Molecular studies on the starch-granule-bound protein (R1 protein) and genetic approaches for its inhibition in the potato tubers

A Ph.D. thesis

Submitted in fulfillment of the requirement

for the award of the degree of

Doctor of Philosophy

In

Biotechnology

By

Anshu Bansal

Regn. No. 9031002

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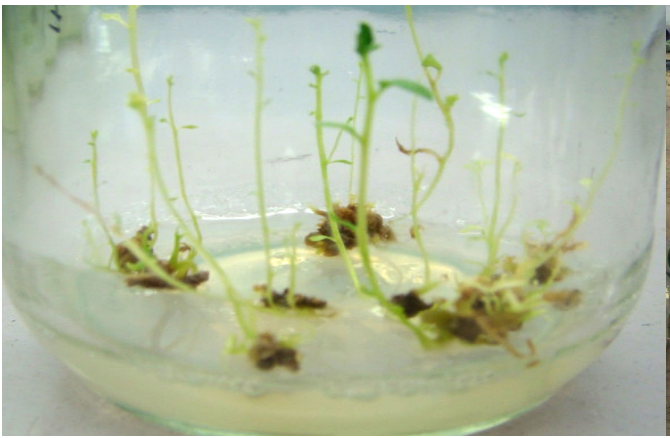
INDIA



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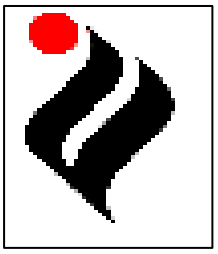

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November 2011

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Certificate

Certified that the thesis **"Molecular studies on the starch-granule-bound protein (R1 protein) and genetic approaches for its inhibition in the potato tubers"** which is submitted by the **Mr. Anshu Bansal**, in fulfillment of the requirement for the award of the degree of Doctor of Philosophy in the Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala is a record of the candidate's independent and original research work carried out by him under my supervision and guidance. The matter embodied in this thesis work has not been submitted in part or full to any other University or Institute for the award of any degree.

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Declaration

I hereby declare that the work which is being present in this thesis **"Molecular studies on the starch-granule-bound protein (R1 protein) and genetic approaches for its inhibition in the potato tubers"** submitted by the undersigned for the award of the degree of Doctor of Philosophy in the Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala, is true record of my own independent and original research work carried out under the supervision of Dr. Niranjan Das, Associate Professor, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University Patiala, Punjab, India. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or Abroad.

Date:

Place: Patiala

Anshu Bansal

(ANSHU BANSAL)

Dedicated to my parents

ACKNOWLEDGMENT

I thank the almighty whose blessings have enabled me to accomplish my thesis work successfully.

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Abbreviations

%	Percent
bp	Base pair
DNA	Deoxyribonucleic acid
FW	Fresh weight
g	Gram
hr	Hour
kb	Kilobase
L	Litre
M	Molar
min	Minute
mL	Mililitre
mM	Milli molar
nm	Nanometer
ºC	Degree centigrade
OD	Optical Density
pH	Hydrogen ion concentration (minus log of)
RNA	Ribonucleic acid
rpm	Rotation per minute
s	Second
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
µg	Microgram
µL	Microlitre

Introduction

1. Introduction

The potato (*Solanum tuberosum* L.) is one of the most important food crops capable of nourishing the world's vast population. Rank-wise, potato comes only after rice, wheat and maize in world food production. The cultivated potato varieties are usually heterozygous and autotetraploid (2n=4x=48) (Ramanna et al. 1979). Morphologically, potato tubers are modified stems (grown underground). The initiation and development of potato in the plant is known as 'tuberization' which is the result of the activity of several interdependent processes; for example, stolon initiation and elongation, subapical swelling, cell division, storage starch biosynthesis along with accumulation of some specific proteins are a few of the contributing processes. The potato is one of the priority crops in our country. Potato tubers serve as important carbohydrate resource for human nutrition, varieties of processed products for human consumption, feed, and renewable resources for industrial uses. Starch is the major component of the dry matter accounting for approximately 70% of the total solids. The other components include total sugars, proteins, fibres, lipids, riboflavin, niacin, vitamin A, vitamin C, minerals, ash, and a very small amount of glycoalkaloids (antinutritional factor). However, harmful glycoalkaloids are within the permissible limit of human consumption in most of the present day potato varieties. Various studies have shown that 100 g of freshly harvested boiled potato with intact skin can nearly 50% of an adult's daily vitamin C requirement. The biological value of potato protein is also very high. Potato is a low energy food because it contains low fat (< 0.1%) and calorie. Taken together, the potato is a highly nutritious, easily digestible and wholesome food.

Most of the major food crops (cereals) are propagated through seeds (products of sexual hybridization). But potato plants are vegetatively propagated through tubers (also known as

'seed tubers' or 'seed potatoes'). This mode of propagation is also known as asexual or vegetative propagation with the following advantages and disadvantages. The main advantage is that a good potato clone can be maintained with high degree of genetic purity. The major disadvantage is that the tubers may accumulate progressively deadly viruses and seed-borne pathogens resulting in the gradual degeneration of a clone. Potato plants can also be propagated through botanical seeds, also known as True Potato Seeds (TPS). TPS is an alternative means of propagation in some areas where seed potato production is not feasible or economic. A potato plant tuberizes under the following environmental conditions: short-day photoperiod and cool night temperature.

In the sub-tropical Indo-Gangetic plains, potatoes are grown in winter season; however, they are also grown in Indian hills during summer. In India, the potato is regarded as short-duration crop with an average growing period of 90-100 days. On the contrary, the potato growing conditions in temperate countries of Europe and North America are very much different from those in Indian subcontinent. A number of processing varieties of potato have been developed in the temperate countries for long-day (about 14-16 hours photoperiod) conditions and long crop duration of 120-180 days. Therefore, these European and American varieties are not suitable to the different agro-climatic conditions of our country. There are more than 40 high-yielding Indian potato cultivars developed through conventional breeding during the last few decades. These potato cultivars vary with regard to maturation time, genetic make up, disease resistance, tuber dry matter, and sugar content. Most of these varieties are not suitable for processing. The varieties meant for processing should have the following parameters: yield 25-30 t/ha; dry matter content 22-23%; reducing sugar level below 250 mg/100 g fresh tuber weight. Only a few of the Indian processing varieties more or less fulfill the above criteria.

In the temperate countries, potato harvest is followed by severe winter. On the contrary, the potato harvest in the plains of India is followed by hot summers. Again post-harvest preservation is a problem because rising temperatures result in sprouting of tubers, weight loss, shrinkage and also tuber rotting. Therefore, in order to avoid sprouting and other undesirable processes during summers potatoes are stored in refrigerated cold stores at ~ 4^0C. The prolonged storage of tubers at such low temperatures leads to the accumulation of reducing sugars-an undesirable process known as 'cold-induced sweetening' or 'Low-temperature sweetening'. Varietal differences exist with regard to the level of reducing sugars during low temperature storage between the cultivars. However, this undesirable phenomenon is more or less a common attribute in almost all the cultivars.

During the last few decades, potato became an important model crop both in terms of basic and applied research as we see its relevance and importance in the current trend of research activities. For example, biochemical and molecular studies on tuber initiation and development, various aspects of starch metabolism both in leaves and somatic storage tissues such as tubers and the involvement of different enzymes, tuber-specific gene expression patterns, and source-sink relationships are, in fact, some of the very fascinating areas of basic research. The other areas such as molecular biology of potato tubers on disease control, starch modifications, improving nutritional quality, repression of cold-sweetening in potato tubers, using potato tubers as factories for production of novel protein represent some of the aspects of applied research in potato (reviewed in Zeeman et al. 2010). Keeping in view of the objectives of this thesis work a few selected aspects of starch metabolism are discussed in the following sections.

