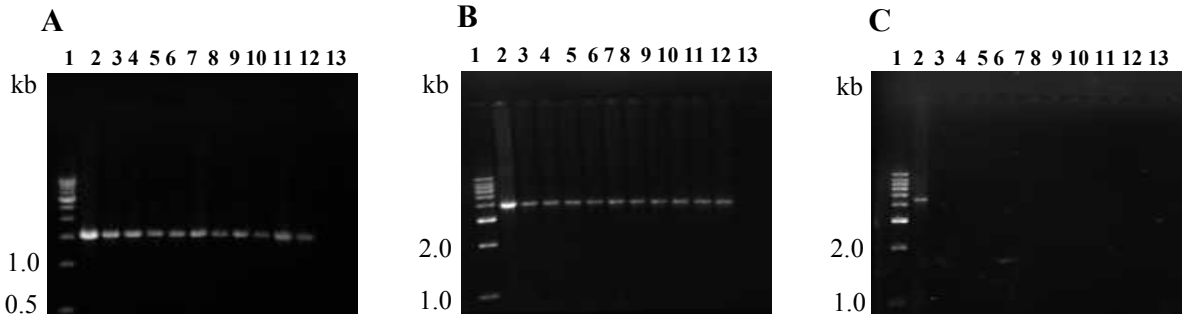

Fig. 27. PCR analyses of VE binary construct-based transgenic potato lines using tuber DNA as template. **A** ~1.5 kb fragment amplified using AF-3501 forward primer (from CaMV 35S promoter region) and AI-F995 reverse primer; **B** ~4.0 kb fragment amplified using NPT-F1 forward primer (from *npt*II promoter region) and AI-F995 reverse primer; **C** ~4.5 kb fragment amplified using ORB-F01 forward primer (from out side right border region) and AI-F995 reverse primer (no DNA band amplified for the transgenic lines).
Lane1 0.5 kb DNA ladder in **A** and 1.0 kb DNA ladder in **B** & **C**; lane 2 in each figure, VE binary construct as template; lanes 3 to 12 different transgenic lines VE-1, VE-2, VE-3, VE-4, VE-5, VE-6 VE-7, VE-8, VE-9, and VE-10; lane 13 Kufri chipsona-1 (CS-1) as control

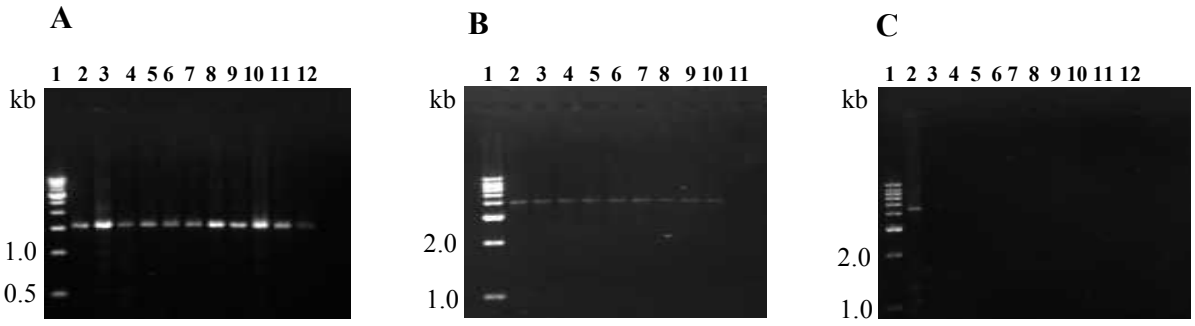

Fig. 28. PCR analyses of VF binary construct-based transgenic potato lines using tuber DNA as template. **A** ~1.5 kb fragment amplified using AF-3501 forward primer (from CaMV 35S promoter region) and AI-2016 reverse primer; **B** ~4.0 kb fragment using NPT-F1 forward primer (from *npt*II promoter region) and AI-2016 reverse primer; **C** ~4.5 kb fragment using ORB-F01 forward primer (from out side right border region) and AI-2016 reverse primer (no DNA band amplified for the transgenic lines).
Lane1 0.5 kb DNA ladder in **A** and 1.0 kb DNA ladder in **B** & **C**; lane 2 in each figure, VF binary construct as template; **A** and **C;** lanes 3 to 12 different transgenic lines VF-1, VF-2, VF-3, VF-4, VF-5, VF-6, VF-7, VF-8, and VF-12; **B** lanes 3 to 10 different transgenic lines VF-1, VF-2, VF-3, VF-4, VF-5, VF-6, VF-7 and VF-12 ; **A** & **C**, lane 12 and **B**, lane 11 Kufri Chipsona-1 (CS-1) as control

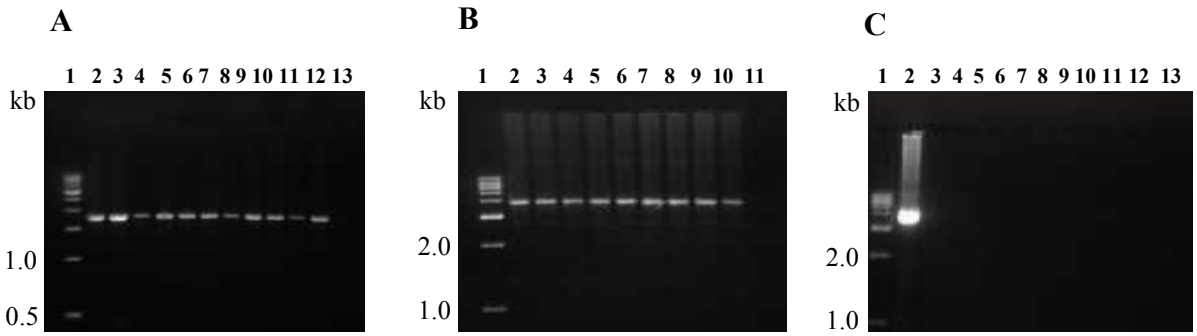

Fig. 29. PCR analyses of VG binary construct-based transgenic potato lines using tuber DNA as template. **A** ~1.8 kb fragment amplified using GB1-F01 forward primer (from *GBSSI* promoter region) and AI-F995 reverse primer; **B** ~4.0 kb fragment amplified using NPT-F1 forward primer (from *npt*II promoter region) and AI-F995 reverse primer; **C** ~4.5 kb fragment amplified using ORB-F01 forward primer (from out side right border region) and AI-F995 reverse primer (no DNA band amplified for the transgenic lines).
Lane1 0.5 kb DNA ladder in **A** and 1.0 kb DNA ladder in **B** & **C**; lane 2 in each figure, VG binary construct as template; **A & C** lanes 3 to 12 different transgenic lines VG-1, VG-2, VG-3, VG-5, VG-8, VG-12, VG-13, VG-14, VG-15 and VG-16; **B** lanes 3 to 10 different transgenic lines VG-1, VG-2, VG-5, VG-8, VG-12, VG-13, VG-14 and VG-15; **A &C** lane 13 and **C** lane 11 Kufri chipsona-1 (CS-1) as control

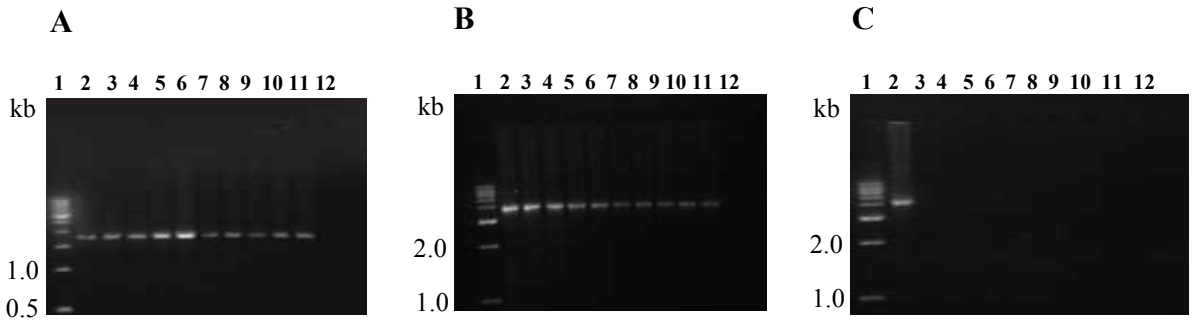

Fig. 30. PCR analyses of VH binary construct-based transgenic potato lines using tuber DNA as template. **A** ~1.8 kb fragment amplified using GB1-F01 forward primer (from *GBSSI* promoter region) and AI-2016 reverse primer; **B** ~4.0 kb fragment amplified using NPT-F1 forward primer (from *npt*II promoter region) and AI-F995 reverse primer; **C** ~4.2 kb fragment amplified using ORB-F01 forward primer (from out side right border region) and AI-F995 reverse primer (no DNA band amplified for the transgenic lines).
Lane1 0.5 kb DNA ladder in **A** and 1.0 kb DNA ladder in **B** & **C**; lane 2 in each figure, VH binary construct as template; lanes 3 to 11 different transgenic lines VH-1, VH-2, VH-3, VH-4, VH-5, VH-6, VH-7, VH-8 and VH-10; lane 12 Kufri chipsona-1 (CS-1) as control

Biochemical analyses of the transgenic potato tubers: *A*fter three weeks of storage at room temperature, the harvested mini tubers were shifted to different experimental temperatures, 4ºC and 25ºC for one month and two months. Tuber slices were frozen in liquid nitrogen and kept at -70^{0}C for further use. Total soluble sugar was extracted from the tuber slices. Crude protein extracts were made from the tuber slices for protein blot analyses.

Biochemical analyses of VA-based transgenic potato tubers: The reducing sugar contents of ten independent transgenic lines are presented in both Table 5 and Fig. 31. Likewise, Table 6 and Fig. 32 provide total soluble sugar contents.

Table 5. Reducing sugar contents in the freshly harvested and stored tubers from VA-based transgenic potato lines at different temperatures

Transgenic Lines of VA	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VA-1	0.97 ± 0.23^{abcd}	1.69 ± 0.05^{a}	1.45 ± 0.05^{bc}	5.42 ± 0.05^{b}	9.14 ± 0.04^{b}
VA-2	1.14 ± 0.05^{abc}	1.04 ± 0.05^{c}	1.39 ± 0.05^{c}	4.38 ± 0.15^{c}	4.76 ± 0.03^{d}
VA-3	1.00 ± 0.15^{abcd}	1.11 ± 0.07^{c}	0.95 ± 0.04^{d}	6.84 ± 0.06^{a}	7.12 ± 0.06^{c}
VA-4	1.27 ± 0.15^{a}	0.63 ± 0.02^{d}	0.74 ± 0.03^{de}	3.75 ± 0.10^{d}	7.75 ± 0.08^{c}
VA-5	0.86 ± 0.03^{cde}	0.75 ± 0.07^{d}	0.87 ± 0.05^{de}	1.25 ± 0.08^{g}	3.56 ± 0.09^{e}
VA-6	0.88 ± 0.09^{bcd}	0.70 ± 0.04^{d}	0.88 ± 0.09^{de}	1.68 ± 0.07^{f}	1.97 ± 0.08^{f}
VA-10	0.77 ± 0.01^{def}	0.73 ± 0.04^{d}	0.89 ± 0.01^{de}	2.21 ± 0.09^{e}	3.66 ± 0.09^{e}
VA-28	0.91 ± 0.15^{bcd}	0.67 ± 0.18^{d}	0.88 ± 0.08^{de}	0.81 ± 0.13^{h}	1.40 ± 0.05^{f}
VA-31	0.53 ± 0.06^{f}	0.67 ± 0.10^{d}	0.71 ± 0.18^{e}	1.14 ± 0.08^{gh}	1.24 ± 0.07^{f}
VA-32	1.22 ± 0.15^{ab}	1.43 ± 0.04^{b}	1.66 ± 0.05^{b}	2.54 ± 0.16^{e}	3.36 ± 0.05^{e}
CS-1	0.60 ± 0.08^{ef}	1.24 ± 0.04^{bc}	2.13 ± 0.01^{a}	3.92 ± 0.35^{d}	13.55 ± 1.16^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

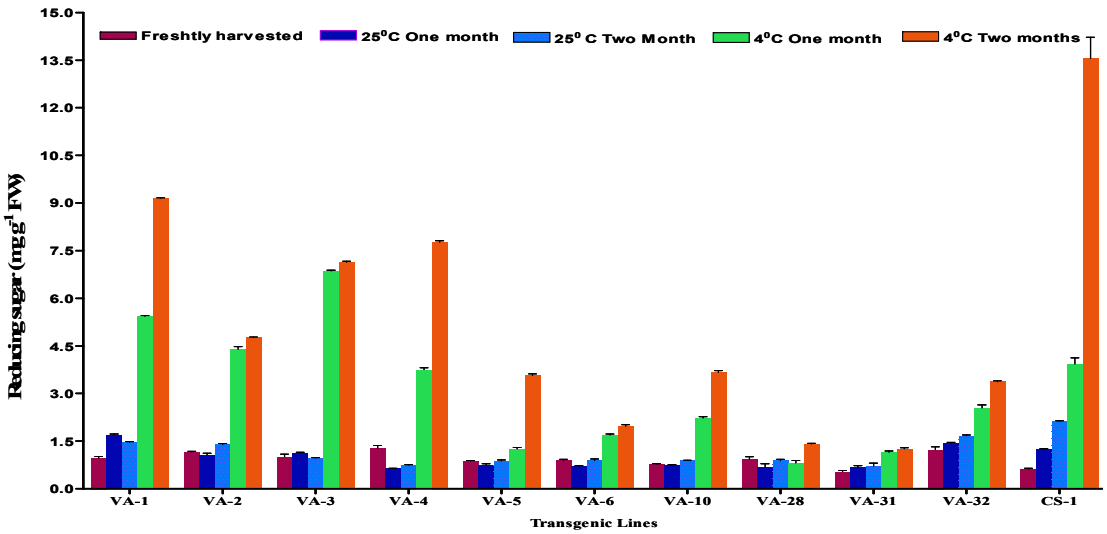

Fig. 31. Reducing sugar contents in the transgenic tubers corresponding to the data as presented in **Table 5**

