Studies on Hexokinases in Potato (*Solanum tuberosum* L.*)* by *In silico* and Biochemical Approaches

A

DISSERTATION

SUBMITTED

FOR

PARTIAL FULFILLMENT FOR THE AWARD OF DEGREE

MASTER OF SCIENCE

in

BIOTECHNOLOGY

By:

Chestha Mittal
M.Sc in Biotechnology
(Regd. No. 302101006)

THAPAR INSTITUTE
OF ENGINEERING & TECHNOLOGY
(Deemed to be University)

Under the Supervision of

Dr. Niranjan Das
Professor

DEPARTMENT OF BIOTECHNOLOGY
Thapar Institute of Engineering & Technology, Patiala

July 2023

CERTIFICATE

TO WHOM IT MAY CONCERN

This is to certify that the dissertation entitled, **Studies on Hexokinases in Potato (*Solanum tuberosum* L.) by *In silico* and Biochemical Approaches,** in the partial of research work undertaken by **Chestha Mittal** (Regd. No. 302101006) under my supervision and guidance between January 2023 to June 2023 for the partial fulfilment of the requirement for the award of the degree of Master of science in Biotechnology, Thapar Institute of Engineering and Technology, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

15/06/2023

(Dr. Niranjan Das)
Professor
Department of Biotechnology
Thapar Institute of Engineering and Technology
Patiala

(Chestha Mittal)

CANDIDATE's DECLARATION

I, hereby declare that the dissertation work which is entitled, **"Studies on Hexokinases in Potato (*Solanum tuberosum* L.) by *In silico* and Biochemical Approaches"** in the partial fulfilment of the requirement for the award of degree of Master of Science in Biotechnology, Thapar University, Patiala, is an authentic record of my own research work carried out under the guidance and supervision of **Dr. Niranjan Das**, Professor, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this dissertation has not been submitted to any other university or institute for award of any other degree.

Place: TIET, Patiala

Date: 15th July, 2023

(Chestha Mittal)

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to each and every one who has helped me throughout the entire project for its successful completion.

I find myself fortunate to acknowledge my guide **Dr. Niranjan Das**, Professor, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, Punjab for his guidance, kindness, motivation and splendid supervision during my work. I express my heartfelt thanks for his patience, support and excellent advice. It was his constant encouragement, constructive criticism and ability to handle the obstacles that has helped me to gain a lot during this period. I express my sincere thanks to **Dr. M.S. Reddy**, HOD, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala for giving me this opportunity to do this project.

I am pleased to show my utmost gratitude towards **Ms. Yadveer Kaur** for her support, continuous motivation to learn and thrive. I also sincerely thank **Ms. Shreya Sharma** for her help. I would like to acknowledge my lab mate **Eakdeep** who helped me a lot to complete the project. I am thankful to the help provided by the non-teaching staff and the lab attendants. I would like to thank **Mr. Ram Nawal Yadav, Mr. Lallan Yadav, Mr. Mohinder and Mr. Surinderpal Kumar**.

I would like to thank my siblings, **Geetika, Nitika, Gagan, Shubham** and **Pulkit** for the continuous dose of motivation and support. I would like to acknowledge my close friends, especially, **Rishika**, **Sahil**, **Shivangi**, **Amritpreet** and **Nehal** who stood by my side when the work felt. The whole credit goes to all the people who always had confidence in me and made me stay motivated.

Chestha

This thesis is dedicated to my father Mr. Suresh Mittal and mother Mrs. Pushpa Mittal.

With all my heart, I thank my family for their affection and faith.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Symbol	Name
µg	Microgram
µL	Microliter
mg	Milligram
ml	Millilitre
rpm	Rotations per minute
cm	Centimeter
in	Inches
mM	Millimolar
aa	Amino acids
ABF	ABA responsive element-binding factors
AGPase	ADP-glucose pyrophosphorylase
AtHXK	*Arabidopsis thaliana* hexokinase
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
CAA	Carbonic anhydrase
CAB	Chl a/b-binding protein
CaHXK	Capsicum annum Hexokinase
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CDS	Coding sequence
cGMP	Cyclic guanosine monophosphate
CK	Cytokinins
CPRI	Central Potato Research Institute
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CS-1	Kufri Chipsona-1
CS-2	Kufri Chipsona-2
De	Desiree
DOF	DNA-binding with one finger factor
EBI	European Bioinformatics Institute
EC	*Enzyme* Commission number
EDTA	Ethylenediaminetetraacetic acid

FRKs	Fructokinases
Fru	Fructose
FT LOCUS	FLOWERING LOCUS T
G6P	Glucose 6 phosphate
G6PDH	Glucose 6 phosphate dehydrogenase
GA	Gibberellic acid
Glc	Glucose
GLK	glucokinase
GRAVY	Grand Average of Hydropathy
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HXK1	Hexokinase isoform 1
HXK2	Hexokinase isoform 2
JA	Jasmonic acid
kbp	Kilo base pair
kDa	Kilo Dalton
KJ	Kufri Jyoti
KNOX	Knotted1-like homeobox
man	Mannose
Mol. Wt.	Molecular weight
MS BASAL	Murashigue and Skoog media
MSA	Multiple sequence alignment
NAD	Nicotinamide adenosine dinucleotide
NADH	nicotinamide adenine dinucleotide (NAD) + hydrogen
NCBI	National Center for Biotechnology Information
NtHXK	Nicotiana thaliana hexokinase
NTP	Nucleotide triphosphate
OD	Optical density
Oxidative PPP	Oxidative pentose phosphate pathway
PDB	Protein data bank
PFK	PhosphoFructokinase
pH	Potential of Hydrogen
PMSF	Phenylmethylsulfonyl fluoride
POTH1	Potato Homobox 1
PR	Kufri Pukhraj
PVP	Polyvinylpyrrolidone

q-PCR	Quantitative Polymerase chain reaction
RBCS	Rubisco small subunit
RMSD	Root Mean Square Deviation
ROS	Reactive oxygen species
SBP	Sedoheptulose bisphosphatase
SlHXK	*Solanum tuberosum* hexokinase
SlHXK1	*Solanum lycopersicum* hexokinase
SP6A	Self-Pruning 6A
StG6PDH	Glucose-6 phosphate dehydrogenase
StGA2ox1	*Solanum tuberosum* gibberellic acid oxidase
StPHYB	Phytochrome B in Solanum tuberosum
SuSy	Sucrose synthetase
TA	Tuberonic acid
TAG	Tuberonic acid glucoside
TALE	Three amino acid loop extension
TALENs	Transcription Activator-Like Effector Nucleases
TF	Transcription factors
STRING	Search Tool for the Retrieval of Interacting Genes
TM	Template modelling score
TPM	Transcripts per million
U/mg	Units/milligram
UDP	Uridine diphosphate
UDPGlc	Uridine diphosphate Glucose
UV	Ultraviolet
ZNFs	Zinc finger factors

ABSTRACT

Potato is one of the most significant tuber crops in terms of nutrition and consumption. It has a promising role in world in terms of food security. The process of tuberization is governed by several TFs and is susceptible to variable temperatures. This indicates the economical and scientific importance of tuber formation for further research. A number of biotechnological and genetic techniques can be used to understand the complex process of tuberization and associated biochemical pathways in order to increase potato yield. Hexokinase (EC 2.7.1.1), an allosteric enzyme belongs to the phosphor-transferases family. It is one of the crucial and rate limiting enzymes involved in the conversion of sucrose to hexose-phosphates during the stolon-to-tuber transition of potatoes. The formation of G6P in presence of HXK is responsible to interconnect various important carbohydrate metabolic pathways. In this study, enzyme extraction and assay were performed from various harvested organs of five potato cultivars. It primarily focusses on the comparative study of HXK isoforms and their functions applying various bioinformatic tools. Genome wide study revealed 6 *HXK* genes localized on 6 chromosomes. The multiple sequence alignment along with the phylogenetic analysis was performed to study the conserved patterns of HXKs in various plant species. Crucial motifs with their functions were retrieved and analysed. Protparam tool was used to predict various physico-chemical properties of HXK. This study would be useful to study the regulation of HXK enzyme in Indian potato cultivars. It would provide insights on the involvement of HXK in the maturation of plant organs. This would help in the right approach for genetic manipulations to raise transgenics with high HXKs activity in various cultivars of potato to satisfy the future global nutritional demands.

Keywords: Potato (*Solanum tuberosum* L.), Tuberization, Hexokinases (HXK), Sequence analysis, Organ extracts, HXK activities

Chapter 1 INTRODUCTION

1.1 About Potato

Root and tuber crops are the third-largest source of carbohydrates in the world. Potato (*Solanum tuberosum* L.) is a significant tuber crop that is grown all over the world. Potato plants are responsible for producing large quantities of tubers that are a good source of nutrients. It is a member of the *Solanaceae* family, which also includes tomatoes, peppers, and eggplants. There are almost 5000 different potato varieties, each with its own size, shape, color, starch content, and flavor. It ranks third worldwide in terms of consumption after wheat and rice (International Potato Centre, 2018). In comparison to cereals, potato tubers are a source of a variety of nutrients such as amino acids, particularly lysine, carbohydrates, vitamins, proteins and minerals. Phytonutrients like carotenoids and phenolic acids are also present in potatoes (Brown et al. 2005; Liu 2013; McGill et al. 2013; Abelenda et al. 2011). These minerals and nutritional elements are known to have the potential to support good health and lower the risk of developing chronic diseases (Beals et al. 2019).

1.2 History of Potato

Eight thousand years ago, potatoes were originally domesticated at the heights of the Peruvian Andes Mountains at the boundary between Bolivia and Peru. Potatoes were first brought to Europe in the 16^{th} century by a Spanish explorer from South America. There, they were cultivated as a temperate crop, and when European nations expanded their colonies, they spread around the world. The Portuguese introduced potatoes to India far earlier than many other nations during the early 17^{th} century. The British also introduced potatoes to the highlands of Northern India and Sri Lanka, where they thrived as an important garden plant in colonial homes. Potato farming was established in 1828 in Simla (now Shimla) hills. In 1830, this crop was established in the Nilgiri highlands. However, until then, potato farming in the nation was still only allowed in a few regions. Sir Herbort Stewart, the Government of India's agricultural advisor in 1945, developed CPRI (Central Potato Research Institute) to conduct a national potato research and development program. The CPRI's headquarters were moved to Shimla in 1956. This program allowed for using a broad potato genetic foundation through hybridization to create superior varieties (Singh et al. 2014). More than 60 varieties of potatoes have been produced by ICAR-CPRI suitable for processing, storage, blight resistance, drought resistance. India and China are currently the leading potato-producing countries (Spooner et al. 2005).

1.3 Morphology of Potato Plant

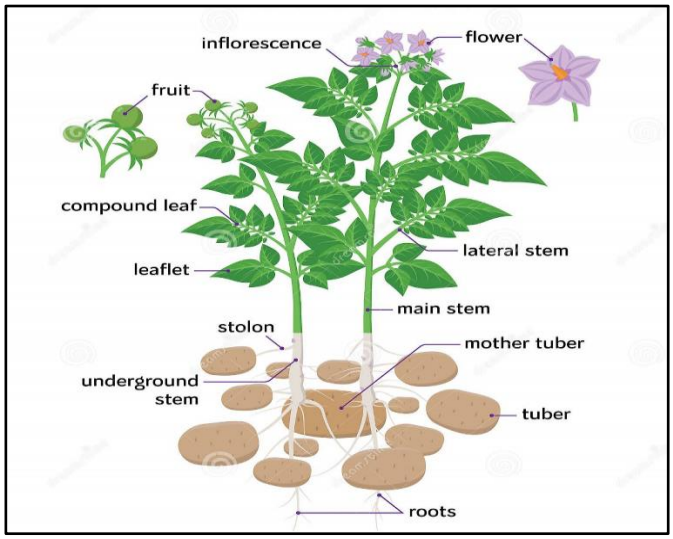

Fig. 1 All the parts of a potato plant (Ref: https://www.dreamstime.com/potato-plant-vector)

The potato plant is an annual, herbaceous, dicotyledonous, and vegetatively propagated plant. It is referred to as a cool weather crop as it is cultivated under temperate, subtropical, and tropical climate conditions. Potato can reach a height of 60 cm (24 in) or more depending on the cultivar. The potato shoot's basal leaves have quite a range in form, from simple to pinnately compound. Potato leaves wither after flowering, fruiting, and tuber formation. Potatoes are propagated through tubers, cut pieces of tubers or seeds. Seeds, often known as "true potato seeds," "TPS," or "botanical seeds," are used to grow several varieties of potatoes. It shows a deep and fibrous root system that requires very good soil condition to survive. (Beukema and Zaag 1979; Struik and Wiersema 1999). Flowering takes about 3-4 weeks after sprouting. Generally, white, pink, or purple flowers with yellow stamens are observed. After flowering a small green fruit is seen with approximately 300 seeds.

The developed tuber stores energy in the form of protein, starch, and water. They produce lateral buds called “eyes," which, in the right circumstances, develop into new plants. Freshly grown potatoes primarily consist of approximately 80% of water along with 16–20% sugars, 2.5–3.2% protein, 0.8–1.2% minerals, 0.1-0.2% fats, 0.6% fibre, and a few vitamins (Yadav et al. 2015).

1.4 Polyploidy in Potato

Solanum tuberosum is a supreme species present worldwide and is a tetraploid with *48* chromosomes (*2n= 4x=48*). It is a combination of the diploid plant *Solanum stentotomum* and the diploid weed *Solanum sparsipilum*, and as a result, it has doubled its chromosome number. *Solanum* species show polyploidy ranging from diploid to hexaploid species. *S. stenotomum*,

S. phureja, *S. goniocalyx*, and *S. Ajanhuiri* are diploid with 24 chromosomes *(2n=2x=24)*. *S. chaucha* and *S. juzepczukii* are triploid species with 36 chromosomes (*2n=3x=36)*. *S. curtilobum* is pentaploid with 60 chromosomes (*2n=5x=60)*. This proves the heterozygosity of the potato plant (Spooner et al. 2014)

1.5 Tuberization in Potato

Under short days and long cool nights, underground stolon develops into an edible storage organ through the process of tuberization. This edible storage organ is referred to as a tuber (Sarkar 2008). The initiation and development of tubers is associated with two distinct processes at morphological and biochemical levels. As reported by Weeda (et al. 2009), the morphological process in tuberization occurs between the I-VIII stages. In stage I, the thickening of the stolon is accompanied by the hook formation with no swelling below the apical portion. Stage II shows slight swelling and opening of the hook. In stage III, the continuous swelling of the stolon takes place, which forces the complete opening of the hook. Stage IV shows the expansion of the developing tuber both radially and longitudinally. Stage V-VIII shows further tuber growth in terms of both weight and size. Approximately 0.6g of tuber develops into a 10g tuber (Fig. 2). At the biochemical level, tuber formation is associated with the accumulation of starch and storage proteins (Visser et al. 1994). Potato tuber is a major sink tissue. The sink strength of growing potato tubers depends on sucrose and starch metabolism (Ewing & Struik 1992; Cutter 1978).

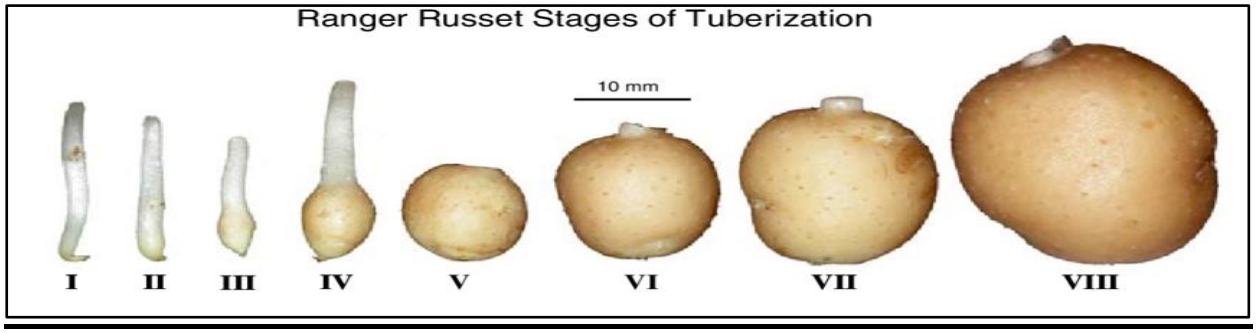

Fig. 2 Stages of tuberization in a potato with morphological descriptions (Weeda et al. 2009)

Carbon partitioning, starch metabolism, growth correlation, and signal transduction are various biochemical pathways involved in tuberization (Gregory 1965). With the benefits of simpler anatomy, large size and ease in transgenic research, potato tubers are considered as an attractive model for biochemical and physiological studies (Geigenberger et al. 2004).

1.6 Factors Affecting Tuberization

The complex process of tuberization is an important survival mechanism for potato's life cycle. It is a highly coordinated morphophysiological process influenced by both extrinsic and intrinsic factors. Considerable efforts have been made by researchers to understand the complex process of tuberization in potato during the last few decades, as discussed in the following section (Sarkar 2008; Dutt et al. 2017).

