Fructokinases (FRKs) of potato (*Solanum tuberosum* L.): Sequence analyses of FRK cDNAs, and studying amplicon profiles in the cultivars using FRK-specific oligonucleotide primers

A

Dissertation

Submitted in the partial fulfillment of the requirement for the award of degree of

Master of Technology
In
Biotechnology

Submitted by:
Harleen Kaur
(Regd. No. 601304007)

Under the guidance of
Dr. N. Das
Professor

Department of Biotechnology
Thapar University, Patiala

July, 2015

CANDIDATE'S DECLARATION

I, hereby declare that the work which is being presented in the thesis entitled, **"Fructokinases (FRKs) of potato (*Solanum tuberosum* L.): Sequence analyses of FRK cDNAs and studying amplicon profiles in the cultivars using FRK-specific oligonucleotide primers"** in the partial fulfillment of the requirement for the award of degree of Master of Technology in Biotechnology, Thapar University, Patiala, is an authentic record of my own research work carried out under the guidance and supervision of **Dr. N. 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.

Date: July 15, 2015

Place: Patiala

Harleen Kaur

(Harleen Kaur)

CERTIFICATE

This is to certify that the dissertation entitled **"Fructokinases (FRKs) of potato (*Solanum tuberosum* L.): Sequence analyses of FRK cDNAs and studying amplicon profiles in the cultivars using FRK-specific oligonucleotide primers"** submitted by **Harleen Kaur** (Regd. No. 601304007) in partial fulfillment of the requirement for the award of the degree of Master of Technology in Biotechnology, to Thapar University is a record of student's own work carried out by her under our guidance and supervision. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

15/07/2015

(Dr. N. Das)
Supervisor
Professor
Department of Biotechnology
Thapar University
Patiala

(Dr. Dinesh Goyal)
Professor and Head
Department of Biotechnology
Thapar University
Patiala

(Dr. S S Bhatia)
Dean of Academic Affairs
Thapar University
Patiala

ACKNOWLEDGEMENT

This is a golden opportunity for me to convey my sincere regards for all those people who enabled me to accomplish my dissertation work successfully.

My utmost gratitude goes to my esteemed advisor **Dr. Niranjan Das**, Professor Department of Biotechnology Thapar University, Patiala for his expertise guidance, kindness, motivation, patience and providing me the opportunity to complete my dissertation work. It is his confidence-imbibing attitude, splendid discussions and endless endeavors through which I have gained a lot and learned a lot building up my future and personality.

A special thanks to all faculty members for their constant encouragement and support throughout the project work.

I am thankful for the help rendered by both PhD scholars throughout my project period without which it would have been difficult for me to achieve my goals. My special thanks to Mrs. Gurpreet Kaur and Mr. Rajneesh Verma. .

I feel lacunae of words to express my most heartfelt and cordial thanks to all my friends especially to Bineypreet Kaur, Akanksha Sharma, Darshleen Kaur, Nitin Kumar and Ritika Ranjan who always stood by my side during all the tough times.

The whole credit of my achievements during the project work goes to my parents and my guide. It was their unshakeable faith in me that has always helped me to proceed further.

Date:

Place: (Harleen Kaur)

LIST OF ABBREVIATIONS

Bp	Base pairs
HXK	Hexokinase
FRK	Fructokinase
UDP	Uridine diphosphate
ADP	Adenosine diphosphate
INV	Invertase
kDa	Kilo Dalton
SUS	Sucrose Synthase
Nts	Nucleotides
aa	Amino acids
NCBI	National centre for Biotechnology Information
UTR	Untranslated region
kb	Kilo base pairs
cDNA	Complementary DNA
dNTP	2′-deoxynucleoside-5′-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
TE	Tris EDTA

ABSTRACT

Hexose kinases are the enzymes which catalyze the phosphorylation of hexose sugars. Fructokinase (FRK) belongs to this enzyme family; it phosphorylate fructose (substrate) to fructose-6-phosphate which then further acts as one of the precursor for starch biosynthesis in plants. FRK is dimeric in nature, and molecular weight is in the range of 75-100 kDa with high specificity only towards fructose (K_m value 0.02-0.1 mM). Different isoforms of FRKs have been identified and their cDNA studies have been done in different plants like tomato, peas, barley and potato. Review of literature revealed that in the S*olanaceae* family, no considerable progress has been made on gene structure and function of FRK till date. The expression patterns of FRK gene(s) during different stages of tuberization are yet to be explored. With regard to the Indian potato cultivars there is no report on FRK is available. In the first instance, the available FRK cDNAs were used for sequence comparison at both nucleotide and amino acid levels. Based on sequence comparison, some salient sequence features were presented in this report including hydropathy characters of FRK. Isolation of good quality genomic DNA was carried out from different potato cultivars, and the quality and quantity were checked by spectrophotometric analyses. A number of oligonucleotide primers were made based on the available FRK cDNA sequences in the database. Polymerase chain reactions (PCR) were carried out using specific primer pairs and different potato genomic DNAs template. Amplicon profiles were noted and analyzed for each PCR. Apart from the expected ones, some cultivar-specific amplicons as found in the study need to be studied in detail at molecular level. This report would be quite useful with regard to understanding structure and function of FRK genes in the *Solanaceae* family, particularly in potato.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

1.1 About potato (*Solanum tuberosum* L.)

Potato (*Solanum tuberosum* L.) is one of the most important food crops after wheat, maize and rice, contributing to food and nutritional security in the world. It is a non-grain food crop belonging to family *Solanaceae*. The word potato may refer to the plant itself as well as the edible tuber. Around 8000 years ago, potato originated in the highlands of the Peruvian Andes-mountains in South America on the border between Bolivia and Peru. In 16th century, through Spanish conquerors potato was introduced to Europe where it developed as temperate crop and with colonial expansion of European countries it got distributed throughout the world. Either through British missionaries or Portuguese traders, potato first came to India in 17th century. Soon it became an important staple food and field crop and became the third largest food crop after rice and wheat.

Potato crop occupied an area of 1.2 million hectare in our country with a total production of 23.5 million tonnes. It is a perishable commodity and its harvest time (March-April) coincides with the rise in temperature in the Indian plains. Potato is the only major tuber crop that is grown in temperate regions. Now when the country's population has crossed one billion marks, there is an urgent need to redesign the potato crop for quality and quantity improvement. Potato is an annual, herbaceous, dicotyledonous and vegetatively propagated plant. However, in some envronments it can grow as perennial and can be propagated through seeds, which are called true potato seeds (TPS). It is a nightshade plant and is susceptible to frost and freezing. Potato plant produces a fibrous root system arising from the underground portion of stem. It has dark green, broad and compound leaves with oval leaflets. They bear white, pink, blue, or purple flowers with yellow stamens. It could have three kinds of stems including sprouts (leafy stems), stolons and tubers (Beukema and Zaag 1979; Struik and Wiersema 1999). Tuber is swollen underground stem used in commercial propagation. The tubers bear lateral buds (eyes) which grow into a new plant under favorable conditions. Potato tubers are sink organs where starch is synthesized from the sucrose (transported from the source leaves).

Solanum tuberosum is a hybrid between the diploid species *Solanum stentotomum* and the diploid weed *Solanum sparsipilum* with subsequent chromosome doubling. It has a series of ploidy levels, based on a haploid number of 12, ranging from diploid (2*n*=2*x*=24) to hexaploid (2*n*=6*x*=72), and including triploids, tetraploids and pentaploids (Dodds 1962). The cultivated potatoes are autotetraploid (2*n*=4*x*=48); many

wild species are diploid, but may range up to hexaploid. The tetraploid cultivated potatoes are not diploid, so that there are four interchangeable genes at each locus. Hence, the plant is heterozygous and shows polyploidy.

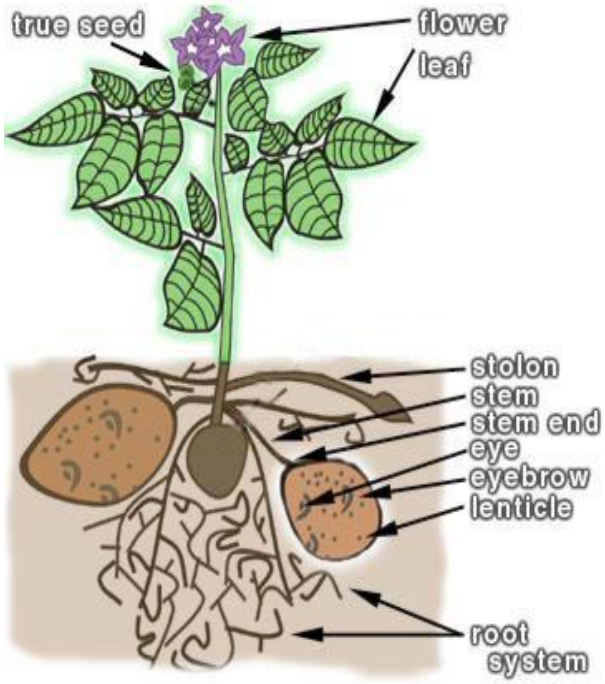


Fig. 1 Schematic view of a potato plant

For different agro-climatic regions of the country with 28 varieties alone for north Indian plains, 47 potato varieties have been bred so far. Varieties have also been developed for north Indian hills and other special problem areas viz. Sikkim, north Bengal hills and south Indian hills. 19 varieties out of the 47 developed varieties possess multiple resistances to different biotic and abiotic stresses. Out of these only nine varieties are suitable for processing and commercial purposes, which are: Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chipsona-3, Kufri Himsona, Kufri Frysona, Kufri Jyoti, Kufri Chandramukhi, Kufri Lauvkar and Kufri Surya (http://cpri.ernet.in/).

Currently, understanding various signals, signal transduction pathways, and particularly sugar-based signaling in potato and other plants are important research areas. All over the world, efforts are being made to produce disease-free and high-yielding potato varieties which can withstand a host of various biotic and abiotic stresses.

1.2 Biosynthesis of starch and sucrose in plants

In plants, sucrose and starch are the major photo assimilates produced during photosynthesis. Photosynthesis provides energy and carbon to both photosynthetic and non-photosynthetic tissues for their growth and development. Therefore, translocation and partitioning of the photo assimilates are basic processes in plants where sucrose plays a very pivotal role. The sugar formation, its translocation, assimilation and storage are controlled by the various factors coupled with number of genes. Sugar form, its transport and quantity in particular tissue or plant organ effects various metabolic and growth activities of plant. This process is highly co-ordinated, and controlled by various mechanisms as underlined below.

During active photosynthesis in bright light a plant leaf produces more carbohydrates (as triose-phosphate such as 3-phosphoglycerate, glyceraldyhyde-3-phosphate) than it needs for generating energy or synthesis precursors. In photo-synthesis, atmospheric carbon dioxide (CO_2) is incorporated into organic molecules via the Calvin cycle in the chloroplast to yield triose-phosphates (Triose-P) and these phosphates may be stored temporarily in the chloroplast as starch (transient starch) or converted to sucrose and exported to non photosynthetic or other parts of the plant. The balance between two processes is tightly regulated, and both must be coordinated with rate of carbon fixation. Transporter sucrose could be used as fuel or stored in other parts of plant. In most of the plants, starch is the main form of sugar storage. The synthesis of sucrose and starch occurs in different cellular compartments (cytosol and plastids, respectively), and the process involves a number of enzymes and are coordinated by a variety of regulatory mechanism that respond to changes in light level and photosynthetic rate.

In the cytoplasm, triose phosphates combine and form fructose 1,6-biphosphate (F1,6BP) from where F1,6BP can yield fructose 6-phosphate (F6P), glucose 6-phosphate (G6P), glucose 1-phosphate (G1P), and the nucleotide sugars UDP-glucose (UDP-G), and ADP-glucose (ADP-G). ADP-glucose serves as precursor of starch biosynthesis and UDP-glucose as a precursor of sucrose biosynthesis (Dennis and Blakeley 2000). The partitioning of triose phosphate between sucrose synthesis and starch synthesis is regulated by the allosteric effects of the enzymes that determine the levels of fructose-6-phosphate. For the use of glucose in metabolic processes it must be phosphorylated to produce G6P. G6P, then can be isomerized into F6P to initiate glycolysis, respiration, and other catabolic and anabolic processes. Alternatively, it may be mutated to G1P and the phosphate (P) group may be replaced by nucleotide diphosphates, such as UDP and ADP, to form the nucleotide sugars UDP-G and ADP-G, which are substrates for many biosynthetic and glycosylation reactions (Bar-Peled and O'Neill 2011). UDP-G may

combine with F6P to form sucrose-phosphate (Suc-P), which is dephosphorylated to produce sucrose. Hence, number of enzymes is required for the sugar metabolism in plants.

Enzymes involved in starch and sucrose metabolism: A number of enzymes and their various forms are involved in starch and sucrose metabolism, and in their inter conversions as well. The various enzymes required for starch biosynthesis are ADP-glucose pyrophosphorylase, starch synthases (SS), starch-branching enzymes (SBEs), and starch de-branching enzymes (DBEs). Starch synthase has several isoforms in the plastid: one isoform, granule-bound starch synthase I [GBSS, starch granule-bound ADP (UDP) glucose: α-1,4-D-glucan 4-α-glucosyl-transferase, EC 2.4.1.21] . It is a protein of nearly 60 kDa, bound exclusively to the starch granule. It is responsible for the biosynthesis of major amylose starch fraction in higher plants, and also contributes to the elongation of amylopectin chains. Second isoform of starch synthase is Starch synthases (SS); its distribution in granular and stromal fractions can vary between species, tissue, and developmental stages. Sucrose-biosynthesis-related proteins (SBRPs) include sucrose-phosphate synthase (SPS), sucrose synthase (SuSy) and sucrose-phosphate phosphatase (SPP).