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Table 6. Total sugar contents in the freshly harvested and stored tubers from VA-based transgenic potato lines at different temperatures

Transgenic Lines of VA	Total sugar (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VA-1	3.56 ± 1.15^{ab}	7.63 ± 0.39^{bc}	7.66 ± 0.05^{a}	15.40 ± 0.20^{b}	16.50 ± 1.15^{a}
VA-2	4.00 ± 0.14^{ab}	8.04 ± 0.11^{b}	3.38 ± 0.59^{efg}	12.73 ± 0.04^{c}	12.63 ± 0.67^{bc}
VA-3	3.63 ± 0.32^{ab}	6.18 ± 0.09^{c}	6.02 ± 0.25^{bc}	16.70 ± 0.19^{a}	14.20 ± 0.36^{b}
VA-4	4.29 ± 0.44^{ab}	2.89 ± 0.02^{d}	4.69 ± 0.09^{cde}	10.20 ± 0.17^{f}	12.03 ± 0.25^{cd}
VA-5	4.64 ± 0.33^{a}	3.08 ± 0.04^{d}	3.13 ± 0.00^{fg}	11.10 ± 0.03^{d}	10.18 ± 1.12^{ef}
VA-6	3.14 ± 0.07^{b}	3.82 ± 1.31^{d}	3.49 ± 0.44^{efg}	11.00 ± 0.02^{de}	9.96 ± 0.06^{ef}
VA-10	4.08 ± 0.15^{ab}	3.10 ± 0.28^{d}	4.37 ± 0.07^{def}	10.10 ± 0.10^{f}	12.03 ± 0.11^{cd}
VA-28	4.16 ± 0.03^{ab}	7.24 ± 0.23^{bc}	5.07 ± 0.50^{cd}	11.10 ± 0.20^{d}	10.76 ± 0.15^{de}
VA-31	4.47 ± 0.04^{a}	3.20 ± 0.51^{d}	2.76 ± 0.51^{g}	11.20 ± 0.09^{d}	7.94 ± 0.17^{g}
VA-32	4.09 ± 0.43^{ab}	10.04 ± 1.11^{a}	6.57 ± 1.12^{ab}	10.12 ± 0.27^{f}	8.86 ± 0.17^{fg}
CS-1	1.85 ± 0.13^{c}	2.45 ± 0.17^{d}	4.36 ± 0.59^{def}	10.51 ± 0.35^{ef}	17.83 ± 0.85^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

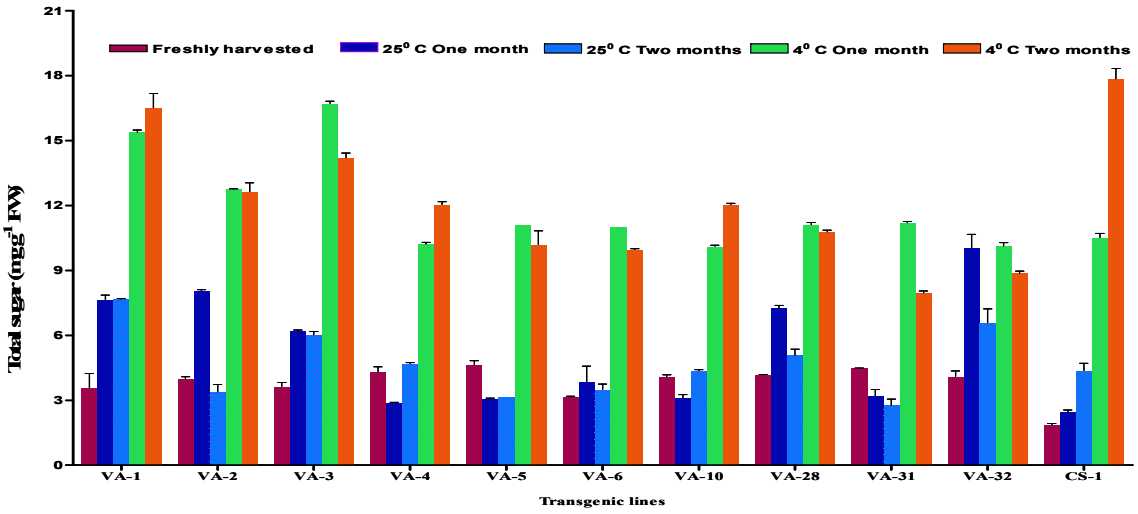

Fig. 32. Total sugar contents in the transgenic tubers corresponding to the data as presented in **Table 6**

Some of the transgenic lines namely, VA-5, VA-6, VA-28 and VA-31 showed reducing sugar contents in the range of 0.81 to 1.68 mg g^{-1} FW of tuber which was considerably less as compared to control CS-1 tubers stored under similar conditions. However the total sugar contents in all the transgenic lines were found to be more or less comparable with control plants.

Biochemical analyses of VB-based transgenic potato tubers: The reducing sugar contents of ten independent transgenic lines are presented in both Table 7 and Fig. 33. Likewise, Table 8 and Fig. 34 provide total soluble sugar contents.

Table 7. Reducing sugar contents in the freshly harvested and stored tubers from VB-based transgenic potato lines at different temperatures

Transgenic Lines of VB	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VB-1	1.20 ± 0.10^{a}	0.55 ± 0.05^{f}	0.88 ± 0.19^{d}	0.88 ± 0.16^{g}	3.48 ± 0.12^{d}
VB-2	1.26 ± 0.03^{a}	1.49 ± 0.09^{b}	1.40 ± 0.19^{bc}	2.92 ± 0.07^{cd}	4.99 ± 0.29^{bc}
VB-3	1.18 ± 0.30^{a}	0.99 ± 0.05^{d}	0.68 ± 0.03^{d}	1.29 ± 0.09^{fg}	2.83 ± 0.16^{de}
VB-5	0.61 ± 0.21^{b}	1.82 ± 0.05^{a}	1.81 ± 0.21^{ab}	3.28 ± 0.12^{c}	3.26 ± 0.06^{de}
VB-6	0.59 ± 0.07^{b}	0.74 ± 0.07^{ef}	0.69 ± 0.08^{d}	3.18 ± 0.24^{cd}	5.69 ± 0.31^{b}
VB-8	0.38 ± 0.14^{b}	0.69 ± 0.07^{ef}	0.84 ± 0.19^{d}	2.46 ± 0.06^{e}	2.61 ± 0.04^{de}
VB-9	0.56 ± 0.10^{b}	1.47 ± 0.07^{bc}	1.00 ± 0.19^{cd}	4.56 ± 0.06^{a}	5.07 ± 0.15^{b}
VB-10	0.40 ± 0.12^{b}	0.80 ± 0.04^{de}	0.62 ± 0.03^{d}	2.78 ± 0.04^{de}	3.78 ± 0.12^{cd}
VB-12	1.11 ± 0.04^{a}	0.56 ± 0.12^{f}	0.58 ± 0.14^{d}	3.05 ± 0.13^{cd}	3.49 ± 0.08^{d}
VB-14	0.63 ± 0.08^{b}	0.79 ± 0.12^{def}	0.96 ± 0.06^{d}	1.46 ± 0.02^{f}	2.13 ± 0.54^{a}
CS-1	0.60 ± 0.08^{b}	1.24 ± 0.04^{c}	2.13 ± 0.01^{a}	3.92 ± 0.35^{b}	13.55 ± 1.16^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

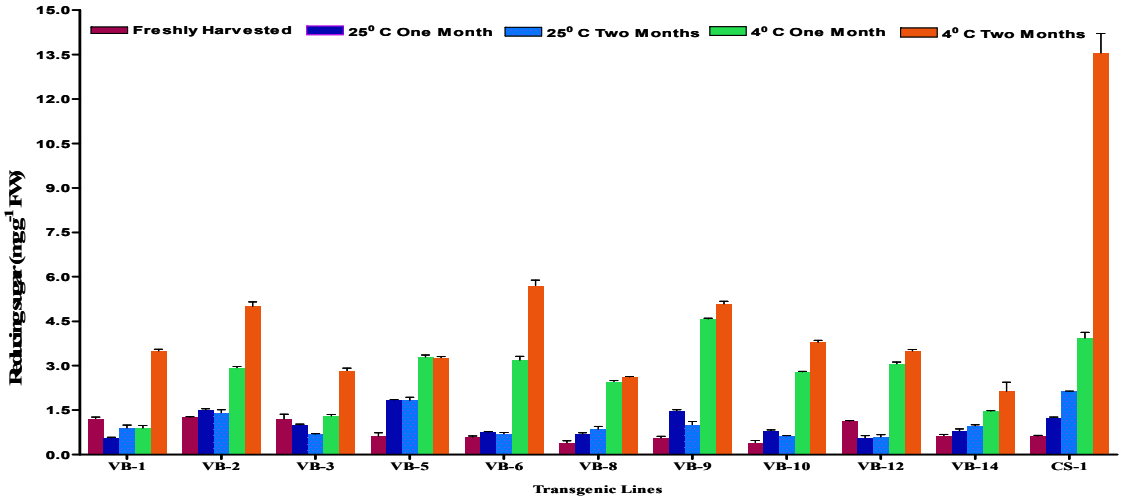

Fig. 33. Reducing sugar contents in the transgenic tubers corresponding to the data as presented in **Table 7**

Table 8. Total sugar contents in the freshly harvested and stored tubers from VB-based transgenic potato lines at different temperatures

Transgenic Lines of VB	Total sugar (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VB-1	4.16 ± 0.03^{ab}	2.51 ± 0.22^{c}	4.89 ± 0.26^{bcd}	13.83 ± 0.30^{a}	17.43 ± 0.19^{ab}
VB-2	3.79 ± 0.13^{ab}	4.81 ± 0.11^{ab}	3.84 ± 0.25^{de}	12.46 ± 0.34^{ab}	15.20 ± 1.61^{abc}
VB-3	4.03 ± 1.28^{ab}	5.23 ± 0.81^{ab}	3.18 ± 0.36^{ef}	11.63 ± 0.28^{abc}	13.96 ± 0.51^{abcd}
VB-5	3.53 ± 0.28^{abc}	5.00 ± 0.42^{ab}	6.12 ± 0.19^{ab}	10.86 ± 0.15^{bc}	10.43 ± 0.15^{de}
VB-6	2.91 ± 0.30^{bc}	5.55 ± 0.27^{ab}	5.52 ± 0.24^{abc}	10.80 ± 0.26^{bc}	13.86 ± 1.41^{bcd}
VB-8	4.33 ± 0.28^{ab}	4.39 ± 0.35^{b}	4.79 ± 0.16^{bcd}	10.40 ± 0.10^{bc}	9.72 ± 0.06^{e}
VB-9	4.55 ± 0.21^{ab}	5.74 ± 0.25^{a}	4.13 ± 0.18^{cde}	9.75 ± 0.04^{cd}	12.06 ± 0.26^{cde}
VB-10	3.42 ± 0.23^{abc}	5.03 ± 0.69^{ab}	1.90 ± 0.17^{f}	7.37 ± 0.09^{d}	13.90 ± 1.47^{bcd}
VB-12	5.04 ± 1.46^{a}	5.10 ± 0.91^{ab}	4.79 ± 1.35^{bcd}	7.59 ± 2.74^{d}	10.86 ± 3.32^{de}
VB-14	3.16 ± 0.04^{bc}	5.92 ± 0.05^{a}	6.44 ± 0.12^{a}	10.40 ± 0.09^{bc}	11.60 ± 0.50^{cde}
CS-1	1.85 ± 0.13^{c}	2.45 ± 0.17^{c}	4.36 ± 0.59^{cde}	10.51 ± 0.35^{bc}	17.83 ± 0.85^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

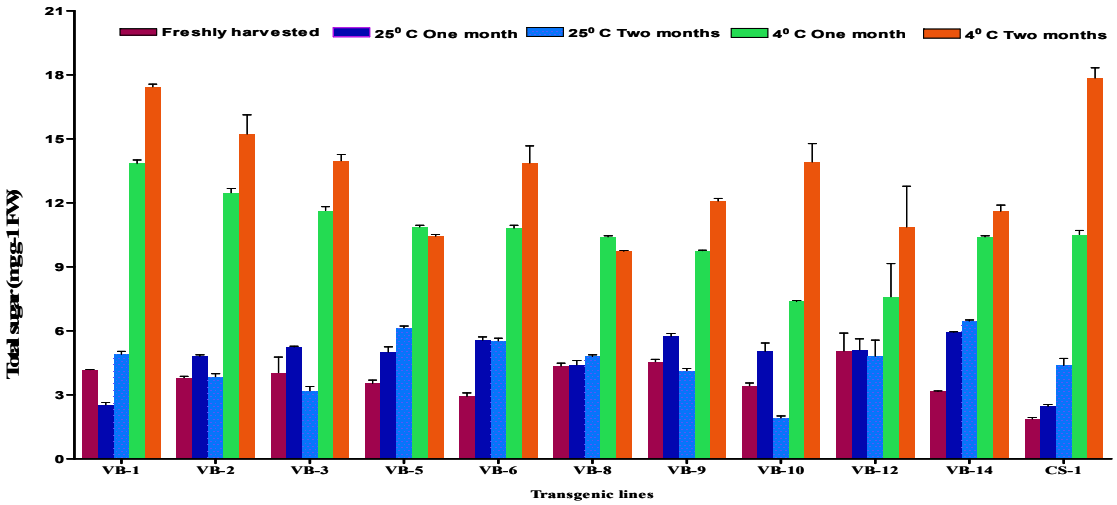

Fig. 34. Total sugar contents in the transgenic tubers corresponding to the data as presented in **Table 8**

The transgenic lines, VB-1, VB-3 and VB-14 showed less reducing sugar contents (0.88-1.46 mg g^{-1} FW) as compared to the control under similar conditions. However the total sugar contents in all the transgenic lines were found to be more or less comparable with control plants.