- **Environmental factors:** Various environmental factors, specifically temperature and Photoperiod, are considered the most crucial cues affecting tuber initiation and development. The ideal temperature for tuber initiation is 22°C, while 15°C is more conducive to tuber induction and tuber growth. A range of 14 to 25°C is better suitable for tuber development. High temperatures negatively affect tuberization (Timlin et al. 2006). Likewise, induction and initiation of tuber formation is strongly influenced by photoperiod. Potato is a short-day crop. It is established that both temperature and photoperiod cues simultaneously control common components of the day-length pathway (Dutt et al. 2017).

- **Nitrogen:** The tuberization process is significantly influenced by soil nitrogen. In 1985 it was shown that cultivating plants in hydroponic culture without nitrogen additions led to active tuberization. However, excessive nitrogen supplementation limits tuber development (Kraus 1985; Singh et al. 2023).

- **Transcription factors/proteins/enzymes:** Various transcription factors like the three-amino acid loop extension (TALE) superclass and MADS-box transcription factors, play crucial role in tuberization (Bahram et al. 2020). TALE superclass encodes TFs that are known to be involved in the regulation of plant developmental events. Unlike animal systems, plants are capable of moving RNA and proteins from the cell of origin to different target cells through the vascular tissue, like phloem, during the developmental processes. Mobile RNAs, namely BEL1 and KNOX protein partner designated as POTH1, have been reported to interact in a tandem complex to regulate the expression of genes involved in tuber initiation and development (Chen et al. 2003). The duration and intensity of the light regulate photosynthesis and the partitioning of dry matter to the tubers, which eventually determines the yield. Photoperiodic control of tuberization shares various common elements with flowering

regulation. FLOWERING LOCUS T (FT) protein was reported to be produced in the leaf vascular bundles and is known to induce floral or tuberization transition. Recently two FT-like paralogs (StSP3D and StSP6A) have been found to respond to independent environmental cues to control tuberization (Navarro et al. 2011). Additionally, cycling DOF (DNA-binding with one finger) factor (CDF), MADS-box and ABFs (ABA responsive element-binding factors) transcription factors are also known to regulate tuberization. A MADS-box potato gene, POTM1 is abundantly expressed in vegetative organs such as leaves, stolons and tubers. This gene is expressed during the early stages of tuber formation (Kang and Hannapel 1995, 1996). ABFs are bZIP transcription factors involved in ABA-mediated plant responses, including tuberization. Muniz Garcia et al. (2014) raised transgenic potato plants by overexpressing the Arabidopsis ABF4 or ABF2 genes. They observed ABA-GA signaling crosstalk during the process of tuberization. Various other proteins, namely acid phosphatase (APase), protein phosphatase type 2A, reactive oxygen species catabolizing enzymes, calcium-dependent protein kinase, and enzymes of sucrose metabolism (sucrose synthase, fructokinase, invertase), have been known to affect tuber initiation and development. Yu et al. (2012) reported that reactive oxygen species metabolizing enzymes, such as superoxide dismutase, ascorbate peroxidise and catalase, were induced during the tuber's initial stages of tuberization.

- **Phytohormones:** Endogenous levels of plant growth regulators such as cytokinins (CK), gibberellins (GA), auxins, and abscisic acid (ABA) play an important role during tuberization in potato. High GA levels are associated with stolon elongation in a non-induced state by promoting longitudinal cell expansion. GA levels get reduced during tuber development and maturation. A key regulatory enzyme of the GA-biosynthetic pathway namely, StGA2ox1, was reported to be upregulated during the early stages of tuber development and hence modify GA levels (Kloosterman et al. 2007). The endogenous levels of GA are controlled by sucrose and ABA. CKs are known to influence the early stages of tuberization by controlling cell cycle events. ABA is reported to antagonize the gibberellins (GAs)-promoted processes in plant development. As discussed earlier, ABFs are ABA-mediated TFs influencing the early stages of tuberization (Muniz et al. 2014). Lipoxygenases (LOXs) are the group of dioxygenases catalyzing the oxygenation of polyunsaturated fatty acids such as linoleic and linolenic acids that results in the synthesis of 9(S)-hydroperoxy linolenic

acid (9(S)-HPOT) and 13(S)-hydroperoxy linolenic acid (13(S)-HPOT). Jasmonic acid, regarded as phytohormone, is synthesized from 3(S)-HPOT. JA is then metabolized into to tuberonic acid (TA) and tuberonic acid glucoside (TAG). These compounds are reported to possess strong tuber-inducing properties (Koda 1997).

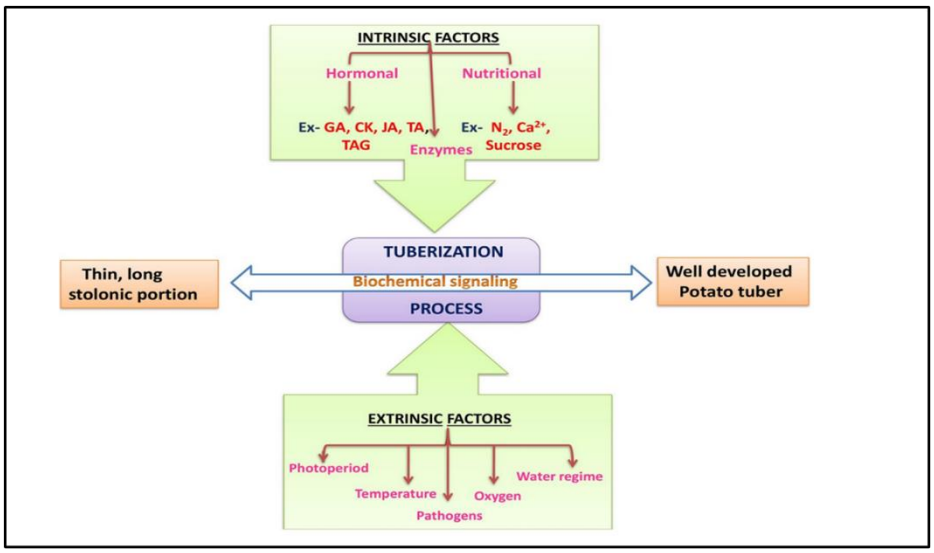

Fig. 3 Critical factors affecting the process of tuberization in potato (Singh et al. 2023)

1.7 Carbohydrate Metabolism during Tuber Development

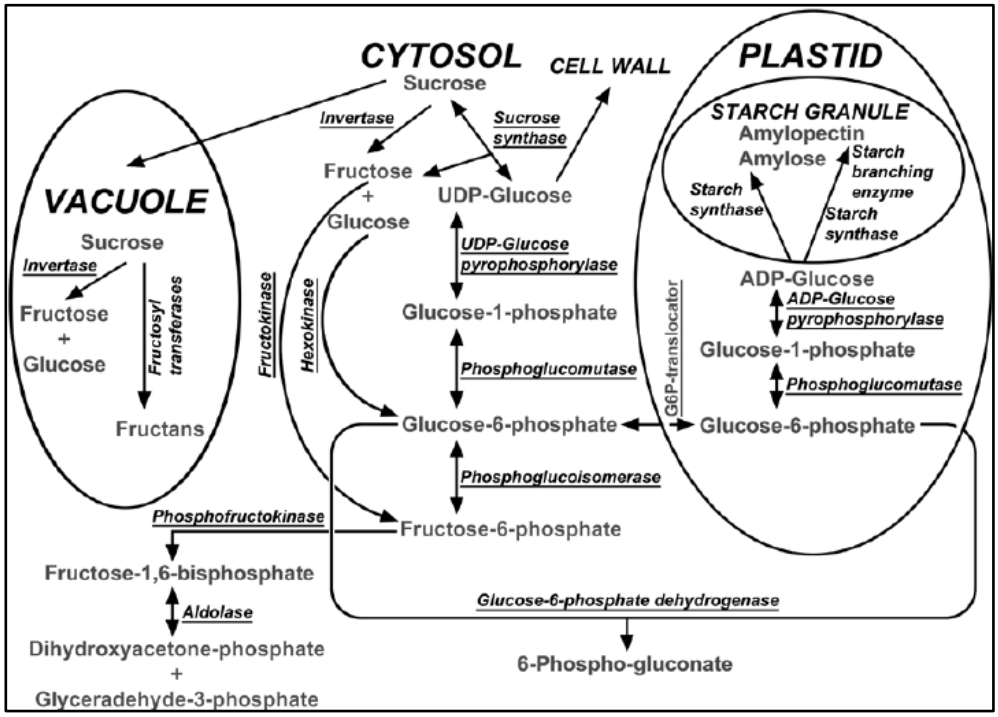

Fig. 4 Key enzymes of primary carbohydrate metabolism (Sergeeva and Vreugdenhil 2002)

Starch is the most common form of carbon in plants. It has high commercial significance. During plant growth, significant changes in mRNA expression levels and enzyme activities that are involved in carbohydrate metabolism occur. All the processes such as glycolysis, starch formation or degradation, carbon partitioning, growth correlation, glucose sensing and more are associated with the expression of a large number of both constitutive and developmentally regulated genes (Appeldoorn et al. 1997).

Sucrose is the starting point of carbohydrate metabolism in developing tubers (Turner and Turner 1980) The carbohydrate metabolism in tuber development is compartmentalized at the vacuolic, cytosolic and plastidic levels (as shown in Fig. 4). SuSy and AGPase are important enzymes for the conversion of sucrose to starch. They are known to be transcriptionally regulated. SuSy is the first key enzyme in the process that transforms sucrose and UDP into UDPGlc and fructose. This reaction is easily reversible. Also, sucrose is hydrolyzed by invertase, resulting in glucose and fructose (Geigenberger and Stitt, 1993). Fructose and Glucose enter glycolysis in the presence of kinase enzymes, fructokinases, and hexokinases respectively to form G6P. G6P as a substrate, regulates various important metabolic pathways such as,

- Downstream regulation of glycolysis and respiration
- Sucrose production
- It is the oxidative PPP's first substrate
- Biosynthesis of starch and trehalose

Hence, G6P produced in the presence of hexokinase is an important substrate that is destined to interconnect sugar metabolism pathways.

Table 1 Key enzymes of primary carbohydrate metabolic functions and activity during development in a tuber plant

Enzymes	Function	Activity during development
Invertases	Sucrolytic activity	Upregulated
Sucrose synthase	Sucrolytic activity	Downregulated
Fructokinase	Sucrose biosynthesis	Downregulated
Hexokinase	Glycolysis	Downregulated
Phosphoglucoisomerase	Glycolysis	Downregulated
Phosphofructokinase	Glycolysis	Downregulated
Aldolase	Glycolysis	Downregulated
ADP-glucose pyrophosphorylase	Starch biosynthesis	Upregulated
phosphoglucomutase	Starch biosynthesis	No change

UDP-glucose pyrophosphorylase	Cell wall biosynthesis	Upregulated
Glucose-6-phosphate dehydrogenase	Oxidative pentose phosphate pathway	Downregulated

Increased starch synthesis and activity of associated enzymes in stolon tips are two characteristics of the tuberization process. In growing tubers, the sucrose synthase pathway dominates sinks that store starch due increase in the activity of Susy activity and decrease in the activity of acidic and alkaline invertases (Renz et al. 1993). Both the content and composition of the soluble sugar pool also show prominent changes during the process of tuberization and there is a significant change in the ratio of hexose to sucrose sugars due to decline in hexoses due to the activity of hexose kinases enzymes. (Wang et al. 1993). Table 1 shows the activity and functions of some crucial enzymes of carbohydrates during tuber development. However, current study focusses on hexokinases. Following section highlight recent advances on this enzyme and their role in potato life cycle.

2.1 Introduction to Hexokinases

The universal carbon sources include glucose and other hexoses in higher plants. These hexoses participate in carbohydrate metabolism. Otto Meyerhof initially made reference to the allosteric enzyme hexokinases (HXKs) in a seminal study in 1927. He discovered it in an extract from baker's yeast. It is the first rate-limiting enzyme in the glycolysis process. Hexokinases belong to the transferases class of enzymes: Phosphotransferases with an alcohol group as an acceptor (EC 2.7.1.1). Hexokinases (HXKs) and fructokinases (FRKs) are the only groups of enzymes found in plants that can phosphorylate hexoses (Granot et al. 2013). HXKs catalyze the phosphorylation of Glucose to Glucose-6-Phosphate (G6P) in the presence of ATP. Multiple HXK isoforms are expressed in various mature and developing plant tissues, each with a unique set of kinetic characteristics and subcellular localization. The HXKs are located in the cytosol, attached to the mitochondrial membrane, or in the stroma of the plastids of a plant cell (Damari-Weissler et al. 2007).

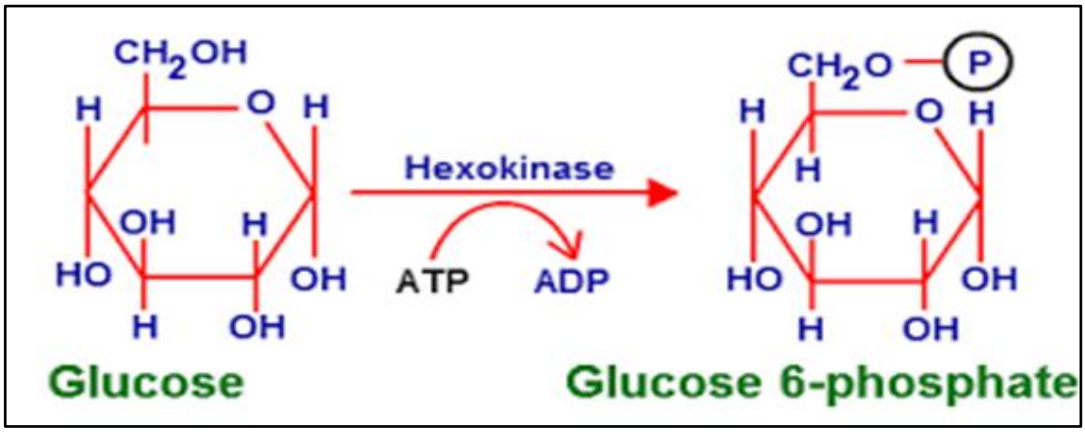

Fig. 5 Conversion of Glucose into G6P. First step of glycolysis in the presence of HXK forming G6P, an important substrate that is destined to interconnect sugar metabolism pathways: Sucrose production, oxidative PPP, Starch biosynthesis, and trehalose biosynthesis (Neuhaus and Emes, 2000).

2.2 Biochemical and Catalytic Properties

Literature suggests that multiple *HXK* isoforms exist in various plant organs distinguished on the basis of their subcellular localizations, and chromatographic and kinetic properties. These isoforms with variable physiochemical properties are suggested to perform specific roles. HXKs follow Michaelis-Menten kinetics with the various sugar substrates (Claeyssen et al. 2007). *Solanum tuberosum* is an important source of sugars and starch. Depending on the substrate, energy, and pH levels, the cell may be able to fine-tune hexose catabolism using its

kinetic properties. In respect to the physiological status of the potato tuber, the kinetic characteristics of the various HXK isoforms proved to be particularly significant for their control *in vivo* (Renz et al. 1993).

- **Nucleotide and ions dependence:** All HXK forms have a distinctly higher affinity for ATP than other NTPs. Altough, HXKs are Mg^{+2} dependent, but are also inhibited by excess free Mg^{+2} and ADP due to the effect of mixed inhibition (Turner and Turner 1980).
- **pH-dependence:** The HXK2 has a broad pH response, whereas the activity of HXK1 decreases quite strongly between pH 7.5 and pH 6.5 (Renz et al. 1993).
- **Hexose dependence:** The glucose-specific forms HXK1 and HXK2 are present at low activities in growing tubers, and increase during storage and sprouting. (Renz et al. 1993). K_m values for hexoses, glucose and fructose are mentioned in Table 2.

Table 2 Kinetic properties of various HXK isoforms in potato

S.No.	Isoforms	Mol. wt (kDa)	K_m Glu (mM)	K_m Fru (mM)	pH optima
1.	StHXK1	54.78	41	11	7.3-8.2
2.	StHXK2	54.56	130	22	7.5-9.0

2.3 Ubiquitous Nature of HXKs

HXKs are found in all phyla. It is one of the major enzymes of the glycolytic pathway. HXKs are known to play crucial role in plant growth and development. Glucokinases (GLKs) and HXKs are functionally distinct: the former only phosphorylates glucose, whereas the latter can phosphorylate a variety of hexoses (e.g., Fru and Man). HXKs have been reported from various organs of the plant species namely tomato fruit (Martinez-Barajas and Randall 1998); maize roots (Galina et al. 1995); maize leaves (Schnarrenberger 1990); castor bean endosperm (Miernyk and Dennis 1983); pea seeds (Turner et al. 1977; Turner and Copeland, 1981); spinach leaves (Baldus et al. 1981; Schnarrenberger 1990); Arabidopsis leaves (Jang et al. 1997); pea leaves (Schnarrenberger 1990); tobacco leaves (Sindelar et al. 1998); soybean nodules (Copeland and Morell, 1985); and avocado (Copeland and Tanner 1988). HXKs could acts as a signal molecule that regulates the growth and expression of several gene groups. The expression patterns, biochemical properties, and subcellular localizations of HXK isoforms may relate to their modes of action (Claeyssen et al. 2007).