Starch biosynthesis: In the leaves, transitory starch metabolism represents a very important cellular process for plant growth and development. Similarly, storage starch metabolism during tuber development and storage is equally important for potato life cycle. Starch, the predominant storage carbohydrate, is synthesized in the amyloplasts of potato tubers; whereas in the leaves, transitory starch is synthesized in the chloroplasts. Starch is widely used in food, feed and different industrial applications. Studies on biotechnological modification of starch are fascinating research areas in the area of plant molecular biology/biotechnology. Starch consists of different polymers of D-glucose arranged into three dimensional, semicrystalline structures - the starch granules (Preiss 1988). The formation of starch granules in plants provides a simple model to study the ordered three dimensional structures of polysaccharides. Starch can be chemically fractionated in to two types of glucan polymers: amylose and amylopectin. Amylose consists of predominantly linear chains of α(1→4)-linked glucose residues, each approximately 1000 residues long. Amylose is usually linear with very less branching pattern. Amylose content usually falls in the range of 20-30% of total starch. Amylopectin, which consists of highly branched glucan chains, makes up approximately 70% of starch. Chains of roughly 20 α(1→4)-linked glucose residues joined by α(1→6) linkages to other branches. The branches themselves form an organized structure. Various evidences suggest that starch biosynthesis in plants requires ADP-glucose and the following enzymes: ADP-glucose pyrophosphorylase (ADPGPPase, EC 2.7.7.23), starch synthases (SS; EC 2.4.1.21), starch branching enzymes (SBEs; EC 2.4.1.28) and starch debranching enzymes (DBEs; EC 3.2.1.68 and EC 3.2.1.142). ADPGPPase catalyzes the condensation of glucose-1-phosphate with ATP to form ADP-glucose (an activated nucleotide sugar). SS then transfers glucose residues from ADP-glucose to the non-reducing end of preexisting starch molecules

that act as primers. In plants, starch synthases are encoded by five gene classes: GBSS (granule-bound starch synthase), SSI, SSII, SSIII, and SSIV. GBSS binds tightly to starch granule and is responsible for amylose synthesis. Again, GBSS has two isoforms, GBSSI and GBSSII. GBSSI [GBSS, starch granule-bound ADP (UDP) glucose: α-1, 4-D-glucan 4-α-glucosyl-transferase, EC 2.4.1.21], a protein of nearly 60 kDa, is bound exclusively to the starch granule and responsible for the biosynthesis of amylose fraction of starch in higher plants, and also contributes to the elongation of amylopectin chains (Denyer et al. 2001). Multiple allelism of different genes is known to affect the morphological aspects and metabolic pathways. The constitutive and tissue-specific expression of different isoforms of GBSSI and GBSSII affect the overall amylose content and the quality of starch granules (van de Wal et al. 2001). GBSS is known to be conserved in higher plants. The other SS isoforms generate the chains in amylopectin and are either soluble in the plastid stroma; or partly soluble and partly associated with the starch granules. The α(1→6) branches of amylopectin polymers are made by SBE. In plant storage organs like potato tuber, starch is synthesized in plastids called amyloplasts. In this case, ATP is required to be imported from the cytosol, probably by an ADP/ATP translocator. Where as glucose-1-phosphate may be directly imported from the cytosol (Tyson et al. 1988) or synthesized in the plastid from glucose-6-phosphate via the action of plastidial phosphoglucomutase (Hill and Smith 1991).

Phosphorylated starch: Starch phosphorylation is an important aspect of starch metabolism. It represents a naturally occurring covalent modification of starch. Starches extracted from different plant species are almost all phosphorylated (Blennow et al. 2000). It has been demonstrated that the introduction of covalently bound phosphate in potato starch proceeds concurrently with the *de novo* synthesis of the polysaccharide chains (Nielsen et al. 1994) and

confined mainly to the amylopectin fraction (Jane et al. 1996). *In vitro* biosynthesis of phosphorylated starch was also shown in the intact amyloplast (Wischmann et al. 1999). Starches from potato tubers and other tuber crops are characterized by a relatively high content of phosphorus in comparison to cereal starches (Tabata et al. 1975). The phosphorus in potato starch is present primarily as phosphate esterified to the glucose residues of the starch. Small starch granules contain 25% more ester-bound phosphate per glucose residue than large starch granules. The majority of the phosphate is bound to the amylopectin fraction of the starch, whereas the extent of phosphorylation of amylose is less significant (Hizukuri et al. 1970). Approximately 60-70% of the phosphate groups are linked to C-6 of the glucose residues, the rest to C-3 positions of glucosyl residues, and only 1% may be linked to C-2. In potato tuber starch, approx. 0.1 - 0.5% of the glucose moieties are found to be phosphorylated. Esterified phosphate groups were also found in glycogen (the animal, fungal and bacterial analogue of starch) suggesting that phosphorylation is a general characteristic for the storage α-glucan metabolism in all classes of organisms (Lomako et al. 1993). Based on the molecular model of phosphorylated starch it is now believed that the free C-3 and C-6 hydroxyl groups of the glucose units are both located at the hydrophilic surface of the double helix. It means that the phosphate groups will align with or protrude from the helix surface which might affect the stability of helices or side-by-side packing of the helices and hence the crystallinity of the starch. The effects of starch phosphorylation on starch granule crystallinity have been shown by differential scanning calorimetry (DSC) and by X-ray crystallography. Moreover, it was also predicted that the observed effects of starch phosphates on starch granule crystallinity result more from C-3 phosphorylation than from C-6 phosphorylation. The level of starch phosphorylation was shown to vary between the starches from different plant species. The

level of phosphate groups in starch influences various physico-chemical properties such as hydration capacity of starch paste after gelatinization, starch-paste peak viscosity and gel-forming capacity. In other words, the level of phosphorylation affects starch functionality for different industrial uses.

During the last more than one decade, many laboratories have been working to understand the pathways by which starch is synthesized and degraded in plants; because clear understanding can only facilitate the improvement of starch crops for both food and nonfood uses. Various factors are believed to control starch metabolism and the structure of starch granules. All these factors vary between the plant species, even between the different tissues like leaves, roots, seeds and tubers. Starch biosynthesis is relatively more understood than the degradation aspects. Literature survey clearly indicates that there has been a considerable progress recently in understanding starch degradation, particularly transitory starch degradation in leaves and storage starch degradation in cereal endosperms. But our current knowledge on storage starch degradation in tubers, roots and noncereal seeds is still inadequate (reviewed in Smith et al. 2005 and Zeeman et al. 2010). Starch phosphorylation is a prerequisite for its degradation. Literature review section mainly focuses on the recent progress in the phosphorylation and degradation aspects of starch metabolism.

Literature Review

&

Objectives

2. Literature Review

2.1 ***Discovery of R1 protein in potato:*** Although starch phosphate esters were known for a long period of time, the biochemical mechanism of starch phosphorylation and its relevance with regard to starch metabolism remained completely unknown until recently (reviewed in Blennow et al. 2002). The discovery of starch-granule-bound protein, named as R1 protein by Lorberth et al. (1998) was a breakthrough in elucidating the enzymatic mechanism for starch phosphorylation. During isolation and characterization of various starch granule associated proteins, they were able to isolate the above protein. For this purpose, they raised antisera against starch-bound proteins which were subsequently used to screen potato tuber cDNA expression library corresponding to the potato cultivar Desiree. Based on the immunological technique, this research group isolated and characterized a 4851 bp cDNA (GenBank Acc No Y09533) encoding a protein, R1 protein, later renamed as **α-glucan, water dikinase (GWD)**. Experimental evidence suggested that it was partially localized on starch granules and involved in the phosphorylation of starch. The entire predicted polypeptide consisted of 1464 amino acids. The presence of transit peptide (amino acids 1-77) was observed by them by the N-terminal sequencing of the mature protein. The calculated molecular weight of the mature protein was ~155.0 kDa. The close inspection of the amino acid sequence revealed that the C-terminal part of the GWD (amino acids 1267-1464) shows considerable homology to bacterial phosphoenolpyruvate (PEP) synthase (amino acids 156-328) from *Pyrococcus furiosus*, which transfers phosphate from ATP in a dikinase reaction to pyruvate and water.

2.2 ***Biochemical and molecular studies on R1 protein (GWD):*** Apart from isolation and characterization of GWD protein, Lorberth et al. (1998) also generated the transgenic potato lines through antisense RNA technology using GWD cDNA which showed reduced GWD

expression level both in the tubers and leaves. In the transgenic lines, the starch-bound phosphate content was drastically reduced by 10%-50% as compared to the wild type (cv. Desiree) starches from both leaf and tubers, indicating that this protein is crucial for starch phosphorylation. Moreover, the reduction of the GWD protein in transgenic potato plants had also led to a starch excess phenotype in the leaves; and cold-induced sweetening was prevented in the tubers, both indicative of reduced rate of starch degradation. Starch degradation was reduced significantly in the above transgenic tubers as evident by approx. nine-fold lowering of reducing sugar level as compared to the wild type tubers even after two months of storage at 4ºC. In order to see the possible involvement of GWD in starch phosphorylation, the full size GWD cDNA was expressed in *E. coli* and its effect on the phosphorylation of glycogen at C-6 position was measured. Interestingly, it was found that the approximately one glucose unit out of 1600 was phosphorylated in presence of GWD as compared to the control *E. coli* extract where only one glucose unit in 4600 was phosphorylated. Therefore, heterologous expression of the GWD in *E. coli* led to increased phosphate content in the glycogen.