The primary end product of photosynthesis is sucrose which is either metabolized in photosynthetic tissues, or exported out of the photosynthetic (source) tissues to non-photosynthetic (sink) tissues, where it acts as an initial substrate for all organic metabolic pathways. This sucrose must be cleaved by invertase (INV) or sucrose synthase (SUS), the only two families of sucrose-cleaving enzyme that have been identified in plants till now, so that it can be metabolized in sink or photosynthetic source tissues (Dennis and Blakeley 2000). The monomer hexoses i.e., glucose and fructose are derived by enzymatic reaction of INV on sucrose whereas SUS cleaves sucrose in the presence of UDP to yield UDP-glucose (UDP-G) and fructose. Starch in photosynthetic tissues (during the dark period) and sink tissues is also degraded to yield glucose monomers (Chia et al. 2004; Smith et al. 2005).

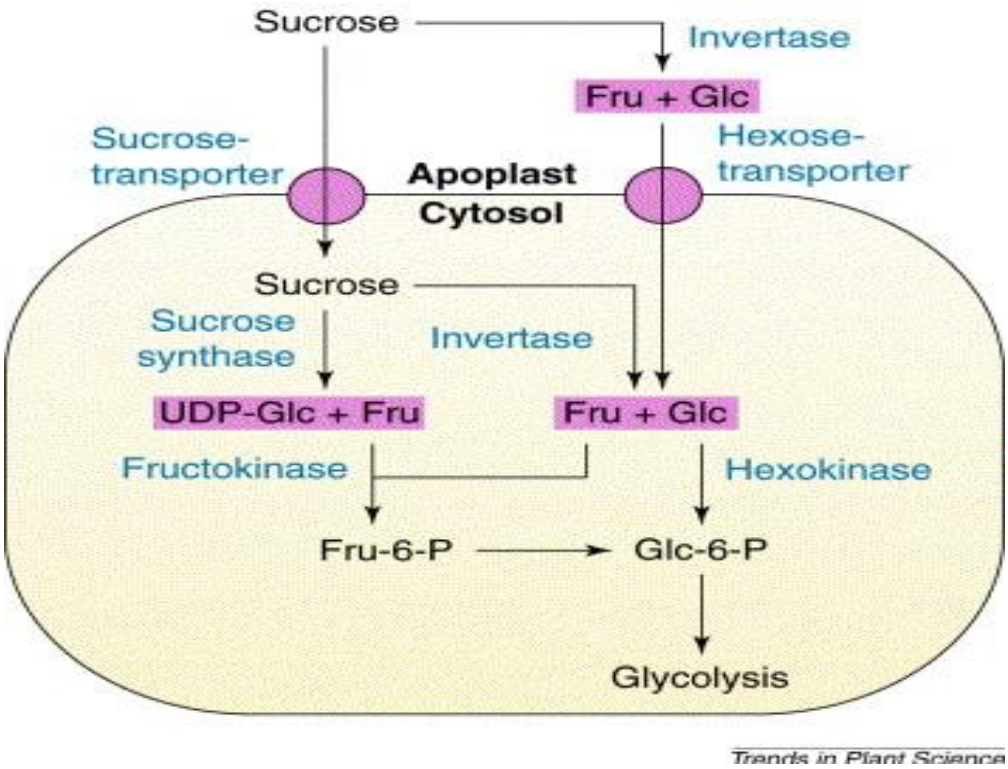


Fig. 2 Sucrose metabolism in plant cells (Pego and Smeekens 2000).

Prior to be used in any metabolic process hexoses, glucose and fructose must be phosphorylated. Hence, hexose-phosphorylating enzymes (**hexose kinases)** potentially play important role in maintaining the flux of carbon from sucrose to starch formation and are essential for all aspects of plant metabolism and development.

1.3 What are Hexose Kinases, Hexokinases (HXKs) and Fructokinases (FRKs)?

Hexose kinases are the hexose (six carbon sugars eg. glucose, fructose, mannose etc) phosphorylating enzymes utilizing NTP as phosphoryl donor (Anich et al. 1990). The hexose kinase having enzymatic catalytic activity for various hexoses is known as hexokinase (HXK) (EC 2.7.1.1) (Purich et al. 1973). This plant HXK is thus distinct from glucokinase (GK), fructokinase (FRK), mannokinase (MK) and galactokinase (GalK) which are highly specific only to glucose, fructose, mannose and galactose, respectively (Ca´rdenas et al. 1998). HXK's specificity for several hexose substrates makes this enzyme a gateway to glycolysis for hexoses arising from D-sucrose or transitory starch degradation. These hexoses must be phosphorylated to glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) for further metabolism. Fructose can be phosphorylated by either hexokinase (HXK) or fructokinase (FRK) while,

glucose can only be phosphorylated by HXK. Interestingly, the reactions catalyzed by these enzymes are of irreversible nature (Granot et al. 2013).

HXKs and FRKs were first purified from protein extracts of sink and source tissues by ion-exchange chromatography in the early 1950s (Millerd et al. 1951; Saltman 1953; Medina and Sols 1956). The potato (*Solanum tuberosum*) FRK was the first hexose-phosphorylating gene to be isolated from a plant (Smith et al. 1993; Taylor et al. 1995) and the first HXK gene was isolated from Arabidopsis (*Arabidopsis thaliana*) (Dai et al. 1995). Since those first discoveries several HXK and FRK genes have been isolated from different plant species. The increased availability of plant genome sequences allowed scientists (Karve et al. 2010) to estimate the number of HXK genes in the moss *Physcomitrella patens*, thelycophyte *Sellaginella mollendorffii*, three eudicot species, and three monocot species. It was concluded that the number of sequences associated with HXK gene families ranged from 11 sequences in *Physcomitrella* to 5–7 sequences for *Sellaginella* and the eudicot species, and 8–10 sequences in the monocot species. Based on genome sequence data, it is observed that there are seven FRK genes in Arabidopsis, three in rice, and eight in *Physcomitrella* (Thelander 2009). It appears that both HXK and FRK exist in species from the main land plant lineages, including mosses, lycophytes, gymnosperms and angiosperms. Only few genes encoding hexose kinases have been cloned from plants.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Hexose Kinases in the *Solanaceae* family

Solanaceae family is an economically important family that includes tomato, pepper, aubergine (eggplant), petunia and tobacco. Two tomato cDNAs encoding FRK were isolated and their ability to complement a yeast triple mutant unable to phosphorylate either glucose or fructose showed that both cDNAs encoded genuine FRK (Kanayama et al. 1997; Kanayama et al. 1998). Two HXK genes from potato (Jang et al. 1997) and one from tobacco (Veramendi et al. 1999) have been cloned so far. By using RT-PCR and Southern Blotting two divergent genes were isolated from tomato (Menu et al. 2001).

2.2 Hexokinases (HXKs) in plants

Catalytic properties and localization of HXKs in plants: HXK is believed to be an 80 year old enzyme but still we need to know much more about this enzyme. In 1922, Noble Prize winner in Physiology or Medicine, Otto Meyerhof was the first person to describe this enzyme which was extracted from baker's yeast (*Saccharomyces cerevisiae*) (Meyerhof 1927) and around a decade later its reaction (Von Euler and Adler 1935; Meyerhof 1935; Colowick and Kalckar 1943) was explained i.e., :

$$\text{Hexose} + \text{ATP} \rightarrow \text{Hexose-6-phosphate} + \text{ADP}$$

In early 1950s, plant HXKs were first identified in sink and source tissues of various plants like wheat (*Triticum* spp.) germ, potato (*Solanum tuberosum*) tubers, spinach (*Spinacea oleracea*) leaves, and pea (*Pisum sativum*), mung bean (*Phaseolus aureus*) and oat (*Avena sativa*) seeds (Millerd et al. 1951; Saltman 1953). In these species it was found that HXKs is capable of phosphorylating glucose, fructose, mannose and glucosamine, but could not phosphorylate galactose (Saltman 1953). Studies done by Turner and Copeland in 1981 showed that HXK has limited ability to phosphorylate galactose and 2-deoxy-D-glucose (the latter is a non-metabolic substrate).

In tobacco (*Nicotiana spp.*) it was reported that more than 80% activity of HXK of leaf tissue was associated with mitochondria-containing particulate fraction (Sindelarova and Sindelar 1988). In pea stems and leaves HXK activity was found in outer membrane of mitochondria (Cosio and Bustamante 1984; Dry et al. 1983; Tanner et al. 1983). Studies done on spinach and pea leaves (Baldus et al. 1981; Schnarrenberger 1990), developing castor bean seeds (Miernyk and Dennis 1983), soybean (Glycine max) nodules (Copeland and Morell 1985) and maize (Zea mays) roots (Galina et al. 1995) proved that apart from mitochondrial association, plant HXKs are also found in cytosol (Claeyssen and Rivoal 2007).

Role of HXKs: To study the physiological role of HXK, its genes have been studied by exposuring plant cell cultures, seeds and seedlings to exogenous sugars and its analog; by mutant selection and modifying expression of HXK in transgenic plants. Gene expression, plant physiology and plant development were analyzed to observe the effects of these treatments. In earlier studies it was hypothesized that plant HXK enzyme also play sugar sensing role in regulation of sugar metabolism in addition to its metabolic functions (Gancedo 1992; Jang and Sheen 1994). This hypothesis was proven when the photosynthetic gene expression of maize protoplasts was monitored after their exposure to exogenous sugars and repressed expression of photosynthetic genes like RBCs and chlorophyll A/B binding protein were taken as markers for studying sugar-sensing in plants (Jang and Sheen 1997; Jang et al. 1997; Moore et al. 2003). The results showed that there was repressed expression of these genes by sugars that are substrates of HXK and no effect by sugar analogs which are not HXK substrates. Therefore, HXK played sugar-sensing role independent of downstream metabolism of G6P and F6P (Jang and Sheen 1997).

After germination, during the reproductive developmental stages of plant it has been established that the repression of microRNA156 (miRNA156) function allows the transition from juvenile to adult state (Wu et al. 2009). This repression of miRNA156 is mediated by leaf-derived signal i.e. sugar (Yang et al. 2011; Yang et al. 2013 and Yu et al. 2013). In plants, where leaves have been removed, showed high levels of miRNA156 and also delayed transition from juvenile to adult state. The role of photosynthesis products in this transition revealed that miRNA156 is partially dependent on HXK signaling in early juvenile state, while at later state its expression is independent of HXK (Yang et al. 2013 and Yu et al. 2013). The role of HXK is to prevent the accumulation of miRNA156 and to promote early juvenile phase transition in response to sugar.

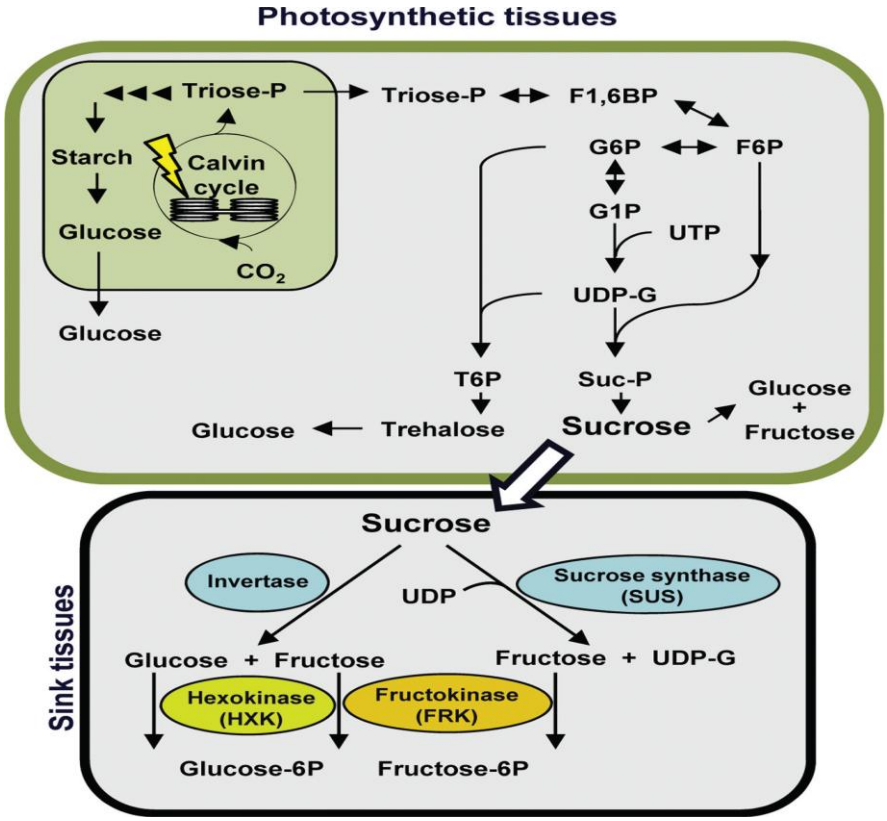


Fig. 3 Sugar metabolism in source and sink tissues. Triose phosphates, the product of photosynthetic CO2 fixation in the Calvin cycle, are exported from the chloroplast to the cytoplasm (Granot et al. 2014).