2.4 Multifaceted Roles of HXKs

HXK is a member of a multigene family. Till date, six isoforms in *Arabidopsis thaliana*, ten in *Oryza sativa*, four in *Solanum lycopersicum*, nine in *Nicotiana tabacum*, and nine in *Zea mays* have been reported (Cho et al. 2006; Damari-Weissler et al. 2006; Karve et al. 2010; Kim et al. 2013; Zhang et al. 2014). The glyoxylate cycle and genes involved in photosynthetic pathway were negatively affected by treatments with glucose. This provided the first experimental proof of HXK's regulatory function in plants. (Sheen 1990). Numerous studies have demonstrated that the activation of *HXK results* in the reduction of expression of photosynthetic genes namely Rubisco small subunit (RBCS), carbonic anhydrase (CAA), sedoheptulose bisphosphatase (SBP), Chl a/b-binding protein (CAB), and the starch-degrading enzyme -amylase which are suppressed by an increase in soluble sugars in Arabidopsis, rice, and maize. HXK are known to protect plants from the harmful effects of reactive oxygen species (ROS). For example, *HXK* regulate the formation of H_2O_2, membrane potential, and cytochrome release in *Nicotiana benthamiana* and *S. tuberosum.* (Kim et al. 2006; Camacho-Pereira et al. 2009). A sugar signaling system in plants relies on the sensory activities of *HXK* (Sheen 1990; Jang et al. 1997). It is well known fact that glucose sugar is a key regulator of numerous crucial processes in plants whose life revolves around sugar production which has been discussed earlier. In 2003, it was reported that in *Arabidopsis,* a variety of glucose responses including root and inflorescence growth, leaf expansion and senescence, and reproduction, are uniquely regulated by HXK1(Moore et al. 2003). Thus, HXKs mediate a number of crucial biological processes, such as seed germination, seedling growth, plant vegetative and reproductive development, stress responses, and senescence (Dai et al. 1999; Rolland et al 2006).

2.4.1 Role of HXKs in Potato

Potato tubers contain a spectrum of three isoforms each of HXKs and FRKs. These enzymes have a very high affinity and selectivity towards glucose and fructose (Renz et al. 1993). *StHXK1* and *StHXK2* are known to express in various potato organs. The substrate dependence and product inhibition of HXKs from growing potato tubers were investigated.

An *in vitro* tuberization system was used to investigate the activity patterns of the enzymes involved in the conversion of sucrose to hexose-phosphates during the stolon-to-tuber transition of potatoes. A prominent tuber-related increase in HXK activity was observed (Appeldoorn et al. 1997). Potato plants transformed with sense and antisense constructs of *HXK1* cDNA exhibited altered enzyme activities and expression of *StHXK1* mRNA confirming the role of *HXK1* carbon export pathway from chloroplasts (Veramendi et al. 2002). After discovering the

role of HXKs in the metabolic and sugar sensing role has resulted in increased interest and raised the question about the physiological and biochemical relevance in *Solanaceae* family with special attention to stages of potato tuberization (Moore et al. 2003). Potato tuber is a highly uniform tissue and the conversion of sucrose to starch comprises the predominant metabolic flow, it makes for an excellent model system for the study of starch metabolism. In recent years, all of the key genes in the potato tuber sucrose to starch pathway were cloned, enabling the production of a collection of antisense transgenic lines in which the activity of each particular enzyme in the system is gradually decreased.

To improve abiotic stress resilience in potato plants, co-expression of *AtHXK1* in guard cells and SP6A was performed. This study explored a sustainable use of limited water resources with a high tuberization capacity under greenhouse conditions and thereby could help to maintain tuber formation and growth under heat, drought, and combined stress conditions (Lehretz et al. 2021).

2.4.2 HXKs in non *Solanaceae* Family Members

- ***Arabidopsis Thaliana***: Till date, six genes coding for *HXK* have been identified. Three of these genes encode for the catalytically active proteins with different cellular/tissue localization. The other three *HXK* genes encode for HXK proteins without catalytic function. The analysis of *Arabidopsis* plants with changed *HXK* levels first revealed the function of a plant *HXK* as an evolutionary conserved glucose sensor. Plants overexpressing *AtHXK1* or *AtHXK2* exhibited glucose hypersensitivity whereas antisense plants were sensitive (Jang et al. 1997). AtHXKs perform a variety of functions such as glc sensor/transductions, metabolic catalysts, and non-catalytic regulatory roles (Karve et al. 2008).

2.4.3 HXKs in other Members of *Solanaceae* Family

- **Tomato *(Lycopersicum esculentum* L.*)***: In tomato, 4 isoforms of *HXK* have been identified. This crop has become a model crop in recent times as it is commonly used in investigating plant growth and development. *HXK* gene expression from tomato were analysed using qPCR and the transcript levels were observed higher in flowers and leaves than in comparison to the other organs. and decreased during leaf and petiole development. (Clayessen et al. 2007). Phylogenetic analysis displayed that SlHXK1 shares higher homology with pepper CaHXK1 (94.4 %) and tobacco

NtHXK1 (91.5 %) than other HXK proteins. The silencing of *SlHXK1* led to the new observation. *SlHXK1* is a significant gene involved in leaf senescence and stunted plant growth and development in tomatoes by affecting starch turnover. *SlHXK1-RNAi* lines displayed advanced leaf senescence and stunted plant growth (Li et al. 2020).

- **Tobacco (*Nicotiana tabacum)*:** It has been investigated that tobacco comprises of at least 9 isoforms of *HXK* out of which only one *HXK* isoform (*NtHXK2*) has been characterized so far and *NtHXK2* was shown to be localized in chloroplast stroma of starch-containing cells (Giese et al. 2005). The suppression of *NtHXK1* showed the strongest effect on plant growth and the highest mRNA expression levels. With further detailed molecular, ultrastructural and biochemical analysis it was observed that this isoform exhibits catalytic activity as well as a sensing function. It also exerts a strong impact on downstream metabolic pathways (Kim et al. 2013).

2.5 Origin of the Problem

The potato tuber development is a complicated process governed by various factors with great economic importance. The key components of tuberization are explored and reviewed from time to time. Potato holds an important position as a crop due to changes in global climate and increasing nutritional demands. In India, the two months of October and March with the short days of the northern hemisphere are more suitable for potato production. The physiological potential of potato productivity in the tropics and sub-tropics substantially decreases in comparison to temperate regions because of a crop's shorter life cycle under short day circumstances and fewer availability of sunlight hours. The interventions in potato research and development face a significant challenge as: how to increase achievable output while maintaining lower potential yield. As climatic conditions are changing, the global potato production is expected to fall by 9–18% in most parts of the world. It is crucial to raise varieties that can withstand the negative impacts of environmental cues. Tuberization is the sensitive stage of potato life cycle. It determines and limits climate-associated geographical distribution and yield of potato. Hence, it is important to understand the biochemical and molecular aspects of tuberization. A number of biotechnological and genomics techniques can be used to understand the complex process of tuberization and associated biochemical pathways in order to increase potato yield. HXKs is an important carbohydrate metabolism enzyme that plays an important role in the regulation of tuberization. Hence, it is important to understand its function and expression in various organs of potato plants. Therefore, keeping all these aspects in view, efforts have been made to explore the biochemical aspects of the HXK enzyme from Indian

cultivars of potato, and the following objectives have been proposed to study the multifaceted roles of HXKs.

2.6 Objectives

The following objectives were framed to carry out this thesis work:

- *In silico* approaches for sequence analyses, multiple sequence alignments, and motif search of the HXKs in the members of *Solanaceae* family and comparison between the HXK isoforms in potato

- Biochemical studies on HXK in the potato organs including developing tubers

3.1 *In silico* Analysis

The Nucleotide Sequence of the cDNA of *Solanum tuberosum* was obtained.

BLASTn was performed using NCBI server

BLASTp was performed using NCBI server

The sequences retrieved were analysed further

Multiple sequence alignment, motifs prediction, genome wide study, expression analysis and string analysis were performed

3.1.1 Multiple Sequence Alignment

The multalin server (http://multalin.toulouse.inra.fr/multalin/) was used to perform MSA. Different nucleotide and protein sequences were chosen after running BLASTn and BLASTp. MSA is performed to align biological sequences at gene or protein level using computational algorithms and further study the evolutionary relationships.

3.1.2 Identification of the Conserved Domains

MY HITS server was used to identify the motifs. The reference protein sequences were uploaded to scan motifs. (https://myhits.sib.swiss/cgi-bin/motif_scan).

3.1.3 Genome-wide Study

The Ensembl plants server (https://plants.ensembl.org/index.html) was used for the genome-wide study of *HXK* gene. A genome browser, Ensembl is for vertebrate genomes that aids studies in comparative genomics, evolution, sequence variation, and transcriptional control. Ensembl performs gene annotation, multiple alignment computation and regulatory function prediction. ProtParam tool of Expert Protein Analysis System (ExPASy) developed by Swiss Institute of Bioinformatics (https://www.expasy.org/) was used. This helps to determine the molecular weight, isoelectric point (pI) and amino acid composition of the predicted proteins.

3.1.4 Expression Atlas

EBI maintained a database, expression atlas (https://www.ebi.ac.uk/gxa/home) was used to retrieve gene expression data. It is an open web server for users to learn more about the expression of genes and proteins. It provides accessible data on the abundance and localisation of RNA (and proteins) in various organs, cell types, developmental stages, illnesses, and other biological contexts across species.

3.1.5 MEGA: Phylogenetic Analysis

Phylogenetic tree was generated using MEGA-X software involving a total of 40 HXK protein sequences from three different plant species: 15 from the potato (StHXK), 13 from tomato (SlHXK) and 12 from Arabidopsis (AtHXK). It is an analytical software which is used for the study of phylogenomics to study evolutionary relationships between various species. (https://www.megasoftware.net/)

3.1.6 Three-dimensional Structure Prediction and Validation

To predict a protein's biological activity, it is very important to predict and understand its structural stability. I- TASSER server (https://zhanggroup.org/I-TASSER/) was used to predict the 3-D structure and PROCHECK server (https://saves.mbi.ucla.edu/) was used to validate the structures by the Ramachandran plots.

3.1.7 STRING Database

The STRING database comprises of experimental data from public sources to analyse known and predicted protein-protein interactions of HXK protein within cell. (https://string-db.org/cgi/input?input_page_show_search=on)

3.2 Plant Materials

Five different potato cultivars i.e., Kufri Chipsona-1 (KC-1), Kufri Chipsona-2 (KC-2), Kufri Jyoti (KJ), Kufri Pukhraj (PR), cultivar Desiree (De) procured from CPRI, Shimla are routinely maintained in our laboratory at TIET, Patiala, under controlled conditions on MS-basal media with 2.5% sucrose (16 h light/8 h dark, light intensity approximately 40-42 μmol m^{-2} s^{-1}, 25-27°C, 70% relative humidity). The micro-propagated plants were then hardened, acclimatized and transferred to the field for 3 months. Various organs at different stages of development were harvested, flash frozen in liquid nitrogen and finally stored at -80°C for the experiments.

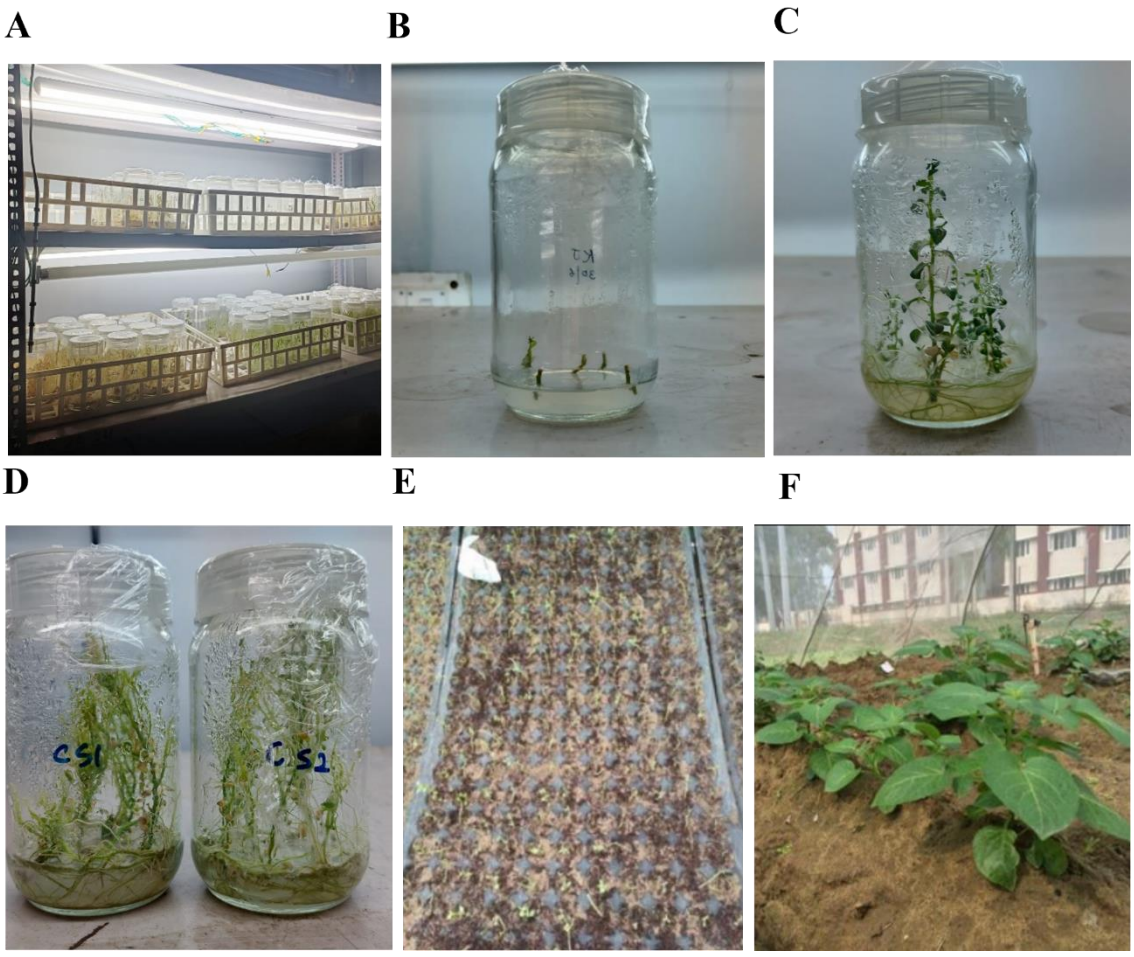

Fig. 6 Steps of plant grown in lab to field conditions a) Micropropagation of potato plant after 7 days of incubation. b) Plant tissue growth room with controlled conditions. c) Potato plant after 30 days of incubation. d) KC-1 and KC-2 cultivars after 45 days of incubation. e) Transfer of potato plantlets in the field

3.2.2 Chemicals and Reagents

Various reagents and buffers such as bis tris, agar, PMSF, Tris HCL and benzamidine were procured from Sigma-aldrich India Pvt. Ltd, Himedia Pvt. Ltd, Mumbai, and SR Bio Solutions, Ludhiana. (Table 3 and 4)

3.3 Biochemical Analysis

3.3.1 Enzyme Extraction

500mg of plant material was homogenized in liquid nitrogen. Then grinding was done in 0.1% PVP (Polyvinylpyrrolidone). 1.2 mL of extraction buffer (Table 3) was added to the homogenate and incubated on ice until the material was fully thawed. Afterward, the homogenate was centrifuged at 15000 rpm for 30 minutes at 4˚ C. The supernatant obtained was transferred in a

fresh tube and then centrifuged at 15000 rpm for 15 mins at 4ºC. Thus, the crude extract was obtained and stored at -20ºC for the determination of enzyme activities. (Bisswanger 2004)

Table 3 Composition of extraction buffer

Component	Composition
PVP	0.1%
Tris HCl	40 mM
$MgCl_2$	3 mM
PMSF	0.1 mM
Benzamidine	1 mM
Beta-mercaptoethanol	14 mM
$NADP^+$	24 µm
EDTA	1 mM

3.3.2 HXK Assay in the Crude Extract

To determine HXK activity, crude extract was incubated with the assay buffer (Table 4). For control reaction, glucose was omitted. HXK activity was determined spectrophotometrically by measuring the absorbance at 340 nm The increase in absorbances was observed due to conversion of NAD^+ to NADH. (Petreikov et al. 2001)

Table 4 Composition of kinetic assay buffer

Component	Composition
Bis tris (pH=8)	50mM
Magnesium chloride	5mM
ATP	0.1mM
NAD	1mM
G-6-PDH	0.8 U
Glucose	5µm
Extract	100 µl

3.3.3 Protein Estimation by the Folin-Lowry Method

A protein assay called Folin-Lowry is a biochemical assay that helps in determining the total protein content in a solution. The basic principle is that a phenolic compound in amino acids namely, tyrosine and tryptophan produce a blue-purple complex, which absorbs at 750 nm. Protein estimation was done using Bovine Serum Albumin (BSA) as a standard.