Biochemical analyses of VC-based transgenic potato tubers: The reducing sugar contents of ten independent transgenic lines are presented in both Table 9 and Fig. 35. Likewise, Table 10 and Fig. 36 provide total soluble sugar contents.

Table 9. Reducing sugar contents in the freshly harvested and stored tubers from VC-based transgenic potato lines at different temperatures

Transgenic Lines of VC	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VC-12	0.36 ± 0.05^{d}	0.55 ± 0.15^{de}	0.34 ± 0.03^{f}	1.09 ± 0.21^{e}	2.61 ± 0.15^{e}
VC-23	0.36 ± 0.08^{d}	1.11 ± 0.07^{ab}	0.57 ± 0.03^{de}	1.58 ± 0.21^{e}	2.99 ± 0.92^{de}
VC-24	0.85 ± 0.02^{a}	1.08 ± 0.03^{ab}	0.83 ± 0.04^{bc}	1.20 ± 0.10^{e}	1.92 ± 0.15^{e}
VC-25	0.21 ± 0.05^{de}	0.43 ± 0.03^{e}	0.33 ± 0.07^{f}	3.39 ± 0.11^{cd}	4.68 ± 0.27^{c}
VC-26	0.20 ± 0.02^{de}	1.10 ± 0.14^{ab}	0.89 ± 0.09^{b}	1.75 ± 0.03^{e}	1.76 ± 0.02^{e}
VC-28	0.65 ± 0.11^{ab}	0.55 ± 0.02^{de}	0.38 ± 0.01^{ef}	1.45 ± 0.30^{e}	2.75 ± 0.16^{e}
VC-31	0.39 ± 0.03^{cd}	0.80 ± 0.08^{bcd}	0.64 ± 0.04^{cd}	3.16 ± 0.02^{d}	4.24 ± 0.08^{cd}
VC-33	0.31 ± 0.06^{de}	0.72 ± 0.04^{cde}	0.74 ± 0.07^{bcd}	5.93 ± 0.05^{b}	5.49 ± 0.11^{bc}
VC-35	0.85 ± 0.14^{a}	0.97 ± 0.23^{abc}	0.86 ± 0.18^{b}	6.90 ± 0.18^{a}	6.07 ± 0.20^{b}
VC-36	0.12 ± 0.02^{e}	1.10 ± 0.14^{ab}	0.60 ± 0.03^{d}	3.41 ± 0.44^{cd}	4.34 ± 0.31^{cd}
CS-1	0.60 ± 0.08^{bc}	1.24 ± 0.04^{a}	2.13 ± 0.01^{a}	3.92 ± 0.35^{c}	13.55 ± 1.16^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

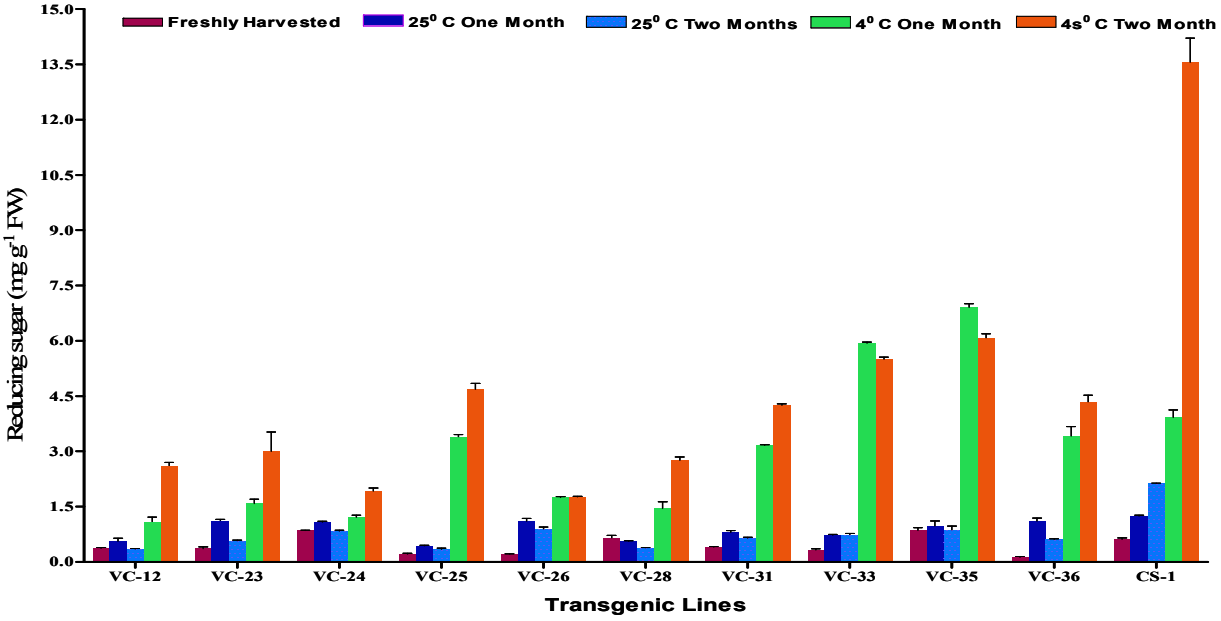

Fig. 35. Reducing sugar contents in the transgenic tubers corresponding to the data as presented in **Table 9**.

Table 10. Total sugar contents in the freshly harvested and stored tubers from VC-based transgenic potato lines at different temperatures

Transgenic Lines of VC	Total sugar (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VC-12	4.64 ± 0.26^{bc}	2.59 ± 0.99^{cde}	3.26 ± 0.32^{ab}	13.93 ± 0.21^{a}	12.10 ± 1.25^{bc}
VC-23	3.20 ± 0.03^{de}	3.53 ± 0.11^{abc}	3.03 ± 0.10^{b}	14.03 ± 0.29^{a}	11.03 ± 1.13^{c}
VC-24	2.99 ± 0.17^{e}	2.78 ± 0.18^{bcde}	3.22 ± 0.31^{ab}	13.53 ± 0.06^{ab}	11.96 ± 0.25^{bc}
VC-25	3.95 ± 0.08^{cd}	4.05 ± 0.08^{a}	3.46 ± 0.48^{ab}	13.06 ± 0.56^{b}	11.26 ± 0.75^{c}
VC-26	3.36 ± 0.01^{de}	3.45 ± 0.05^{bcd}	3.47 ± 0.95^{ab}	11.80 ± 0.01^{c}	10.20 ± 0.17^{c}
VC-28	4.77 ± 0.14^{bc}	2.33 ± 0.07^{de}	2.77 ± 0.14^{b}	11.40 ± 0.40^{cd}	12.36 ± 0.55^{bc}
VC-31	4.77 ± 0.26^{bc}	2.39 ± 0.45^{de}	3.67 ± 0.43^{ab}	9.25 ± 0.11^{g}	12.13 ± 0.25^{bc}
VC-33	6.14 ± 0.18^{a}	2.12 ± 0.05^{e}	3.89 ± 0.05^{ab}	10.93 ± 0.05^{de}	11.13 ± 0.85^{c}
VC-35	3.55 ± 0.80^{de}	2.79 ± 0.67^{bcde}	3.50 ± 0.34^{ab}	12.0 ± 0.25^{c}	12.86 ± 1.75^{bc}
VC-36	5.42 ± 0.32^{ab}	3.90 ± 0.16^{ab}	4.01 ± 0.27^{ab}	9.91 ± 0.34^{fg}	14.93 ± 2.70^{ab}
CS-1	1.85 ± 0.13^{f}	2.45 ± 0.17^{cde}	4.36 ± 0.59^{a}	10.51 ± 0.35^{ef}	17.83 ± 0.85^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

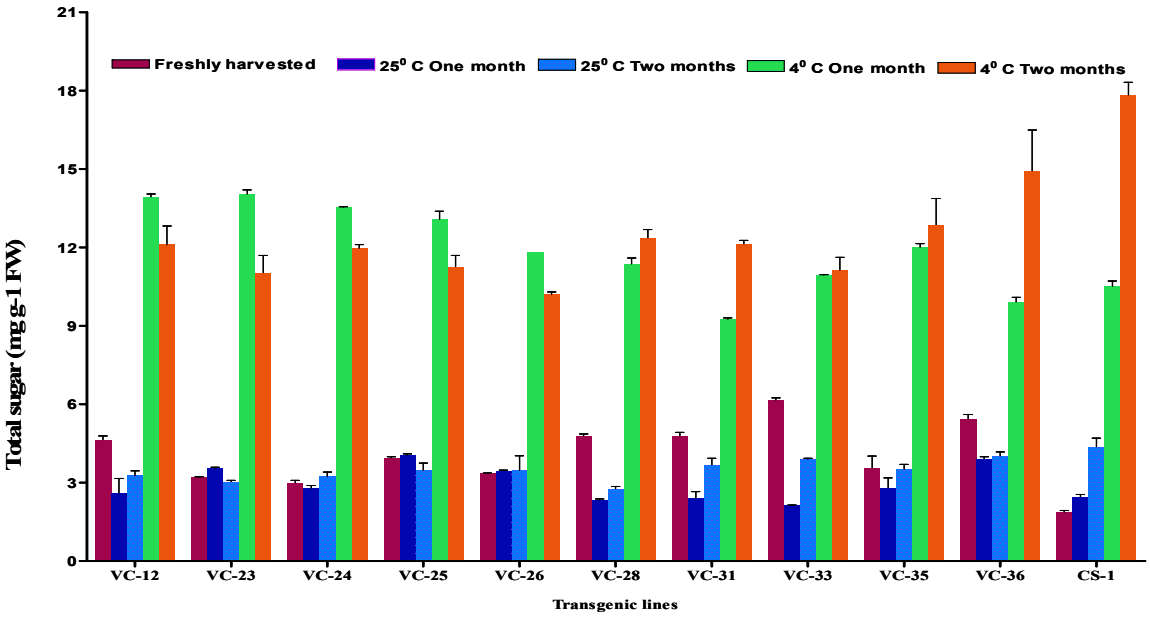

Fig. 36. Total sugar contents in the transgenic tubers corresponding to the data as presented in **Table 10**

Here, the transgenic lines namely VC-12, VC-23, VC-24, VC-26 and VC-28 showed less reducing sugar level ranging from 1.09-1.75 mg g^{-1} FW of tuber as compared to control tubers stored under similar conditions. However, the total sugar content in the above transgenic lines was more or less similar when compared to control.

Biochemical analyses of VD-based transgenic potato tubers: The reducing sugar contents of four independent transgenic lines are presented in both Table 11 and Fig. 37. Likewise, Table 12 and Fig. 38 provide total soluble sugar contents.

Table 11. Reducing sugar contents in the freshly harvested and stored tubers from VD-based transgenic potato lines at different temperatures

Transgenic Lines of VD	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VD-1	0.40 ± 0.06^{b}	0.72 ± 0.05^{c}	0.53 ± 0.03^{c}	4.46 ± 0.09^{a}	6.98 ± 0.02^{b}
VD-2	0.50 ± 0.07^{ab}	0.54 ± 0.07^{d}	0.53 ± 0.08^{c}	2.49 ± 0.11^{c}	3.31 ± 0.06^{c}
VD-3	0.46 ± 0.07^{ab}	0.52 ± 0.04^{d}	0.78 ± 0.05^{b}	2.10 ± 0.26^{c}	3.39 ± 0.05^{c}
VD-4	0.39 ± 0.02^{b}	0.96 ± 0.09^{b}	0.89 ± 0.01^{b}	3.52 ± 0.02^{b}	6.40 ± 0.25^{b}
CS-1	0.60 ± 0.08^{a}	1.24 ± 0.04^{a}	2.13 ± 0.01^{a}	3.92 ± 0.35^{ab}	13.55 ± 1.16^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

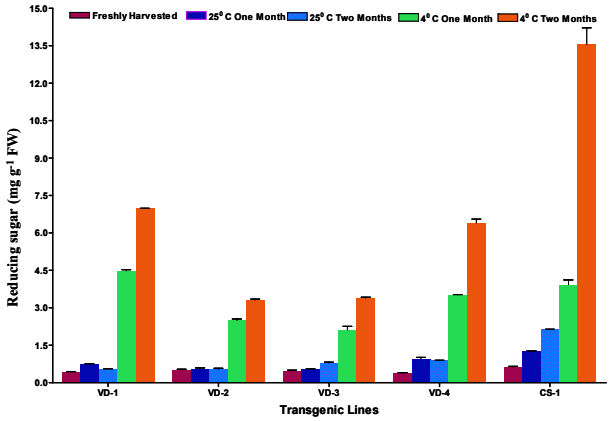

Fig. 37. Reducing sugar contents in the transgenic tubers corresponding to the data as presented in **Table 11**