The role of photosynthesis products in this transition revealed that miRNA156 is partially dependent on HXK signaling in early juvenile state, where as at later state its expression is independent of HXK (Yang et al. 2013; Yu et al. 2013). The role of HXK is to prevent the accumulation of miRNA156 and to promote early juvenile phase transition in response to sugar.

In the latest studies, it has been found that HXK along with abscisic acid (ABA) mediates the closure of stomata by sucrose within the guard cells (Kelly et al. 2013). These findings supported the hypothesis that feedback-inhibition mechanism is mediated by sucrose and HXK (Outlaw 2003; Kang et al. 2007). When the rate of sucrose production exceeds the rate of sucrose loading in phloem then, the surplus sucrose is taken towards the stomata with the help of transpiration stream. It stimulates stomatal closure via HXK. This prevents the water loss in plant system and thus also controls the uptake of water and minerals by the plant. HXK also helps in breakdown of carbohydrates to fuel respiration and provides carbon intermediated to various anabolic pathways, thus known as glycolytic enzymes (Plaxton 1996).

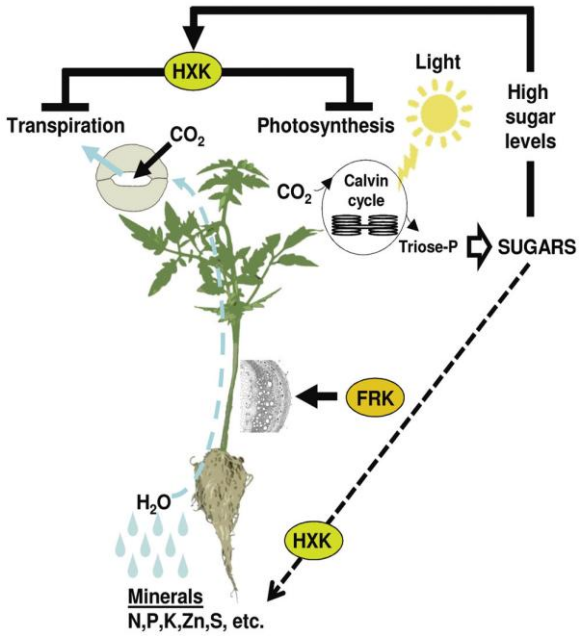


Fig. 4 The direct effects (solid lines) of HXK on photosynthesis and transpiration, and how stomatal closure by HXK reduces the uptake of CO_2, water, and minerals (dashed blue line). HXK also enhances mineral uptake by roots (dashed black line). HXK also enhances mineral uptake by roots (dashed black line) (Granot et al. 2014).

2.3 Fructokinases (FRKs) in plants

The phosphorylation of free hexoses not only is the initial step of glycolysis but is also required for the mobilization of all hexoses taken up by the cell. Fructose phosphorylation by fructokinase (FRK, EC 2.7.1.4) is an irreversible and near rate-limiting reaction in vivo, and could thus be an important site for the regulation of carbohydrate metabolism. Fructokinase (EC 2.7.1.4) efficiently catalyses the phosphorylation of fructose to fructose 6-phosphate. Hexose phosphorylation is especially important in plants since the use of free phosphates is particularly complex (Kruger 1990).

Localization of FRKs: Through the studies of efficient movement of sucrose into plastids, plastidic INV has been identified which explained the presence of fructose in plastids thus it was implied that fructose phosphorylation by FRK does occurs in plastids (Gerrits et al. 2001; Vargas et al. 2008). GFP fusion proteins of four tomato FRKs were studied to check intracellular localization of FRK isozymes. Three out of these four tomato FRK isozymes were found to be located in cytosol and one within plastids and stromules (Damari-Weissler et al. 2006). In eudicots HXK is associated with mitochondria and FRK is localized in cytosol, which suggested that phosphorylation of glucose occurs adjacent to mitochondria,

whereas fructose might be phosphorylated either adjacent to mitochondria by HXK or in cytosol FRKs present in cytoplasm.

Advances on FRKs in plants at biochemical and molecular level: Activities of FRK have been purified from various plant species and tissues, by ion exchange chromatography. Number of peaks was reported between one to three and it varies among different tissues and species as well. Order of elution dictates the designation attributed to the activity peaks, which leads to diversity in designations to different plant FRK proteins. In potato, maize, barley three activity peaks were found, designated as FRK1, FRK2, FRK3; FRK0, FRK1 and FRK2 and FRK1a, FRK1b and FRK2, respectively (Pego and Smeekens 2000).

Enzymatic attributes of FRKs: To determine the molecular weight of plant FRK, SDS gel analysis and gel filtration assays studies were implied. SDS–PAGE analysis revealed proteins of~36 kDa which shows a close agreement with the values obtained based on the deduced amino acid sequence in potato, tomato and sugar beet. Through gel filtration assays the dimeric nature of FRK proteins was revealed as it shows a native molecular weight of 75-105 kDa. Most of the plant FRKs is present as dimmers but in pea seed and barley leaf, the FRK-2 is of monomeric in nature. Exceptionally, pea isoform has showed almost twice weigh on SDS- PAGE i.e., 72 kDa from other species. FRK1 and FRK2 generally have same attributes with some differences. Optimum pH is 8 for both isoforms and the km value ranges from 41 µM to 220 µM for fructose. Principal substrate is ATP for FRK unless they are in cellular region with high GTP or UTP concentration. Mg ion is an indispensable cofactor for FRK activity. Sucrose gradient centrifugation revealed that FRK2 is present in cytosol and FRK1 is associated with the chloroplast (Pego and Smeekens 2000).

Role of FRKs: Unlike HXK, the physiochemical role of isozymes of FRK in plants is less known. The information about FRK isozyme's function has been collected mostly from transgenic plants and also via gene expression profile analysis under different growth conditions. Earlier it was believed that FRK affects the starch accumulation in different plant species (eg. tomato) (Schaffer and Petreikov 1997b) but by examining FRK2- and FRK1-antisense tomato and potato plants it was reported that starch accumulation is not affected by FRK (Dai et al. 2002a; Odanaka et al. 2002; Davies et al. 2005). By studying the reduced expression of FRK2 in antisense plants it was found that FRK2 was essential for vascular development because with its reduced expression the plants had deformed vasculature, smaller cell size in xylem and phloem, reduced cambium activity and secondary walls in vessels, and small sieve elements with low levels of callose deposition (German et al. 2003 and Damari-Weissler et al. 2009). In

the vascular system, the development of xylem and phloem depends on sucrose metabolism, therefore, sucrose must be first cleaved by either INV or SUS so that it can be metabolized. In the vascular tissues of tomato stems SUS1, SUS2 and FRK2 are highly expressed (German et al. 2003; Goren et al. 2011). Fructose and UDP-G are the products of sucrose cleavage by SUS and both are believed to be central for development of vascular system. In cellulose and cell wall synthesis UDP-G is used, while fructose is phosphorylated so that it can be either utilized in energy production or fed into other metabolic pathways. The exceeded concentration of fructose from 0.5-1 mM (Schaffer and Petreikov 1997a) causes feedback inhibition of SUS. Thus the phosphorylation of fructose by FRK2 might be necessary for the sucrose cleavage, sugar metabolism, and cell wall synthesis that are essential for proper development of the vascular tissues (German et al. 2003 and Damari-Weissler et al. 2009). The FRK plant mutants suggested that FRK genes are either essential or have highly redundant functions under normal growth conditions.

When the reduced FRK activity in potato, by antisense suppression of *St*FRK1 (the potato homolog of tomato FRK2), was checked it was reported that there is reduced tuber yield in potato (Davies et al. 2005).

In anther development, it has been seen that FRKs play an important role as developing anther is not photosyntheically active and thus for development it requires sucrose as energy source. FRK provides F6P and facilitates UDP-G in cellulose synthesis for enlongation of cell wall, by stopping feedback inhibition of SUS by fructose (Karni and Aloni 2002).

2.4 Competition between HXK and FRK for Fructose

The cleavage of sucrose by INV or SUS produces fructose (hexose sugar) which can serve as substrate for both HXK and FRK. It is seen that in early tuber development in potato there is high INV and HXK activity at subapical region of non-tuberizing stolon (Appeldoorn et al. 2002) and later during tuberization FRK does most of the hexose phosphorylating activity due to predominant SUS function in sucrose cleavage (Renz et al. 1993; Appeldoorn et al. 2002; Davies et al. 2005) thus the carbon flow via INV and SUS routes at different stages of tuber development correlates the activities of HXK and FRK, respectively.

2.5 Comparison between enzymatic activities of HXK and FRK

In plants, HXK is capable of phosphorylating glucose, fructose, mannose and glucosamine (reviewed by Granot 2007 & 2008) whereas FRK activity is specific to fructose. The measured HXKs affinity for glucose is in 0.02−0.1 mM range whereas its affinity for fructose is calculated to be in 2−120 mM range

(Granot 2007) similarly, the affinity of FRKs for fructose is very high, within same range as the affinity of HXKs for glucose (Pego and Smeekens 2000; Granot 2007). With this reports it has been concluded that HXKs phosphorylate mainly glucose whereas fructose is phosphorylated primarily by FRKs in *in vivo* conditions.

2.6 Advances on FRKs in potato till date

Purification and studies on the properties of fructokinase have been done from developing tubers of Potato (*Solanum tuberosum* L.) (Gardner et al. 1992). Hexose metabolism pathways in developing tubers of potato have been explored (Davies et al. 2005). Primary structure and characterization of cDNA clone of fructokinase from potato has been done, and its gene expression has been characterized (Smith et al. 1993). Features of cDNA, G+C content and structural features of protein has been identified (Taylor et al. 1995). Modulation of fructokinase activity in potato tuber metabolism has been studied (Davies et al. 2005). Kinetic studies on fructokinase isoforms of potato has been carried out (Gardner et al. 1992). Characterization of antisense lines and over expression of fructokinase gene in potato has been analyzed (Davies et al. 2005). Role of Fructokinase in catalyzing the sugar metabolism required for the development of vascular tissues in the potato tubers (sink tissues) (Granot et al. 2014).

Origin of the problem

As discussed in the earlier sections, in plants FRK is a crucial enzyme with regard to carbohydrate metabolism, more precisely, sucrose and starch biosynthesis. Literature review revealed that till date most of the work was carried out on potato FRKs at cDNA and enzyme level only. With regard to structure and function of FRK gene(s), there is no substantial report in the database and literature as well. Regulation and differential expression of FRK gene(s) are an important area of research. Molecular and biochemical studies of gene structure and functions of different forms of fructokinases in different potato cultivars are yet to be carried out. There was no comprehensive study for different patterns of expression of fructokinase gene(s) particularly during different phases of tuber development. Likewise biochemical attributes of different forms of fructokinases are yet to be studied in detail. We need to have in-depth understanding on fructokinases with regard to their role during sucrose-starch interconversions. In this context, it may be noted that there are many commercially important Indian potato cultivars. However, no considerable progress had been made on FRKs in these cultivars. Biochemical, molecular and genetic studies on different FRK forms in potato could help in improving these cultivars through transgenics. Keeping in view of above review of literature following objectives were framed for the present study.

Objectives

- Sequence analyses of the FRK cDNAs, and the corresponding predicted polypeptides as available in the database
- Isolation and purification of genomic DNA from different potato cultivars
- Analyses of the amplicon profiles in the potato cultivars using potato FRK-specific oligonucleotide primers under various conditions

CHAPTER 3: MATERIALS AND METHODS

3.1 Plants and Materials

Potato germplasm procurement: The germplasm of various Indian potato cultivars such as Kufri Chipsona-1 (CS-1), Kufri Chipsona-2 (CS-2), Kufri Chandramukhi (KCM), Kufri Jyoti (KJ), Kufri Ashoka (AS) and Kufri Pukhraj (PR) (names are abbreviated for convenience) were procured from Central Potato Research Institute (CPRI), Shimla, India. These germplasm are routinely maintained on basal MS medium in our laboratory.

Buffers:

Gel loading buffer (5X)

Sucrose - 35 % (w/v)

EDTA - 50 mM (pH 8.0)

Tris – 25 mM

Bromophenol blue - 0.2 % (w/v)

TBE (5X) buffer

Tris Base - 54 gL^{-1}

Boric acid - 28 gL^{-1}

EDTA - 3.8 gL^{-1}

The pH of the buffer was set at 8.0

TE buffer

Tris.HCl - 10 mM (pH 8.0)

EDTA - 1 mM (pH 8.0)

3.2 Methods

Genomic DNA isolation from potato cultivars: Genomic DNA was isolated from the potato cultivars by the protocol described by Kumari et al. (2012). 0.2 to 1 g of plant material was taken from fresh *in vitro* cultures and grounded to fine powder in the presence of liquid nitrogen using mortar and pestle. The fine

powder was then transferred to conical flask containing 5 mL extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA(pH 8.0), 250 mM NaCl and 15% (w/v) sucrose) maintained at 65°C. Contents were mixed properly and incubated at 65°C for 20 min with intermittent gentle shaking. The mixture was then cooled and centrifuged at 5500 rpm for 15 minutes. The supernatant was taken in fresh tube and one-third volume of 5.0 M potassium acetate solution was added, mixed vigorously and incubated further on ice for 20 min and then centrifuged at 5500 rpm at 4°C for 25 min. The supernatant was filtered through a fine muslin cloth and equal volume of isopropanol was added, mixed gently and incubated at 4°C for 1 hour. DNA was extracted by centrifugation at 10000 g at 4°C for 25 min. The crude DNA pellet was washed with ice cold 70% ethanol, air dried and suspended in 40 µL of TE buffer (10 mM Tris-HCl pH 8.0 and 1.0 mM EDTA pH 8.0). Further purification of DNA was done by treatment with DNase –free RNase followed by solvent extraction step twice using mixture of phenol:chloroform:isoamyl alcohol (25:24:1). DNA was then precipitated using one-tenth the volume of 3.0 M sodium acetate (pH 5.5) and equal volumes of isopropanol and the DNA was finally recovered by dissolving in 200 µL of TE buffer and stored at -20°C. The quality and quantity of DNA was initially checked through nano-drop spectrophotometric method by measuring the A_{260}/A_{280} ratio and 260 nm respectively.