The reversible binding of R1 protein to assimilatory starch granules using the recombinant R1 protein was explained by Ritte et al. (2000a). The R1 protein binds more effectively to the native starch granules isolated from darkened leaves as compared to the illuminated potato leaves when incubated with recombinant R1 protein. The data clearly suggested that changes in the properties of starch granule surface depending upon the metabolic state of cells, affects the binding of R1 protein. Ritte et al. (2000b) analyzed the leaf starch proteins and tuber starch proteins that the major portion of the total proteins located at the surface of leaf starch granule as compared to surface of tuber starch granule. R1 exists in three different states;

enclosed inside starch granules; associated with granule surface; and soluble in the plastids in both leaves and tubers of potato. The R1 protein was located at the surface of the starch granule in assimilatory starch (isolated from darkened leaves) but occurs predominantly in an internalized form in the starch from growing tubers. As reported earlier by Lorberth et al. 1998, R1 protein involved in starch phosphorylation, there must be a positive correlation between R1 and phosphate content in the plant tissue but in case of dry seeds of pea, maize, barley, and mature banana fruits, R1 was present whereas covalently bound phosphate was present in very low amounts or not detectable. However, turmeric starch was found to be highly phosphorylated but R1 was not detected immunologically. Therefore, analysis of various starch containing tissues from a variety of plant species did not reveal a positive correlation between the R1 level and starch phosphate content. The immunological detection of R1 homologues in various plants tissues i.e. tubers of sweet potato and yam, seeds of maize and barley, and banana fruits revealed that R1 is ubiquitous and exerts general function throughout the plant kingdom, even though some plants (e.g. maize and barely) synthesize storage starch with a low or undetectable phosphate content.

In order to further substantiate the early notions on starch phosphorylation, the R1 (GWD) protein was further expressed in *Escherichia coli* and purified to apparent homogeneity. Incubation of the purified protein with various phosphate donor and acceptor molecules showed that GWD phosphorylates the glucosyl residues of α-glucans at both the C-6 and the C-3 positions in a ratio similar to that occurring naturally in starch (Ritte et al. 2002). They demonstrated that phosphorylation occurs in a dikinase-type reaction in which three substrates, a α-polyglucan, ATP, and water are converted into three products, an

α-polyglucan-P, AMP, and orthophosphate. The use of ATP radiolabeled at either the γ or β position showed that solely the β phosphate is transferred to the α-glucan.

Ritte et al. (2003) developed the protocol for quantification of α-glucan, water dikinase (GWD) activity in crude plant extracts by radio-labeling assay using soluble starch, and ^{33}P-labeled ATP at β-position as phosphate acceptor and donor, respectively. The maximum rate of starch phosphorylation was 27 pmol $min^{-1}mg^{-1}$ protein found in extracts from leaves of wild type *Arabidopsis thaliana*. No significant incorporation of phosphate was observed with leaf extracts from GWD deficient mutants whereas less activity was found in leaf extract from transgenic potato lines expressing antisense constructs as compared to the wild type. The R1 protein phosphorylates itself with β phosphate when incubated with radio labeled ATP in the absence of α-glucan. The autocatalytic phosphorylation of R1 supports the occurrence of a phosphohistidine intermediate (containing the β-P) as reported for both PEP-synthase and PPDK. In the potato R1 protein and *Arabidopsis* SEX1, a conserved stretch of amino acids similar to the phosphohistidine domains of PEP synthase and PPDK had been identified and site directed mutagenesis of the histidine residue (at 992 of GWD His to Ala) resulted in an inactive enzyme and loss of autophosphorylation (Mikkelsen et al. 2004).

Ritte et al. (2004) showed that starch phosphorylation occur concurrently with starch biosynthesis during photosynthesis in *Chlamydomonas* cells without detectable turnover. However phosphorylation occurs at an even higher rate during the transitory starch breakdown. Phosphorylation of the *Chlamydomonas* starch under the conditions of net starch breakdown is transient, as it is subjected to a substantial turnover. Probably, this is due to the fact that both phosphorylation and degradation take place at the granule surface. During the starch degradation, the increase in starch phosphorylation revealed that GWD plays important

role during degradation. Ritte et al. (2000a) demonstrated that the binding of GWD to the starch granules is increased, due to the surface modifications of the starch granules, by *in vitro* binding assays using purified recombinant GWD which lead to the enhanced phosphorylating activity. Those granules that under *in vitro* conditions are more effective GWD substrates already display an elevated phosphorylation level at their surface. Thus, starch phosphorylation might be a self-enhancing process. The increase in the surface bound phosphate is, therefore, not (solely) responsible for an enhanced in vitro phosphorylation. Possibly, phosphorylation and degradation of starch are interdependent processes. Phosphorylation of the granule surface by GWD could facilitate the attack of degrading enzymes. By the activity of the latter, new phosphorylation targets could become accessible. These phosphorylation targets are not yet clearly defined.

2.3 ***Different isoforms of GWD in Arabidopsis:*** Although biochemical and molecular studies on GWD were initiated in the potato system, subsequently it was more focused on *Arabidopsis* model to gain more in-depth understanding of this gene function as it is related to starch phosphorylation and degradation. The three isoforms, AtGWD1 (GenBank Acc No NP_563877), AtGWD2 (GenBank Acc No AAO42141) and AtGWD3 (GenBank Acc No AAU93516) were identified from *Arabidopsis thaliana*. AtGWD1 and AtGWD3 are localized in the chloroplast and involved in the degradation of transient starch from leaves and AtGWD2 is non-chloroplastic and its function is not known. The three *Arabidopsis* gene products along with glucan-related dikinases from other plant species as well share a common overall structure: they possess a C-terminal nucleotide binding domain, a short conserved sequence containing the phosphohistidine residue, indispensable for glucan phosphorylation, and a carbohydrate binding domain at the N-terminal region. The AtGWD1/SEX1 (STARCH

EXCESS) shows considerable amino acid identity (66%) with the potato GWD and responsible for phosphorylation at C-6 position of glucose units. But the AtGWD3, designated as PWD (Phosphoglucan, water dikinase) phosphorylates the C-3 position of the glucose units preferably pre-phosphorylated amylopectin substrate with long side chains. With the help of improved methods (^{31}P NMR and HPLC), it has been demonstrated that C-6 and C-3 phosphorylation were selectively catalyzed by GWD and PWD, respectively in *Arabidopsis thaliana* (Yu et al. 2001; Mikkelsen et al. 2004; Baunsgaard et al. 2005; Kotting et al. 2005; Ritte et al. 2006; Glaring et al. 2007).

2.4 ***Different domains of R1 (GWD):*** Mikkelsen et al. (2005) showed that, GWD comprised of five discrete domains of 37, 24, 21, 36 and 38 kDa as revealed by the analysis of proteolytic fragments of GWD, peptide microsequencing and the generation of deletion mutants. The catalytic histidine, which mediates the phosphoryl group transfer from ATP to starch, is located on 36 kDa fragment, whereas the 38 kDa C-terminal fragment contains the ATP-binding site. Binding of the glucan molecule appears to be confined to regions containing the three N-terminal domains.

Recently, it has been established that the intracellular enzymes like α-glucan, water dikinases (GWDs, EC 2.7.9.4), the α-amylases (AMYs, EC 3.2.1.1), present primarily in photosynthesizing organisms, contain small families of noncatalytic starch-binding domains (SBDs), named CBM45 (http://www.cazy.org, Glaring et al. 2011). Based on the recombinant expression, Glaring et al. (2011) showed that the CBM45s represent low-affinity starch-binding domains, a feature relevant to plastidial starch metabolism. Based on the alignment of the available CBM45s across all species, each CBM45 domain contains five widely conserved aromatic amino acids (a few of them are necessary for starch binding). In potato, the

carbohydrate-binding modules (CBMs) i.e., CBM45-1 and CBM45-2 belong to the 37 and 24 kDa domains, respectively(Mikkelsen and Blennow 2005; Mikkelsen et al. 2006; Glaring et al. 2011).