Table 12. Total sugar contents in the freshly harvested and stored tubers from VD-based transgenic potato lines at different temperatures

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Transgenic Lines of VD	Total sugar (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VD-1	2.33 ± 0.15^{c}	5.35 ± 0.70^{a}	2.97 ± 0.06^{bc}	10.90 ± 0.04^{ab}	19.73 ± 0.05^{a}
VD-2	3.36 ± 0.13^{b}	5.22 ± 0.08^{a}	2.59 ± 0.34^{c}	11.23 ± 0.35^{a}	10.70 ± 0.06^{d}
VD-3	4.17 ± 0.16^{a}	3.60 ± 0.23^{b}	3.60 ± 0.08^{ab}	10.20 ± 0.10^{bc}	11.03 ± 0.11^{d}
VD-4	3.47 ± 0.14^{b}	5.56 ± 0.50^{a}	3.88 ± 0.04^{a}	9.38 ± 0.61^{c}	13.16 ± 0.26^{c}
CS-1	1.85 ± 0.13^{d}	2.45 ± 0.17^{c}	4.36 ± 0.59^{a}	10.51 ± 0.35^{ab}	17.83 ± 0.85^{b}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

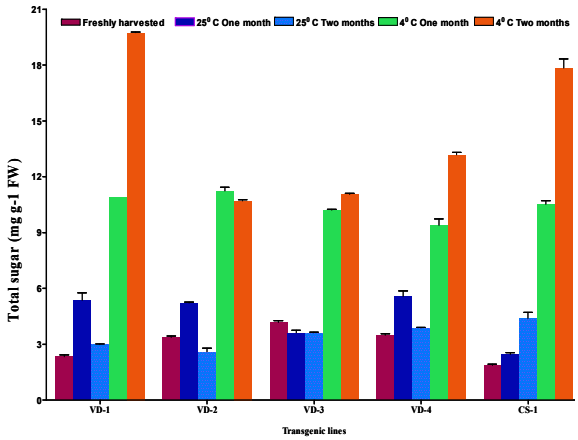

Fig. 38. Total sugar contents in the transgenic tubers corresponding to the data as presented in **Table 12**

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Some of the transgenic lines namely VD-2 and VD-3 showed less reducing sugar level between 2.10 to 2.49 mg g^{-1} FW of tuber which was considerably less as compared to control tubers stored under similar conditions. However, the total sugar content in the above transgenic lines was more or less similar when compared to control.

Biochemical analyses of VE-based transgenic potato tubers: The reducing sugar contents of ten independent transgenic lines are presented in both Table 13 and Fig. 39. Likewise, Table 14 and Fig. 40 provide total soluble sugar contents.

Table 13. Reducing sugar contents in the freshly harvested and stored tubers from VE-based transgenic potato lines at different temperatures

Transgenic Lines of VE	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VE-1	1.34 ± 0.13^{bc}	0.69 ± 0.03^{ef}	0.84 ± 0.01^{cd}	5.62 ± 0.09^{a}	8.33 ± 0.20^{b}
VE-2	1.02 ± 0.08^{cd}	1.21 ± 0.17^{ab}	1.03 ± 0.01^{bc}	1.56 ± 0.11^{e}	1.64 ± 0.17^{d}
VE-3	4.25 ± 0.25^{a}	0.76 ± 0.05^{de}	0.81 ± 0.05^{cd}	2.66 ± 0.02^{c}	2.08 ± 0.09^{d}
VE-4	0.85 ± 0.08^{de}	1.42 ± 0.03^{a}	1.26 ± 0.09^{b}	2.13 ± 0.08^{d}	3.76 ± 0.13^{c}
VE-5	0.82 ± 0.14^{de}	0.94 ± 0.05^{cd}	0.70 ± 0.04^{d}	1.00 ± 0.05^{f}	1.29 ± 0.03^{d}
VE-6	0.80 ± 0.18^{de}	1.03 ± 0.06^{bc}	0.81 ± 0.07^{cd}	0.92 ± 0.13^{f}	1.15 ± 0.11^{d}
VE-7	1.53 ± 0.04^{b}	0.95 ± 0.17^{cd}	0.80 ± 0.07^{cd}	0.94 ± 0.11^{f}	1.34 ± 0.02^{d}
VE-8	1.06 ± 0.07^{cd}	1.10 ± 0.04^{bc}	0.71 ± 0.01^{d}	1.07 ± 0.06^{f}	1.29 ± 0.26^{d}
VE-9	0.73 ± 0.09^{de}	0.48 ± 0.02^{f}	0.70 ± 0.21^{d}	0.94 ± 0.04^{f}	1.21 ± 0.08^{d}
VE-10	0.60 ± 0.06^{e}	0.99 ± 0.05^{bcd}	0.80 ± 0.04^{cd}	0.95 ± 0.04^{f}	1.31 ± 0.03^{d}
CS-1	0.60 ± 0.08^{e}	1.24 ± 0.04^{ab}	2.13 ± 0.01^{a}	3.92 ± 0.35^{b}	13.55 ± 1.16^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

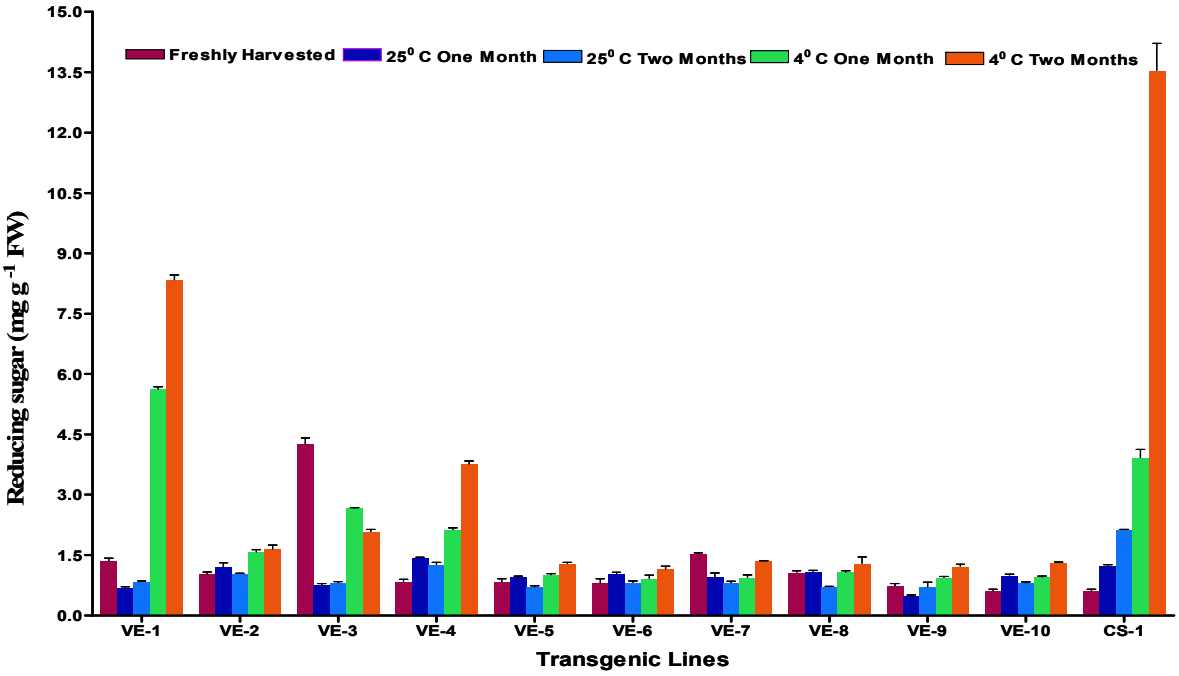

Fig. 39. Reducing sugar contents in the transgenic tubers corresponding to the data as presented in **Table 13**

Table 14. Total sugar contents in the freshly harvested and stored tubers from VE-based transgenic potato lines at different temperatures

Transgenic Lines of VE	Total sugar (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VE-1	4.66 ± 2.25^{bc}	3.78 ± 0.18^{ab}	4.64 ± 1.51^{a}	10.39 ± 0.91^{a}	17.10 ± 1.45^{a}
VE-2	5.59 ± 0.10^{ab}	4.74 ± 0.12^{a}	4.11 ± 0.01^{a}	10.43 ± 0.21^{a}	9.47 ± 0.24^{c}
VE-3	7.00 ± 0.25^{a}	2.96 ± 0.15^{b}	4.41 ± 0.08^{a}	9.69 ± 0.10^{abc}	9.84 ± 0.39^{c}
VE-4	4.84 ± 0.01^{bc}	3.66 ± 0.42^{ab}	4.31 ± 0.42^{a}	10.19 ± 0.82^{ab}	12.36 ± 0.25^{b}
VE-5	3.03 ± 0.01^{cd}	2.96 ± 0.61^{b}	4.25 ± 0.18^{a}	8.83 ± 0.66^{cd}	9.68 ± 0.10^{c}
VE-6	6.25 ± 0.05^{ab}	3.14 ± 0.83^{b}	3.03 ± 0.15^{c}	9.06 ± 0.11^{bcd}	8.28 ± 0.63^{c}
VE-7	5.90 ± 0.06^{ab}	2.97 ± 0.86^{b}	4.66 ± 0.68^{a}	8.70 ± 0.31^{cd}	8.56 ± 0.45^{c}
VE-8	4.94 ± 0.36^{bc}	3.71 ± 0.04^{ab}	3.99 ± 0.13^{ab}	8.81 ± 0.06^{cd}	9.72 ± 0.27^{c}
VE-9	4.28 ± 0.17^{bc}	2.83 ± 0.52^{b}	4.31 ± 0.21^{a}	8.93 ± 0.19^{bcd}	9.43 ± 0.18^{c}
VE-10	4.54 ± 0.25^{bc}	3.64 ± 0.55^{ab}	4.57 ± 1.13^{a}	8.09 ± 0.03^{d}	9.08 ± 0.25^{c}
CS-1	1.85 ± 0.13^{d}	2.45 ± 0.17^{b}	4.36 ± 0.59^{a}	10.51 ± 0.35^{a}	17.83 ± 0.85^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

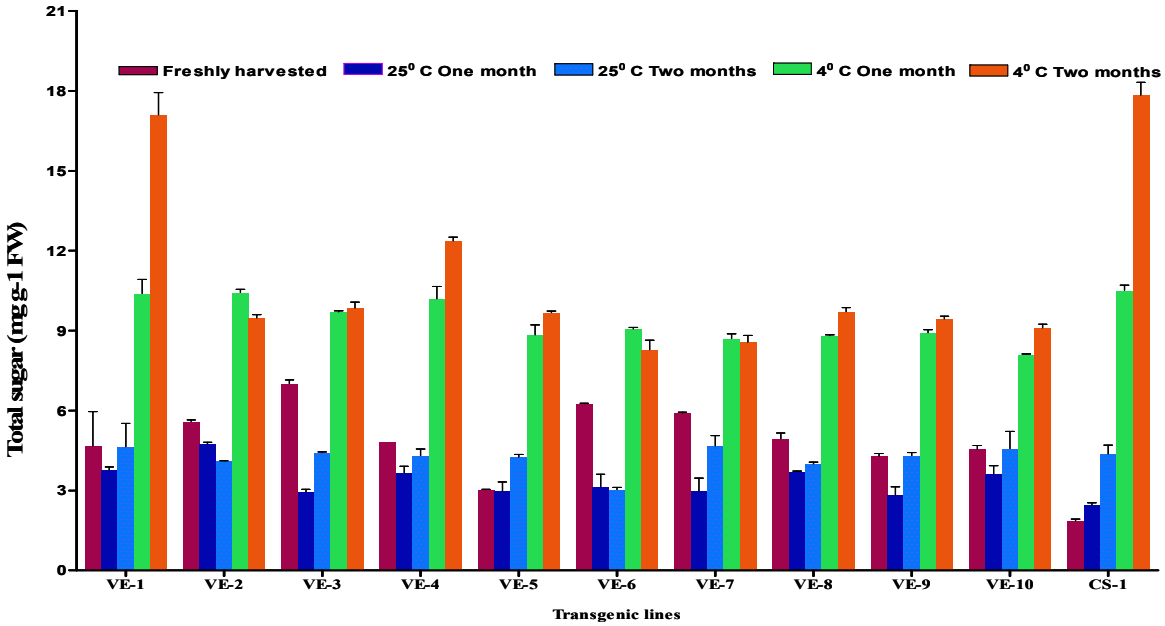

Fig. 40. Total sugar contents in the transgenic tubers corresponding to the data as presented in **Table 14**

The transgenic potato lines namely, VE-5, VE-6, VE-7, VE-8, VE-9 and VE-10 showed less reducing sugar level ranging from 0.92 to 1.07 mg g^{-1} FW of tuber which was considerably less as compared to control tubers stored under similar conditions. However, the total sugar content in the above transgenic lines was almost same when compared to control.

Biochemical analyses of VF-based transgenic potato tubers: The reducing sugar contents of nine independent transgenic lines are presented in both Table 15 and Fig. 41. Likewise, Table 16 and Fig. 42 provide total soluble sugar contents.