`*Primer Designing*: The oligonucleotide primers were designed based on the available genome sequence corresponding to fructokinase gene in GenBank databse (Accession numbers Z12823 and JX576230 from Smith et al. 1993 and Gangadhar et al. 2014, respectively). GenBank ID Z12823, refers to fructokinase cDNA sequence cloned from *S. tuberosum* L. cv Record, contains ORF of 960 nts (i.e.319-aa). Apart from this NCBI database search revealed GenBank accession number JX576230 which also encodes for 969 bp cDNA sequence of fructokinase consisting polypeptide of 256-aa.To ensure minimum chances of non-specific amplification, important factors kept in consideration while designing the primers were:

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

Keeping all these parameters in mind following primers were designed using cDNA sequences of FRK present in database:

- 5′ CATCGTCGCCATGGCAGTTA 3′ (F1A-FK01; Bases 1-20 of Z12823)
- 5′ TCGAGTTCCACATCGCTGAC 3′ (R1-FK606; complementary to the bases 587-606 of Z12823)
- 5′ AACATGATGTTTCTATGCTC 3′ (R2-FK982; complementary to the bases 963-982 of Z12823. Note: This sequence is also complementary to the bases 765-784 of JX576230)
- 5′ TGATGGACCGTATCACAACA 3′ (R3-FK1110; complementary to the bases 1091-1110 of Z12823)
- 5′ GATGCTCGCCGGGATTCTGA 3′ (F2B-FK01; Bases 1-20 of JX576230)
- 5′ ATAGTTCCAGATGCTCTTGA 3′ (RU-FK373; complementary to the bases 354-373 of JX576230)

Polymerase Chain Reaction (PCR): PCR was used to amplify a specific DNA sequence in a simple, rapid and automated manner using forward and reverse primer. PCR is repeated cycling of three steps: heat denaturation of template DNA (94°C); annealing of primers to the complementary sequences in template DNA (55°C & 50°C); extension of annealed primers by a thermo stable DNA polymerase (72°C). [Note: Prior to applying the thermal cycling parameters, the reaction mixture was incubated for 1 min at 94ºC and then Taq DNA Polymerase was added.]

The genomic DNA preparations from the following potato cultivars were used in PCR:

- Kufri Chipsona-1 (CS-1)
- Kufri Chipsona-2 (CS-2)
- Kufri Jyoti (KJ)
- Kufri Chandramukhi (KCM)
- Kufri Ashoka (AS)
- Kufri Pukhraj (PR)
- Désirée (De)

Different primer pairs were used to carry out PCR under different conditions.

Composition of PCR reaction:

Total reaction volume: 50µL	
Template DNA	3 µL
Buffer 10X	5 µL
Forward primer	10 pmoles
Reverse primer	10 pmoles
dNTP's	25 mM
Sterile deionized water	volume made up to 50 µL
Taq DNA polymerase	3U/µL

The thermal cycling parameters were as given below:

Step	*Time*
Denaturation	1 min
Annealing	2 min
Polymerization	2-3 min

The reaction was carried out for 30 cycles with final extension at 72°C for 5 minutes.

***Agarose gel electrophoresis*:** Agarose gel electrophoresis was performed using standard methods (Sambrook- a laboratory manual). 1% agarose gel was made in 0.5X TBE buffer and ethidium bromide dye (0.5 µg mL^{-1}) was added to it. Gel was then casted in the casting tray. The gel was first allowed to solidify and then DNA samples were loaded. Electrophoresis was carried out in 0.5X TBE (running buffer) at 2 – 5 Volt per cm till the tracking dye covered two-third of the gel length. Finally, the DNA bands were visualized under UV light.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Sequence analyses of FRK cDNAs

During the last few decades, considerable progress had been made on FRKs in the different plant species including the members of the *Solanaceae* family. FRK has several forms as evident from both biochemical and cDNA cloning studies. With regard to sequence analyses and comparison, no comprehensive report is available in the database. One of the objectives includes sequence analyses and comparison between the FRK cDNAs at both nucleotide and amino acid levels. The results of the sequence analyses as carried out in the study are provided in following sections. NCBI database search revealed a few cDNA sequences encoding FRK and FRK-like protein in potato.

Analyses of FRK and FRK-like cDNA sequences: Carefully analyzing the sequences listed some interesting attributes which are given below:

FRK-specific cDNA sequence (GenBank ID: Z12823): This refers to 1143-bp cDNA sequence cloned from *S. tuberosum* L. cv Record. The cDNA contains an ORF of 960 nucleotides encoding a form of FRK consisting of 319-aa (Protein ID- CAA78283).

FRK-specific cDNA sequence (GenBank ID: JX576230): This ID referred to 969-bp cDNA sequence, clone from potato which contained 771 nucleotides ORF which is encoding another form of FRK. In this case FRK consisted of 256-aa. Therefore, it was concluded that these two cDNAs (GenBank IDs: Z12823 and JX576230) encodes two distinct isoforms of FRK in potato.

FRK-like cDNA sequence (GenBank ID: JX576279): Apart from previous two GenBank IDs, NCBI database search also revealed this ID which referred to 1097-bp cDNA encoding for FRK-like protein in potato. This cDNA contains ORF of 801 nucleotides encoding 266-aa polypeptide of FRK-like protein

BLAST analyses of FRK and FRK-like sequences: All the cDNA sequences were further analyzed at both nucleotide and amino acids sequence levels using double alignment tool in BLAST. The salient features of these analyses are listed below:

Comparison between GenBank IDs, Z12823 and JX576230: At nucleotide level, the results showed that there was 96% sequence identity with query coverage of 79%, thus there is significant difference between them. The corresponding deduced polypeptides of both FRKs were found to show 93% sequence identity with a query coverage of 77% indicating again that there is considerable sequence difference at level of

amino acids also. Interestingly, the N-terminal region appears to be highly divergent as reflected by presence a unique 73-aa extension present only in Z12823.

Comparison between GenBank IDs, Z12823 and JX576279: At nucleotide level, the BLAST results between these two GenBank IDs showed that there is 99% sequence identity between them with 86% query coverage. Therefore, this cDNA is more close to Z12823 than JX576230 but still there is significant divergent between them. The deduced polypeptides of FRK and FRK-like showed 94% sequence identity with 78% query coverage depicting the difference between at amino acid level also. It was seen that the presence of 63-aa more in Z12823 at C-terminal gives the significant amount of variation between the two forms of FRK in potato.

```
StFRK-L    GCTCCTCCTCTTCTCTCTCTATTACTATATAAAACCTATAGATACATATATTTTCTCTAT     60

StFRK-L    TCATCGTCGCCATGGCAGTTAACGGTAGTGCTCCTTCTTCTGGTTTGATCGTCAGTTTCG    120
STFRK01     CATCGTCGCCATGGCAGTTAACGGTAGTGCTCTTTCTTCCGGTTTGATCGTCAGTTTCG     59
            F1A-FK01
            ..............................  ......  ...................

StFRK-L    GTGAGATGTTGATCGATTTCGTTCCGACAGTCTCCGGCGTATCCCTTGCCGAGGCTCCCG    180
StFRK01    GTGAGATGTTGATCGATTTCGTTCCGACAGTCTCCGGCGTATCCCTGGCCGAGGCTCCCG    119
           ..............................................  ............

StFRK-L    GATTTTTGAAAGCTCCGGGCGGTGCACCGGCGAACGTCGCCATCGCGGTGACAAGGCTCG    240
StFRK01    GATTTTTGAAAGCTCCCGGCGGTGCACCGGCGAACGTCGCCATCGCGGTGACAAGGCTCG    179
           ................  ........................................

StFRK-L    GAGGGAAGTCGGCGTTCGTCGGGAAACTCGGCGACGATGAGTTTGGTCACATGCTCGCCG    300
StFRK01    GAGGGAAGTCGGCGTTCGTCGGGAAACTCGGCGACGATGAGTTTGGTCACATGCTCGCCG    239
StFRK02                                                  GATGCTCGCCG     11
                                                         F2A-FK01
           ..........................................................**********

StFRK-L    GGATTCTGAAAACGAACGGCGTACAAGCCGACGGAATCAATTTTGACAAGGGCGCCAGGA    360
StFRK01    GGATTCTGAAAACGAACGGCGTACAAGCCGACGGAATCAATTTTGACAAGGGCGCCAGGA    299
StFRK02    GGATTCTGAAAACGAACGGCGTACAAGCCGACGGAATCAATTTTGACAAGGGTGCCAGGA     71
           ************************************************.*******

StFRK-L    CGGCTTTGGCGTTCGTGACTCTACGCGCCGACGGAGAGCGTGAGTTTATGTTTTACAGAA    420
StFRK01    CGGCTTTGGCGTTCGTGACTCTACGCGCCGACGGAGAGCGTGAGTTTATGTTTTACAGAA    359
StFRK02    CGGCTTTGGCGTTCGTGACTCTACGCGCCGACGGAGAGCGTGAGTTTATGTTTTACAGAA    131
           ************************************************************

StFRK-L    ATCCCAGTGCTGATATGTTGCTCACGCCCGATGAGTTGAATCTTGATCTTATTAGATCTG    480
StFRK01    ATCCCAGTGCTGATATGTTGCTCACGCCCGATGAGTTGAATCTTGATCTTATTAGATCTG    419
StFRK02    ATCCCAGTGCTGATATGTTGCTCACGCCCGATGAGTTGAGTCTTGATCTTATTAGATCTG    191
           ************************************.***********************
```

```
StFRK-L   CTAAGGTGTTCCACTACGGATCAATTAGTTTGATCGTGGAGCCATGTAGATCAGCACATT   540
StFRK01   CTAAGGTGTTCCACTATGGATCAATTAGTTTGATCGTGGAGCCATGTAGATCAGCACATT   479
StFRK02   CTAAGGTGTTCCACTATGGATCAATTAGTTTGATCGTGGAGCCATGTAGATCAGCACATT   251
          ****************.*********************************************

StFRK-L   TGAAGGCAATGGAAGTAGCAAAGGAGGCAGGGGCATTGCTCTCTTATGACCCAAACCTTC   600
StFRK01   TGAAGGCAATGGAAGTAGCAAAGGAGGCAGGGGCATTGCTCTCTTATGACCCAAACCTTC   539
StFRK02   TGAAGGCAATGGAAGTAGCAAAGGAGGCAGGGGCATTGCTCTCTTATGACCCAAATCTTC   311
          ******************************************************.*****
StFRK-L   GTTTGCCGCTGTGGTCTTCGGAAGAAGAGGCCAGGAAGCAAATCAAGAGCATCTGGAACT   660
StFRK01   GTTTGCCGCTGTGGTCTTCGGAAGCAGAGGCCAGGAAAGCA-------------------   580
StFRK02   GTTTGCCGCTGTGGTCTTCGGAAGAAGAGGCCAGGAAGCAAATCAAGAGCATCTGGAACT   371
                                                    RU-FK373
          ************************:************:::*:::::::::::::::::::

StFRK-L   ATGCTGATGTGATCAAGGTCAGCGATGTGGAACTCGAATTCCTAACTGGAAGCAACAAGA   720
StFRK01   -----------ATCAAGGTCAGCGATGTGGAACTCGAATTCCTCACTGGAAGCGACAAGA   629
                      R1-FK606
StFRK02   ATGCTGATGTGATCAAGGTCAGCGATGTGGAACTCGAATTCCTCACTGGAAGCAACAAGA   431
          :::::::::::**********************************▪*********:******

StFRK-L   TTGATGATGAATCCGCCATGTCCTTGTGGCATCCTAATTTGAAGCTCCTCTTGGTCACTC   780
StFRK01   TTGATGATGAATCCGCCATGTCCTTGTGGCATCCTAACTTGAAGCTCCTCTTGGTCACTC   689
StFRK02   TTGATGATGAATGCGCCATGTCCTTGTGGCATCCTAACTTGAAGCTCCTCTTGGTCACTC   491
          ************.************************▪**********************

StFRK-L   TTGGTGAAAAGGGTTGCAATTACTACACCAAGAAATTCCATGGAAGCGTTGGAGGATTCC   840
StFRK01   TTGGTGAAAAGGGTTGCAATTACTACACCAAGAAATTCCATGGAAGCGTTGGAGGATTCC   749
StFRK02   TTGGTGAAAAGGGTTGCAATTACTACACCAAGAAATTCCATGGAAGCGTTGGAGGATTCC   551
          ************************************************************

StFRK-L   ACGTGAGACTGTTGACA-CCACTG-AGCTGGTGATTCTTTTGTGTGCTCTCTACAGATG-   897
StFRK01   ACGTGAAGACTGTTGACACCACTGGAGCTGGTGATTCTTTTGTTGGTGCCCTTCTAACCA   809
StFRK02   ACGTGAAGACTGTTGACACCACTGGAGCTGGTGATTCTTTTGTTGGTGCCCTTCTAACCA   611
          ******▪▪▪▪▪▪*▪▪▪▪▪▪********▪************************▪▪*▪▪*▪**▪*▪▪*▪▪▪

StFRK-L   ----TGATGATCAGCATTCTGAGAT-----------------GAGCAGTGAGAGTACTAG   936
StFRK01   AGATTGTTGATGACCAAGCCATTCTTGAGGATGAAGCAAGGTTGAAGGAAGTACTAAGGT   869
StFRK02   AGATTGTTGATGACCAAGCCATTCTTGAGGATGAAGCAAGGTTGAAGGAAGTACTAAGGT   671
          ▪▪▪▪**▪****▪*▪**▪▪*▪▪▪▪▪*▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪*▪▪▪▪*▪**▪▪▪▪

StFRK-L   TTTCATGTGCATGTGAGCATCACACACAGAAGGAGCATCCA----------GCTCTGCTA   986
StFRK01   TTTCATGTGCATGTGGAGCCATCACAACCACCAAGAAAGGAGCAATTCCAGCTCTGCCTA   929
StFRK02   TTTCATGTGCATGTGGAGCCATCACAACCACCAAGAAAGGAGCAATCCCAGCTCTGCCTA   731
          ***************▪▪▪▪▪▪▪▪****▪▪▪*▪▪▪**▪*▪▪▪*▪▪▪▪▪ ▪▪▪▪▪▪▪▪▪▪***

StFRK-L   CTGAATCTGAGCCTCACTGCTCAGGAA------------GGACAGAACATCAGGTAACTT   1034
StFRK01   CTGAATCTGAAGCACTCACTTTGCTCAAGGGAGGAGCATAGAAACATCATGTTATCTTTT   989
                                              R2-FK982
StFRK02   CTGTATCTGAAGTCCTCACTTTGCTCAAGGGAGGAGCATAGAAACATCATGTTATCTTTT   791
          ***.*******▪▪· *▪*▪▪▪*▪▪▪▪▪*▪▪▪▪▪▪▪▪▪▪▪▪▪**▪*▪*▪***▪▪▪▪*▪▪▪**

StFRK-L   TCATTTTTCATCCAATCCCCCTTAGACTACTCACGTGGAGCTCATAGTGATAGTTTTGGG   1094
StFRK01   TAATTTTTTTTTCCATCTCCATATA-TT---CCCCCCTCTATGAGTTTTTTTACTTTCAA   1045
StFRK02   TATTTTTTTTTTCCATCTCCATATA-TT---CCCCCCTCTATGAGTTTTTTTACTTTCAA   847
          *▪·*****▪▪*▪*▪*******▪*▪▪▪ ▪*   *▪*▪▪▪▪▪▪*▪*▪▪▪*▪▪*▪▪▪***▪▪▪
```