Starch biosynthesis, phosphorylation and degradation are now considered as the main biochemical aspects of starch metabolism. Growing body of experimental evidences as accumulated during the last decade clearly substantiated that the glucan phosphorylation is a prerequisite for starch degradation. Transitory starch metabolism in the leaves and storage starch metabolism in other plant organs such as tubers, roots, pollen grains, seeds, and fruits play a crucial role with respect to the plant growth and development.

2.5 ***Transitory starch degradation in Arabidopsis leaves:*** Starch phosphorylation by glucan, water dikinase (GWD; EC 2.7.9.4) is an essential step in the breakdown of native starch particles, but the underlying mechanisms have remained obscure. Transitory starch formed in the chloroplasts of leaves during the day and degraded during night. Transitory starch provided the carbohydrates during the night to the plant when sugars cannot be made by photosynthesis, so act as energy reserve. Degradation of transitory starch occur by hydrolytically and phosphorolytically. Hydrolytic and phosphorolytic pathways shared the initial steps of starch degradation. Glc-1-P (G1P) the product of phosphorolytic pathway is normally converted to Glc-6-P (G6P). Maltose and glucose, the products of hydrolytic pathway are produced by the action of β-amylase and D-enzyme (DPE1; Smith et al. 2005). The hydrolytic pathway of starch breakdown has recently been shown to be of primary importance for starch conversion to sucrose. Maltose and Glucose were the main metabolites exported from the chloroplast as evident from the isolated chloroplasts breaking down the starch in the night. *Arabidopsis* plants deficient in enzymes required for maltose export from

the chloroplast and metabolism in the cytosol exhibit a maltose and starch-excess phenotype and stunted growth. However, mutants lacking the plastid starch phosphorylase enzyme exhibited no such phenotype. Therefore starch degradation to sugars, which are exported from chloroplast at night is by hydrolytic pathway. But phosphorolytic starch degradation may be supply carbon for metabolism inside the chloroplast, as chloroplasts have sufficient enzymatic capacity to metabolize all the products of starch degradation through the pentose phosphate pathway (Nittyla et al. 2004; Weise et al. 2004; Zeeman et al. 2004).

2.6 *Current understanding on the mechanism of starch degradation and the role of GWD:*

Starch degradation involves different and complex pathways, varies in different plant organs. A number of studies have been carried out to understand and find out the distinct mechanism or pathway of starch degradation by different research groups in the last more than a decade. According to the current model for transient starch degradation in *Arabidopsis* leaves, starch degradation involves transient amylopectin phosphorylation that proceeds concurrently with glucan hydrolysis (Zeeman et al. 2010; Kötting et al. 2010; Santelia and Zeeman 2011). Similar type of mechanism is believed to be involved in glycogen degradation as glycogen phosphatase (laforin), similar to SEX4, catalyzes dephosphorylation (Tagliabracci et al. 2007). The reversible phosphorylation of glucans at the surface of starch granules by GWD convert the highly ordered structure of glucans to less ordered state and solublize the granule surface which is accessible to hydrolases for further degradation (Hejazi et al. 2008). The degradation of phosphate-free starch from *sex1* mutants of *Arabidopsis* by β-amylases and Debranching Enzymes (DBEs) was increased in the presence of recombinant GWD and ATP due to phosphorylation of granule surface (Edner et al. 2007). In other words, phosphorylation leads to the structural disruption of crystalline lamella of amylopectin allowing progressive

degradation and dephosphorylation by the hydrolytic enzymes and phosphoglucan phosphatases, respectively (Yu et al. 2001). Recently, Kötting et al. (2009) showed that the loss of a glucan-binding phosphatase encoded at the *SEX4* locus, also designated as PTPKIS1 (protein-tyrosine phosphatase kinase interaction sequence) and DSP4 (dual specificity phosphatase 4) of *Arabidopsis* decreases the leaf starch degradation. Recombinant SEX4 is capable to remove phosphate groups from glucans and semicrystalline amylopectin at either the C-6 or C-3 position of glucosyl residues (Hejazi et al. 2010). The need for dephosphorylation during starch degradation probably relates to the substrate requirements of β-amylases, since these enzymes can not act on a glucan chain carrying a phosphate group close to the nonreducing end. The rate of starch degradation by β-amylase and DBE is enhanced by the addition of GWD and ATP, and is further enhanced when SEX4 is also added (Kötting et al. 2009).

The degradation of leaf transitory starch and cereal endosperm storage starch has been understood more clearly as compared to the other starch containing parts such as tubers and roots. The transitory starch degradation in chloroplasts involves the hydrolysis of the glucans to the maltose and glucose at the surface of starch granule catalyzed by the β-amylases (α-1,4-glucan malto hydrolase; EC: 3.2.1.2). Glucose and maltose exported to the cytosol and metabolized in the cytosol. However, in case of cereal endosperm, four starch-degrading enzymes, α-amylase, β-amylase, DBE (specifically LDA), and α-glucosidase (maltase; EC:3.2.1.3) are present and involved in starch degradation. During germination, α-amylase synthesized in the aleurone and scutellum was secreted into the endosperm and attack at specific sites on the surface of starch granules and releases the mixture of soluble linear and branched oligosaccharides. These oligosaccharides further degraded to maltose and short

malto-oligosaccharides by α-amylase, DBE and β-amylase (Fincher 1989). Maltose and short malto-oligosaccharides further degraded to the glucose. Therefore, initial stage of transitory leaf starch degradation is different from the cereal endosperm starch degradation.

There was a growing interest on the isolation and characterization of α-glucan/glucan-like, water dikinases (GWDs) from plants, particularly agriculturally important crops, and photosynthesis-competent eukaryotes. In plants, apart from potato and *Arabidopsis thaliana*, glucan-related dikinases were also found in other plant species such as *Solanum lycopersicum* (*Lycopersicon esculentum)* (Nashilevitz et al. 2009), *Citrus reticulata* (Li et al. 2003), *Lotus japonicus* (Vriet et al. 2010), the bread wheat *Triticum aestivum* (GenBank Acc No GU250878). Moreover, based on the annotated genomic sequence data, ESTs, and documentation of NCBI's Annotation Process, glucan-related dikinases (GWDs) were also predicted in other plant species, such as *Ricinus communis* (GenBank Acc No XM_002527856), *Populus trichocarpa* (GenBank Acc No XM_002315643), *Oryza sativa* Indica Group (GenBank Acc No EEC80673, CM000131); *Vitis vinifera* (GenBank Acc No XM_002270449). All these data clearly suggest that glucan-related dikinases are widely distributed in different plant species as mentioned above, and also in photosynthesis-competent eukaryotes (Fettke et al. 2009). Currently, GWD, PWD, and SEX4 have become the targets of applied research as they are related to the desirable agronomic traits.

2.7 ***Relevance of molecular and biochemical studies on R1 (GWD) in potato:*** Studies on starch phosphorylation has emerged as new front line of starch research because of the following reasons: various physico-chemical properties depend on the extent of starch phosphorylation by GWD, secondly to elucidate the underlying molecular mechanism. The latter aspect gained a considerable importance as it is involved in the pathways of starch

degradation and related to desirable agronomic traits (Blennow et al. 2002). In comparison to *Arabidopsis* transitory leaf starch metabolism, the overall information on the roles of various enzymes involved in starch phosphorylation and its degradation is scarce in nonphotosynthetic heterotrophic organs of plants. At present, it is not possible to deduce the pathway of starch degradation clearly because of the following reasons: individual isoforms of the enzymes and their subcellular locations remain vastly unknown, and the precise biochemical role of the individual enzymes needs to be assessed. The biochemical mechanism of storage starch phosphorylation in potato is less understood, because of scarce experimental data, as compared to the similar aspects of leaf transitory starch as we know in the *Arabidopsis* model currently. It is now believed that the pathways of starch degradation in leaves and other starch storage organs have some common features, but also have major differences. In leaves, starch degradation could be studied at biochemical and molecular levels over a short and controllable period. But similar type of in-depth studies in other plant organs like roots, tubers are relatively difficult. One of the reasons being starch degradation may occur over long periods, during which the concerned organs undergo many conspicuous developmental changes. The nature of the starch granules and their appearances during degradation differ substantially between plant organs. A host of developmental, metabolic, and environmental factors influence starch degradation in various plant organs. Moreover, the biochemical roles of the individual isoforms of the concerned enzymes still remain vastly unknown, and the precise biochemical role of the individual enzymes needs to be assessed. Although GWD was discovered in the potato cultivar, still we lack in-depth knowledge on storage starch phosphorylation and its degradation aspects. For example, still we do not know clearly the number of GWD isoforms involved in starch phosphorylation in potato. In the

tetraploid heterozygous potato cultivar genomes, we can not rule out the multiplicity of the *GWD* genes and their allelic variations. It is likely that more number of GWD isoforms is involved in potato starch metabolism which remains to be elucidated.