Table 15. Reducing sugar contents in the freshly harvested and stored tubers from VF-based transgenic potato lines at different temperatures

Transgenic Lines of VF	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VF-1	1.22 ± 0.03^{e}	1.16 ± 0.06^{bcd}	1.04 ± 0.09^{bc}	0.85 ± 0.03^{d}	1.51 ± 0.17^{d}
VF-2	3.62 ± 0.10^{a}	1.99 ± 0.49^{a}	1.24 ± 0.23^{b}	2.08 ± 0.25^{c}	5.05 ± 0.35^{bc}
VF-3	3.19 ± 0.04^{ab}	1.41 ± 0.10^{abc}	1.30 ± .03^{b}	2.85 ± 0.10^{b}	6.00 ± 0.77^{b}
VF-4	3.30 ± 0.06^{a}	1.96 ± 0.40^{a}	1.03 ± 0.21^{bc}	2.73 ± 0.19^{b}	4.88 ± 0.28^{bc}
VF-5	1.76 ± 0.14^{cd}	0.86 ± 0.15^{cd}	0.94 ± 0.03^{bc}	0.88 ± 0.01^{d}	1.10 ± 0.26^{d}
VF-6	1.44 ± 0.11^{de}	1.01 ± 0.29^{bcd}	1.00 ± 0.18^{bc}	1.04 ± 0.15^{d}	3.80 ± 0.43^{c}
VF-7	1.58 ± 0.29^{de}	0.52 ± 0.05^{d}	0.53 ± 0.38^{c}	0.86 ± 0.18^{d}	1.90 ± 0.53^{d}
VF-8	2.79 ± 0.24^{b}	0.84 ± 0.04^{cd}	0.78 ± .04^{bc}	2.24 ± 0.42^{bc}	5.14 ± 0.60^{bc}
VF-12	2.23 ± 0.25^{c}	1.66 ± 0.27^{ab}	0.99 ± 0.17^{bc}	1.82 ± 0.02^{c}	5.56 ± 0.33^{b}
CS-1	0.60 ± 0.08^{f}	1.24 ± 0.04^{bc}	2.13 ± 0.01^{a}	3.92 ± 0.35^{a}	13.55 ± 1.16^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

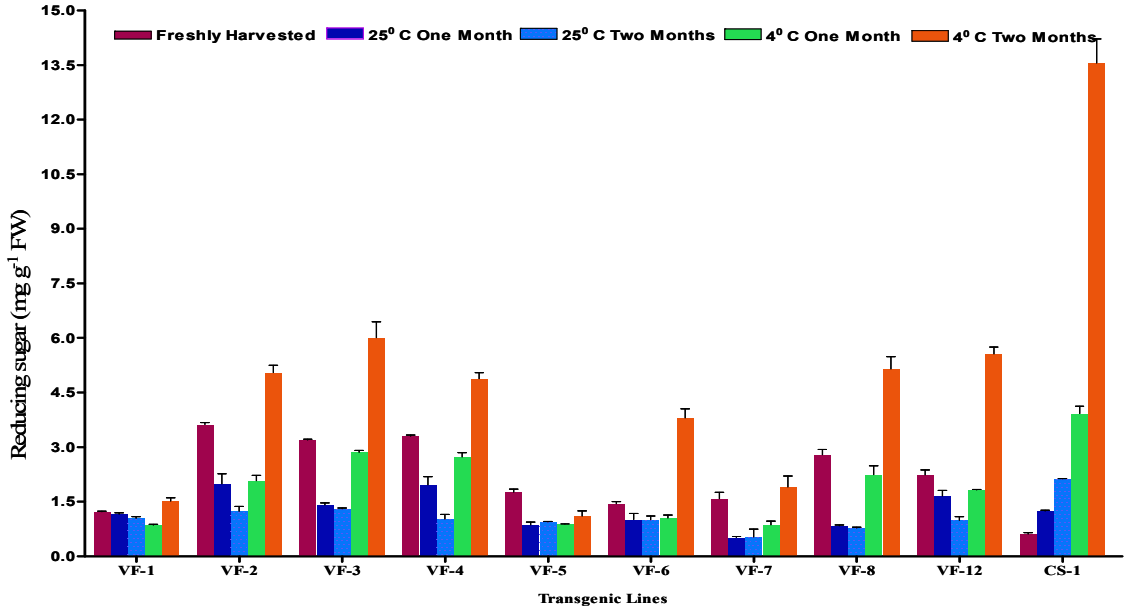

Fig. 41. Reducing sugar contents in the transgenic tubers corresponding to the data as presented in **Table 15**

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Table 16. Total sugar contents in the freshly harvested and stored tubers from VF-based transgenic potato lines at different temperatures

Transgenic Lines of VF	Total sugar (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VF-1	3.24 ± 0.49^{ab}	4.74 ± 0.34^{cd}	3.12 ± 0.09^{b}	10.47 ± 0.03^{a}	13.24 ± 0.62^{bc}
VF-2	4.88 ± 0.08^{a}	5.88 ± 0.60^{ab}	4.12 ± 0.36^{a}	8.47 ± 0.21^{cd}	14.48 ± 1.26^{ab}
VF-3	4.33 ± 0.41^{a}	6.28 ± 0.01^{a}	4.26 ± 0.19^{a}	8.85 ± 0.02^{bcd}	12.62 ± 0.86^{bc}
VF-4	4.51 ± 0.86^{a}	5.06 ± 0.08^{c}	3.97 ± 0.29^{ab}	9.44 ± 0.38^{abcd}	12.56 ± 1.54^{bc}
VF-5	3.87 ± 1.10^{a}	5.36 ± 0.39^{bc}	3.86 ± 0.08^{ab}	8.34 ± 0.32^{d}	11.34 ± 1.05^{bc}
VF-6	4.02 ± 0.40^{a}	4.79 ± 0.16^{cd}	3.21 ± 0.17^{b}	8.79 ± 0.55^{bcd}	12.53 ± 2.96^{bc}
VF-7	4.35 ± 0.06^{a}	3.69 ± 0.14^{e}	3.62 ± 0.30^{ab}	9.19 ± 0.07^{bcd}	9.37 ± 1.85^{c}
VF-8	4.07 ± 0.47^{a}	4.17 ± 0.25^{de}	3.15 ± 0.40^{b}	9.84 ± 1.00^{ab}	14.45 ± 2.13^{ab}
VF-12	4.08 ± 0.72^{a}	3.99 ± 0.09^{de}	3.22 ± 0.15^{b}	9.68 ± 0.10^{abc}	13.87 ± 0.97^{abc}
CS-1	1.85 ± 0.13^{b}	2.45 ± 0.17^{f}	4.36 ± 0.59^{a}	10.51 ± 0.35^{a}	17.83 ± 0.85^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

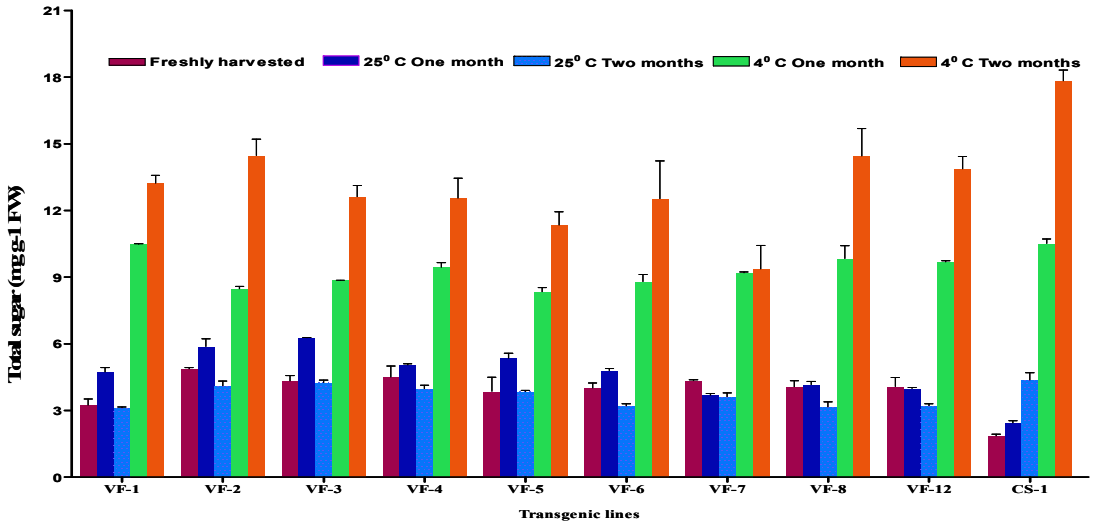

Fig. 42. Total sugar contents in the transgenic tubers corresponding to the data as presented in **Table 16**

The transgenic lines namely VF-1, VF-5, VF-6 and VF-7 showed less reducing sugar level ranging from 0.85 to 1.04 mg g^{-1} FW of tuber which was considerably less as compared to control tubers stored under similar conditions. However, the total sugar content in the above transgenic lines was more or less similar when compared to control.

Biochemical analyses of VG-based transgenic potato tubers: The reducing sugar contents of ten independent transgenic lines are presented in both Table 17 and Fig. 43. Likewise, Table 18 and Fig. 44 provide total soluble sugar contents.

Table 17. Reducing sugar contents in the freshly harvested and stored tubers from VG-based transgenic potato lines at different temperatures

Transgenic Lines of VG	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VG-1	0.85 ± 0.05^{e}	0.87 ± 0.03^{bc}	1.20 ± 0.32^{b}	2.01 ± 0.38^{de}	2.69 ± 0.04^{cd}
VG-2	0.81 ± 0.14^{ef}	1.68 ± 0.68^{a}	1.08 ± 0.29^{b}	1.86 ± 0.10^{def}	1.78 ± 0.10^{de}
VG-3	2.30 ± 0.07^{a}	0.71 ± 0.05^{bc}	1.09 ± 0.08^{b}	2.22 ± 0.15^{cd}	3.89 ± 0.32^{b}
VG-5	1.05 ± 0.08^{de}	0.67 ± 0.05^{bc}	0.93 ± 0.16^{b}	1.39 ± 0.12^{fgh}	1.98 ± 0.02^{de}
VG-8	1.50 ± 0.03^{bc}	0.83 ± 0.14^{bc}	1.34 ± 0.35^{b}	1.25 ± 0.20^{gh}	1.30 ± 0.21^{e}
VG-12	1.56 ± 0.07^{b}	0.72 ± 0.14^{bc}	1.08 ± 0.27^{b}	1.49 ± 0.17^{efg}	1.66 ± 0.03^{de}
VG-13	1.26 ± 0.03^{cd}	0.67 ± 0.20^{bc}	1.18 ± 0.10^{b}	0.88 ± 0.14^{h}	1.99 ± 0.40^{de}
VG-14	1.63 ± 0.11^{b}	0.64 ± 0.20^{bc}	0.90 ± 0.02^{b}	2.71 ± 0.06^{bc}	1.15 ± 0.06^{e}
VG-15	1.10 ± 0.03^{d}	0.65 ± 0.08^{bc}	0.86 ± 0.15^{b}	1.64 ± 0.22^{defg}	1.66 ± 0.05^{de}
VG-16	0.60 ± 0.09^{f}	0.51 ± 0.08^{c}	1.02 ± 0.07^{b}	2.96 ± 0.06^{b}	3.53 ± 0.13^{bc}
CS-1	0.60 ± 0.08^{f}	1.24 ± 0.04^{ab}	2.13 ± 0.01^{a}	3.92 ± 0.35^{a}	13.55 ± 1.16^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

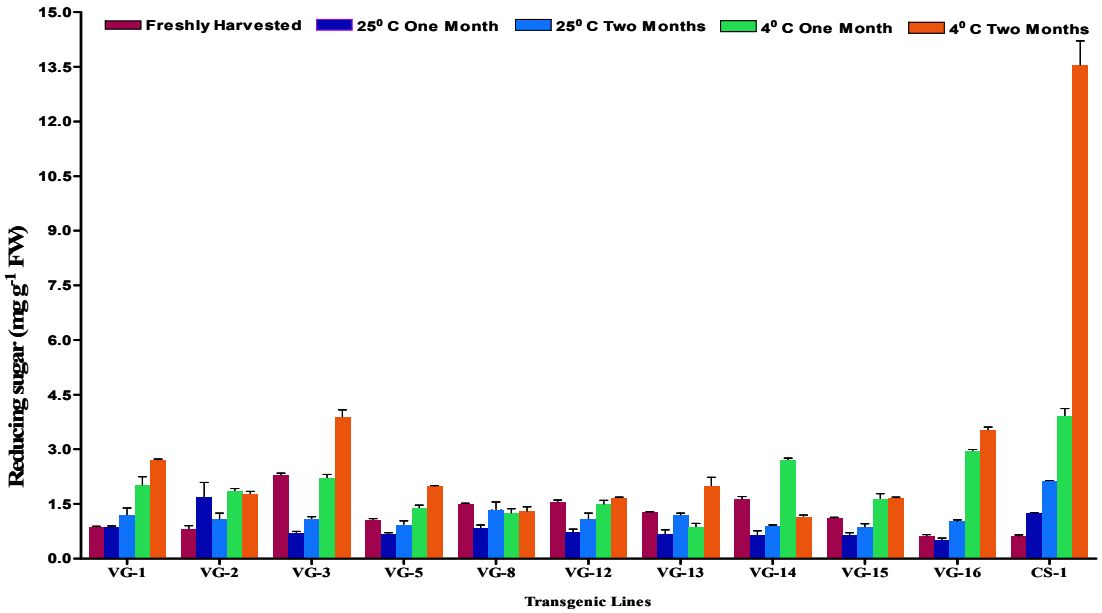

Fig. 43. Reducing sugar contents in the transgenic tubers corresponding to the data as presented in **Table 17**

Table 18. Total sugar contents in the freshly harvested and stored tubers from VG-based transgenic potato lines at different temperatures