Table 5 Chemicals and reagents used in protein estimation

S.No.	Chemical Name
Solution I	0.1M alkaline Sodium Carbonate (2% Sodium carbonate in 0.1M NaOH)
Solution II	Freshly prepared copper sulphate-sodium potassium tartrate solution.
Solution A	Solution I: Solution II (50:1)
Solution III	Folin-Ciocalteu reagent: distilled water (1:1)

Distilled water (900 µL) was added in all the test tubes except blank. Blank was set with the distilled water. 100 µL of protein samples from all 5 cultivars in different test tubes were added individually. Then 5 mL of solution A was added in all the test tubes including the blank and incubated in the dark for 20 minutes, followed by the addition of 500 µL of Folin reagent and then vortexed for 10 seconds and incubated in the dark for 30 minutes. The absorbance was taken at the wavelength of 750 nm and the standard graph was plotted. Thus, total protein content is estimated in different plant organs such as young leaves, mature leaves, young and mature stems, and all stages of tubers.

4.1 *In silico* Analysis

In this study, we obtained the 1619 bp long *HXK* cDNA encoding 498 amino acids of a potato by exploring NCBI database (GenBank ID: X94302). This is basically a transcription factor involved in the process of glycolysis and tuber development in potatoes. The size of the ORF in *StHXK1* is 1497 i.e., from 19th to 1515th bp. We investigated various sequence aspects, biochemical characteristics, and conserved motifs through *in silico* characterization of this important gene- *StHXK1*. Additionally, a comparison with other members of the *Solanaceae* family using multiple sequence alignment was also done. The structural and functional aspects were linked and understood of any protein. It is crucial for speculating on a protein's biological function. Therefore, a 3D model was created to better comprehend the StHXK1 structure. Ramachandran plot analyses were used to further validate these 3D structures.

4.1.1a Sequence Analysis of *StHXK1* by BLASTn

To compare the nucleotide sequence of *StHXK1* with other plant species, 1619 nucleotide ORF of *StHXK1* was taken and BLASTn was performed. The result suggested significant *StHXK1* sequence dissimilarity with other plant species belonging to both *Solanaceae* and non-*Solanaceae* family members. From all the sequences, we selected 9 nucleotide sequences belonging to potato family for further analysis. The *StHXK1* gene showed 99% query coverage and 94.96% sequence identity with *Solanum lycopersicum* (NM_001247028), 98% query coverage and 89.45% percentage identity with the nucleotide sequence of *Nicotiana tabacum* (NM_001325809), 97% query coverage and 90.75% sequence identity with *Capsicum annum* (XM_016707155), 99% query coverage and 65.96% sequence similarity with *Arabidopsis thaliana*.

Significant variation between two isoforms of potato HXK (*StHXK1* and *StHXK2*, NM_001287912) was also observed with 92% query coverage and 83.54% sequence identity.

Table 6 Description *HXK1* nucleotide sequences and their homology with other sequences as available in the database

S. No.	Accession No.	Species	Isoform	Length(bp)	ORF	Query Coverage	E-value	% Identity	aa
1	NM_119057.4	***Arabidopsis Thaliana***	*AtHXK1*	2128	19-1515	99%	0.0	65.86%	496
2	NM_001247028	***Solanum lycopersicum***	*SlHxk1*	1848	125-1621	99%	0.0	94.96%	498
3	NM_001325809	***Nicotiana tabacum***	*NtHxk1*	1853	82-1575	98%	0.0	89.45%	497

4	XM_016707155	***Capsicum annum***	*CaHxk1*	1927	272-1678	97%	0.0	90.75%	468
5	AJ401153.1	***Lycopersicon esculentum***	*LeHXK1*	1862	139-1635	99%	0.0	94.96%	498
6	DQ177440	***Solanum chacoense***	Nd	1793	50-1540	92%	0.0	83.54%	496
7	NM_001287912	***Solanum tuberosum***	*StHXK2*	1874	64-1554	92%	0.0	83.28%	496
8	NM_001247477	***Solanum lycopersicum***	*SlHXK2*	1908	234-1724	92%	0.0	83.04%	496
9	AF208543.1	***Solanum esculentum***	*LeHXK2*	1770	138-1628	92%	0.0	82.77%	496

4.1.1b Sequence Analysis of StHXK1 by BLASTp

To compare the polypeptide sequence of StHXK1 with other plant species, 499 aa long protein sequence of StHXK1 was taken. Then, BLASTp was performed. The result showed significant StHXK1 sequence dissimilarity/divergence with respect to other plant species belonging to both *Solanaceae* and non-*Solanaceae* family members. From all the sequences, we selected 8 polypeptide sequences belonging to potato family for further analysis. The StHXK1 protein which is 499 aa long showed 100% query coverage and 90.00% sequence identity with polypeptide sequence of *Nicotiana tabacum* (AAT77515), 100% query coverage and 89.80% percentage identity with the polypeptide sequence of *Solanum stenotomum* (XP_049395017), 95% query coverage and 88.70% sequence identity with *Solanum lycopersicum* (NP_001233957), 95% query coverage and 84.70% sequence similarity with Capsicum annum, 95% query coverage and 85.12% sequence similarity with *Datura stramonium* (MCD9640265) and 95% query coverage and 76.15% sequence similarity with wild potato- *Solanum chacoense* (ABA01010.1).

When data was analysed then there was prominent sequence dissimilarity between 2 isoforms of HXK for example, 95% query coverage and 75.94% sequence identity with polypeptide sequence of isoform 2 of *Solanum tuberosum* (NP_001274841).

Table 7 Description HXK1 protein sequences and their homology with other sequences as available in the database

S. No.	Protein ID	Species	Isoform	Protein length	Query Coverage	E-value	Percentage Identity
1.	AAT77515	***Nicotiana tabacum***	HXK7	497 aa	100%	0.0	90.00%
2.	XP_049395017	***Solanum stenotomum***	HXK1	497 aa	100%	0.0	89.80%
3.	NP_001312563	***Nicotiana tabacum***	HXK1	497 aa	100%	0.0	82.60%

4.	NP_001233957	***Solanum lycopersicum***	HXK1	498 aa	95%	0.0	88.70
5.	MCD9640265	***Datura stramonium***	HXKA	495 aa	95%	0.0	85.12%
6.	KAF3627642	***Capsicum annuum***	HXK1	495 aa	95%	0.0	84.70%
7.	ABA01010.1	***Solanum chacoense***	HXK1	496	95%	0.0	76.15%
8.	NP_001274841	***Solanum tuberosum***	HXK2	496	95%	0.0	75.94%

4.1.1c Sequence Analysis of *StHXK2* by BLASTn

The nucleotide sequence of StHXK2 were compared with other plant species using BLASTn server. The 1874 nucleotide ORF of *StHXK2* (NM_001287912) was retrieved to perform BLASTn. The result showed significant *StHXK2* sequence dissimilarity with other plant species belonging to both *Solanaceae* and non-*Solanaceae* family members. From all the sequences, we selected 5 nucleotide sequences belonging to potato family for further analysis. The *StHXK2* gene which is 1874 bp long showed 97% query coverage and 90.63% sequence identity with the nucleotide sequence of *Nicotiana tabacum* (XM_016609530), 94% query coverage and 98.99% sequence identity with wild potato-*Solanum chacoense* (DQ177440),90% query coverage and 90.60% sequence similarity with *Capsicum annum* (XM_016723108), 88% query coverage and 96.43% sequence similarity with *Solanum lycopersicum* (NM_001247477) and 86% query coverage and 96.18% sequence similarity with *Lycopersicum esculentum* (AF208543).

Table 8 Description *HXK2* nucleotide sequences and their homology with other sequences as available in the database

S. No.	Accession No.	Species	Isoform	Acc. Length (bp)	ORF	Query Coverage	E-value	%age Identity	No. of residues
1	DQ177440.	***Solanum chacoense***	StHXK2	1793 bp	50-1540	94%	0.0	98.99%	496 aa
2	NM_001247477	***Solanum lycopersicum***	SlHxk2	1908 bp	234-1724	88%	0.0	96.43%	496 aa
3	XM_016609530	***Nicotiana tabacum***	NtHxk2	2014 bp	206-1699	97%	0.0	90.63%	497 aa
4	XM_016723108	***Capsicum annum***	CaHxk2	1943 bp	222-1721	90%	0.0	90.60%	499 aa
5	AF208543.1	***Lycopersicon esculentum***	LeHXK2	1770bp	138-1628	86%	0.0	96.18%	496 aa

4.1.1d Sequence Analysis of StHXK2 by BLASTp

The polypeptide sequence of StHXK2 was compared with other plant species using BLASTp server. The 496 aa long protein sequence of StHXK2 (NP_001274841) was retrieved to perform BLASTp. The result showed significant StHXK2 sequence dissimilarity with respect to various plant species belonging to both *Solanaceae* and non-*Solanaceae* family members. From all the sequences, we selected 7 polypeptide sequences belonging to potato family for further analysis. The StHXK2 protein showed 100% query coverage and 99.40% sequence identity with polypeptide sequence of wild potato *Solanum chacoense* (ABA01010.1), 100% query coverage and 96.98% percentage identity with the polypeptide sequence of *Solanum lycopersicum* (AAG35735.1), 100% query coverage and 92.94% sequence identity with *Nicotiana tabacum* (NP_001311844), 100% query coverage and 93.75% sequence similarity with *Nicotiana tomentosiformis* (XP_009622660), 100% query coverage and similarity with *Capsicum annum* ***(***APW83749.1).

Table 9 Description HXK2 protein sequences and their homology with other sequences as available in the database

S. No.	Protein ID	Species	Isoform	Protein length	Query Coverage	E-value	%age Identity
1.	ABA01010.1	***Solanum chacoense***	HXK2	496	100%	0.0	99.40%
2.	AAG35735.1	***Solanum lycopersicum***	HXK2	496	100%	0.0	96.98%
3.	NP_001311844.1	***Nicotiana tabacum***	HXK2-like	497	100%	0.0	92.94%
4.	XP_009622660.1	***Nicotiana tomentosiformis***	HXK2	497	100%	0.0	93.75%
5.	APW83749.1	***Capsicum annum***	HXK2	499	95%	0.0	93.01%
6.	APW83749.1	***Lycium barbarum***	HXK	497	100%	0.0	91.33%
7.	AAT77515.1	***Nicotiana tabacum***	HXK7	497	100%	0.0	83.67%

4.1.2 Multiple Sequence Alignment

Comparison of 8 HXK protein sequences representing two isoforms of HXKs- HXK1 and HXK2 from potato and other members of *Solanaceae* family such as tomato, tobacco and capsicum. These sequences were analysed by MultAlin that revealed significant no. of conserved amino acids between them (Fig. 7). Isoforms 1 and 2 are significantly divergent from each other specifying functional and structural differences at protein level. MSA revealed that most of the nucleotides were highly conserved. However, at some places species-specific insertion/deletion mutations could be seen. The observations were made using StHXK1 as the reference sequence. It is observed that amino acid at the 342nd position is missing in all the sequences except StHXK1. This confirms insertional mutation at 342nd position in all the

sequences of tomato, capsicum and tobacco with respect to StHXK1. In Fig. 7 all the important motifs and domains have been highlighted and it can be seen that the HXK domain and other binding domains are highly conserved in all the polypeptide sequences. However, the C-terminal region shows maximum conserved region as compared to the N-terminal.

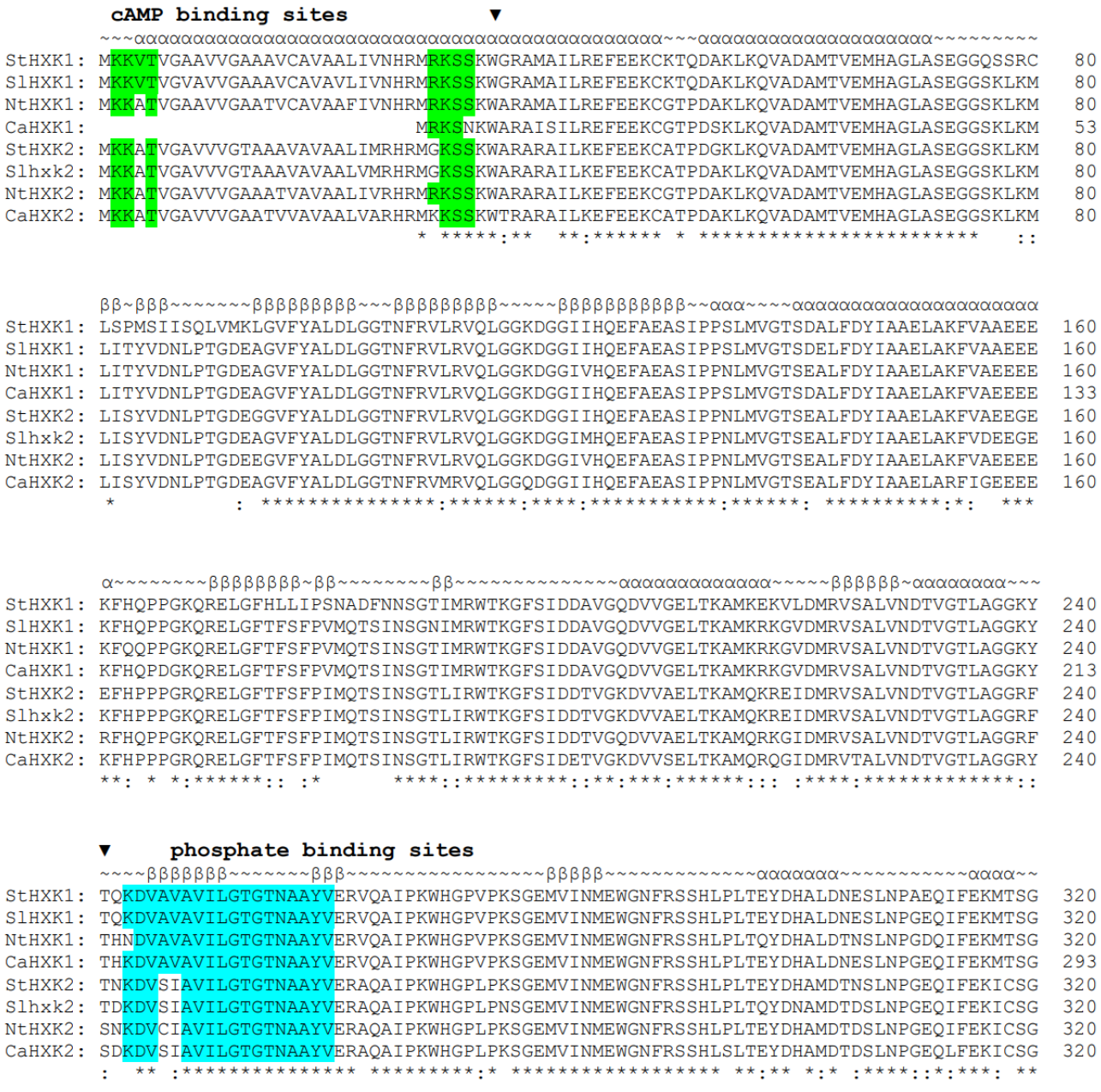

Fig. 7 Multiple sequence alignment was done using MultAlin software. Gaps arisen during alignment are indicated by dashes. Asterisks '*' represent the conserved amino acids and semicolon ':' represent the conservative substitution. The signature HXK domain (35-242) is shown by a downward arrow. Highlighted motifs present are phosphate binding site by blue, cAMP and cGMP dependent phosphorylation sites by green and ATP binding site by yellow. 'α', 'β' and '~' denote the tendency of the individual amino acids of StHXK1 to form alpha helix, beta-sheet and random coil formation, respectively. GeneBank accession numbers are StHXK 1 (CAA63966), SlHXK 1 (NP_001233957), NtHXK 1(NP_001312738), CaHXK 1 (XP_016562641) and StHXK 2 (AAF14186), SlHXK 2 (AAG35735), NtHXK 2 (NP_001311844), CaHXK 2 (XP_016578594).

4.1.3 Motif Scan

A section of a protein sequence with a particular structure that can serve as the basis for classifying proteins is called a motif. They might also help in predicting the function of proteins. Important protein motifs that were identified using amino acid sequences of chosen HXK1 and HXK2 proteins from the *Solanaceae* family, with the help of MY HITS program are described as follows:

- **cAMP- and cGMP-dependent protein kinase phosphorylation site:**
  cAMP-dependent protein kinase is a ubiquitous intracellular secondary messenger that regulates multiple processes. Adenyl cyclase converts ATP into cAMP, which is then

hydrolysed by cAMP-specific phosphodiesterase to provide adenosine 5′-monophosphate.

cGMP-dependent protein kinase is a serine/threonine-specific protein kinase that is activated by cGMP. It phosphorylates a number of biologically significant targets.

- **HXK domain:** HXK domain is present at both N- and C- terminal and is involved in the catalysing role of hexose phosphorylation It provides a site for sugar binding.
- **ATP binding domain:** They are also referred as ABC (ATP-binding cassettes), are a large superfamily of membrane proteins which perform ATP hydrolysis to allow translocation of various molecules across biological membranes.