2.8 ***Origin of the problem:*** As revealed by literature survey, there is a growing interest in the studies on starch phosphorylation and degradation aspects as these biochemical processes are related to the traits of agronomic value. GWD has become a target of both basic and applied research in the area of starch metabolism. Decreasing the activity of GWD in potato can increase starch content both in leaves and tubers, and prevent undesirable starch degradation in the cold-stored tubers. On the other hand, increasing its activity can elevate starch-bound phosphate content which has profound influence on its physico-chemical properties which in turn lead to some of the desirable attributes for industrial uses. It is to be noted here that the phosphate content in potato tubers is very high as compared with other plant storage organs. It is believed that more tuber starch phosphorylation leads to more degradability of starch into the hexose sugars. In order to understand the relevance of GWD gene function, the phenomenon of cold induced sweetening in potato tuber is briefly discussed here. After harvest, the storage of potato tubers at low temperatures results in high accumulation of reducing sugars such as glucose and fructose a phenomenon recognized as low-temperature sweetening (LTS) or cold-induced sweetening. The free hexoses react with free amino groups of proteins/amino acids which resulting in the formation of complex of large molecular weight molecules, known as Maillard reaction. By this, processing quality of potato tubers is severely compromised as the objectionable dark brown colour develops in potato chips during frying. Different enzymes such as starch degrading enzymes, sucrose-phosphate-synthase, invertase etc. are thought to be involved in this undesirable phenomenon i.e., starch-hexose

conversions in the cold-stored potato tubers. Moreover, the overall status of tuber starch phosphorylation is also implicated in the process of cold sweetening of potato tubers as supported by the recent studies. Starch with less bound phosphate could be achieved through the inhibition of GWD gene functions. Currently many laboratories are working to understand the biochemical & molecular mechanisms involved in the process of cold-induced sweetening in the potato tubers. Therefore, manipulation of GWD gene function appeared to be quite promising for the crop improvement through transgenic molecular breeding approaches.

Till to date, only two potato GWD cDNA sequences were submitted to the database by other research groups; one is from the cv. Desiree (GenBank Acc No Y09533), and the other one from the cv. Prevalent (GenBank Acc No AY027522). Common potato cultivars are autotetraploid, heterozygous. Multiple allelism occurs for potato genes that affect morphological characteristics, and various metabolic pathways (van de Wal et al. 2001). The copy number of the GWD and other related genes, and their allelic composition are not known clearly in potato. Therefore, it is likely that more isoforms corresponding to GWD and glucan-related dikinases are involved in the potato starch metabolism. There are more than 40 high yielding Indian Potato cultivars including some processing varieties released through conventional breeding during the last few decades, which are suitable to different agro-climatic conditions of our country. These potato cultivars vary with regard to genetic make up, maturation time, dry matter content, level of soluble sugars both reducing and nonreducing during storage at low temperature and growth in different agro-climatic zones of the Indian subcontinent. The genetic resources of various potato cultivars could be explored for identifying the concerned genes, their allelic composition, and expression pattern of the individual isoforms. GWD gene function is not studied in these cultivars till date at

biochemical and/or molecular level. Moreover, for improving the potato crop GWD gene inhibition studies may be carried out in these cultivars through transgenic means to see the effect with regard to cold-induced sweetening phenomenon during low temperature storage of potato tubers. Keeping the above points in view the following objectives were proposed to carry out Ph.D. thesis work.

3. Objectives

1) Studies on the status of starch phosphorylation and temperature dependence of sugar accumulation in potato tubers from various cultivars

2) Isolation and characterization of cDNA and/or genomic clones corresponding to starch-granule bound protein R1 protein from potato cultivars

3) Use of different fragments from R1 cDNA and/or genomic clones for making various genetic constructs for inhibition of R1 gene function

4) Transformation of potato plants using above constructs followed by screening of transgenic potato lines

Materials and Methods

4. Materials and Methods

4.1 Materials

4.1.1 ***Plant materials:*** The high-yielding Indian potato cultivars used in this study were procured from Central Potato Research Institute (CPRI), Shimla, India namely Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chandramukhi, Kufri Jyoti, Kufri Ashoka, and Kufri Pukhraj. These cultivars vary with regard to genetic make up, maturation time, and growth in different agro-climatic zones of the Indian subcontinent. The cultivars Kufri Chandramukhi and Kufri Ashoka are early maturing, whereas the remaining cultivars are medium maturing. All these cultivars along with Desiree (a late maturing exotic cultivar) were routinely micropropagated in our laboratory under controlled conditions (16 h light/8 h dark, 25-27ºC, 70% relative humidity) for four to five weeks on MS-Basal medium. The aseptically grown micropropagated potato plantlets of the above cultivars were used for genomic DNA isolation. After proper hardening and acclimatization of the aseptically grown micropropagated potato plantlets, all these cultivars were grown in the field. Leaves and mini tubers were collected from the mature potato plants and used in this study. Kufri Chipsona-1 (a processing Indian potato cultivar) was used in this study for *Agrobacterium*-mediated genetic transformation.

4.1.2 ***Bacterial strains and plasmid vectors:*** For routine molecular cloning experiments pUC19 (Acc No X02514) was used as a vector, and *E. coli* DH5α (*sup*E44 Δ*lac*U169 (Φ80 *lac*ZΔ*M*15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) was used as host. This is a common bacterial strain, routinely maintained in our laboratory. The binary vector pBI121 with CaMV35S-GUS fusion (Acc No AF485783) and pAN-GB01 binary vector with GBSSI-GUS gene fusion (constructed in this study) were used for making of different antisense and sense genetic constructs for potato genetic transformation. During triparental mating,

E. coli pRK2013 and *Agrobacterium tumefaciens* LBA4404 strains were used as helper and recipient, respectively. The Ti- plasmid in *E. coli* is mobilized to the recipient *Agrobacterium* strain due to the mobilization function of the *E. coli* pRK2013 (broad host range plasmid). *Agrobacterium tumefaciens* LBA4404 is the most commonly used strain for co-cultivation, carrying the pAL4404 plasmid (resident plasmid with the *vir* genes). LBA4404 strain is resistant to antibiotics rifampicin (due to chromosomal selection) and streptomycin (due to the presence of pAL4404).

4.1.3 ***Designing of oligonucleotide primers:*** Number of different oligonucleotide primers were used to carry out the different experiments is described below:

*R1 (GWD) cDNA-specific primers***:** The different primers were designed based on the cDNA sequence encoding R1 protein (GWD) from potato as available in the database (Genbank Accession no. Y09533). Forward primers: A20R1, 5'-TTCATCGAATTTCTCGAAGC-3' corresponding to the bases 5-24; R1-F3539, 5'-TTACGCAATATCAGCAGATG-3' corresponds to the bases 3539-3558; Reverse primers: B20R1, 5'-TTAGCCTTGCTCGAA TGTCC-3' complementary to the bases 921-902; C20R1, 5'-ATCGGATCATCAATC TGCTC-3' complementary to the bases 1363-1344; R1-4810, 5'-TTCAGTGCATAATGC AATAC-3' complementary to the bases 4810-4791

GBSSI gene-specific primers: The different primers were designed based on the available granule-bound starch synthase (GBSSI) gene sequence in the database (GenBank Accession No. X58453) corresponding to the wild type monoploid potato clone, AM79.7322. Forward primers: GB1-F01, 5'-AATGCAACAGTATCTTGTAC-3' corresponding to the bases 54-73 and GB1-F02, 5'-AGACATAGGAATGTCAAGTG-3′ corresponds to the bases 514-533; Reverse primers: GB1-R01, 5'-AAGAACATCACCTAGTCCAC-3' complementary to the bases 1363-1382 and corresponds to the N-terminus of mature peptide; GB1-R02,

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

Constitutive gene-specific primers: Actin (Mr ~ 41,800) is widely distributed in eukaryotic cells, often being the most abundant protein and commonly making up of 10 percent of the total cell protein. The following primers were designed which are specific to constitutive actin gene and used in the present study, also described by Dohmann et al. (2005). Forward primer Actin-FW**,** 5'-ATTCAGATGCCCAGAAGTCTTGTTC-3' and reverse primer Actin-RV, 5'-GCAAGTGCTGTGATTTCTTTGCTCA-3'.