Transgenic Lines of VG	Total sugar (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VG-1	4.60 ± 0.07^{b}	3.17 ± 0.52^{abc}	3.67 ± 0.32^{c}	9.12 ± 0.08^{cde}	11.70 ± 0.23^{bcd}
VG-2	4.46 ± 0.47^{b}	4.21 ± 0.72^{a}	3.11 ± 1.08^{c}	9.67 ± 0.05^{bcd}	12.40 ± 0.51^{bc}
VG-3	4.58 ± 0.22^{b}	2.54 ± 0.27^{bc}	3.76 ± 1.22^{c}	9.09 ± 0.21^{cdef}	13.16 ± 0.66^{b}
VG-5	4.36 ± 0.04^{b}	2.07 ± 0.06^{c}	3.33 ± 0.18^{c}	8.43 ± 0.14^{efg}	9.58 ± 0.01^{def}
VG-8	5.39 ± 0.28^{ab}	2.68 ± 0.33^{bc}	4.04 ± 0.02^{b}	8.09 ± 0.30^{fg}	8.68 ± 0.22^{ef}
VG-12	5.45 ± 0.29^{ab}	2.90 ± 0.35^{bc}	3.92 ± 0.11^{c}	7.97 ± 0.16^{g}	7.70 ± 2.12^{f}
VG-13	5.05 ± 0.44^{ab}	2.92 ± 0.39^{bc}	3.21 ± 0.44^{c}	8.71 ± 0.44^{defg}	10.53 ± 0.86^{cde}
VG-14	5.83 ± 0.41^{a}	3.29 ± 0.46^{ab}	3.57 ± 0.57^{c}	8.14 ± 0.05^{efg}	8.31 ± 0.16^{ef}
VG-15	5.17 ± 0.28^{ab}	2.77 ± 0.10^{bc}	3.94 ± 1.40^{c}	11.10 ± 0.42^{a}	12.16 ± 0.14^{bc}
VG-16	5.47 ± 0.92^{ab}	2.67 ± 0.54^{bc}	3.29 ± 0.26^{c}	9.93 ± 0.80^{bc}	13.40 ± 0.74^{b}
CS-1	1.85 ± 0.13^{c}	2.45 ± 0.17^{bc}	4.36 ± 0.59^{a}	10.51 ± 0.35^{ab}	17.83 ± 0.85^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

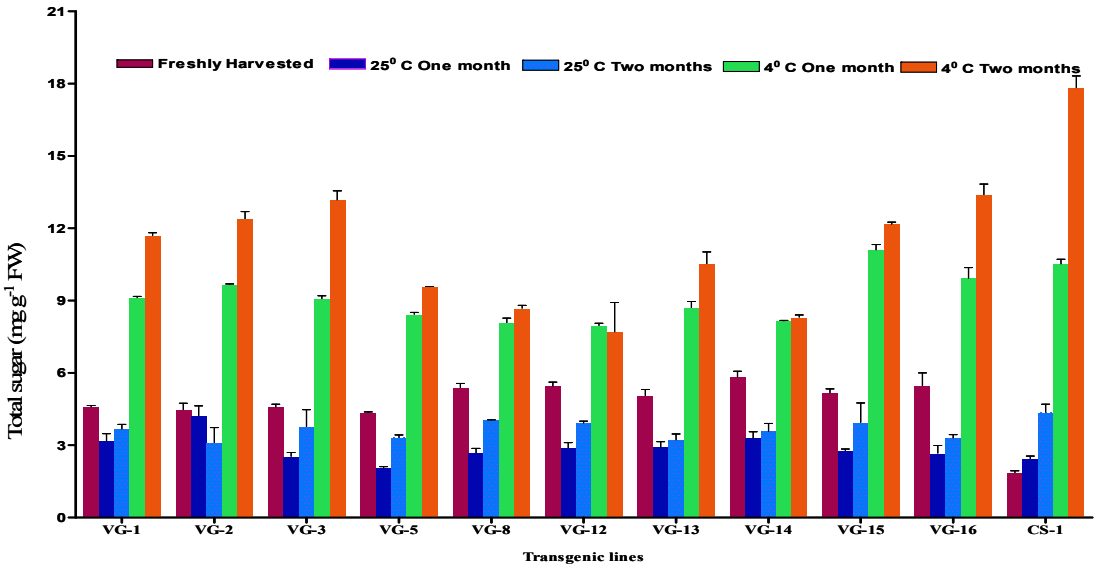

Fig. 44. Total sugar contents in the transgenic tubers corresponding to the data as presented in **Table 18**

Transgenic lines VG-5, VG-8, VG-12, VG-13 and VG-15 have sugar content in the range of 0.88 to 1.64 mg g^{-1} FW of tuber weight which was considerably less as compared to control tubers stored under similar conditions. However, the total sugar content in the above transgenic lines was more or less similar when compared to control.

Biochemical analyses of VH-based transgenic potato tubers: The reducing sugar contents of nine independent transgenic lines are presented in both Table 19 and Fig. 45. Likewise, Table 20 and Fig. 46 provide total soluble sugar contents.

Table 19. Reducing sugar contents in the freshly harvested and stored tubers from VH-based transgenic potato lines at different temperatures

Transgenic Lines of VH	Reducing sugars (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VH-1	1.57 ± 0.05^{f}	1.15 ± 0.09^{cd}	1.87 ± 0.37^{a}	1.51 ± 0.12^{ef}	2.15 ± 0.05^{de}
VH-2	5.77 ± 0.06^{a}	1.90 ± 0.07^{b}	1.76 ± 0.09^{a}	2.63 ± 0.33^{bc}	3.10 ± 0.06^{bcd}
VH-3	1.29 ± 0.07^{f}	1.34 ± 0.54^{bcd}	1.16 ± 0.12^{b}	2.23 ± 0.72^{cde}	3.10 ± 0.13^{bcd}
VH-4	4.36 ± 0.02^{c}	1.37 ± 0.06^{bcd}	0.92 ± 0.31^{bc}	2.48 ± 0.11^{bcd}	3.65 ± 0.21^{bc}
VH-5	2.09 ± 0.07^{e}	1.63 ± 0.08^{bc}	0.88 ± 0.26^{bc}	2.15 ± 0.34^{cde}	3.81 ± 0.15^{bc}
VH-6	0.67 ± 0.16^{g}	2.69 ± 0.02^{a}	0.32 ± 0.02^{d}	1.65 ± 0.21^{de}	2.85 ± 0.16^{cd}
VH-7	2.53 ± 0.04^{d}	0.88 ± 0.17^{d}	0.73 ± 0.04^{bcd}	3.63 ± 0.14^{a}	2.13 ± 0.28^{de}
VH-8	5.32 ± 0.21^{b}	1.85 ± 0.12^{b}	1.15 ± 0.09^{b}	0.66 ± 0.21^{f}	1.27 ± 0.08^{e}
VH-10	2.52 ± 0.16^{d}	1.14 ± 0.18^{cd}	0.45 ± 0.08^{cd}	3.32 ± 0.04^{ab}	4.19 ± 0.15^{b}
CS-1	0.60 ± 0.08^{g}	1.24 ± 0.04^{cd}	2.13 ± 0.01^{a}	3.92 ± 0.35^{a}	13.55 ± 1.16^{a}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

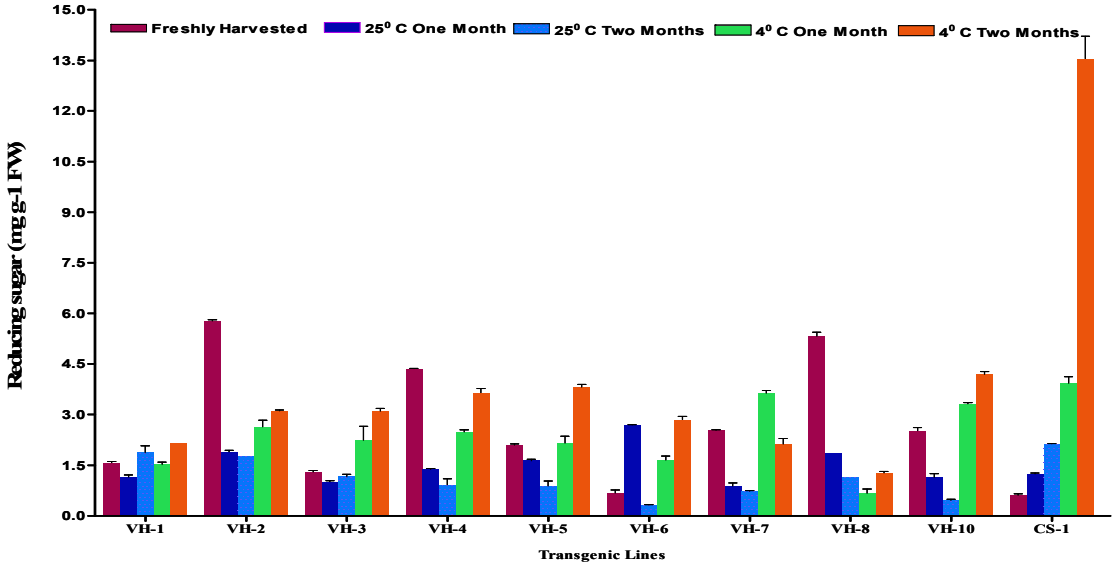

Fig. 45. Reducing sugar contents in the transgenic tubers corresponding to the data as presented in **Table 19**

Table 20. Total sugar contents in the freshly harvested and stored tubers from VH-based transgenic potato lines at different temperatures

Transgenic Lines of VH	Total sugar (mg g^{-1} FW)				
	Freshly harvested	25ºC one month	25ºC two months	4ºC one month	4ºC two months
VH-1	3.53 ± 0.47^{c}	2.96 ± 0.09^{de}	4.48 ± 0.70^{ab}	9.91 ± 0.19^{ab}	8.85 ± 0.83^{de}
VH-2	11.43 ± 1.10^{a}	4.63 ± 0.07^{a}	4.37 ± 0.17^{ab}	9.60 ± 0.90^{ab}	10.13 ± 0.46^{cd}
VH-3	3.62 ± 0.04^{c}	2.94 ± 0.04^{de}	4.25 ± 0.14^{ab}	9.37 ± 0.83^{ab}	11.05 ± 0.30^{c}
VH-4	5.59 ± 0.09^{b}	3.23 ± 0.06^{cd}	5.79 ± 0.62^{a}	8.59 ± 0.40^{b}	10.53 ± 0.35^{c}
VH-5	4.05 ± 0.27^{c}	3.22 ± 0.08^{cd}	4.44 ± 0.47^{ab}	8.86 ± 0.92^{ab}	11.64 ± 0.62^{bc}
VH-6	4.17 ± 0.46^{c}	5.14 ± 0.02^{a}	3.85 ± 0.54^{b}	8.83 ± 0.18^{ab}	10.46 ± 0.35^{c}
VH-7	4.02 ± 0.16^{c}	3.65 ± 0.23^{bc}	4.43 ± 0.44^{ab}	9.31 ± 0.16^{ab}	7.86 ± 0.34^{e}
VH-8	11.43 ± 0.67^{a}	4.02 ± 0.07^{b}	3.09 ± 0.27^{c}	10.26 ± 0.25^{ab}	12.96 ± 0.15^{b}
VH-10	4.63 ± 0.29^{bc}	3.52 ± 0.47^{bc}	4.69 ± 0.50^{ab}	10.04 ± 1.24^{ab}	11.03 ± 0.65^{c}
CS-1	1.85 ± 0.13^{d}	2.45 ± 0.17^{e}	4.36 ± 0.59^{ab}	10.51 ± 0.35^{a}	17.83 ± 0.85^{a}

Values sharing a common lowercase letter within the column are not significant at $P \leq 0.05$. Values are the mean ± SD of n =3 independent tubers.

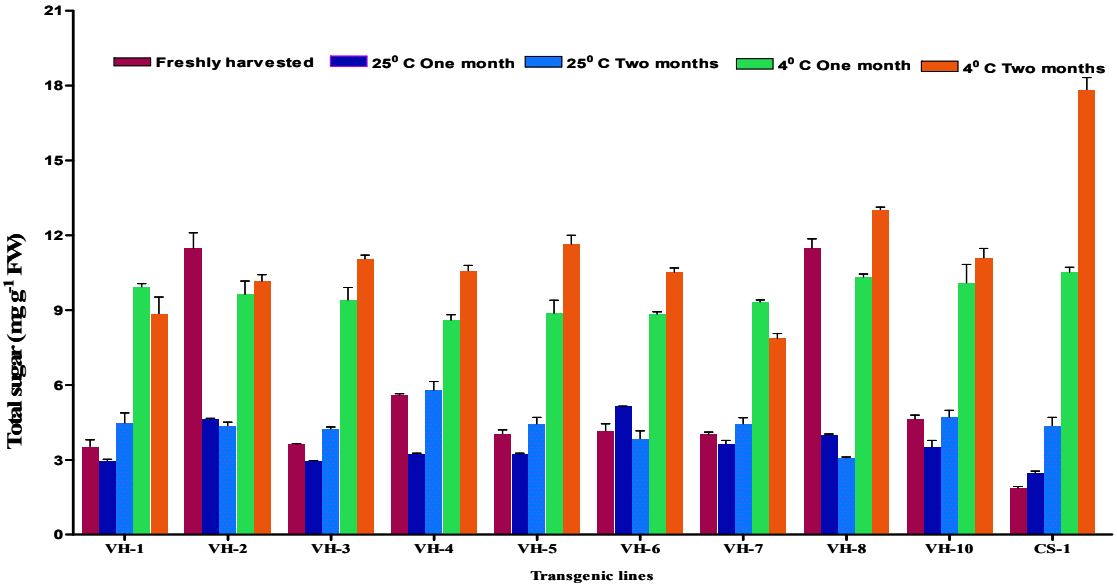

Fig. 46. Total sugar contents in the transgenic tubers corresponding to the data as presented in **Table 20**

Some of the transgenic lines namely, VH-1, VH-6 and VH-8 showed less reducing sugar content in the range of 0.66 to 1.65 mg g^{-1} FW of tuber which was considerably less as compared to control tubers stored under similar conditions. However, the total sugar content in the above transgenic lines was more or less similar when compared to control.