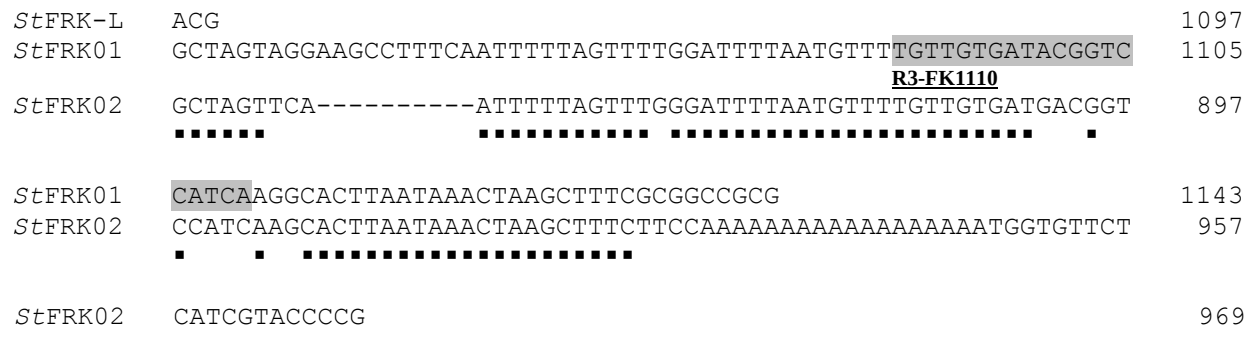


Fig. 5 Multiple sequence alignment of the nucleotide sequences of FRK and FRK-like proteins. *St*FRK01, *St*FRK02 and *St*FRK-L refer to GenBank IDs Z12823, JX576230 and JX576279, respectively. This alignment is based on CLUSTAL OMEGA tool. *Dashes* in the nucleotide sequences indicate gaps arising during the alignment; *dots* refer to match between the sequences *St*FRK01 and *St*FRK-L; *asterisk* is for common nucleotides in all three sequences; *solid box '▪'* denotes identical sequence between *St*FRK01 and *St*FRK02; and *colon':'* refers to sequence identical between *St*FRK02 and *St*FRK-L. Labeled are regions from where oligonucleotide primers are designed

Studies and analyses for attributes of FRK protein by using ExPASy Resource Portal: The 319-aa FRK sequence (GenBank ID: Z12823) was analyzed by using ProtParam tool of ExPASy resource portal under Swiss Institute of Bioinformatics (SIB) which revealed couple of attributes about the deduced polypeptide. The molecular weight of predicted 319-aa polypeptide was ~33.8 kDa. The total number of negatively charged residues (Asp+Glu) was 39 and the total number of positively charged residues (Arg+Lys) was 32. This data clearly indicated that the protein is acidic in nature which was further proven by its theoretical pI i.e., 5.47. The amino acid composition data showed that some of the amino acids such as Gly (10%), Ala (11%) and Leu (12.5%) occurred more frequently as compared to their average occurrence; whereas amino acids Gln (0.6%), Trp (0.6%) and Tyr (1.6%) occurred less frequently (Doolittle 1989). The instability index (II) was computed to be 20.75 thus the protein was classified as stable. The average occurrence of Gly was found to be more frequent which implied that the protein showed less propensity towards the alpha-helix formation. The half life was estimated to be 30 hours.

The hydropathy profile (Fig. 6) of FRK (Z12823) deduced polypeptide (319-aa) was generated with 9-aa running window using the ProtScale tool based on the Kyte-Doolittle scale. According to this plot it can be seen that around first 50 amino acids are showing high hydrophobicity and from 51st to 200th amino acids hydrophilic amino acids are predominant though some portions of hydrophobic amino acids can be seen. Interestingly from around 201th to 319th hydrophobic and hydrophilic amino acids are present periodically. This may indicate that hydrophilic amino acid residues are in contact with solvent, or water,

and that they are therefore likely to reside on the outer surface of the protein, or the stretch of hydrophobic amino acids may be part of alpha-helix across lipid bilayer, composed of hydrophobic fatty acids.

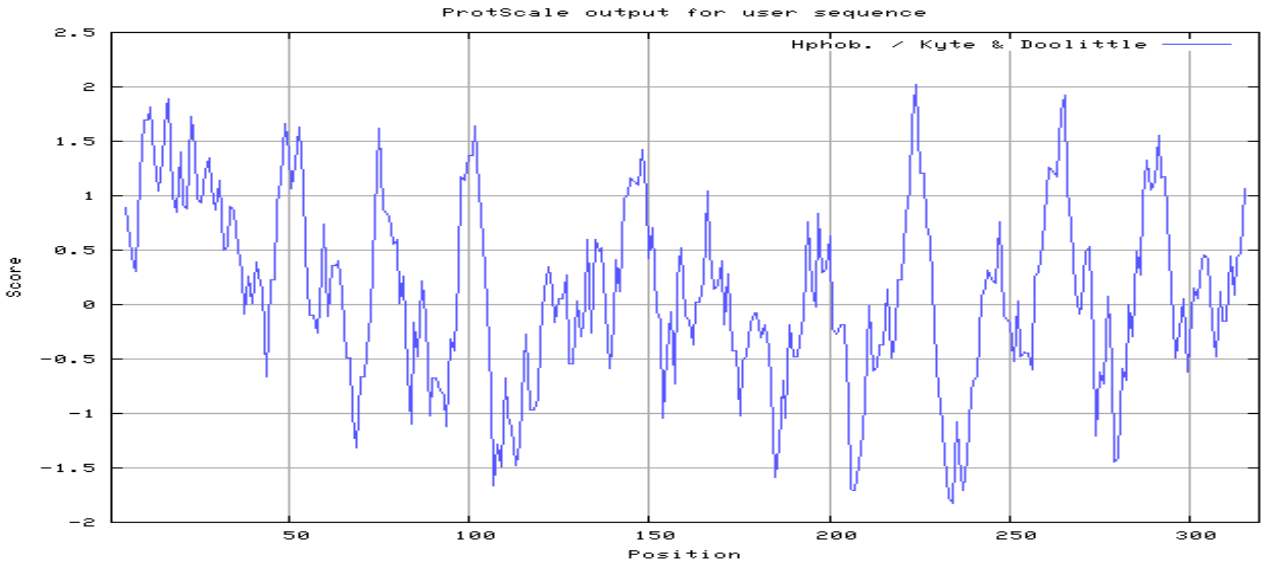


Fig. 6 Hydropathy plots of the predicted amino acid sequences of FRK (Z12823) based on based on the Kyte and Doolittle scale.

Multiple sequence alignment using CLUSTAL OMEGA tool: The multiple sequence alignment of amino acids sequences of FRK and FRK-like proteins (Fig. 7) discovered that first 73-aa were absent in P*St*FRK02 at N-terminal. In P*St*FRK01 a fragment of amino acid sequence (from 191th to 200th) was found to be missing. It can be hypothesized that during the course of evolution mutation (insertion/deletion) may have occurred in P*St*FRK01. Interestingly it was also observed that 63-aa amino acids were missing in P*St*FRK-L towards the C-terminal. From this data we can interpret that two forms of FRK-specific were more divergent towards the N-terminal and FRK-like protein has more variation towards C-terminal though significant part of sequences show consensus pattern.

Comparison with potato Hexokinase: To understand the relation and difference between HXK and FRK, a comparison of amino acid sequences of HXK (Menu et al. 2001) and FRK sequences was done (Fig. 7). This analyses showed us that, although isoforms of FRK shows substantial similarities in regulatory motifs like ATP motif, phosphate motifs and sugar binding motifs, but when compared with HXK, there was huge divergence but a few critical amino acids in these different motifs were conserved in HXKs and FRKs, for example in ATP motif amino acids like proline (P) and two glycine (G) residues were conserved; in sugar binding motif amino acids glutamic acid (E), leucine (L) and phenylalanine (F) were found to be consensus; in phosphate 1 binding segment three glycine (G),

alanine (A), asparagine (N) and leucine (L) conserved residues were present; and phosphate 2 binding domain had aspartic acid (D), valine (V) and glycine (G) amino acids conserved in it. This data showed that although HXK and FRK are referred to as hexose kinases, still there are significant changes present in specific motifs which account for diversification between them which can be use and exploited for protein engineering studies of FRKs.