Binary vector specific primers: The different primers were designed based on the available pBI121 binary vector sequence in the database (GenBank Acc No AF485783) as given below: CaMV35S promoter-specific primers: Forward primer AF35-01, 5'-ATTCAAATAG AGGACCTAAC-3' corresponding to the bases 5291-5310, and reverse primer BR3502, 5'-CCGTGTTCTCTCCAAATGA-3' complementary to the bases 5791-5809.

GUS gene-specific primers: Forward primer US-F5832 5'-TGGTCAGTCCCTTATGTTAC-3' corresponding to the bases 5832-5851 and two reverse primers US-R6747, 5'-CAAGTCCGC ATCTTCATGAC-3' complementary to the bases 6728-6747 and US-R7719, 5'-TTCGAGC TCGGTAGCAATTC-3' complementary to the bases 7700-7719.

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

pBI121 vector specific primers: Forward primer ORB-F1, 5'-TTGGCATGCACATAC AAATG-3'corresponding to the bases 2364-2383 outside the right border.

4.2 Methodology

Here first few sections are for different molecular biology techniques followed by biochemical techniques as adopted in this study:

4.2.1 ***Isolation of genomic DNA from potato cultivars:*** Different amounts of plant material ranging from 0.1 g to 2.0 g were used for total DNA isolation using a modified method based on the protocol of Dellaporta et al. 1983. One of the steps involved potassium acetate to remove carbohydrates and other bulk impurities. Briefly the steps are given below: around 2.0 g of plant material was ground to fine powder in the presence of liquid nitrogen, then transferred quickly to a conical flask containing 15 mL extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 250 mM NaCl, and 15% sucrose) maintained at 65^{o}C. This was mixed well and incubated at 65^{o}C for 20 min with intermittent gentle shaking. 5.0 mL of 5.0 M potassium acetate solution was added, mixed vigorously and incubated further on ice for 20 min and then centrifuged at 4000 g, 4^{o}C for 20 min. The supernatant was filtered through two layers of fine muslin cloth and 0.70 vol of isopropanol was added, mixed gently and incubated at -20^{o}C for four to five hrs followed by centrifugation at 10000 g, 4^{o}C for 15 min. The crude DNA pellet was washed with ice cold 70% ethanol, then air dried and suspended in 500 µL of TE buffer (10 mM Tris-HCl pH 8.0 and 1.0 mM EDTA pH 8.0). For further purification of DNA, DNase-free RNase treatment was carried out followed by solvent extraction twice using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1), the DNA was precipitated using 0.1 vol of 3.0 M sodium acetate (pH 5.5) and 2.0 vol of ethanol, finally dissolved in 200-250 µL of TE buffer. For 0.1 to 0.2 g plant materials, the same protocol was adopted by scaling down the necessary steps accordingly. The quality and quantity of the potato DNA samples were initially checked spectrophotometrically by

measuring the A_{260}/A_{280} ratio and absorbance at 260 nm, respectively. The A_{260}/A_{280} ratio appeared to be 1.70 to 1.90 for most of the DNA preparations. Further quality checking was done by restriction analyses.

4.2.2 ***Agarose gel electrophoresis:*** Agarose gel electrophoresis was performed using standard methods (Sambrook et al. 1989). 0.8 % agarose gel was made in 0.5X TBE / 1X TAE buffer to which ethidium bromide dye was added (working conc. 0.5-1.0 μg/mL). The DNA samples were loaded after mixing well with the gel loading buffer and electrophoresis was carried out at 2-5 V/cm till the tracking dye covered two-third of the gel length. Finally, the DNA bands were visualized under UV light.

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

4.2.4 ***RNA extraction from the potato plant materials by Lithium chloride / SDS Phenol method:*** Total RNA was isolated from the tubers and leaves of the eight to nine weeks old field grown potato plants including all seven potato cultivars (six Indian potato cultivars, and the reference cultivar Desiree) by SDS-Phenol method essentially as described by Gilman et al. 1987. Briefly, the plant materials (0.5 to 2.0 g) were frozen in liquid nitrogen and pulverized to a fine powder, and homogenized further in a buffer containing lithium chloride and SDS (the composition of the RNA extraction buffer: 100 mM LiCl, 100 mM Tris-HCl pH 8.7, 10 mM EDTA pH 7.40, 1.0% SDS, 0.2% β-mercaptoethanol) followed by direct

extraction with phenol:chloroform (1:1). In order to remove DNA impurities, RNA was selectively precipitated from the aqueous phase by adding one-third volume of 8.0 M LiCl under ice-cold condition followed by incubation for at least two hours. The RNA pellet was further purified by another round of solvent extraction followed by ethanol precipitation. In order to remove the DNA impurities, the aqueous RNA solution was treated with RNase-free DNase, followed by solvent extraction. Finally, the RNA was precipitated with ethanol, washed and dissolved in deionized water, and kept in aliquots at -70^{0}C. The A_{260}/A_{280} ratio of the RNA samples was measured spectrophotometrically. For checking the intactness of RNA, both normal and formaldehyde agarose gel electrophoresis along with RT-PCR using different potato gene-specific primers were carried out.

4.2.5 ***Formaldehyde agarose gel electrophoresis:*** RNA samples were denatured by treatment with formamide and separated by electrophoresis through agarose gels containing formaldehyde (Sambrook et al. 1989). For 100 mL of 1.5 % agarose gel containing formaldehyde: 1.5 g of agarose in 62 mL of sterile water was boiled and cooled to 55ºC. 20 mL of 5X MOPS electrophoresis buffer and 18 mL of deionized formaldehyde was added and casted the gel in gel casting apparatus and gel was allowed to set for one hour at room temperature. 1.5 % agarose gel is suitable for resolving RNAs in the 0.5-8.0 kb size range. The gel was submerged in the 1X MOPS electrophoresis buffer. For denaturation of RNA samples, 10.0 µL of RNA, 2.0 µL of 5X MOPS electrophoresis buffer, 3.0 µL of formaldehyde, 7.0 µL formamide and 1.0 µL ethidium bromide (200 µg mL^{-1}) were mixed and incubated at 65ºC for 15 min. The samples were chilled on ice, 3.0 µL of formaldehyde gel loading buffer was added and loaded in the gel and electrophoresis was carried out at 4-5 V/cm until the bromophenol blue migrated to end. The composition of buffers used; 5X

MOPS buffer: 0.1 M MOPS pH 7.0, 40 mM Sodium acetate, 5 mM EDTA pH 8.0; Formaldehyde gel loading buffer: 50 % glycerol, 1.0 mM EDTA (pH 7.5), 0.25% Bromophenol blue.

4.2.6 ***Reverse Transcription (RT) followed by Polymerase chain reaction (PCR) for amplification of R1 cDNA:*** First strand cDNA was synthesized using Revert Aid H Minus M-MuLV reverse transcriptase. The enzyme lacks ribonuclease H activity specific to RNA in RNA-DNA hybrids. Therefore degradation of RNA does not occur during first strand cDNA synthesis, resulting in higher yields of full-length cDNA from long templates upto 13 kb. Reverse transcription (RT) was performed using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit from Fermentas Life Sciences containing M-MuLV reverse transcriptase and the gene-specific reverse primer, R1-4810. For each RT reaction, approx. 2.0 μg of total RNA either from tuber or leaf from Indian potato cultivars was used as template. All the steps of reverse transcription were carried out according to the manufacturer's instructions. In order to isolate full-length GWD cDNA, PCR was carried out using the individual RT product as template, R1 (GWD) cDNA-specific primers, A20-R1 and R1-4810, and high fidelity XT-5 PCR System (Bangalore Genei). After initial denaturation at 94ºC for 1 min 30 s, the thermal cycling parameters during PCR were: denaturation at 94ºC for 1 min, annealing at 55ºC for 2 min; polymerization at 72ºC for 3 min for 30 cycles followed by final extension at 72ºC for 5 min.