Table 10 Important domains/motifs in HXK in members of *Solanaceae* family

Site	Hexokinase 1 domain	ATP binding domain	cAMP phosphorylation site	Hexokinase signature domain
Species				
Solanum tuberosum	95-174 186-242	24-242	2-5 29-32	172-197
Solanum lycopersicum	35-242	23-242	2-5 29-32	172-197
Capsicum annum	8-215	5-215	-	145-170
Nicotiana tabacum	35-242	24-242	2-5 29-32	172-197

When the motifs and domains were scanned with the help of reference polypeptide sequence of StHXK1, the major motifs observed were 2 cAMP dependent phosphorylation site, 1 phosphate binding site, 1 Nucleotide binding domain and a signature HXK domain. These motifs are seen to be highly conserved in the first isoform of HXK polypeptide sequences. But there are insertional mutations in the isoform 2 of HXK in all 4 species. (Fig. 7)

4.1.4 Genome Wide Study

A close examination of potato genome database was done. It revealed a total of 6 *HXK* genes encoding 14 multiple forms including *HXK1*, *HXK2*, *HXKRP1*, *HXK7*. ProtParam tool was used to predict various physicochemical properties of the proteins such as molecular weight, chromosomal location, exons, aliphatic index, instability index, and GRAVY (Table 11). The HXKs family was observed to be localised on 6 chromosomes. The gene length ranges from 24.15 kbp-29.63 kbp. Amino acids number varies from 107-533 aa and molecular weight ranged from approximately 16 kDa- 60 kDa.

The protein's stability in a test tube can be estimated using the instability index. A protein is considered to be stable if its instability index is less than 40; if it is greater than 40, the protein may be unstable. The instability indices range from 26-55. Thus, gene *HXK1* and *HXK2* are stable as compared to other genes with instability index less than 40. The aliphatic index is the volume taken up by aliphatic side chains such as alanine, valine, isoleucine, and leucine in relation to other side chains. Greater the aliphatic index, greater is the thermostability of the protein. All proteins with aliphatic index more than 70 are thermally stable. Thus, all the genes of *HXK* retrieved are thermostable. Proteins with a negative GRAVY value are non-polar, while those with a positive value are polar. All genes except PGSC0003DMT400042601, and PGSC0003DMT400000795 are non-polar with negative GRAVY. The low GRAVY index indicates the hydrophilic nature of a protein.

Table 11 Description of *HXK* members of potato extracted from potato genome databases

(Please refer to the Table in the next page)

Gene	Gene ID	Transcript ID	Amino acids	Mol. Wt. (kDa)	Chromosomal Localization	Exons	Base pairs	Gene Length	pI	Instability index	Aliphatic Index	GRAVY
HXKRP1	PGSC0003DMG400009861	PGSC0003DMT400025526	533	58.184	4	8	1602	24.15	5.81	40.14	89.25	-0.024
		PGSC0003DMT400025527	250	27.088	4	9	2003		5.38	38.93	95.12	-0.043
		PGSC0003DMT400025528	499	54.113	4	9	1999		5.73	41.89	90.64	-0.04
-	PGSC0003DMG400030624	PGSC0003DMT400078702	491	54.022	2	9	1740	25.32	5.52	48.58	96.13	-0.099
		PGSC0003DMT400078703	371	40.599	2	9	1623		4.83	44.17	95.18	-0.119
		PGSC0003DMT400078704	152	16.694	2	5	1320		4.75	36.3	101.38	-0.008
HXK2	PGSC0003DMG400016521	PGSC0003DMT400042598	496	53.736	6	9	2032	24.68	6.07	29.74	90.48	-0.081
		PGSC0003DMT400042601	235	25.29	6	4	1203		6.13	26.45	89.7	0.02
		PGSC0003DMT400042599	214	23.572	6	4	955		5.57	38.12	94.35	-0.162
		PGSC0003DMT400042600	137	14.733	6	2	951		6.42	26.89	101.09	-0.047
HXK1	PGSC0003DMG400002525	PGSC0003DMT400006474	497	53.781	3	9	1978	25.48	6.11	30.79	89.88	-0.08

-	PGSC0003DMG400000295	PGSC0003DMT400000795	499	54.114	12	9	5989	29.63	5.48	27.76	98.26	0.015
		PGSC0003DMT400000797	107	11.502	12	1	1811		4.95	14.88	104.86	-0.05
-	PGSC0003DMG400013187	PGSC0003DMT400034299	512	25.46	11	9	2319	25.46	5.74	53.54	95.21	-0.025

4.1.5 Expression Patterns of HXKs in Potato

The *in silico* approach was adopted to predict the organ-specific expression patterns of 6 *StHXK* genes listed in the Table 11. In order to achieve this data (represented in Fig. 8), the expression atlas database was examined to create a heat map that made it very evident that *HXK* genes are ubiquitous in nature and have a significant role in plant growth and development. The heat map clearly showed that *StHXK* was highly expressed in young tubers with 40 TPM in comparison to the mature tubers having an expression level of 26 TPM.

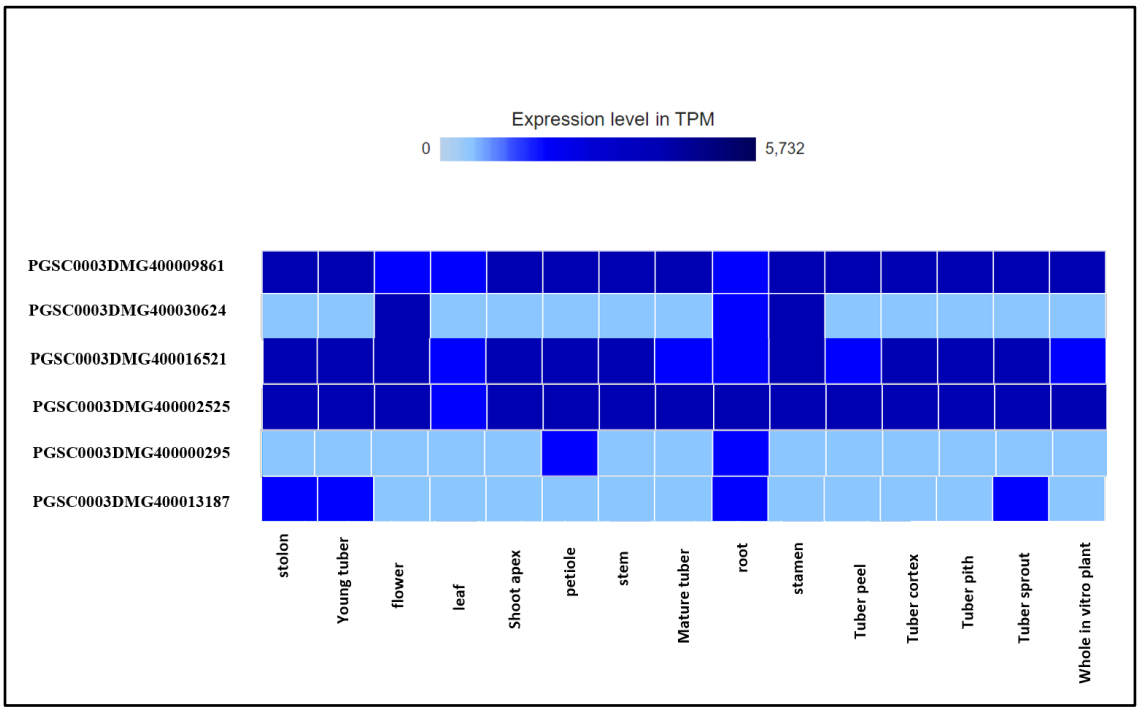

Fig. 8 The heatmap of *HXK* genes retrieved from Potato Genome Sequencing Consortium (PGSC) database

4.1.6 Phylogenetic Analysis

The homology between StHXK1 and StHXK2 (as discussed in the section 4.1.1) is approximately 75% which is very low. Similarly, the homology between SlHXK1 and SlHXK2 is reported to be 83%. Which shows indicates structural and functional differences between isoforms in same species. The StHXK1 and SlHXK1 show high percentage similarity (approx. 95%). This implies that there is high number of conserved sequences in potato and tomato HXK protein. When compared to Arabidopsis, the ATHXK shows high divergence with respect to HXK of *Solanaceae* family members. (Fig. 9)

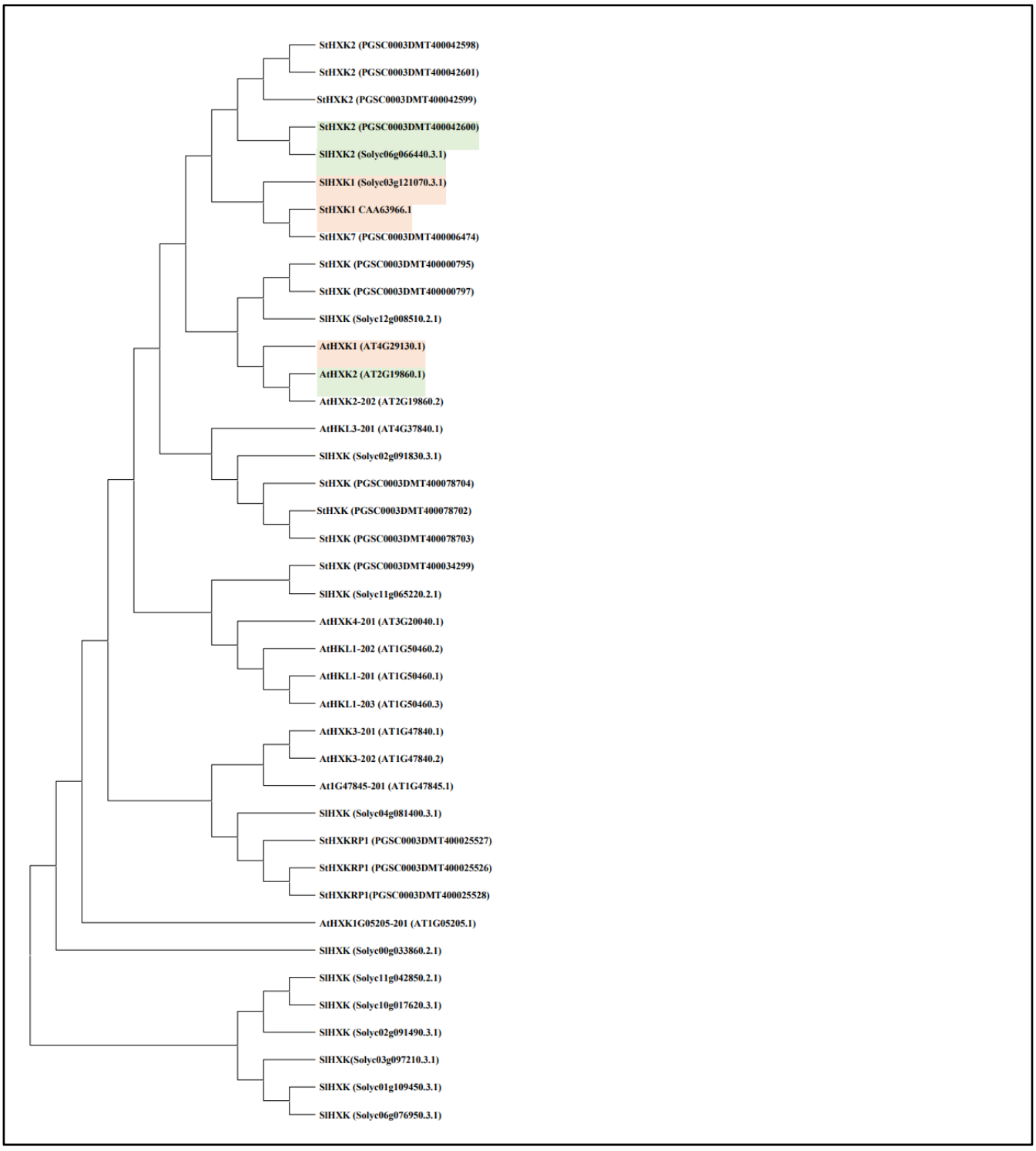

Fig. 9 Phylogenetic tree analysis. It represents 40 HXK protein sequences from three different plant species: 15 from the potato (StHXK), 13 from tomato (SlHXK) and 12 from Arabidopsis (AtHXK). The genes marked in green represent the isoform 2 of HXK and red are the isoform 2 of HXK

4.1.7 Three-dimensional Protein Structure Prediction

The overall three-dimensional structure is the polypeptide's tertiary structure. A thorough knowledge of a protein's 3D structure is necessary in order to annotate its biological function. With the aid of a protein structure, we may better comprehend how a protein functions and

devise novel strategies to control, manage, or alter it. The three-dimensional configuration of its atoms is responsible for the determination of biological and functional aspect of a protein. The entire protein sequences of *Solanum tuberosum*, *Solanum lycopersicum*, *Capsicum annum* and *Nicotiana tabacum* were modelled and configured using I-TASSER server. (Fig. 10 and 11)

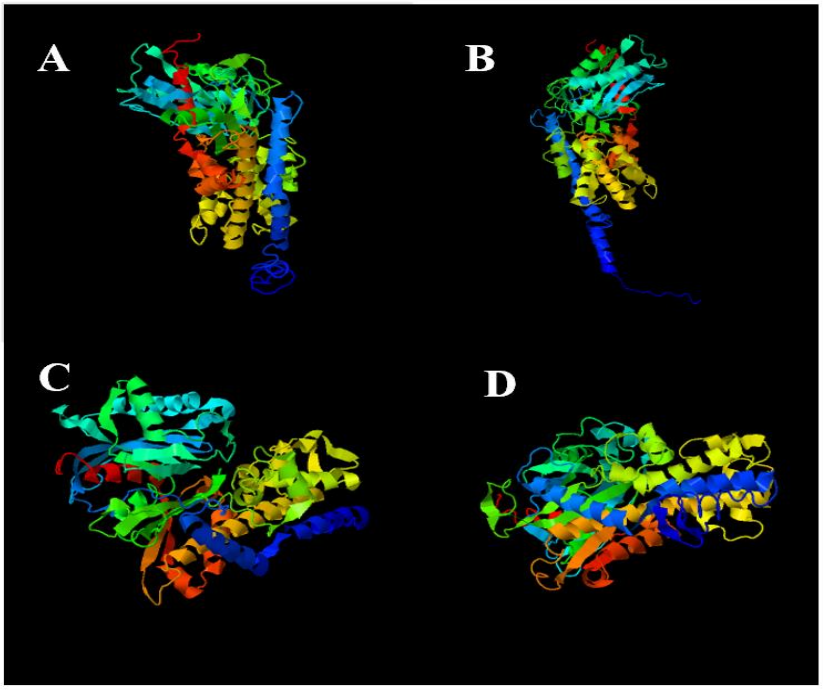

Fig. 10 3-D protein structure of HXK-1 of four different species of *Solanaceae* family

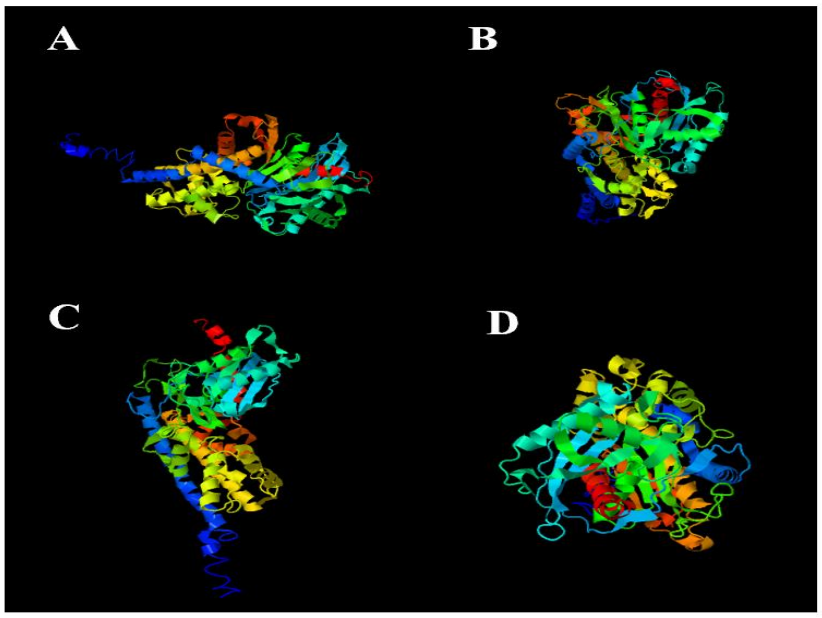

Fig. 11 3-D protein structure of HXK-2 of four different species of the *Solanaceae* family

- **Confidence score (C-score)**

I-TASSER uses the C-score, a confidence score, to evaluate the quality of predicted models. the C-score falls between [-5, 2], with a higher C-score denoting a more confident model and vice versa. The model performs better when the C-score is higher.

- **Quality estimation**

The 3-D structure must be accurately estimated in order to ascertain the protein's applications. This is accomplished by going through and analysing the Z-score numbers that I-TASSER provides. The effectiveness of threading alignment is frequently assessed using the Z-score of the alignment. It is the difference between the raw and average scores' standard deviations. The superior alignment is represented by a normalized Z-score <1. The normalized Z- score of the models obtained is shown in the Tables 12 and 13.