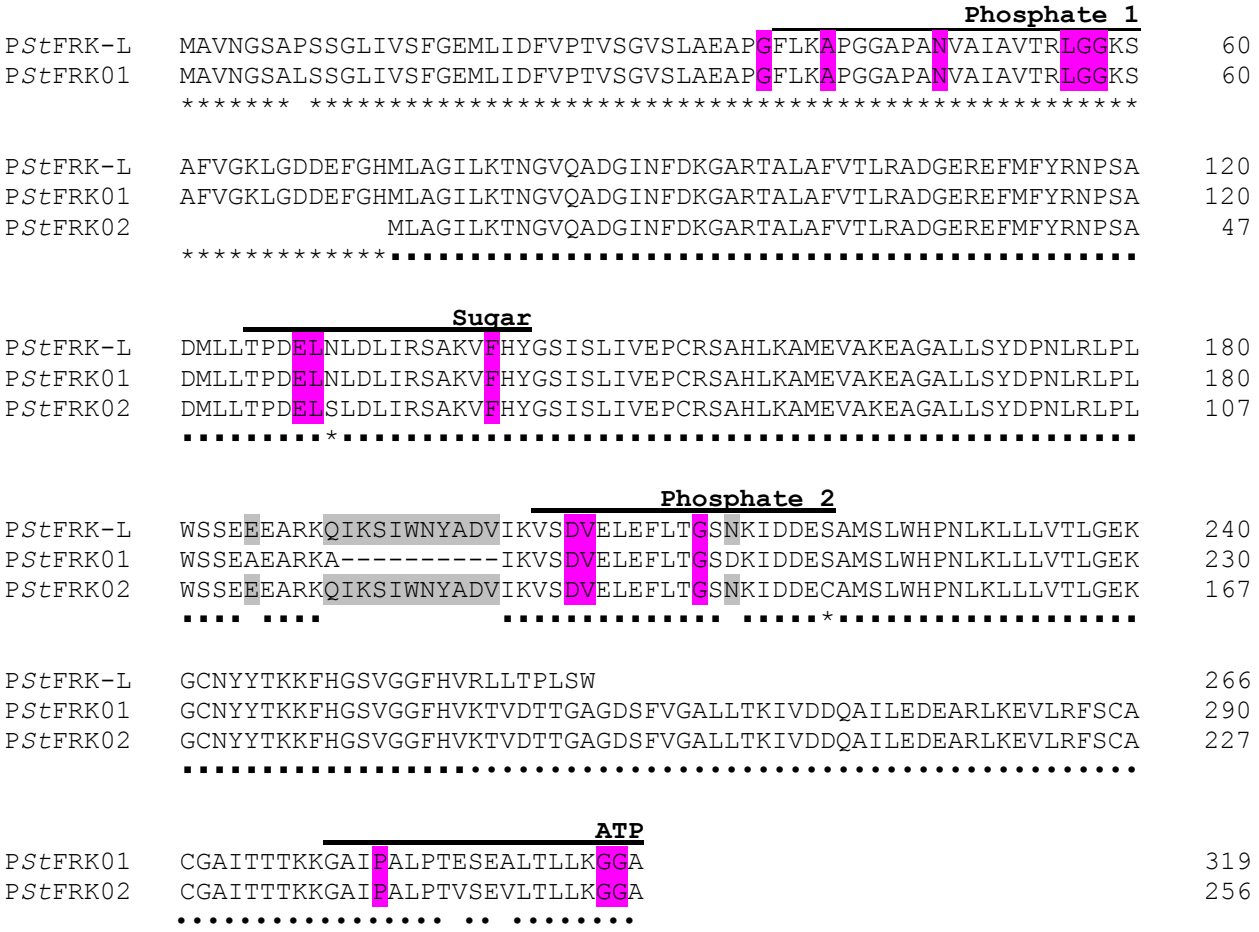
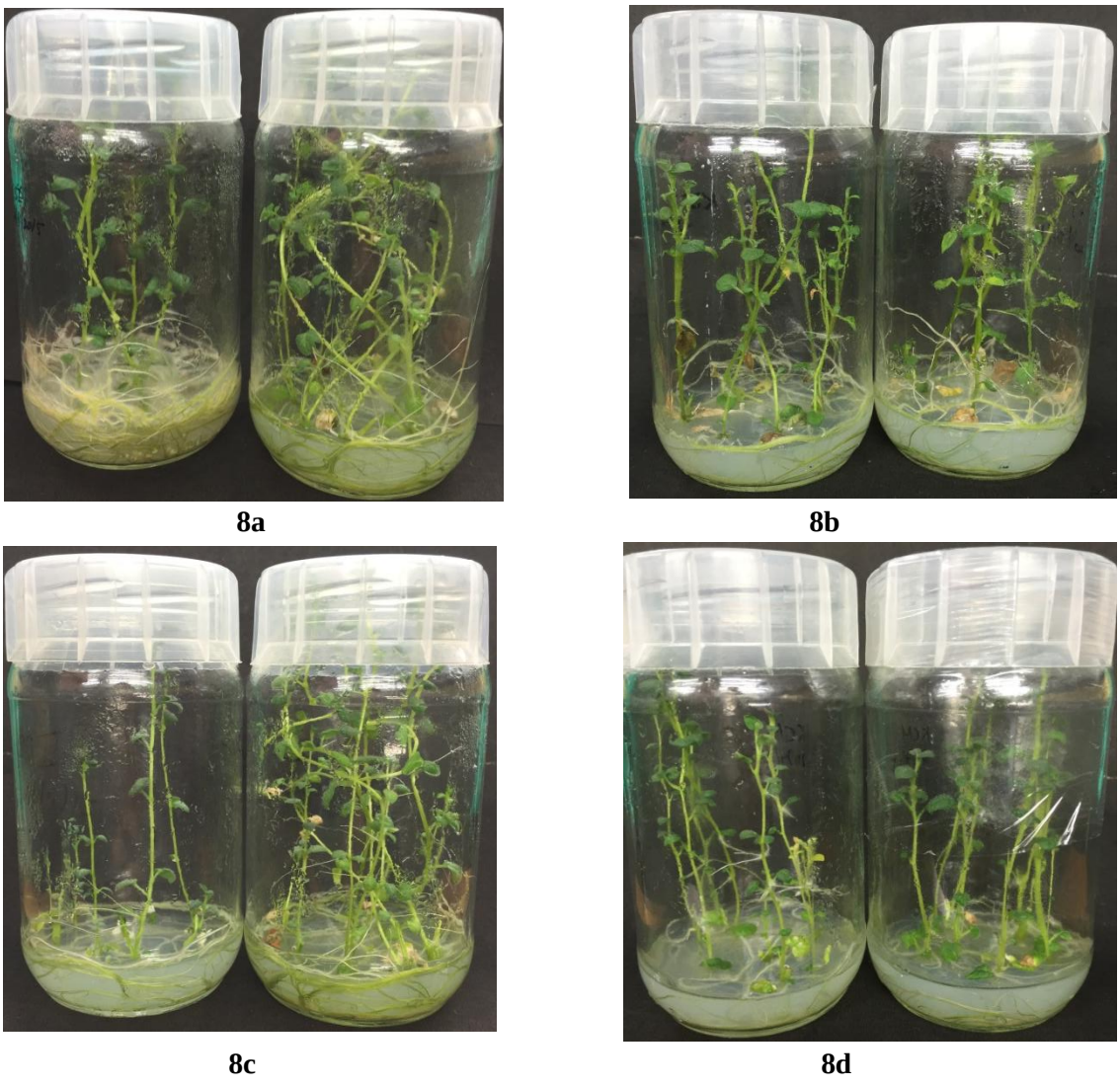


Fig. 7 Multiple sequence alignment of the amino acid sequences of FRK and FRK-like proteins. PS*t*FRK01, PS*t*FRK02 and PS*t*FRK-L refer to deduced polypeptide sequence of GenBank IDs Z12823, JX576230 and JX576279, respectively. This alignment is based on CLUSTAL OMEGA tool. *Dashes* in sequences indicate gaps arising during the alignment, *asterisk* is for common amino acids between PS*t*FRK-L and PS*t*FRK01; *shaded grey* portion refers to identical amino acids between PS*t*FRK-L and PS*t*FRK02; *symbol bullet '•'* indicates common residues between PS*t*FRK01 and PS*t*FRK02 and *symbol solid square '▪'* denotes the consensus residues in all three sequences. The conserved amino acids with phosphate 1, sugar, phosphate 2 and ATP motifs of *St*HXK1 (Menu et al. 2001) are highlighted *pink.*

4.2 Isolation of genomic DNA from different cultivars

Total genomic DNA was isolated from 3 weeks old *in vitro* potato cultures maintained in proper laboratory conditions using method described in 3.2.1. The quality of DNA and band was check using agrose gel electrophoresis and lambda DNA. Both the quality and quantity of the potato genomic DNA preparations were further assessed by nano-drop spectrophotometer.



8a **8b**

8c **8d**

Fig. 8 3-weeks old potato cultures of Kufri Chipsona-1(CS-1) (8a), Kufri Jyoti (KJ) (8b), Kufri Chipsona-2 (CS-2) (8c) and Kufri Chandramukhi (KCM) (8d).

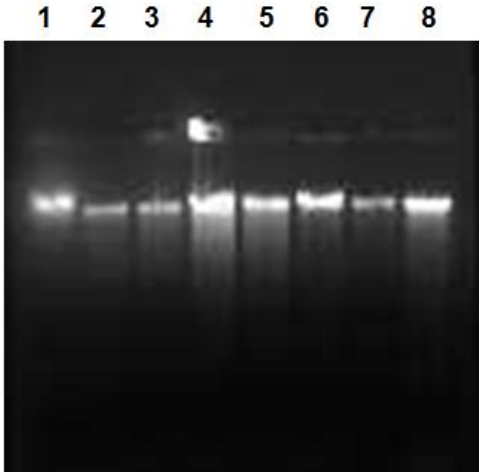


Fig. 9 Agrose gel electrophoresis of genomic DNA preparations from the potato cultivars.
Lane 1- λ DNA; *Lane-2* Kufri Chipsona-1 (CS-1); *Lane-3* Kufri Chipsona-2 (CS-2); *Lane-4* Kufri Jyoti (KJ); *Lane-5* Kufri Chandramukhi (KCM); *Lane-6* Kufri Ashoka (AS); Lane-7 Kufri Pukhraj (PR); Lane-8 Désirée (De)

Table 1 Spectrophotometeric quantification of potato genomic DNAs

Sr. No.	Potato cultivar	Amount of plant material (g)	Volume of DNA soln. (μL)	Ratio of absorbance (A_{260}/A_{280})	Conc. (ng/ μL)
1.	Kufri Chipsona-1	0.94	500	1.62	348.0
2.	Kufri Chipsona-2	0.92	500	1.67	625.7
3.	Kufri Jyoti	0.92	500	1.61	230.3
4.	Kufri Chandramukhi	0.93	500	1.59	545.8

4.3 FRK-specific oligonucleotides primer and analyses of Amplicon profiles between different potato cultivars at varying conditions

Some relevant details about the oligonucleotide primers used in the study: One of the objectives of the study was to study the amplicon profiles using different primer combinations. The individual primer sequences were analyzed by BLAST search, and some relevant details are provided below:

F1A-FK01 (forward primer): BLASTn search revealed that this 20-mer primer not only showed 100% sequence identity with FRK cDNA (GenBank ID: Z12823) of potato but also with number of

other cDNA sequences encoding FRK-like proteins in potato (GENBANK IDs: JX576279, DQ235199, DQ235181) and tomato (GenBank ID: AY325501).

R1-FK606 (reverse primer): Search showed that apart from 100% sequence identity with cDNA of FRK in potato (GenBank IDs: JX576230, Z12823), it also had 100% sequence identity with FRK-like proteins in potato (GENBANK IDs: JX576279, DQ235199, DQ235181).

R2-FK982 (reverse primer): This 20-mer oligonucleotide primer exhibit 100% sequence identity with FRK cDNA (GenBank ID: JK576230), FRK-like cDNA (GenBank IDs: DQ235199, DQ235181), and interestingly also with FrK-2 cDNA sequence in tomato (*Lycopersicon hirsutum*) (GenBank ID: AY325501).

R3-FK1110 (reverse primer): BLASTn search of this particular 20-mer primer suggested that this primer is very unique to a particular form of cDNA encoding FRK (GenBank ID: Z12823) in potato as it showed 100% identity only this FRK. Considering the region from which this primer was designed it is very likely that cDNAs encoding FRK and FRK-like proteins have significant divergence in 3' UTR region.

F2B-FK01 (forward primer): Apart from 100% identity with FRK cDNA (GenBank ID: JX576230) this 20-mer oligonucleotide primer showed nearly 95% sequence identity with some cDNA sequences that encode FRK cDNA in potato (GenBank ID: Z12823) from bases 230 to 248, FRK-like protein in potato (GENBANK IDs: DQ294257, DQ235199, DQ235181), and also with Frk-2 sequence in tomato (GenBank ID: AY325501).

RU-FK373 (reverse primer): This oligonucleotide primer from FRK cDNA (JX576230) did not show any reflection in FRK cDNA in Z128230. But, BLASTn search revealed that this sequence shows 100% sequence identity with FRK-like cDNAs in potato (GENBANK IDs: DQ294257, JX576279, DQ235181).

PCR amplification using FRK-specific primer pairs

Individual PCR was carried out using specific primer pair and potato genomic DNA as template under different annealing temperatures. The amplicons were analyzed by 1.0% agarose gel electrophoresis. The details of PCR are given below:

a) PCR using the primer pair F1A-FK01 and R1-FK606

PCR results revealed multiple band patterns in different potato cultivars. Amplicon profile showed that a DNA band of ~1.6 kb is common in KJ, KCM, AS and De whereas, less intense DNA band of ~0.7 kb was found for CS-1 and CS-2 which were near the expected size of bands i.e., ~0.6 kb. However, CS-2 showed a distinct band of ~1.4 kb which was absent in other cultivars and no amplicon was found in PR.

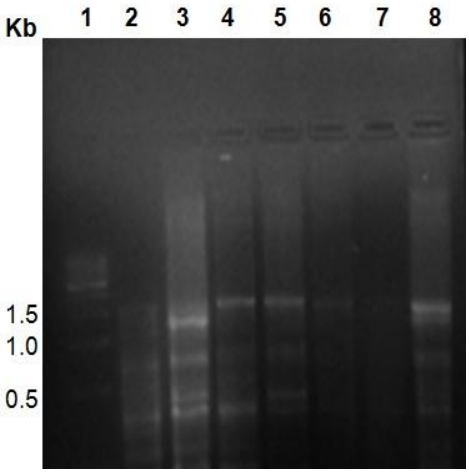


Fig. 10 PCR amplification using the primers, F1A-FK01 & R1-FK606 (annealing temp. 55ºC), and DNA templates from different potato cultivars as indicated lane wise. *Lane 1* 500 bp DNA ladder; *Lane 2* CS-1; *Lane 3* CS-2; *Lane 4* KJ; *Lane 5* KCM; *Lane 6* AS; *Lane 7* PR and *Lane 8* De

Table 2 Sizes of amplicons at annealing temp. of 55ºC (Expected size of the amplicon for the primer pair F1A-FK01 and R1-FK606 is approx. 0.6 kb)

Sr. No.	Potato Variety	Observed band size at annealing temperature 55ºC (in kb)
1.	CS-1	**~0.7**
2.	CS-2	~1.4, **~0.7**
3.	KJ	~1.6
4.	KCM	~1.6
5.	AS	~1.6
6.	PR	Nil
7.	De	~1.6

b) PCR using the primer pair F1A-FK01 and R3-FK1110

PCR results at annealing temperature 50ºC revealed that a DNA band of ~1.2 kb is common for all the potato cultivars. However, the band is more intense in KCM followed by CS-2. Similar band pattern, lesser then the expected size was observed in KJ, AS, PR and De. At annealing temperature 55ºC, PCR products showed distinct and intense band patterns. Bands of sizes ~1.2 Kb and ~0.9 kb were found in all potato cultivars, though the bands for CS-1, CS-2 and De were more intense as compared with other cultivars. Interestingly, it was noted that at both annealing temperatures all the varieties of potato showed at least one band near the expected size i.e., ~1.1 kb. Thus, we can strongly say that this form of FRK is found common in most of the varieties. Bands found below the expected size may be spurious in nature.