4.2.7 ***Klenow enzyme treatment and DNA purification:*** Prior to ligation, the termini of the target DNA fragment and the vector should be compatible. Generally, the PCR-amplified DNA products are not truly blunt-ended as the enzyme *Taq* DNA polymerase has a tendency to add an extra 'A' residue at the 3' end of both the strands. Moreover, the PCR-amplified

DNA may have 3' recessed termini. As a polishing step here, *Klenow* treatment served two purposes: firstly, removal of the extra 'A' residue at the 3' ends; secondly, filling up the recessed 3' termini in the amplified DNA products. For this purpose, RT-PCR amplified DNA products were treated with Klenow enzyme in 40 µL of reaction volume (buffer, dNTP-mix and finally 1-2 unit of *Klenow* enzyme) as per manufacturers' instructions. After klenow treatment, the coveted DNA band from agarose gel was purified through Electro-elution method using the dialysis membrane. Apart from this, QIAEX II Gel Extraction kit (Qiagen) was also used as per manufacturers' instructions.

4.2.8 ***Ligation reaction:*** A ligation reaction was set up in order to ligate the purified DNA fragment into the vector, using the enzyme T4 DNA ligase. It catalyzes the formation of phosphodiester bond between the juxtaposed 5'-phosphate and 3'-OH termini in the duplex DNA. It can join blunt as well as the cohesive end termini. The main components of a ligation reaction are: vector (~ 0.3 µg), insert (~ 0.3 to 0.6 µg) and T4 DNA Ligase enzyme (1-10 units). T4 DNA Ligase buffer containing PEG 8000 was used in case of blunt end ligation. The reaction volume was made up to 15 µL and the reaction was set up at ~25°C for cohesive end ligation and at 21°C for blunt end ligation. It was carried out for 2-3 hours.

4.2.9 ***Transformation of E. coli DH5α with plasmid vectors:*** A single bacterial colony was inoculated in 25 mL of Luria Broth and incubated at 125 rpm at 37°C for overnight. A small aliquot of overnight-grown culture was used to re-inoculate 25 mL of fresh Luria Broth and then incubated at 37°C with shaking and grown up to an O.D. of around 0.4-0.6 at 590 nm. The culture was kept on ice to arrest the cell growth, and cells were harvested. The cells were washed and resuspended in 1.0 mL of ice-cold 100 mM $CaCl_2$ and kept in ice for 2½ hours for preparation of competent cells. *E. coli* DH5α was transformed with the ligation mix using the

standard $CaCl_2$ method (Mandel and Higa, 1970). The transformants were screened on the basis of alpha-complementation (Blue/White colour selection) in case of pUC19 vector. The white colonies are the ones that contain plasmid with insert and the blue colonies have only plasmid.

4.2.10 ***Plasmid isolation by boiling and alkali lysis methods:*** Plasmid isolation in mini scale was carried out by boiling method as described by Holmes and Quigley, 1981. Cells were harvested from 1.5 mL overnight grown culture and resuspended in 800 µL of STET buffer. After addition of 30 µL of lysozyme (stock 10 mg ml^{-1}), the bacterial suspension kept in boiling water bath for 90 seconds and centrifuged (12,000 rpm) for 15 minutes. The supernatant was treated with RNase followed by solvent extraction twice using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1), the DNA was precipitated using 0.1 vol of 3.0 M sodium acetate (pH 5.5) and 1.0 vol of isopropanol, finally dissolved in 200-250 µL of TE buffer. In some cases plasmid isolation was also done by Alkali lysis method as described by Birnboim and Doly 1979.

4.2.11 ***Sequence analyses of R1 (GWD) cDNA:*** The nucleotide sequence of the cDNA was analyzed by NCBI Blast tools. The deduced amino acid sequence was predicted by the open reading frame (ORF) finder available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). In order to calculate the theoretical molecular weight, isoelectric point (pI), and amino acid composition of the deduced amino acid sequence the ProtParam tool of ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB; URL: http://expasy.org/tools/) was used. CFSSP: Chou & Fasman Secondary Structure Prediction Server (http://www.biogem.org/tool/chou-fasman/) along with the ProtScale tool of ExPASy were used for prediction of the different secondary

structures. Multiple sequence alignment was done using ClustalW2, an EMBL-EBI sequence analysis tool with its default parameters (http://www.ebi.ac.uk/Tools/). In order to generate phylogenetic tree, multiple sequence alignment was done first by the *MultAlin* software (Corpet 1998; http://www.multalin.toulouse.inra.fr/multalin/), followed by generation of the phylogenetic tree by the neighbor-joining method using MEGA5 software (Saitou and Nei 1987; Tamura et al. 2011). For this purpose a total of eleven predicted GWD sequences corresponding to different plant species were used (accession number of the protein id in parenthesis): three members from *Solanum tuberosum* (ACC93586, CAA70725, and AAK11735), *Solanum lycopersicum* (ACG69788), *Arabidopsis thaliana (*AAG47821), *Citrus reticulata* (AAM18228), *Oryza sativa (*EEC80673), *Ricinus communis* (XP_002527902), *Populus trichocarpa* (XP_002315679), *Vitis vinifera* (XP_002270485), *Triticum aestivum* (ADG27838).

4.2.12 ***Semi-quantitative PCR:*** Semi-quantitative PCR was carried out to see the GWD gene expression pattern in the tubers and leaves collected from the seven field-grown potato cultivars. From each sample 2.0 μg of total RNA (free from DNA impurities) was used for reverse transcription in a reaction volume of 20 μL using the cDNA Synthesis Kit from Fermentas Life Sciences as mentioned earlier. 3.0 μL of each RT mixture was used as template in PCR (50 μL reaction volume) using the GWD cDNA specific forward and reverse primers, A20-R1 and C20-R1, respectively and 1.0 unit of Taq DNA polymerase (Bangalore Genei). After initial denaturation at 94ºC for 1 min 30 s, the thermal cycling parameters during PCR were: denaturation at 94ºC for 1 min, annealing at 55ºC for 2 min; polymerization at 72ºC for 2 min for 30 cycles followed by final extension at 72ºC for 5 min. As a control, the primers Actin-FW (forward primer), and Actin-RV (reverse primer) specific to the

housekeeping actin gene were used to amplify ~250 bp fragment using the same 3.0 μL individual RT mixture as template. After initial denaturation at 94ºC for 1 min 30 s, the PCR amplification conditions were 94ºC for 1 min, 55ºC for 2 min; 72ºC for 1 min for 30 cycles followed by final extension at 72ºC for 5 min. Both the GWD and actin specific RT-PCR products were resolved in 1.0% agarose gel electrophoresis.

4.2.13 ***Protein gel blot analysis:*** The entire deduced amino acid sequence of GWD was examined for the prediction of antigenic peptide using the MIF (Molecular Immunology Foundation) Bioinformatics tool based on the method of Kolaskar and Tongaonkar (1990) (http://immunax.dfci.harvard.edu/Tools/ antigenic.pl). The current website for the same is http://imed.med.ucm.es/Tools/antigenic.html under server of Dr. Reche's Immunomedicine Group, Universidad Complutense Madrid. A 16-mer polypeptide NIELQVDVRPPTSGDV, corresponding to the amino acids 19 to 34 of the mature KC-GWD protein, having predicted antigenic determinants was synthesized by Biomatrix Technologies, New Delhi, India. Polyclonal antibody was raised in the rabbit using the Keyhole Limpet Hemocyanin (KLH)-conjugated peptide immunogen by Bangalore Genei (India) Pvt. Ltd., India. Total protein from potato tubers and leaves (~0.20 g of plant material) of different potato cultivars were isolated using the extraction buffer (50 mM MOPS-NaOH pH 7.5, 10 mM $MgCl_2$, 1.0 mM EDTA, 0.2% 2-mercaptoethanol, 0.1% Triton X100, 1.0 mM PMSF). Equal amount of each protein samples was loaded on 6.5% denaturing polyacrylamide gel and blotted onto PVDF membrane. Immunodetection was carried out using the above polyclonal antibody with the help of Goat anti-rabbit IgG-horse radish peroxidase (HRP) conjugated secondary antibody using 3, 3′-diaminobezidine (DAB) system (Bangalore Genei (India) Pvt. Ltd., India).