5.4.6. Vacuolar invertase assay in the transgenic potato mini tubers: Here vacuolar invertase activities in the extracts of the transgenic mini tubers along with control were measured. Extraction and assay were carried out according to the protocol as described by Greiner et al. (1999). All data are provided below:

Table 21. Vacuolar invertase activities in the VA-based transgenic potato tubers along with control in the freshly harvested and cold-stored tubers (four weeks at 4^0C)

Transgenic lines of VA	Vacuolar invertase activity [nmol (min.mg)$^{-1}$]	
	Freshly harvested	4^0C one month
CS-1	0.048 ± 0.001^{abc}	$1.586 \pm .096^{a}$
VA-5	0.062 ± 0.006^{ab}	0.099 ± 0.001^{bc}
VA-6	0.079 ± 0.002^{a}	0.105 ± 0.008^{b}
VA-28	0.032 ± 0.003^{bc}	0.065 ± 0.001^{c}
VA-31	0.021 ± 0.002^{c}	0.058 ± 0.001^{c}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

Table 22. Vacuolar invertase activities in the VB-based transgenic potato tubers along with control in the freshly harvested and cold-stored tubers (four weeks at 4^0C)

Transgenic lines of VB	Vacuolar invertase activity [nmol (min.mg)$^{-1}$]	
	Freshly harvested	4^0C one month
CS-1	0.048 ± 0.001^{b}	1.586 ± 0.096^{a}
VB-1	0.079 ± 0.010^{a}	0.115 ± 0.005^{b}
VB-3	0.072 ± 0.002^{a}	0.116 ± 0.005^{b}
VB-8	0.068 ± 0.003^{a}	0.189 ± 0.010^{b}
VB-14	0.067 ± 0.004^{a}	0.161 ± 0.015^{b}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

Table 23. Vacuolar invertase activities in the VC-based transgenic potato tubers along with control in the freshly harvested and cold-stored tubers (four weeks at 4^0C)

Transgenic Lines of VC	Vacuolar invertase activity [nmol (min.mg)$^{-1}$]	
	Freshly harvested	4^0C one month
CS-1	0.048 ± 0.001^{d}	1.586 ± 0.096^{a}
VC-12	0.057 ± 0.002^{c}	0.069 ± 0.005^{b}
VC-23	0.102 ± 0.002^{a}	0.128 ± 0.021^{b}
VC-24	0.065 ± 0.004^{b}	0.099 ± 0.004^{b}
VC-28	0.072 ± 0.002^{b}	0.122 ± 0.001^{b}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

Table 24. Vacuolar invertase activities in the VD-based transgenic potato tubers along with control in the freshly harvested and cold-stored tubers (four weeks at 4^0C)

Transgenic Lines of VD	Vacuolar invertase activity [nmol (min.mg)$^{-1}$]	
	Freshly harvested	4^0C one month
CS-1	0.048 ± 0.001^{b}	1.586 ± 0.096^{a}
VD-1	0.110 ± 0.011^{a}	0.208 ± 0.019^{b}
VD-2	0.107 ± 0.007^{a}	0.205 ± 0.005^{b}
VD-3	0.102 ± 0.087^{a}	0.154 ± 0.018^{b}
VD-4	0.123 ± 0.053^{a}	0.216 ± 0.014^{b}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

Table 25. Vacuolar invertase activities in the VE-based transgenic potato tubers along with control in the freshly harvested and cold-stored tubers (four weeks at 4^0C)

Transgenic Lines of VE	Vacuolar invertase activity [nmol (min.mg)$^{-1}$]	
	Freshly harvested	4^0C one month
CS-1	0.048 ± 0.001^{c}	1.586 ± 0.096^{a}
VE-5	0.112 ± 0.015^{ab}	0.090 ± 0.005^{b}
VE-6	0.043 ± 0.030^{c}	0.073 ± 0.014^{b}
VE-9	0.067 ± 0.014^{bc}	0.087 ± 0.003^{b}
VE-10	0.102 ± 0.003^{ab}	0.083 ± 0.005^{b}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

Table 26. Vacuolar invertase activities in the VF-based transgenic potato tubers along with control in the freshly harvested and cold-stored tubers (four weeks at 4^0C)

Transgenic Lines of VF	Vacuolar invertase activity [nmol (min.mg)$^{-1}$]	
	Freshly harvested	4^0C one month
CS-1	0.048 ± 0.001^{c}	1.586 ± 0.096^{a}
VF-1	0.100 ± 0.004^{b}	0.114 ± 0.006^{b}
VF-5	0.124 ± 0.013^{ab}	0.095 ± 0.005^{b}
VF-6	0.129 ± 0.008^{ab}	0.157 ± 0.010^{b}
VF-7	0.143 ± 0.011^{a}	0.124 ± 0.002^{b}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

Table 27. Vacuolar invertase activities in the VG-based transgenic potato tubers along with control in the freshly harvested and cold-stored tubers (four weeks at 4^0C)

Transgenic Lines of VG	Vacuolar invertase activity [nmol (min.mg)$^{-1}$]	
	Freshly harvested	4^0C one month
CS-1	0.048 ± 0.001^{b}	1.586 ± 0.096^{a}
VG-5	0.112 ± 0.004^{a}	0.105 ± 0.005^{b}
VG-8	0.131 ± 0.017^{a}	0.102 ± 0.010^{b}
VG-13	0.108 ± 0.015^{a}	0.093 ± 0.007^{b}
VG-15	0.144 ± 0.036^{a}	0.154 ± 0.038^{b}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

Table 28. Vacuolar invertase activities in the VH-based transgenic potato tubers along with control in the freshly harvested and cold-stored tubers (four weeks at 4^0C)

Transgenic Lines of VH	Vacuolar invertase activity [nmol (min.mg)$^{-1}$]	
	freshly harvested	4^0C one month
CS-1	0.048 ± 0.001^{d}	1.586 ± 0.096^{a}
VH-1	0.093 ± 0.010^{bc}	0.103 ± 0.011^{b}
VH-5	0.105 ± 0.005^{b}	0.152 ± 0.006^{b}
VH-6	0.083 ± 0.004^{c}	0.104 ± 0.011^{b}
VH-8	0.300 ± 0.003^{a}	0.089 ± 0.010^{b}

Values sharing a common lowercase letter within the column are not significant at P≤0.05. Values are the mean ± SD of n =3 independent tubers.

Analyses of vacuolar invertase activities data as presented in the above Tables 21-28 revealed that extractable enzyme activity was very less (negligible) in the freshly harvested tubers (both in control and transgenic); whereas, it was markedly increased in the control tubers, i.e., 1.586 ± 0.096 nmol $(min.mg)^{-1}$ if stored at 4^0C for four weeks. But some of the transgenic potato tubers as generated in this study showed considerably reduced level of vacuolar invertase activities even after storage at low temperature as summarized here: The transgenic lines namely VA-5, VA-6, VA-28, VA-31, VB-1, VB-3, VB-8, & VB-14 showed very low enzyme activities, in the range 0.058-0.189 nmol $(min.mg)^{-1}$. Likewise, the transgenic lines namely VC-12, VC-23, VC-24, VC-28, VD-1, VD-2, VD-3, VD-4 showed the values between 0.069-0.216 nmol $(min.mg)^{-1}$. In the cases of the transgenic lines namely VE-5, VE-6, VE-9, VE-10, VF-1, VF-5, VF-6, VF-7, VG-5, VG-8, VG-13, VG-15, VH-1, VH-5, VH-6, VH-8 the level of vacuolar invertase activities were found to be in the range of 0.073-0.157 nmol $(min.mg)^{-1}$ slightly lower values as compared to the above.

***5.4.7. Protein gel blot analyses*:** Protein gel blot analyses were also carried out to know the vacuolar invertase expression patterns in some of the transgenic lines (Figs. 47 & 48). In the cold-stored tuber extracts (four weeks at 4^0C) of all the selected transgenic lines, a very faint band of ~65 kDa, an expected size of vacuolar invertase, was detected; whereas, in the case of freshly harvested potato tubers the immuno-detected band was found to be faint or negligible suggesting very less expression of vacuolar invertase gene.

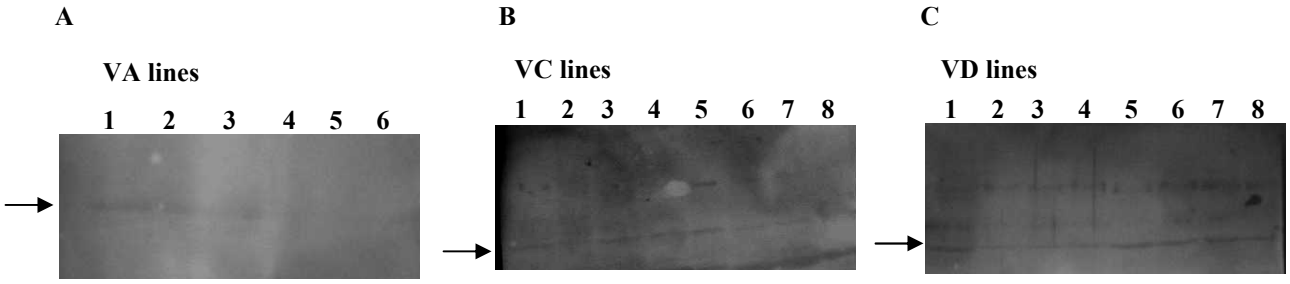

Fig. 47. Protein blot analyses in the transgenic tubers along with control. **A** lane 1 freshly harvested control tuber; lanes 2 & 3 freshly harvested VA-5 and VA-6 tubers; lanes 4-6 cold-stored tubers with same order; **B** lane 1 freshly harvested control tuber; lanes 2-4 freshly harvested VC-12, VC-24 and VC-26 tubers; lanes 5-8 cold-stored tubers with same order; **C** lane 1 freshly harvested control tuber; lanes 2-4 freshly harvested VD-2, VD-3 and VD-4 tubers; lanes 5-8 cold-stored tubers with same order

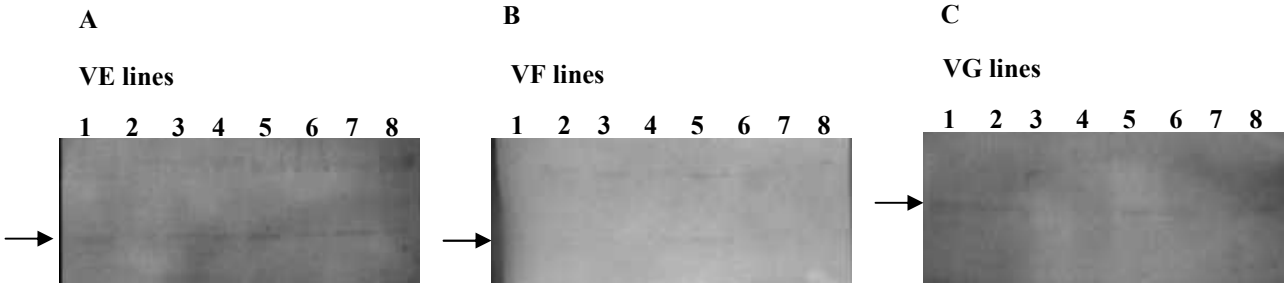

Fig. 48. Protein blot analyses in the transgenic tubers along with control. **A** lane 1 freshly harvested control tuber; lanes 2-4 freshly harvested VE-5, VE-6 and VE-7 tubers; lanes 5-8 cold-stored tubers with same order; **B** lane 1 freshly harvested control tuber; lanes 2-4 freshly harvested VF-5, VF-6 and VF-7 tubers; lanes 5-8 cold-stored tubers with same order; **C** lane 1 freshly harvested control tuber; lanes 2-4 freshly harvested VG-5, VG-8 and VG-12 tubers; lanes 5-8 cold-stored tubers with same order

Literature survey revealed that considerable progress has been made in the area of plant invertase research both at biochemical and molecular level. Inhibition of cold-induced sweetening in the potato tubers is an attractive area of applied research for many laboratories. Soluble acid invertases along with other proteins/enzymes are considered to play important roles for this undesirable phenomenon. Particularly, acid invertase gene functions are implicated in hexose accumulation in the cold-stored tubers. Different strategies were adopted by many laboratories as described previously. For inhibition of vacuolar invertase gene functions in the potato cultivar, various molecular strategies were adopted in this study. Some of the promising transgenic potato lines were generated. The outcomes of the transgenic approaches are briefly mentioned below:

Soluble sugars were extracted from mini tubers stored at different temperatures i.e., 4ºC, and 25ºC for four weeks and eight weeks. At 25°C, there were little changes in the reducing sugar contents of all transgenic lines during the dormant periods; the contents were in the range of 0.3-2.6 mg g^{-1} FW of tuber fresh weight in most of the cases. This suggests a very low metabolic activity during the dormant period. A slight increase in the total sugar content was observed at the time of sprouting in all transgenic lines, which was in agreement with the results described by Burton (1989). Here, in control (nontransgenic) potato tubers, the reducing sugar level was found to be in the range of 3.80-4.20 mg g^{-1} FW. Transgenic potato tubers corresponding to a number of transgenic lines as generated by different antisense and sense binary genetic constructs showed significant ($P<0.05$) lower accumulation of reducing sugars during storage at 4^0C. The reducing sugar level was found to be the range 0.81-1.68 mg g^{-1} FW of tuber for the following transgenic lines: VA-5, VA-6, VA-28 and VA-31 for four weeks; whereas, increased from 1.24 to 3.56 mg g^{-1} FW of tuber after four weeks of storage at low temperature. Invertase assay of above selected transgenic lines showed very low activity which was in the range of 0.058-0.105 nmol $(min.mg)^{-1}$ which was considerably less as compared to control i.e., 1.58 nmol $(min.mg)^{-1}$ tubers stored under similar conditions due to the inhibition of vacuolar invertase. This data was further supported by protein blot analysis. The other lines of VA binary vector showed considerable accumulation of reducing sugars; due to the remaining soluble acid invertase activity that might have resulted from inefficient antisense inhibition of the gene or from the presence of several genes encoding soluble acid invertases.