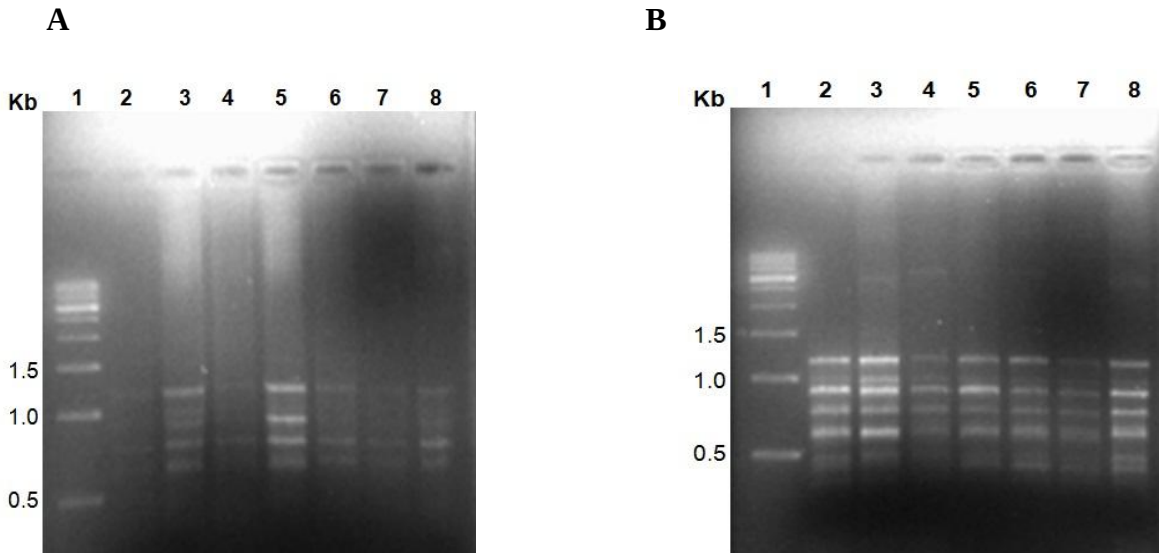


Fig. 11 PCR amplification using the primers, F1A-FK01 & R3-FK1110, and DNA templates from different potato cultivars as indicated lane wise **A** annealing temperature 50ºC *Lane 1* 500 bp DNA ladder; *Lane 2* CS-1; *Lane 3* CS-2; *Lane 4* KJ; *Lane 5* KCM; *Lane 6* AS; *Lane 7* PR and *Lane 8* De. **B** annealing temperature 55ºC *Lane 1* 500 bp DNA ladder; *Lane 2* CS-1; *Lane 3* CS-2; *Lane 4* KJ; *Lane 5* KCM; *Lane 6* AS; *Lane 7* PR and *Lane 8* De.

Table 3 Sizes of amplicons at annealing temp. of 55ºC and 50ºC (Expected size of the amplicon for primer pair F1A-FK01 and R3-FK1110 is approx. 1.1 kb)

Sr. No.	Potato Variety	Observed band size at annealing temperature 50ºC (in kb)	Observed band size at annealing temperature 55ºC (in kb)
1.	CS-1	**~1.2**	~0.9,**~1.2**
2.	CS-2	**~1.2**	~0.9,**~1.2**
3.	KJ	**~1.2**	~0.9,**~1.2**
4.	KCM	**~1.2**, ~0.9	~0.9,**~1.2**
5.	AS	**~1.2**	~0.9,**~1.2**
6.	PR	**~1.2**	~0.9,**~1.2**
7.	De	**~1.2**	~0.9,**~1.2**

c) PCR using the primer pair F2A-FK01 and R3-FK1110

In PCR products at annealing temperature 50ºC, it was detected that in case of CS-1 and KJ there is only one band of ~1.15 kb size, and for the remaining cultivars multiple amplicon bands were observed all

with band of size ~1.15 kb. KCM, AS, PR and De showed similar band patterns of sizes ~1.1 kb, ~1.0 kb and ~0.9kb. However, band intensity was more in KCM and AS. CS-2 showed intense band profile of sizes ~1.05 kb, ~0.9 kb and ~0.8 kb which was very distinct from other cultivars. At annealing temperature 55ºC, amplicon profile showed that AS, PR and De has similar band pattern with band size of ~1.2 kb. However, for CS-1 and KCM no amplicon bands were observed. CS-2 and KJ showed different band pattern from other cultivars. In CS-2, bands of sizes ~1.0 kb, ~0.9 kb, ~0.7kb and ~0.6 kb were seen and for KJ light bands of size ~1.15 kb and ~0.7 kb were observed. We took this primer pair knowingly, that both primers belong to distinct cDNAs, and surprisingly results showed very distinct amplicon profile. Promising amplicon bands were seen at CS-2 at varying conditions. We can suggest that these bands may represent another distinct form of FRK present in CS-2. As both the primers belonged to different cDNAs the expected band size could not be calculated.

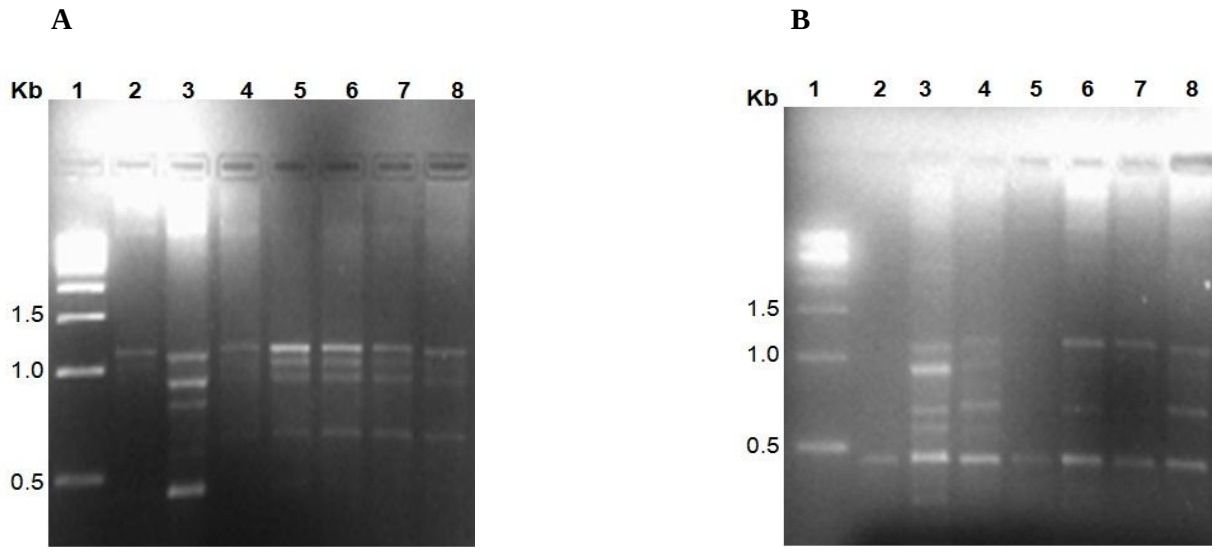


Fig. 12 PCR amplification using the primers, F2A-FK01 & R3-FK1110, and DNA templates from different potato cultivars as indicated lane wise **A** annealing temperature 50ºC *Lane 1* 500 bp DNA ladder; *Lane 2* CS-1; *Lane 3* CS-2; *Lane 4* KJ; *Lane 5* KCM; *Lane 6* AS; *Lane 7* PR and *Lane 8* De. **B** annealing temperature 55ºC *Lane 1* 500 bp DNA ladder; *Lane 2* CS-1; *Lane 3* CS-2; *Lane 4* KJ; *Lane 5* KCM; *Lane 6* AS; *Lane 7* PR and *Lane 8* De.

Table 4 Sizes of amplicons at annealing temp. of 55ºC and 50ºC

Sr. No.	Potato Variety	Observed band size at annealing temperature 50ºC (in kb)	Observed band size at annealing temperature 55ºC (in kb)
1.	CS-1	~1.15	Nil
2.	CS-2	~1.15, ~1.05, ~0.9, ~0.8	~1.0, ~0.9, ~0.7, ~0.6
3.	KJ	~1.15	~1.15, ~0.7
4.	KCM	~1.15, ~1.1, ~1.0, ~0.9	Nil
5.	AS	~1.15, ~1.1, ~1.0, ~0.9	~1.2
6.	PR	~1.15, ~1.1, ~1.0, ~0.9	~1.2
7.	De	~1.15, ~1.1, ~1.0, ~0.9	~1.2

d) PCR using the primer pair F1A-FK01 and RU-FK373

This primer combination also belongs to two different cDNAs and the cross using of primers for PCR amplification at annealing temperature of 55ºC revealed some very unique band pattern again suggesting presence of different isoforms of FRK which are to be further studied. Careful examination of the gel picture revealed similar band pattern of size ~0.7 kb for KJ, CS-2, KCM, AS and PR. Whereas, potato cultivar KJ showed two distinct and intense bands of sizes ~1.7 kb and ~1.2 kb and, single band of size ~1.2 kb was observed for CS-1. No amplicon was found in De.

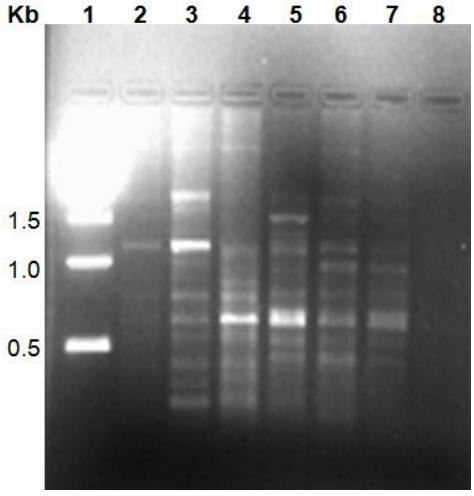


Fig. 13 PCR amplification using the primers, F1A-FK01 & RU-FK373 (annealing temp. 55ºC), and DNA templates from different potato cultivars as indicated lane wise. *Lane 1* 500 bp DNA ladder; *Lane 2* CS-1; *Lane 3* KJ; *Lane 4* CS-2; *Lane 5* KCM; *Lane 6* AS; *Lane 7* PR and *Lane 8* De.

Table 5 Sizes of amplicons at annealing temp. of 55ºC

Sr. No.	Potato Variety	Observed band size at annealing temperature 55ºC (in kb)
1.	CS-1	~1.2
2.	CS-2	~0.7
3.	KJ	~1.7, ~1.2, ~0.7
4.	KCM	~0.7
5.	AS	~0.7
6.	PR	~0.7
7.	De	Nil

e) PCR using the primer pair F2A-FK01 and RU-FK373

PCR gel picture showed that band of size ~1.3 kb is consistent in all varieties of potato. Interestingly, it was seen that only CS-2 showed a distinct band of size ~1.0 kb. Amplicon profile in AS, PR and De seems to be similar in nature. Though, the expected band size of this pair was calculated approx. 0.3 kb, the bands observed were of much bigger sizes indicating genotypic variations between potato varieties.

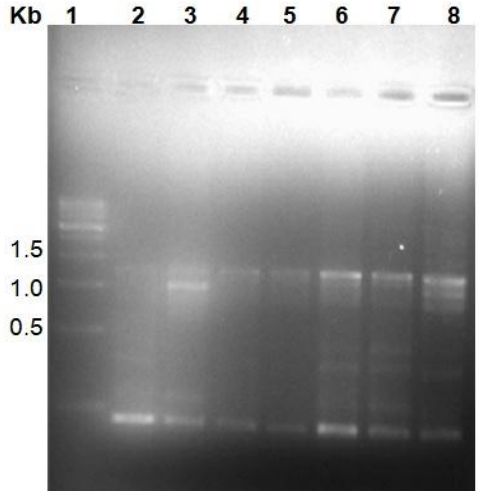


Fig. 14 PCR amplification using the primers, F2A-FK01 & RU-FK373 (annealing temp. 55ºC), and DNA templates from different potato cultivars as indicated lane wise. *Lane 1* 500 bp DNA ladder; *Lane 2* CS-1; *Lane 3* CS-2; *Lane 4* KJ; *Lane 5* KCM; *Lane 6* AS; *Lane 7* PR and *Lane 8* De.