Table 12 Models of Normalized Z-score of HXK-1 of A. *Solanum tuberosum,* B. *Solanum lycopersicum,* C. *Capsicum annum, and* D. *Nicotiana tabacum*

A

Rank	PDB Hit	Norm. Z-score
1	4qs7A	2.85
2	4qs7A	6.18
3	7t78A	2.73
4	5zqt	2.24
5	1dgk	2.04
6	4qs7A	4.32
7	1dgk	3.06
8	3o8mA	8.66
9	4qs7A	3.06
10	7t78A	3.92

B

Rank	PDB Hit	Norm. Z-score
1	4qs7A	2.96
2	5zqtA	6.48
3	7t78A	2.78
4	5zqt	2.34
5	1dgk	2.04
6	4qs7A	4.42
7	1dgk	3.07
8	1czaN	9.03
9	4qs7A	3.12
10	7t78A	4.06

C

Rank	PDB Hit	Norm. Z-score
1	4qs7A	3.08
2	4qs7A	6.33
3	7t78A	2.90
4	5zqt	2.29
5	1dgk	2.02
6	4qs7A	4.65
7	1dgk	3.06
8	1ig8A	9.50
9	4qs7A	3.25
10	7t78A	4.18

D

Rank	PDB Hit	Norm. Z-score
1	4qs7A	2.97
2	5zqtA	6.27
3	7t78A	2.76
4	5zqt	2.35
5	1dgk	2.03
6	4qs7A	4.41
7	1dgk	3.07
8	4qs8A	9.19
9	4qs7A	3.11
10	7t78A	4.02

Table 13 Models of Normalized Z-score of HXK-2 of A. *Solanum tuberosum,* B. *Solanum lycopersicum,* C. *Capsicum annum, and* D. *Nicotiana tabacum*

A

Rank	PDB Hit	Norm. Z-score
1	5zqtA	6.24
2	7t78A	2.76
3	5zqt	2.33
4	1dgk	2.03
5	4qs7A	4.45
6	1dgk	3.07
7	4qs8A	9.25
8	5zqtA	3.15
9	7t78A	4.06
10	5zqtA	8.51

B

Rank	PDB Hit	Norm. Z-score
1	4qs7A	6.12
2	7t78A	2.75
3	5zqt	2.33
4	1dgk	2.04
5	4qs7A	4.39
6	1dgk	3.06
7	4qs8A	9.17
8	5zqtA	3.06
9	7t78A	3.96
10	5zqtA	8.37

C

Rank	PDB Hit	Norm. Z-score
1	5zqtA	6.18
2	7t78A	2.78
3	5zqt	2.36
4	1dgk	2.03
5	4qs7A	4.37
6	1dgk	3.08
7	4qs8A	9.14
8	4qs7A	3.04
9	7t78A	3.93
10	5zqtA	8.35

D

Rank	PDB Hit	Norm. Z-score
1	5zqtA	6.18
2	7t78A	2.80
3	5zqt	2.35
4	1dgk	2.04
5	4qs7A	4.43
6	1dsk	3.07
7	3o8mA	9.50
8	5zqtA	3.15
9	7t78A	4.04
10	5zqtA	8.45

- ***Structural similarity***

The TM-score can be used to compare the structural or topological similarity of two structures. The top PDB proteins with the greatest TM-score and the most structural similarity to the predicted I-TASSER models are listed by the Tables below.

All potential PDB structures that are near to the target proteins are listed in Tables 14 and 15 A, B, C, and D. A valid topological model is suggested by a TM-score of >0.5, whilst a TM-score of 0.17 denotes random similarity. These cutoffs are unaffected by the length of the protein.

The average distance between all residue pairs in two structures is known as the RMSD which prone to local inaccuracy. This inaccuracy is solved by TM value. if the global topology is accurate, a local error will lead to a high RMSD value. The TM-score, on the other hand, makes the outcome less vulnerable to local modelling errors because the small distance is weighted more heavily than the vast distance.

Table 14 Models of TM score of HXK-1 models of *A. Solanum tuberosum, B. Solanum lycopersicum, C. Capsicum annum, and D. Nicotiana tabacum*

A.

Rank	PDB Hit	TM Score
1	4qs7A	0.909
2	5zqtA	0.905
3	1dgkN	0.880
4	3b8aX	0.854
5	2nztA	0.850
6	3f9mA	0.848
7	1bdgA	0.845
8	3hm8A	0.836
9	1hkgA	0.828
10	6r2nA	0.800

B.

Rank	PDB Hit	TM Score
1	5zqtA	0.906
2	1dgkN	0.903
3	4qs7A	0.900
4	3b8aX	0.858
5	2nztA	0.856
6	3f9mA	0.853
7	1bdgA	0.850
8	3hm8A	0.841
9	1hkgA	0.833
10	3o08B	0.804

C.

Rank	PDB Hit	TM Score
1	4qs7A	0.961
2	5zqtA	0.958
3	1dgkN	0.927
4	3b8aX	0.909
5	3f9mA	0.908
6	1bdgA	0.903
7	2nztA	0.894
8	3hm8A	0.894
9	1hkgA	0.880
10	6r2nA	0.851

D.

Rank	PDB Hit	TM score
1	5zqtA	0.908
2	4qs7A	0.904
3	1dgkN	0.875
4	3b8aX	0.858
5	3f9mA	0.852
6	1bdgA	0.850
7	2nztA	0.845
8	3hm8A	0.842
9	1hkgA	0.833
10	6r2nA	0.802

Table 15 Models of TM score of HXK2 models of *A. Solanum tuberosum, B. Solanum lycopersicum, C. Capsicum annum, and D. Nicotiana tabacum.*

A.

Rank	PDB Hit	TM Score
1	5zqtA	0.907
2	1dgkN	0.904
3	4qs7A	0.902
4	2nztA	0.864
5	3b8aX	0.861
6	3f9mA	0.858
7	1bdgA	0.854
8	3hm8A	0.847
9	1hkgA	0.835
10	3o08B	0.807

B.

Rank	PDB Hit	TM Score
1	5zqtA	0.910
2	1dgkN	0.908
3	4qs7A	0.902
4	2nztA	0.871
5	3b8aX	0.858
6	3f9mA	0.855
7	1bdgA	0.852
8	3hm8A	0.846
9	1hkgA	0.834
10	6r2nA	0.805

C.

Rank	PDB Hit	TM Score
1	5zqtA	0.905
2	4qs7A	0.899
3	1dgkN	0.897
4	2nztA	0.861
5	3b8aX	0.856
6	3f9mA	0.851
7	1bdgA	0.847
8	3hm8A	0.839
9	1hkgA	0.830
10	3o08B	0.802

D.

Rank	PDB Hit	TM Score
1	5zqtA	0.896
2	4qs7A	0.890
3	1dgkN	0.882
4	3b8aaX	0.864
5	3f9mA	0.863
6	2nztA	0.857
7	1bdgA	0.854
8	3hm8A	0.851
9	1hkgA	0.836
10	3o08B	0.821

4.1.8 Ramachandran Plot

The Ramachandran plot depicts the statistical distribution of the backbone dihedral angle combinations and the energetically permissible and forbidden dihedral angle areas. The PROCHECK examines the overall model geometry as well as the geometry of each individual residue to determine the stereochemical quality of a predicted model. The protein sequence was uploaded on the PROCHECK server and the Ramachandran plots as shown in Fig. 12 and 13 were obtained. The regions that are shown in red color i.e., A, B, and L contain the most favored residues. Residues in additional and generously allowed regions are also shown.

Table 16 and 17 displays the plot statistics for the structure of HXK1 and HXK2 respectively of various plant species. It shows the percentage of different amino acids in each location. It can be seen that the bulk of the amino acids are present in the most preferred regions and additional permitted regions for all four anticipated protein forms. This supports the 3-D protein structures' validity. A better understanding of the projected 3-D protein structures is made possible by these statistic data.

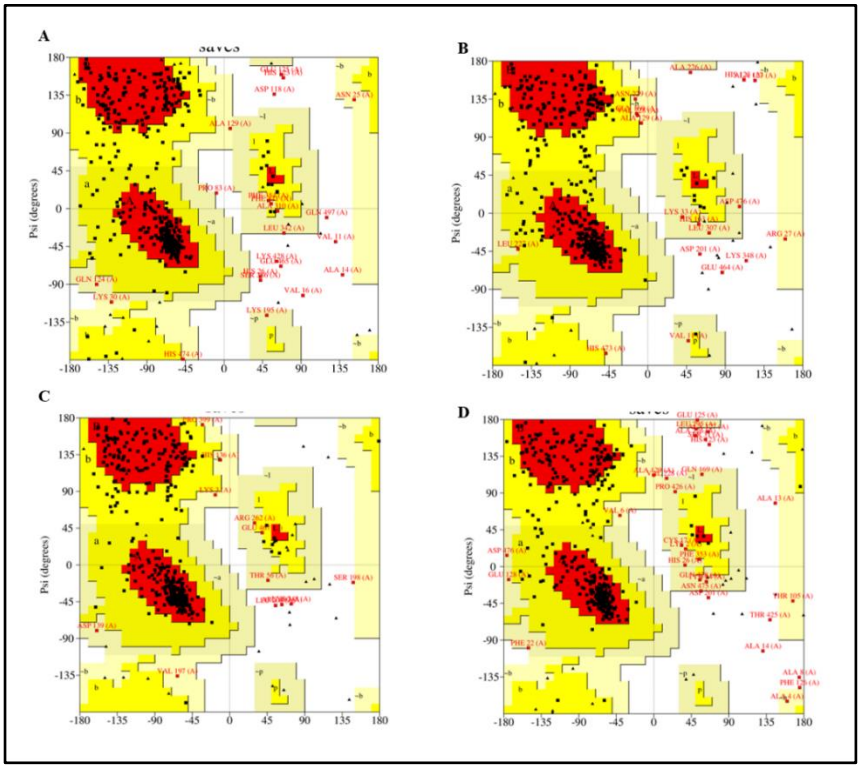

Fig. 12 Ramachandran plot of HXK-1 protein in different plant species of Solanaceae family. A. *Solanum tuberosum*, B. *Solanum lycopersicum*, C. *Capsicum annum*, D. *Nicotiana tabacum*

Table 16 Ramachandran plot statistics of HXK1 enzyme of 4 different plant species of *Solanaceae* family

Number of residues				
	Solanum tuberosum	***Solanum lycopersicum***	***Capsicum annum***	***Nicotiana tabacum***
In most favoured regions [A,B,L]	336 (76.5%)	340	335	335
		78.7%	83.1%	78.1%
In additional allowed regions [a,b,l,p]	82 (18.7%)	74	57	66
		17.1%	14.1%	15.4%
in generously allowed regions [~a,~b,~l,~p]	10	12	7	17
	2.3%	2.8%	1.7%	4.0%
In disallowed regions	11	6	4	11
	2.5%	1.4%	1.0%	2.6%
Non-glycine and non-proline residues	439	432	403	429
	100%	100%	100%	100%
End-residues (Excl. pro and glyc)	2	2	2	2
Glycine residues	43	48	46	48
Proline residues	14	16	17	18
Total Residues	498	498	468	497

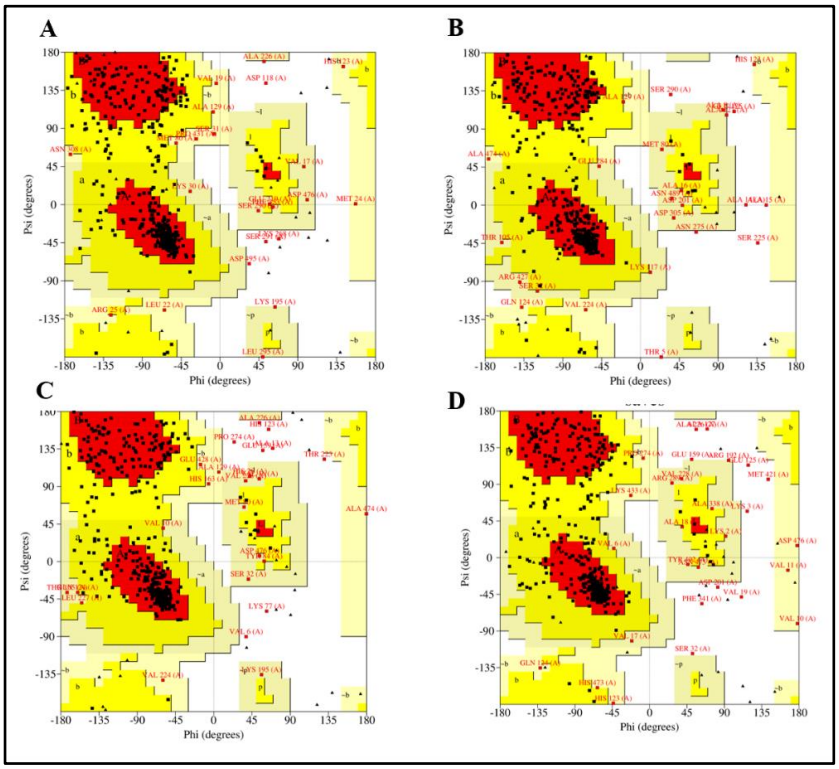

Fig. 13 Ramachandran plot of HXK-2 protein in different plant species of Solanaceae family. A. *Solanum tuberosum*, B. *Solanum lycopersicum*, C. *Capsicum annum*, D. *Nicotiana tabacum*

Table 17 Ramachandran plot statistics of HXK2 enzyme of 4 different plant species of *Solanaceae* family

	Number of residues			
	Solanum tuberosum	***Solanum lycopersicum***	***Capsicum annum***	***Nicotiana tabacum***
In most favoured regions [A,B,L]	323 (75.5%)	338 (78.6%)	338 (77.9%)	319 S(73.7%)
In additional allowed regions [a,b,l,p]	83 (19.4%)	68 (15.8%)	72 (16.6%)	87 (20.1%)
in generously allowed regions [~a,~b,~l,~p]	17 (4.0%)	17 (4.0%)	17 (3.9%)	17 (3.9%)
In disallowed regions	5 1.2%	7 (1.6%)	7 (1.0%)	10 (2.3%)
Non-glycine and non-proline residues	428 (100.0%)	430 (100.0%)	434 (100.0%)	433 (100.0%)
End-residues (Excl. pro and glyc)	2	2	2	2
Glycine residues	48	46	47	46
Proline residues	18	18	16	16
Total Residues	496	496	499	497

4.1.9 Study of Protein- Protein Interactions

Networks of protein-protein interactions are crucial for understanding biological activities at the system level. These networks offer a simple framework for annotating the structural, functional, and evolutionary characteristics of proteins as well as for filtering and evaluating functional genomics data. The STRING analysis of StHXK displayed functional association with other enzymes involved in carbohydrate metabolism. This implies the interdependency of other enzymes on the activity of HXK. It confirms the inevitable role of the enzyme in the starch accumulation during plant growth and development It's important to note that G6P inhibits HXK and if other carbohydrate pathways get downregulated then the HXK regulation is affected. On the inhibition of PFK, G6P gets accumulated to inhibit HXK. G6PDH gives negative feedback to the HXK enzyme.

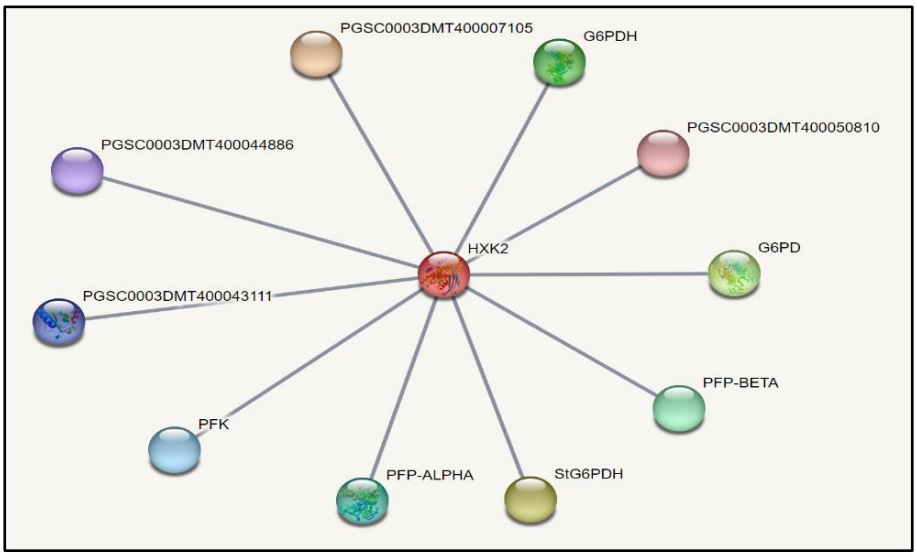

Fig. 14 String database of HXK1 with all the possible protein interactions between StHXK1 and various other proteins within Potato Genome Sequencing Consortium (PGSC) network: Glucose-6-phosphate dehydrogenase (PGSC0003DMT400007105, G6PDH, StG6PDH), PFK (PGSC0003DMT400050810, PGSC0003DMT400044886, PGSC0003DMT400043111, PFK, PFP-ALPHA, PFP-BETA)

4.2 Plant Materials

In the field of TIET, Patiala, numerous potato varieties were planted. Different organs, including leaves, stems and tubers, were collected and at first held at ambient temperatures as depicted in the Fig. 15 and 16. The plant materials relevant to each cultivar were kept in storage at various experimental temperatures.