Some transgenic lines VB-1, VB-3 and VB-14 showed less reducing sugar level 0.88-1.46 mg g^{-1} FW of tuber after one month storage at 4ºC as compared to control due to repression of

invertase in these lines. The same transgenic lines showed slight increase in the reducing sugar after two months of storage from 2.13-3.48 mg g^{-1} FW of tuber which was very less as compared to control under similar conditions. This is due to the repression of vacuolar invertase in the above lines and less accumulation of reducing sugars. At the same time total sugar contents were increased in four to eight weeks of storage due to increase in the reducing sugar level. This could be due to the overexpression of the transcripts which might lead to repression of gene function. Invertase activity of selected transgenic lines namely VB-1, VB-8, and VB-14 showed very low activity from 0.115-0.189 nmol $(min.mg)^{-1}$ FW of tuber which was considerably less as compared to control tubers. Some of the transgenic lines VC-12, VC-23, VC-24, VC-26 and VC-28 showed less reducing sugar level (1.09-1.75 mg g^{-1} FW) after one month storage at 4^0C as compared to control due to the inhibition of invertase gene function (the tuber-specific *GBSSI* promoter was used in these lines). These transgenic lines also showed slight increase in the reducing sugar after two months of storage i.e., 1.76-2.99 mg g^{-1} FW of tuber. Invertase assay in the above transgenic lines showed considerably less activity which was also supported by protein blot analysis.

The transgenic lines VE-5, VE-6 VE-7, VE-8, VE-9 and VE-10 showed less reducing sugar level (0.92-1.07 mg g^{-1} FW) after one month of storage at 4ºC. Again, the same transgenic lines showed slight increase in the reducing sugar level (the range was 1.15-1.34 mg g^{-1} FW) even after two months of storage. Vacuolar invertase activities in these lines were found to be low, 0.073-0.090 nmol $(min.mg)^{-1}$. This was further supported by protein blot analysis. The transgenic lines VF-1, VF-5, VF-6 and VF-7 showed less reducing sugar level (0.85-1.04 mg g^{-1} FW) after one month storage, and showed slight increase in the reducing sugars after two months of storage from 1.10-3.80 mg g^{-1} FW of tuber, which was further supported by

enzyme assay and protein blot analysis. The transgenic lines VG-5, VG-8, VG-12, VG-13 and VG-15 showed less reducing sugar level (0.88-1.64 mg g^{-1} FW) after one month storage at 4ºC. The same transgenic lines showed only a slight increase in the reducing sugars after two months of storage (found in the range 1.30-1.99 mg g^{-1} FW). Invertase activities in these lines were found to be in the range of 0.093-0.154 nmol $(min.mg)^{-1}$ FW of tuber. This data was further supported by protein blot analysis. The transgenic lines VH-1, VH-6 and VH-8 showed less reducing sugar level (0.66 to 1.65 mg g^{-1} FW) after one month storage at 4°C. The same transgenic lines showed only slight increase in the reducing sugar level (1.27-2.85 mg g^{-1} FW) after two months of storage. In these lines, vacuolar invertase activities was found to be low i.e., 0.089-0.152 nmol $(min.mg)^{-1}$ FW of tuber which was supported by protein blot analysis. In all these transgenic potato lines as generated in this study, the level of reducing sugars was found to be considerably reduced by up to 80% if compared with control tubers under similar conditions. But the total sugar contents were found to be more or less comparable between the transgenic and nontransgenic if stored under the same conditions. Some of the transgenic tubers corresponding to the individual genetic constructs as used in this study showed considerable accumulation of reducing sugars during low temperature storage along with higher level of vacuolar invertase activities. This could be due to inefficient inhibition of acid invertase gene functions in the cold-stored tubers.

A few observations were made based on sugar data analyses: a) the independent transgenic lines corresponding to the individual genetic constructs showed variations in the reducing sugar level during low temperatures storage, b) reducing sugar level was found to be comparable in some of the transgenic lines generated by both antisense and sense binary genetic constructs, c) the constitutive CaMV 35S and the tuber-specific *GBSSI* promoter

appeared to exhibit more or less similar effects in terms of inhibition of invertase gene function in the cold-stored tubers, and d) use of invertase cDNA fragment towards the 3′ terminus in the binary genetic constructs in either orientation appeared to be more effective. This study will help in developing desired transgenic potato lines with reduced hexose accumulation during low temperature storage.

In conclusion, different molecular biological approaches as adopted in this study appeared to be considerably effective in terms of inhibition of invertase gene function in the potato tubers as compared to the other strategies reported in the literature. Some transgenic potato lines under this study were quite promising. This study would provide some clues for in-depth understanding of not only structure-function relationships between vacuolar invertases but also inhibition of cold-induced sweetening phenomenon in the potato tubers both at biochemical and molecular levels. In other words, this thesis work would be quite relevant and useful with regard to both basic and applied aspects of invertase research in plants.

Summary
&
References

Summary

In order to fulfill the objectives of this thesis work, different experiments were carried out which are summarized below:

- Micropropagation of some of the high-yielding Indian potato cultivars such as Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chandramukhi, Kufri Jyoti, Kufri Pukhraj, Kufri Ashoka along with Desiree as a reference cultivar. After hardening and acclimatization of the plantlets they were grown in the filed, mature mini tubers were harvested, kept at room temperature for three weeks, then transferred to various experimental temperatures, 4ºC and 25 ºC for four to eight weeks.
- The reducing sugar levels of the stored potato tubers of different potato cultivars at 4ºC were found to be in the range of 3.92-8.50 mg g^{-1} FW of tuber; whereas, the reducing sugar levels in the freshly harvested tubers were found in the range of 0.55-1.80 mg g^{-1} FW of tuber.
- With the help of semi-quantitative RT-PCR approach it was shown that the level of vacuolar invertase transcripts was low in the freshly harvested tubers, but considerably increased in the cold-stored tubers in all the potato cultivars as studied here.
- Available vacuolar invertase activities were measured. In case of the freshly harvested tubers total extractable activities of vacuolar invertase in the different potato cultivars were found to be negligible, ranging from 0.048 ± 0.001 nmol $(min.mg)^{-1}$ (Kufri Chipsona-1) to 0.089 ± 0.002 nmol $(min.mg)^{-1}$ (Desiree); whereas, in the cold-stored tubers vacuolar invertase activity was increased significantly, ranging from 0.935 ± 0.034 nmol $(min.mg)^{-1}$ (Desiree) to 3.411 ± 0.028 nmol $(min.mg)^{-1}$ (Kufri Jyoti). The results of

vacuolar invertase assay were more or less consistent with that of semi-quantitative RT-PCR. Hexose accumulation in the cold-stored tubers in different potato cultivars could be correlated with soluble acid invertase activity. Based on the observations made by Matsuura-Endo et al. (2004), the potato cultivars as used in this study belong to category of 'type I' as the levels of reducing sugars kept on increasing during storage at low temperature.

- Good quality total RNA was isolated from potato plantlets and tubers using a simple and efficient phenol/SDS method (A_{260}/A_{280} ratio was ~2.0).
- In the present study, two cDNA clones (*AI-01*, 2013 bp; *AI-02*, 1945 bp, sharing 99% sequence identity) encoding vacuolar invertase isoforms were isolated from a commercially important Indian potato cultivar, Kufri Chipsona-1 by RT-PCR approach. The sequence information was submitted to the NCBI data base under the GenBank Accession Numbers EU622806 (protein id ACC93584) and EU622807 (protein id ACC93585), respectively (reported for the first time from Indian potato cultivar). The corresponding predicted proteins consisted of 635 amino acids (designated as *KC-VIN1*, lacking a few amino acids at N-terminus) and 639 amino acids (designated as *KC-VIN2*), respectively. They showed 99% identity and found to vary at eight locations with mostly nonconservative substitutions. Multiple sequence alignment of eleven vacuolar invertase homologs covering four *Solanaceae* family members provided a clear view for comparison of amino acid sequences between them, and also revealed some notable distinguishing sequence features (signature-type sequences) not documented in the earlier reports. Based on the sequence alignment using 45 vacuolar invertase sequences from 27 taxonomically different plant species, consensus sequence was predicted. A phylogenetic

tree was generated to know the evolutionary relatedness between them. Hydrophobic characters were predicted and compared in different plant species. All these data would be useful in further biochemical characterization and elucidating the structure-function relationships of the individual vacuolar invertase forms.

- We have optimized and employed a simple genomic DNA isolation protocol suitable for a variety of plant materials covering *in vitro* grown tender plantlets to relatively complex plant tissues such as field grown mature potato leaves and tubers. Unlike other methods, no detergent was included in the isolation steps. This protocol, based on Dellaporta's method as reported earlier, worked efficiently both at small and miniscale during handling large number of plant materials. DNA yield was found to be in the range of 70 to 120 μg per gram of the plant material, sufficient for most of the molecular techniques.
- Similarly, two partial *GBSSI* alleles with promoter regions were isolated and characterized from the potato cultivar Kufri Chandramukhi, designated as GKC1 and GKC2. The sequence information were submitted to the NCBI under the GenBank Acc. Nos. EU548081 and EU548082, respectively. This is also first report from the Indian potato cultivar. The promoter of GKC1 allele was tuber-specific as evident from functional characterization. This promoter was used in making the binary vector pAN-GB01 having *GBSSI*-GUS genetic fusion by replacing of the CaMV 35S promoter of pBI121 binary vector.
- A total of eight binary genetic constructs were made using different *AI-01* and *AI-02* cDNA fragments under both constitutive CaMV 35S and tuber-specific *GBSSI* promoter that included four antisense (VA, VC, VE and VG), and four sense (VB, VD, VF and VH) constructs.

- *Agrobacterium tumefaciens* strain (LBA4404) was transformed with the each of the above genetic constructs by either of the following methods: electroporation & triparental mating.

- Each of the *Agrobacterium* transformants was co-cultivated with the internodal stem segments of the potato plantlets cv. Kufri Chipsona-1 followed by the selection of the independent transgenic lines on selective shoot inducing medium. The presence of transgene (s) was checked through PCR using suitable primers.

- After hardening and acclimatization of the transgenic potato plants corresponding to each binary genetic construct and control (nontransgenic) plants, they are grown in the restricted experimental plots for production of mini tubers. The harvested mini tubers were kept three weeks at room temperature, and then transferred to different experimental temperatures (4ºC and 25 ºC) for four to eight weeks. Tuber slices were frozen in liquid nitrogen and stored at -70^{0}C for further biochemical and molecular studies.

- Soluble sugars were extracted from mini tubers stored at different temperatures i.e., 4ºC, and 25ºC for four weeks and eight weeks. In control (nontransgenic) potato tubers, the reducing sugar level was found to be in the range of 3.80-4.20 mg g^{-1} FW. Transgenic potato tubers corresponding to a number of transgenic lines as generated by different antisense and sense binary genetic constructs showed significant ($P<0.05$) lower accumulation of reducing sugars during storage at 4^{0}C. The reducing sugar level was found to be the range 0.66-1.75 mg g^{-1}FW for the following transgenic lines: VA-5, VA-6, VA-28, VA-31, VB-1, VB-3, VB-14, VC-12, VC-23, VC-24, VC-26, VC-28, VE-5, VE-6, VE-7, VE-8, VE-9, VE-10, VF-1, VF-5, VF-6, VF-7, VG-5, VG-8, VG-12, VG-13, VH-1 and VH-8. The level of reducing sugars was reduced by 50-80% in these trangenic tubers as

compared to control. The activity of vacuolar invertase was found to be ~1.60 nmol $(min.mg)^{-1}$ in control potato tubers after storage for four weeks at 4ºC; whereas, the values for the above transgenic tubers were considerably low; found to be in the range of 0.058-0.161 nmol $(min.mg)^{-1}$.

- Protein gel blot analyses were also carried out to know the vacuolar invertase expression patterns in some of the transgenic lines. In the cold-stored tuber extracts (four weeks at 4^0C) of all the selected transgenic lines, a very faint band of ~65 kDa, an expected size of vacuolar invertase, was detected; whereas, in the case of freshly harvested potato tubers the immuno-detected band was found to be faint or negligible suggesting very less expression of vacuolar invertase gene.
- In all these transgenic potato lines as generated in this study, the level of reducing sugars was found to be considerably reduced by up to 80% if compared with control tubers under similar conditions. But the total sugar contents were found to be more or less comparable between the transgenic and nontransgenic if stored under the same conditions. Some of the transgenic tubers corresponding to the different genetic constructs showed considerable accumulation of reducing sugars during low temperature storage along with higher level of vacuolar invertase activities. This could be due to inefficient inhibition of acid invertase gene functions in the cold-stored tubers.

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