Table 6 Sizes of amplicons at annealing temp. of 55ºC (Expected size of the amplicon for primer pair F2A-FK01 and RU-FK373 is approx. 0.3kb)

Sr. No.	Potato Variety	Observed Band size at annealing temperature 55ºC (in kb)
1.	CS-1	~1.3
2.	CS-2	~1.3, ~1.0
3.	KJ	~1.3
4.	KCM	~1.3
5.	AS	~1.3
6	PR	~1.3
7.	De	~1.3

Efforts were made to use R2-FK982 as reverse primer in some PCR using the primer either F1A-FK01 or F2A-FK01, but no amplicon was found in these cases. It may be likely that the primer R2-FK982 corresponds to exon-intron boundary region of FRK gene.

Concluding Remarks

Fructokinase belongs to hexose kinases enzyme family, which catalyze the phosphorylation of fructose that is either fed in carbohydrate metabolic pathways or in energy production. To prevent feedback inhibition of sucrose synthase in starch synthesis, fructokinase plays a very crucial role in tuber development in potato. The biochemical and molecular attributes of this enzyme have been studied at both cDNA and enzymatic levels but with regard to structure and function of FRK genes very little progress has been made till to date. Also, no comprehensive report is available with regard to FRK isoforms and their expression patterns in different potato tissues, particularly in the different stages of tuber development. In this context, this study is quite relevant. The sequence analyses and comparison of different forms of fructokinases at both nucleotide and amino acid levels gave a clear and comprehensive idea about the sequence relatedness between them. Moreover, it helped to know about the salient sequence features. Some conserved amino acids were found by comparing between FRKs and HXK. Such type of information is not only helpful in understanding the evolutionary consequences but also important with regard to gene manipulation and protein engineering. In this study, the good quality genomic DNA was isolated from different commercially important Indian potato cultivars, and both quality and quantity were assessed by spectrophotometric analysis. Use of different FRK-specific primers helped not only to distinguish genotypic variations between these cultivars, some of the cultivar-specific amplicons could be further studied in detail at molecular level. It is likely that some of the amplicons may correspond to some of the allelic variants of the FRK gene in potato which are still not known. In conclusion, this report made a consolidated base for further studying structure and function of FRK genes in potato.

REFERENCES

Anich M, Fanta N, Mancilla M, Kettlun AM, Valenzuel MA and Traverso-Cori A (1990) Apyrase activity and changes in metabolites during germination and tuberization. Phytochemistry 29:1411-1415

Appeldoorn NJ, Sergeeva L, Vreugdenhil D, VanDerPlas LH and Visser RG (2002) *In-situ* analysis of enzymes involved in sucrose to hexose-phosphate conversion during stolon-to-tuber transition of potato. Physiol Plant 115:303–310

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

Bar-Peled M and O'Neill MA (2011) Plant nucleotide sugar formation, interconversion, and salvage by sugar recycling. Annu. Rev. Plant Biol. 62:127–155

Beukema HP and Vander Zaag DE (1979) Introduction to Potato Production. PUDOC Wageningen The Netherlands: 76

Ca´rdenas ML, Cornish-Bowden A and Ureta T (1998) Evolution and regulatory role of the hexokinases. Biochim. Biophys. Acta 1401:242–264

Chia T, Thorneycroft D, Chapple A, Messerli G, Chen J, Zeeman SC, Smith SM and Smith AM (2004) A cytosolic glucosyl transferase is required for conversion of starch to sucrose in Arabidopsis leaves at night. Plant Journal 37: 853–863

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

Colowick SP and Kalckar HM (1943) The role of myokinase in transphosphorylations. I. The enzymatic phosphorylation of hexoses by adenyl pyrophosphate. J. Biol. Chem. 148:117–126

Copeland L and Morell M (1985) Hexose kinases from the plant cytosolic fraction of soybean nodules. Plant Physiol 79:114–117

Cosio E and Bustamante E (1984) Subcellular localization of hexokinase in pea leaves. Evidence for the predominance of a mitochondrially bound form. J. Biol. Chem. 259:7688–7692

Dai N, Kandel M, Petreikov M, Levine I, Ricard B, Rothan C, et al. (2002a) The tomato hexokinase LeHXK1: cloning, mapping, expression pattern and phylogenetic relationships. Plant Sci. 163:581–590

Dai N, Schaffer AA, Petreikov M and Granot D (1995) *Arabidopsis thaliana* hexokinase cDNA isolated by complementation of yeast cells. Plant Physiol. 108:879–880

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

Damari-Weissler H, Rachamilevitch S, Aloni R, German MA, Cohen S, Zwieniecki MA et al. (2009) LeFRK2 is required for phloem and xylem differentiation and the transport of both sugar and water. Planta 230:795–805

Davies H, Louise V, Burrell M, Carrari F, Urbanczyk W, Leisse A, Hancock R, Taylor M, Viola R, Ross H, McRae D, Willmitzer L, and Fernie A (2005) Modulation of Fructokinase Activity of Potato *(Solanum tuberosum).* Plant Cell Physiol 46: 1103–1115

Dennis DT and Blakeley SD (2000) "Carbohydrate metabolism," in Bio-chemistry and Molecular Biology of Plants, eds BB Buchanan, W Gruissem and R.L.Jones (Rockville, MD: American Society of Plant Physiologists) 676–728

Dodds, JH, Huaman Z and Lizarraga R (1962) Potato germplasm conservation. In: J.H. Dodds (Ed.), *In vitro* methods for conservation of plant genetic resources. Chapman & Hall,London: 93-109

Doolittle RF (1989) Redundancies in protein sequences. In: Fasman GD (ed) Prediction of protein structure and the principles of protein conformation. Plenum Press, New York: 599–623

Dry IB, Nash D and Wiskich JT (1983) The mitochondrial localization of hexokinase in pea leaves. Planta 158:152–156

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

Gancedo JM (1992) Carbon catabolite repression in yeast. Eur. J. Biochem. 206:297–313

Gangadhar BH, Yu JW, Sajeesh k and Park SW (2014) A systematic exploration of high-temperature stress-responsive genes in potato using large-scale yeast functional screening. Mol Genet Genomics 289:185–201

Gardener A, Davies H and Burch L (1992) Purification and properties of fructokinase from developing tubers of potato *(Solanum tuberosum* L.*).* Plant Physiol. Vol. 100:178–183

German MA, Dai N, Matsevitz T, Hanael R, Petreikov M, Bernstein N, et al (2003) Suppression of fructokinase encoded by LeFRK2 in tomato stem inhibits growth and causes wilting of young leaves. *Plant J.* 34:837–846

Gerrits N, Turk SC, vanDun KP, Hulleman SH, Visser RG, Weisbeek PJ, et al (2001) Sucrose metabolism in plastids. Plant Physiol 125:926–934

Goren S, Huber SC and Granot D (2011) Comparison of a novel tomato sucrose synthase, SlSUS4, with previously described SlSUS Isoforms reveals distinct sequence features and differential expression patterns in association with stem maturation. Planta 233:1011–1023

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

Granot D, Kelly G, Stein O and Schwartz R (2014) Substantial roles of hexokinase and fructokinase in the effects of sugars on plant physiology and development. Journal of Experimental Botany 65:809–819

Granot D (2007) Role of tomato hexose kinases. Funct. Plant Biol. 34:564–570

Granot D (2008) Putting plant hexokinases in their proper place. Phytochemistry 69:2649–2654

Jang JC and Sheen J (1994) Sugar sensing in higher plants. Plant Cell 6:1665–1679

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

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

Kanayama Y, Dai N, Granot D, Petreikov M, Schaffer A and Bennett AB (1997) Divergent fructokinase genes are differentially expressed in tomato. Plant Physiol 113:1379–1384

Kanayama Y, Granot D, Dai N, Petreikov M, Schaffer A, Powell A, et al. (1998) Tomato fructokinases exhibit differential expression and substrate regulation. Plant Physiol 117:85–90

Kang Y, Outlaw WH Jr., Andersen PC and Fiore GB (2007) Guard-cell apoplastic sucrose concentration-a link between leaf photosynthesis and stomatal aperture size in the apoplastic phloem loader Vicia faba L. Plant, Cell & Environment 30:551–558

Karni L and Aloni B (2002) Fructokinase and hexokinase from pollen grains of bell pepper (*Capsicum annuum* L.): possible role in pollen germination under conditions of high temperature and CO2 enrichment. Ann. Bot. 90:607–612

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

Kelly G, Moshelion M, David-Schwartz R, Halperin O, Wallach R, Attia Z, Belausov E and Granot D (2013) Hexokinase mediates stomatal closure. The Plant Journal 75:977–988

Kooch KE (1996) Carbohydrate-modulated gene expression in plants. Annu.Rev. Plant Physiol. Plant Mol. Biol. 47: 509–540

Kruger NJ (1990) Carbohydrate synthesis and degradation. In Plant Physiology, Biochemistry and Physiology (Dennis, D.T. and Turpin, D.H., eds): 59–76, Longmann Scientific

Kumari V, Bansal A, Aminedi R, Taneja D and Das N (2012) Simplified extraction of good quality genomic DNA from a variety of plant materials. African J Biotech 11: 6420-6427

Medina A and Sols A (1956) A specific fructokinase in peas. Biochim. Biophys. Acta 19:378–379

Menu T, Rothan C, Dai N, Petreikov M, Etienne C, Destrac-Irvine A, et al. (2001) Cloning and characterization of a cDNA encoding hexokinase from tomato. PlantSci 160:209–218

Meyerhof O (1927) U¨ ber die enzymatische Milchsa¨urebildung im Muskelextrakt. III. Mitteilung: Die Milcha¨urebildung aus den ga¨rfa¨higen Hexosen. Biochem. Z. 183:176–215

Meyerhof O, (1935) U¨ ber die Wirkungsweise der Hexokinase. Naturwissenschaften 23:850–851

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

Millerd A, Bonner J, Axelrod B and Bandurski R (1951) Oxidative and phosphorylative activity of plant mitochondria. Proc. Natl. Acad. Sci. U.S.A. 37:855–862

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

Odanaka S, Bennett A and Kanayama Y (2002) Distinct Physiological Roles of Fructokinase Isozymes Revealed by Gene-Specific Suppression of Frk1 and Frk2 Expression in Tomato. Plant Physiol 129:1119-1126

Outlaw WH (2003) Integration of cellular and physiological functions of guard cells. Critical Reviews in Plant Sciences 22:503–529

Pego JV and Smeekens SC (2000) Plant fructokinases: a sweet family get-together. Trends in Plant Science 5:531–536

Plaxton WC (1996) The organization and regulation of plant glycolysis. Annu. Rev. Plant Physiol Plant Mol. Biol. 47:185–214

Purich DL, Fromm HJ and Rudolf FB (1973) The hexokinases: kinetic, physical and regulatory properties. Adv Enzymol 39:249-326

Renz, A, Merlo L and Stitt M (1993) Partial purification from potato tubers of three fructokinases and three hexokinases which show differing organ and developmental specificity. Planta 190:156–165

Saltman P (1953) Hexokinase in higher plants. J. Biol. Chem. 200:145–154

Schaffer AA and Petreikov M (1997a) Inhibition of fructokinase and sucrose synthase by cytosolic levels of fructose in young tomato fruit undergoing transient starch synthesis. Physiol Plant 101:800–806

Schaffer AA and Petreikov M (1997b) Sucrose-to-starch metabolism in tomato fruit undergoing transient starch accumulation. Plant Physiol 113:739–746

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

Sindelarova M and Sindelar L (1988) Hexokinases of tobacco leaves: subcellular localization and characterization. Biol. Plant 30:275–284

Smith AM, Zeeman SC and Smith SM (2005) Starch degradation. Annual Review of Plant Biology 56:73–98

Smith S, Taylor M, Burch L and Davies H (1993) Primary Structure and Characterization of a cDNA Clone of Fructokinase from Potato (*Solanum tuberosum* cv record). Plant Physiol 102:1043

Struik PC and Wiersema SG (1999) Seed Potato Technology. Wageningen Press Wageningen 383

Tanner GJ, Copeland L and Turner JF (1983) Subcellular localization of hexose kinases in pea stems: mitochondrial hexokinase. Plant Physiol 72:659–663

Taylor MA, Ross HA, Gardner A and Davies HV (1995) Characterization of a cDNA encoding fructokinase from potato (*Solanum tuberosum* L.). J. Plant Physiol 145:253–256

Thelander M, Nilsson A and Ronne H (2009) "Carbon and energy metabolism, " in The Moss Physcomitrella Patens, eds C.Knight, P.F. Perroud, and D. Cove(Ames, IA: Blackwell) 211–245

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

Vargas WA, Pontis HG and Salerno GL (2008) New insights on sucrose metabolism: evidence for an active A/N-Inv in chloroplasts uncovers a novel component of the intracellular carbon trafficking. Planta 227:795–807

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

VonEuler H, Adler E (1935) U¨ ber die Komponenten der Dehydrasesysteme. 6. Dehyrierung von Hexosen unter Mitwirkung von Adenosintriphosphorsa ¨ure. Z. Physiol. Chem. 235:122–153

Wu G, Park MY, Conway SR, Wang JW, Weigel D and Poethig RS (2009) The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell 138:750–759

Yang L, Conway SR and Poethig RS (2011) Vegetative phase change is mediated by a leaf- derived signal that represses the transcription ofmiR156. Development 138:245–249

Yang L, Xu M, Koo Y, He J and Poethig RS (2013) Sugar promotes vegetative phase change in Arabidopsis thaliana by repressing the expression of MIR156A and MIR156C. eLife 2

Yu S, Cao L, Zhou C-M, Zhang T-Q, Lian H, Sun Y, Wu J, Huang J, Wang G and Wang JW (2013) Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. eLife 2