Fig. 15 Harvested tuberising stolons

Fig. 16 Collecting the stored organs of potato plant

4.3 Enzyme Activity Estimation

4.3.1 Total Protein Estimation in Potato Cultivars

Total protein extracts were prepared by using the method mentioned earlier in materials and methods. BSA (Bovine Serum Albumin) was used to construct a standard curve of proteins at different concentrations by taking absorbance at 750 nm. Absorbance at various concentrations is tabulated below (Table 18 and Fig. 17)

Table 18 BSA at different concentrations and absorbance (750 nm)

Concentration (µg)	**A_{750} nm Value**
0	0
200	0.4
300	0.573
400	0.697
500	0.859
600	0.956
700	1.078
800	1.202
900	1.305

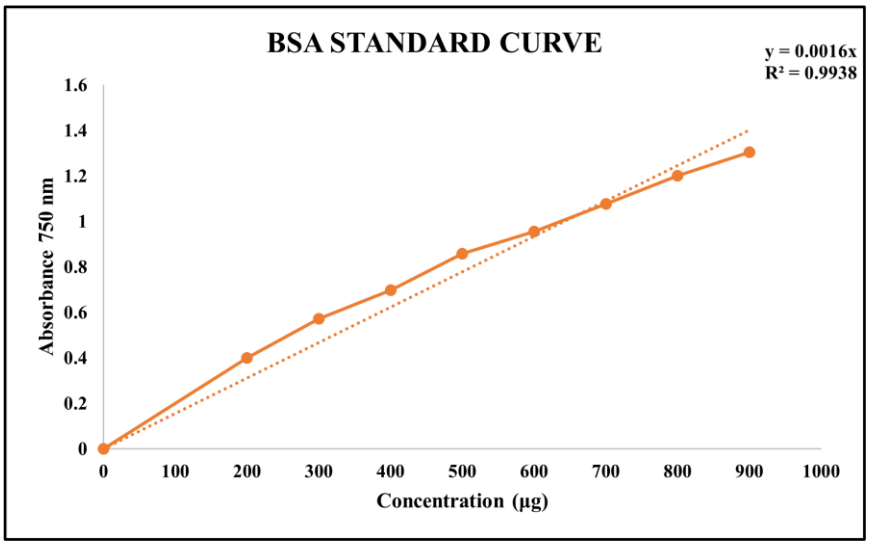

Fig. 17 Standard curve of BSA

4.3.2 HXK Activities in Different Potato Cultivars

The three organs of potato namely, leaves, stem and tubers were taken to estimate HXK activity using glucose as a substrate as mentioned in material and methods. Glucose converting to glucose-6-phosphate in presence of formation of NADH from NAD^+ was recorded for 5 min. Further specific activity was expressed in U/g of proteins.

The Fig. 18 represents the HXK activity in the different stages of tuber. The tuber size increases with the time due to the formation of starch and break down of sugars in presence of enzymes as discussed before. Here, the activity of HXK is observed to be maximum in the early stage of tuber development. Also the activity in the reference cv. (De) is seen to be higher when compared to the Indian cv. KC-1 and less when compared to cv. KC-2.

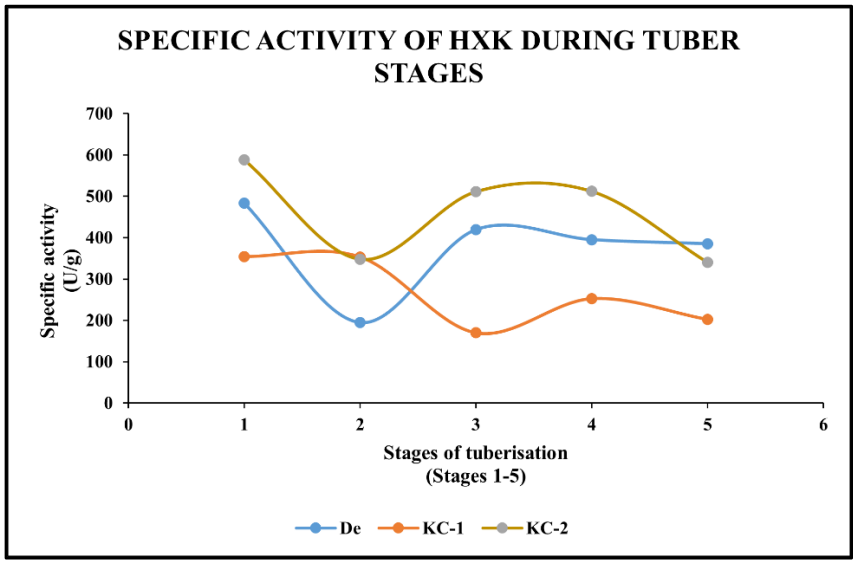

Fig. 18 HXK activity in all stages of tuber in cultivar De, KC-1 and KC-2

4.3.2a HXK Activity in Leaves: HXK activities were observed to be highest in young leaves in comparison to the mature leaves. Among all the cultivars, PR (3202.45 U/ mg protein), PR (1578.46 U/ mg protein) and KC-1 (1473.47) in young leaves showed the highest HXK activity in comparison to the other cultivar. From the data, it was observed that specific activity of HXK decreased approximately one and a half to two folds near maturation. This implies that HXK is directly responsible in the carbohydrate metabolism that further helps in plant growth and development by increased starch production. Due to decreased development at the leaf tips as the photosynthetic apparatus becomes functional, the requirement for carbohydrate metabolism and oxidative activity of enzymes declines (Croxdale and Vanderveer 1986).

Table 19 Comparison of HXK activity in the leaves of various potato cultivars at different growth stages of leaves (the experimental data is presented as mean of three independent leaf extracts)

Cultivars / **Stage**	**KC-1**	**KC-2**	**KJ**	**PR**	**De**
	HXK activity in leaves (U/g of protein)				
Young	1473.47	809.26	1578.46	3202.45	891.36
Mature	508.24	637.10	1264.40	2885.87	829.28

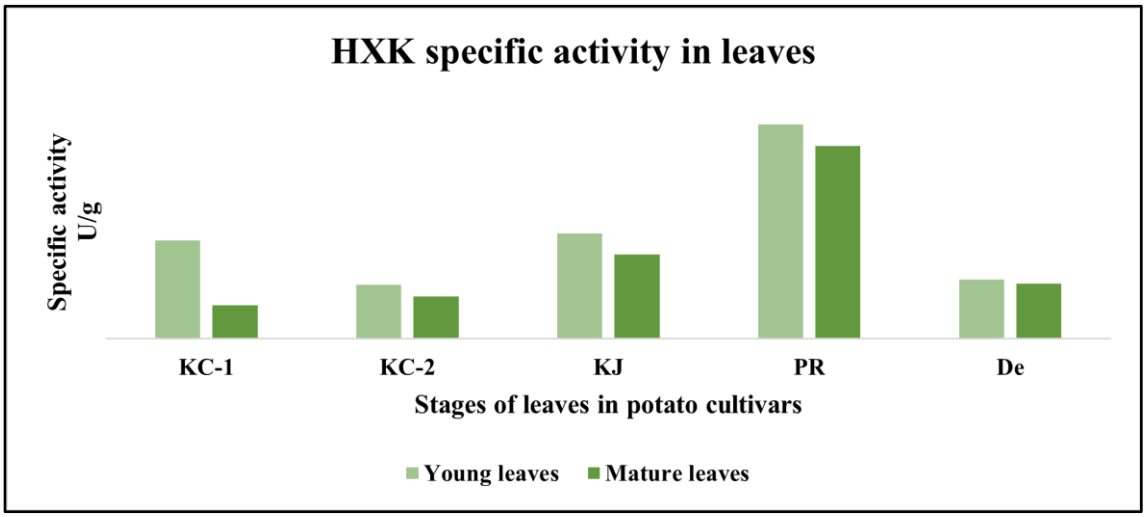

Fig. 19 Comparative specific activities of HXK in leaves of potato cultivars

4.3.2b HXK Activity in Stem: HXK activity was compared between young and matured stem as shown in Fig. 20. The activity was observed to be highest in the young stem in comparison to the mature stage. Among all the cultivars HXK activity was highest in KJ (3622.34 U/g) and De (758.99 U/ mg protein) KC-2 (226.28 U/g). It was lowest in KC-1 (16.02 U/ mg protein, PR

(79.11 U/ mg protein), KC-2 (159.01U/g) and De (275.99 U /mg protein) mature stems. From the Table 8 and Fig. 12, it was observed that specific activity of HXK decreased approximately two folds near maturation. This decline in activity could be due to the decreased energy needs of mature stem (Croxdale and Vanderveer 1986).

Table 20 Comparison of HXK activity in the stems of various potato cultivars at different growth stages of stem (the experimental data is presented as mean of three independent stem extracts)

Cultivars / **Stage**	**KC-1**	**KC-2**	**KJ**	**PR**	**De**
	HXK activity in stem (U/g of protein)				
Young	72.20	226.28	3622.34	212.70	758.99
Mature	16.02	159.01	2400.83	79.11	275.99

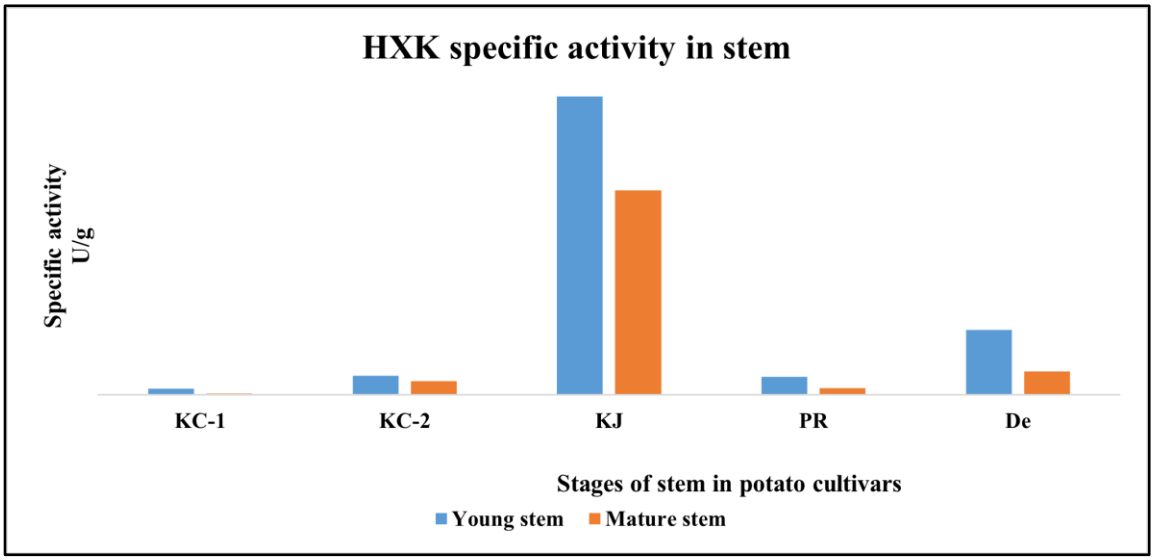

Fig. 20 Comparative specific activities of HXK in stem of potato cultivars

4.3.2c HXK Activity in Tubers: Enzyme activity was observed in all the stages of tuber development. As evident from literature, the subapical, mitotically active region of the uninduced tuberizing stolon was found to have high HXK activities (Appeldoorn et al. 2002). It was observed that specific activity of HXK in tubers showed significant yet variable activity at different stages of tuberization. It was highest in young tubers with highest activity in KJ at both stage 1 (ST-1: 911.93 U/g) of tuber and tuberising stolon (TS: 752.55 U/g) in comparison to the other cultivar. HXK activity showed a significant drop in the growing tubers from ST-2 to ST-4 and then slight increase till ST-5. The least HXK activity observed was in PR at ST-4 (79.07 U/g) and ST- 5 (129.5 U/g). Other activities observed variable at various points, KC-1

(170.27 U/g), PR (181.65 U/g), De (419.18 U/g), KC-2 (511.29 U/g) and KJ (949.78 U/g). However, mature tubers (ST-5) showed a slight increase in HXK activity in cultivars namely KC-1 (202.57 U/g), KC-2 (340 U/g), KJ (584.80 U/g), and De (419.18 U/g). (Fig. 21 and Table 21)

Table 21 Comparison of HXK activity in various potato cultivars at different growth stages of tuber (the experimental data is presented as mean of three independent tuber extracts)

Cultivars / **Stage**	**KC-1**	**KC-2**	**KJ**	**PR**	**De**
	HXK activity in tubers (U/g of protein)				
TS	410.40	599.99	752.55	370.35	202.57
ST-1	354.1	588.84	911.93	100.66	483.26
ST-2	352.04	347.82	534.63	330.05	194.13
ST-3	170.27	511.29	484.20	181.65	419.18
ST-4	252.61	512.04	567.23	79.07	394.99
ST-5	202.57	340.34	436.13	129.5	385.22

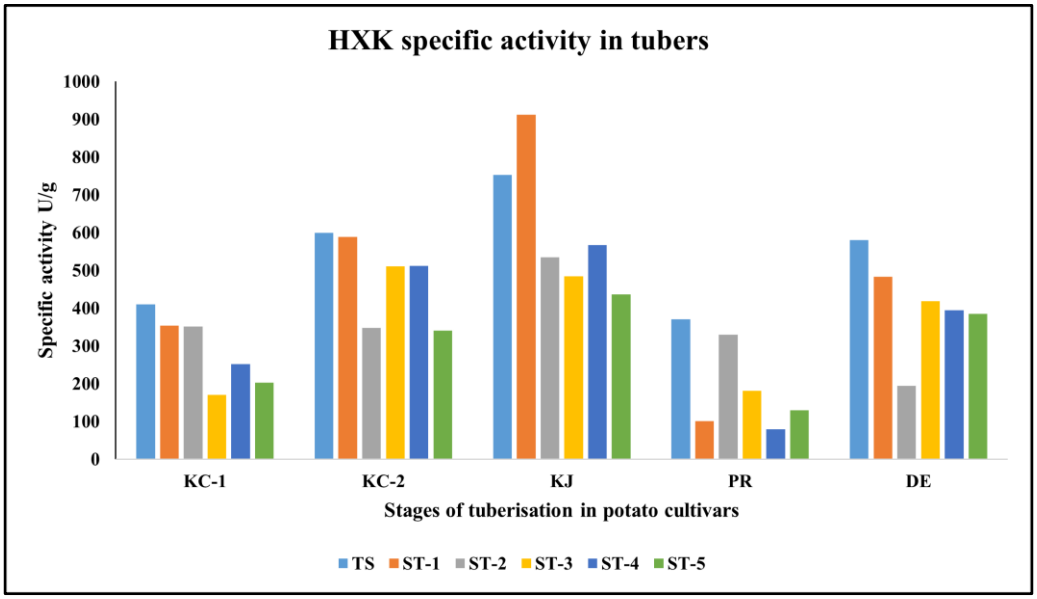

Fig. 21 Comparative specific activities of HXK in tubers of potato cultivars

Concluding Remarks

The salient aspects of the thesis work are briefly stated below:

- Potato is a significant non-grain food crop in developing countries. Tubers are rich source of carbohydrates.
- HXKs, member of phosphotransferase multigene family, was found to be maximum during the young and growing stages of various potato organs such as young tubers, leaves and stems indicating crucial role of this enzyme in the plant growth and development.
- Various bioinformatic tools were employed to analyse available multiple HXK isoforms at both gene and protein level.
- Genome wide study revealed a total of 6 *HXK* genes localized on six chromosomes encoding mostly stable isoforms of HXKs.
- Multiple sequence alignment revealed significant divergence at N-terminal region. However, crucial domains namely nucleotide binding domain, phosphate binding domain and sugar binding domain were found to be mostly conserved. Phylogenetic analysis was performed to reveal the relatedness of potato HXK members with other plant species.
- Protein-protein interactions showed association of HXK with carbohydrate metabolism enzymes namely glucose-6-phosphate dehydrogenase, phosphofructokinase and PFP indicating their role during starch accumulation.
- This data could be useful to raise transgenics with upregulated activity of HXK in the Indian cultivars of potato to satisfy the future global nutritional demands using tools like ZNFs, TALENs and CRISPR by making site specific breaks in DNA to improve crop quality and yield.
- The expression analysis and biochemical assays confirmed the regulatory and catalytic role of HXKs enzymes in the early stages of tubers.

Future Perspectives

- After reviewing the biochemical properties of HXK in Indian cultivars of potato, the significant kinetic and catalytic activities were explored.
- Comparative study of pH profiling and storability of crude extract from De and KC-1 were analysed.
- Denaturing SDS-PAGE revealed the two bands around 51-54 kDa in the crude extract from the cv. De and KC-1 (data not shown here).
- All the potato varieties available in the country are not suitable for processing. For making superior quality processed products, the potatoes must possess certain minimum morphological and biochemical quality character.
- Further these studies could be used to raise Indian cultivars transgenics in terms of starch production and tuber enhancement using various gene editing tools such as CRISPR or enhancing environmental factors which affect the HXK activity.
- Also, the study done so far can be used to further analyse the glucose sensing role of HXK in potato cultivars, which is a crucial concern in the field of agriculture.

References

Abelenda JA, Navarro C, Prat S (2011) From the model to the crop: genes controlling tuber formation in potato. Current Opinion in Biotechnology, 22:287–292

Acevedo-Siaca LG, Głowacka K, Driever SM, Salesse-Smith CE, Lugassi N, Granot D, Long SP, Kromdijk J (2022) Guard-cell-targeted overexpression of Arabidopsis Hexokinase 1 can improve water use efficiency in field-grown tobacco plants. Journal of Experimental Botany, 73:5745–5757

Appeldoorn NJ, de Bruijn SM, Koot-Gronsveld EA, Visser RG, Vreugdenhil D, van der Plas LH (1997) Developmental changes of enzymes involved in conversion of sucrose to hexose-phosphate during early tuberisation of potato. Planta, 202:220–226

Appeldoorn NJ, Sergeeva L, Vreugdenhil D, Van Der Plas LH, Visser RG (2002) In situ analysis of enzymes involved in sucrose to hexose-phosphate conversion during stolon-to-tuber transition of potato. Physiologia plantarum, 115:303–310

Appeldoorn NJG, De Bruijn SM, Koot-Gronsveld EA, Visser RGF, Vreugdenhil D, Plas LVD (1999) Developmental changes in enzymes involved in the conversion of hexose phosphate and its subsequent metabolites during early tuberization of potato. Plant, Cell & Environment, 22:1085–1096

Atanasov V, Fürtauer L, Nägele T (2020) Indications for a central role of hexokinase activity in natural variation of heat acclimation in *Arabidopsis thaliana*. Plants, 9: 819

Baldus B, Kelly GJ, Latzko E (1981) Hexokinases of spinach leaves. Phytochemistry 20:1811–1814

Beals KA (2019) Potatoes, Nutrition and Health. Am J Potato Res 96:102–110

Beukema HP, Van Der Zaag (1979) Potato improvements. Some factors and facts. In: International Agricultural centre, Wageningen pp 56

Bisswanger H (2004) Enzyme reactions. In: Aehle W (ed) Wiley VCH, Weinheim, Practical enzymology, pp 7-163

Brown CR, Culley D, Yang CP, Durst R, Wrolstad R (2005) Variation of anthocyanin and carotenoid contents and associated antioxidant values in potato breeding lines. J Am Soc Hortic Sci 130:174– 180

Camacho-Pereira J, Meyer LE, Machado LB, Oliveira MF, Galina A (2009) Reactive oxygen species production by potato tuber mitochondria is modulated by mitochondrially bound hexokinase activity. Plant Physiol, 149:1099-1110

Chen H, Rosin FM, Prat S, Hannapel DJ (2003) Interacting transcription factors from the TALE superclass regulate tuber formation. Plant Physiol 132:1391–1404

Cho YH, Sheen J, Yoo SD (2010) Low glucose uncouples hexokinase1-dependent sugar signaling from stress and defense hormone abscisic acid and C2H4 responses in *Arabidopsis*. Plant Physiol. 152:1180–1182

Cho YH, Yoo SD, Sheen J (2006) Regulatory functions of nuclear hexokinase1 complex in glucose signaling. Cell, 127:579-589

Cho YH, Yoo SD, Sheen J (2006b) Regulatory functions of nuclear hexokinase1 complex in glucose signaling. Cell, 127:579–589

Claeyssen É, Dorion S, Clendenning A, He JZ, Wally O, Chen J, Auslender EL, Moisan MC, Jolicoeur M, Rivoal J (2013) The futile cycling of hexose phosphates could account for the fact that hexokinase exerts a high control on glucose phosphorylation but not on glycolytic rate in transgenic potato (*Solanum tuberosum*) roots. PLoS One, 8:e53898

Claeyssen E, Rivoal J (2007) Isozymes of plant hexokinase: occurrence, properties and functions. Phytochemistry, 68:709–731

Copeland L, Morrell M (1985) Hexose kinases from the plant cytosolic fraction of soybean nodules. Plant Physiol 79:114–11

Copeland L, Tanner GJ (1988) Hexose kinases of avocado. Physiol Plant 74:531–536

Croxdale JG, Vanderveer PJ (1986) Quantitative measurements of hexokinase activity in the shoot apical meristem, leaf primordia, and leaf tissues of Dianthus chinensis L. Plant physiology, 81:186-191.

Cutter EG (1978) Structure and development of the potato plant. In: Harris PM (ed) The Potato Crop. Springer, Boston, pp 70-152

Dai N, Schaffer A, Petreikov M, Shahak Y, Giller Y, Ratner K, Levine A et al. (1999) Overexpression of Arabidopsis hexokinase in tomato plants inhibits growth, reduces photosynthesis and induces rapid senescence. Plant Cell, 11:1253–1266

Damari-Weissler H, Ginzburg A, Gidoni D, Mett A, Krassovskaya I, Weber AP, Granot D et al. (2007) Spinach SoHXK1 is a mitochondria-associated hexokinase. Planta, 226: 1053-1058

Damari-Weissler H, Kandel-Kfir M, Gidoni D, Mett A, Belausov E, Granot D (2006) Evidence for intracellular spatial separation of hexokinases and fructokinases in tomato plants. Planta 224:1495–1502

Dutt S, Manjul AS, Raigond P, Singh B, Siddappa S, Bhardwaj V, & Kardile HB (2017) Key players associated with tuberization in potato: potential candidates for genetic engineering. Critical reviews in biotechnology, 37:942–957

Dutt S, Manjul AS, Raigond P, Singh B, Siddappa S, Bhardwaj V, Kawar PG, Patil VU, Kardile B (2017) Key players associated with tuberization in potato: potential candidates for genetic engineering. Crit Rev Biotechnol 37:942–957

Ewing EE, Struik PC (1992) Tuber formation in potato: induction, initiation, and growth. Am Soc Hortic Sci 14:89–198

Galina A, Reis M, Albuquerque MC, Puyou AG, Puyou MTG, Demeis L (1995) Different properties of the mitochondrial and cytosolic hexokinases in maize roots. Biochem J 309:105–112

Geigenberger P, Stitt M (1993) Sucrose synthase catalyses a readily reversible reaction in vivo in developing potato tubers and other plant tissues. Planta, 189:329-339

Geigenberger P, Stitt M, & Fernie AR (2004) Metabolic control analysis and regulation of the conversion of sucrose to starch in growing potato tubers. Plant, Cell & Environment, 27:655-673

Giese JO, Herbers K, Hoffmann M, Klosgen RB, Sonnewald U (2005) Isolation and functional characterization of a novel plastidic hexokinase from Nicotiana tabacum. FEBS Lett, 579:827–831

Granot D, David-Schwartz R, Kelly G (2013) Hexose kinases and their role in sugar-sensing and plant development. Frontiers in Plant Sci, 4:44

Gregory LE (1965) Physiology of tuberization in plants. In: Ruhland W (ed) Encyclopedia of Plant Physiology vol 15. Springer, Berlin, pp 1328-1354

Harrington GN, & Bush DR (2003) The bifunctional role of hexokinase in metabolism and glucose signaling. The Plant Cell, 15:2493–2496

Jammer A, Gasperl A, Luschin-Ebengreuth N, Heyneke E, Chu H, Cantero-Navarro E, Großkinsky DK, Albacete AA, Stabentheiner E, Franzaring J, Fangmeier A & Roitsch T (2015) Simple and robust determination of the activity signature of key carbohydrate metabolism enzymes for physiological phenotyping in model and crop plants. Journal of Experimental Botany, 66:5531–5542

Jang JC, León P, Zhou L, Sheen J 1997. Hexokinase as a sugar sensor in higher plants. Plant Cell 9:5–19

Jang JC, Sheen J (1997) Sugar sensing in plants. Trends Plant Sci, 2:208–214

Kang SG, Hannapel DJ (1996) A novel MADS-box gene of potato (*Solanum tuberosum* L.) expressed during the early stages of tuberization. Plant Mol Biol 31:379–386

Karve A, Rauh BL, Xia X, Kandasamy M, Meagher RB, Sheen J et al. (2008) Expression and evolutionary features of the hexokinase gene family in Arabidopsis. Planta, 228:411–425

Karve R, Lauria M, Virnig A, Xia X, Rauh BL, Moore B (2010) Evolutionary lineages and functional diversification of plant hexokinases. Mol Plant 3:334-346

Kim M, Lim JH, Ahn CS, Park K, Kim GT, Kim WT et al. (2006) Mitochondria-associated hexokinases play a role in the control of programmed cell death in Nicotiana benthamiana. Plant Cell, 18:2341–2355

Kim YM, Heinzel N, Giese JO, Koeber J, Melzer M, Rutten T et al. (2013) A dual role of tobacco hexokinase 1 in primary metabolism and sugar sensing. Plant, Cell & Environment, *36*:1311-1327

Kloosterman B, Navarro C, Bijsterbosch G, Lange T, Prat S, Visser RGF, Bachem CWB (2007) StGA2ox1 is induced prior to stolon swelling and controls GA levels during potato tuber development. The Plant Journal, 52:362–373

Koda Y (1997) Possible involvement of jasmonates in various morphogenetic events. Physiol Plant, 100:639–646

Krauss A (1985) Interaction of nitrogen nutrition, phytohormones, and tuberization. Potato physiology, 209-230

Lehretz GG, Sonnewald S, Lugassi N, Granot D, Sonnewald U (2021) Future-proofing potato for drought and heat tolerance by overexpression of hexokinase and SP6A. Frontiers in Plant Science, 11:614534

Li J, Chen G, Zhang J, Shen H, Kang J, Feng P et al. (2020) Suppression of a hexokinase gene, SlHXK1, leads to accelerated leaf senescence and stunted plant growth in tomato. Plant Science, 298:110544

Liu RH (2013) Health-promoting components of fruits and vegetables in the diet. Adv Nutr 4:384–392

Martinez-Barajas E, Randall DD (1998) Purification and characterization of a glucokinase from young tomato (*Lycopersicum esculentum* L. Mill.) fruit. Planta 205:567–573

McGill CR, Kurilich AC, Davignon J (2013) The role of potatoes and potato components in cardiometabolic health: a review. Ann Med 45:467–473

Miernyk JA, Dennis DT (1983) Mitochondrial, plastid, and cytosolic isozymes of hexokinase from developing endosperm of *Ricinus communis*. Arch Biochem Biophys 226:458–468

Moisan MC, Rivoal J (2011) Purification to homogeneity and characterization of nonproteolyzed potato (*Solanum tuberosum)* tuber hexokinase 1. Botany, 89:289–299

Moore B, Zhou L, Rolland F, Hall Q, Cheng WH, Liu YX et al. (2003) Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science, 300:332-336

Muñiz García MN, Stritzler M, Capiati DA (2014). Heterologous expression of Arabidopsis ABF4 gene in potato enhances tuberization through ABA-GA crosstalk regulation. Planta, 239:615-631

Navarro C, Abelenda JA, Cruz-Oró E, Cuéllar CA, Tamaki S, Silva J et al. (2011) Control of flowering and storage organ formation in potato by FLOWERING LOCUS T. *Nature*, *478*:119-122

Neuhaus HE, Emes MJ (2000) Nonphotosynthetic metabolism in plastids. Annual review of plant biology, 51:111-140

Petreikov M, Dai N, Granot D, Schaffer AA (2001) Characterization of native and yeast-expressed tomato fruit fructokinase enzymes. Phytochemistry 58:841–847

Renz A, Stitt M (1993) Substrate specificity and product inhibition of different forms of fructokinases and hexokinases in developing potato tubers. Planta, 190:166-175

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu Rev Plant Biol, 57:675–709

Sarkar D (2008) The signal transduction pathways controlling in planta tuberization in potato: An emerging synthesis. Plant Cell Reports, 27:1–8

Schnarrenberger C (1990) Characterization and compartmentation in green leaves of hexokinases with different specificities for glucose, fructose and mannose and for nucleoside triphosphates. Planta 181:249–255

Sergeeva LI, Vreugdenhil D (2002) In situ staining of activities of enzymes involved in carbohydrate metabolism in plant tissues. Journal of Experimental Botany, 53:361-370

Sheen J (1990) Metabolic repression of transcription in higher plants. Plant Cell 2:1027–1038

Sheen J, Zhou L, Jang JC (1999) Sugars as signalling molecules. Curr Opin Plant Biol, 2:410–418

Sindelar L, Sindelarova M, Burketova L (1998) Hexokinases of tobacco leaves: influence of plant age on particulate and soluble isozyme composition. Biol Plant 40:469–474

Singh BP, Rana RK (2014) History of potato and its emerging problems in India. In: Singh BP, Rana RK, Dalamu, Kumar R, Singh DK (eds) Souvenir–National Seminar on Emerging Problems in Potato, Indian Potato Association, Shimla, India, pp 7-21

Singh P, Arif Y, Siddiqui H, Upadhyaya CP, Pichtel J, Hayat S (2023) Critical Factors Responsible for Potato Tuberization. The Botanical Review, 1-17

Spooner DM, Ghislain M, Simon R, Jansky SH, Gavrilenko T (2014) Systematics, diversity, genetics, and evolution of wild and cultivated potatoes. The botanical review, 80:283-383

Spooner DM, McLean K, Ramsay G, Waugh R, Bryan GJ (2005) A single domestication for potato based on multilocus amplified fragment length polymorphism genotyping. Proceedings of the National Academy of Sciences, 102:14694–14699

Timlin D, Lutfor Rahman SM, Baker J et al. (2006) Whole plant photosynthesis, development, and carbon partitioning in potato as a function of temperature. Agronomy Journal 98:1195–1203

Turner JF, Chensee QJ, Harrison DD (1977) Glucokinase of pea seeds. Biochim Biophys Acta 480:367–375

Turner JF, Copeland L (1981) Hexokinase II of pea seeds. Plant Physiol 68:1123–1127

Turner JF, Turner DH (1980) The regulation of glycolysis and the pentose phosphate pathway. In: Davies DD (ed) The biochemistry of plants, vol 2. Academic Press, London, New York. pp 279–316

Veramendi J, Fernie AR, Leisse A, Willmitzer L et al. (2002) Potato hexokinase 2 complements transgenic Arabidopsis plants deficient in *hexokinase 1* but does not play a key role in tuber carbohydrate metabolism. *Plant Molecular Biology*, *49*:491-501

Veramendi J, Roessner U, Renz A, Willmitzer L, & Trethewey RN (1999) Antisense repression of hexokinase 1 leads to an overaccumulation of starch in leaves of transgenic potato plants but not to significant changes in tuber carbohydrate metabolism. Plant Physiol, 121:123–134

Visser RG, Vreugdenhil D, Hendriks T, Jacobsen E (1994) Gene expression and carbohydrate content during stolon to tuber transition in potatoes (*Solanum tuberosum*). Physiologia plantarum, 90:285-292

Wang F, Sanz A, Brenner ML, Smith A (1993) Sucrose synthase, starch accumulation, and tomato fruit sink strength. Plant Physiology, 101:321-327

Weeda SM, Mohan Kumar GN, Richard Knowles N (2009) Developmentally linked changes in proteases and protease inhibitors suggest a role for potato multicystatin in regulating protein content of potato tubers. Planta, 230:73-84

Yadav SK Srivastava AK (2015) International Journal of Applied and Pure Science and Agriculture, 1:26-34

Yu JW, Choi JS, Upadhyaya CP, Kwon SO, Gururani MA, Nookaraju A et al. (2012) Dynamic proteomic profile of potato tuber during its in vitro development. Plant science, 195:1-9

Document Information

Analyzed document	MSc Thesis_Plagiarism_Chestha Mittal.docx (D172109935)
Submitted	7/15/2023 1:38:00 PM
Submitted by	Niranjan Das
Submitter email	ndas@thapar.edu
Similarity	9%
Analysis address	ndas.thapar@analysis.urkund.com

Sources included in the report

SA	**Thapar Institute Of Engineering And Technology / Plagiary Checking_Simran Shrimal.docx** Document Plagiary Checking_Simran Shrimal.docx (D111873774) Submitted by: ndas@thapar.edu Receiver: ndas.thapar@analysis.urkund.com	1
SA	**Thapar Institute Of Engineering And Technology / MSc Dissertation_Ravneet Kaur_Jun 2022.docx** Document MSc Dissertation_Ravneet Kaur_Jun 2022.docx (D141431912) Submitted by: ndas@thapar.edu Receiver: ndas.thapar@analysis.urkund.com	24
SA	**Thapar Institute Of Engineering And Technology / Plagiary Checking_Nidhi.docx** Document Plagiary Checking_Nidhi.docx (D111871943) Submitted by: ndas@thapar.edu Receiver: ndas.thapar@analysis.urkund.com	1
W	URL: https://academic.oup.com/pcp/article/58/7/1151/3753635 Fetched: 6/20/2021 9:21:33 AM	2
W	URL: https://www.frontiersin.org/articles/10.3389/fpls.2020.614534/full Fetched: 1/4/2022 7:27:08 AM	1
SA	**s41598-020-68407-2.pdf** Document s41598-020-68407-2.pdf (D79477788)	1

Entire Document

15/7/23

N. Das
15/07/2023
Supervisor