4.2.14 ***Isolation and sequence analyses of GBSSI alleles from potato:*** In this study, PCR were carried out for amplification of GBSSI genes using individual potato DNA as template and the following oligonucleotide primers GB1-F01 and GB1-R01. After initial denaturation at 94ºC for 1 min 30 s, the thermal cycling parameters were: denaturation at 94ºC for 1 min, annealing at 55ºC or 50ºC for 2 min; polymerization at 72ºC for 2 min or 1 min for 30 cycles followed by final extension at 72ºC for 5 min. Out of seven potato cultivars, the PCR amplified products (~1.3 kb and 1.4 kb) corresponding to the potato cultivar Kufri Chandramukhi were purified and cloned into the *Sma*1 site of pUC19. The individual sequence information was submitted to the GenBank data base under the Accession Numbers EU548081 and EU548082. These two partial GBSSI alleles were designated as GKC1 and GKC2, respectively. The nucleotide sequences of the partial GBSSI alleles, GKC1 and GKC2, were analyzed by NCBI BLAST tools (http://www.ncbi.nlm. nih.gov/BLAST). The deduced amino acid sequences were predicted by the open reading frame (ORF) finder available at the NCBI website. G+C content analyses were carried out by DNADynamo software (http://www.bluetractorsoftware.co.uk/). Multiple sequence alignment was done using ClustalW2, an EMBL-EBI sequence analysis tool, with its default parameters (http://www.ebi.ac.uk/Tools/msa/clustalw2). For phylogenetic analysis multiple sequence alignment was done first by the *MultAlin* software (Corpet 1998; http://www.multalin.toulouse.inra.fr/multalin/), followed by generation of the phylogenetic tree by the neighbor-joining method using MEGA 5.0 software (Tamura et al. 2011). Basal promoter and putative *cis*-regulatory sequence motifs in the GBSSI alleles were detected by sequence comparison with the published reports and other databases namely PlantCARE

(Lescot et al. 2002) (http://bioinformatics.psb.ugent.be/webtools/plantcare) and PLACE (Higo et al. 1999) (http://www.dna.affrc.go.jp/PLACE/).

4.2.15 ***Construction of binary vector with GBSSI promoter, potato transformation and histochemical GUS assay:*** The 767 bp GKC1 promoter was cloned into the upstream of GUS gene of pBI121 binary vector by replacing the constitutive CaMV35S promoter including its own TSS. The resulting binary vector having GBSSI-GUS gene fusion was designated as pAN-GB01. All the *in vitro* grown independent transgenic potato plantlets (corresponding to the pBI121 and pAN-GB01 binary vector) along with non transgenic ones (as control) were hardened and acclimatized, and then individual plants were grown in the restricted experimental plot where the ambience of field grown conditions was maintained. After 70 to 80 days of vegetative growth, mini tubers and other plant materials were collected. For the purpose of histochemical staining, free hand thin sections were made from different explants of potato, namely tuber, leaf, stem and root. β-glucuronidase (GUS) assay was carried out using the protocol essentially as described by Jefferson (1987) and Jefferson et al. (1987). Briefly the steps were: the thin tissue sections were incubated in the GUS assay buffer containing the chromogenic substance 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) for ~1.0 h at 37ºC for development of blue color. The above sections were then washed with 70% ethanol (this step was particularly useful to remove chlorophyll pigments from leaf sections). The stained sections of plant materials were examined under a light microscope (Nikon Eclipse 50i), and photographed.

4.2.16 ***Transformation of Agrobacterium through Tri parental mating:*** In Tri parental mating, the donor strain (*E. coli* harboring Ti plasmid with gene of interest) mate with conjugal helper strain (*E. coli* harboring broad host range plasmid pRK2013) and a recipient

Agrobacterium strain (harboring *vir* plasmid). The Ti plasmid in *E. coli* is mobilized to recipient *Agrobacterium* strain due to mobilization function of pRK2013 (broad host range plasmid). After mating *Agrobacterium tumefaciens* strains harboring the engineered plant transformation vector (Ti plasmid with gene of interest) are selected by growth in the presence of antibiotics for which resistance is provided by genetic markers unique to those recipient *Agrobacterium* and Ti plasmid vector (Ti plasmid with gene of interest). The steps involved are: The recipient *Agrobacterium tumefaciens* strain LBA4404 was grown on Luria-Agar medium containing rifampicin (15 μg mL^{-1}) at 28ºC. The donor *E. coli* strain harboring engineered Ti plasmid (i.e. different genetic constructs) and conjugal helper *E. coli* strain (pRK2013) were grown on Luria-Agar medium containing kanamycin (50 μg mL^{-1}) at 37ºC. A single colony of each freshly grown strain was patched separately and closely to each other on Luria-Agar plates. The three patches were mixed with sterile loop and plates were incubated at 28ºC for 24 hours. The small part of Tri parental patch was picked with help of loop and made serial dilution in 100 μL of 0.9% saline water and spread on Luria-Agar containing antibiotics rifampicin (15 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) and incubated at 28ºC. Single colonies of transformed *Agrobacterium* were streaked on YEM medium containing rifampicin (15 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) for further experiments. Apart from Tri parental mating technique, in some cases electroporation technique for transformation of *Agrobacterium* was also used as described by the manufacturers' instructions.

4.2.17 ***Agrobacterium mediated co-cultivation:*** The well characterized transformed *Agrobacterium* strain (corresponding to individual genetic constructs) was used for co-cultivation. The single colony of transformant strain was grown in YEM broth for 24 hrs.

upto 0.4-0.5 O.D and then 1ml of culture was diluted in 10 ml of MS basal medium. Inter-nodal stem segments of five to six weeks old potato plantlets (Kufri Chipsona-1), grown in MS medium, were co-cultivated with diluted culture for 10 min., blot the internodal stem segments on sterile filter paper, placed horizontally on MS-basal medium and incubated in dark for 48 hrs in growth room. Internodal stem segments were washed in cefotaxime (250 mg L^{-1}) and shifted to the selective shoot regeneration medium (MS medium containing Zeatin 2.5 mg L^{-1}, GA_3 3.0 mg L^{-1}, IAA 0.01 mg L^{-1} supplemented with kanamycin 80 mg L^{-1} and cefotaxime 250 mg L^{-1}) for regeneration and primary selection of the transgenic potato lines. The initial shoots were further transferred to the rooting media i.e., MS medium supplemented with IAA (0.05 mg L^{-1}), kanamycin (80 mg L^{-1}) and cefotaxime (250 mg L^{-1}), to obtain complete potato plantlets. Around ten independent transgenic potato lines corresponding to each binary genetic construct were screened by PCR approach using different sets of gene-specific primer pairs. The well characterized tissue-cultured transgenic plantlets after hardening and acclimatization were grown in polybags for mini-tuber production in the restricted experimental plots. Mature potato mini tubers were harvested and used for biochemical analyses.

4.2.18 ***Extraction of soluble sugars:*** Total soluble sugars were extracted from the freshly harvested tubers and tubers stored at different temperatures (3ºC, 4ºC, 10ºC & 25ºC) for four and eight weeks. The potato tuber slice (~ 200 mg) was crushed thoroughly using mortar and pestle to make fine paste, 1.0 mL of 80% alcohol was added, mixed well and then transferred the slurry into microfuge tube and rinsed with 1.0 mL of 80% alcohol and transferred to the same tube. Then incubated in water bath at 80ºC for 45 min, centrifuged for 10 min and the supernatant containing soluble extract was collected in a beaker. The pellet was re-extracted

with 80 % alcohol and the supernatant obtained was collected in the same beaker. The supernatant kept at 65ºC for nearly two hours to ensure complete evaporation of solvent. The residual matter in the beaker was thoroughly solubilized in a total volume of 1.5 mL sterile water and the soluble extract was transferred to a clean and fresh microfuge tube. Soluble sugar extract was stored at -20ºC for determination of total soluble sugars by Anthrone method and reducing sugars by Nelson-Somogyi's Method.

4.2.19 ***Estimation of reducing sugars by Nelson-Somogyi's Method:*** The reducing sugars when heated with alkaline copper tartarate reduce the copper from Cu^{2+} to cuprous oxide form. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. The blue color developed is compared with standards and the intensity of color is read at 620 nm. The color is stable for at least 18 hours.

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

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

100 µL and 200 µL aliquots of each soluble sugar extract were taken in clean test tubes. Standards prepared by taking different aliquots of D-glucose (stock 0.1 mg mL^{-1}) so that the tubes contained the following amounts 10, 20, 40, 60, 80, 100, 120, 140,150 µg along with blank. The volume in each of the samples was made up to 2.0 mL with water followed by addition of 1.0 mL of alkaline copper tartarate reagent. [Alkaline copper tartarate: Solution A - 2.5 g of anhydrous Na_2CO_3, 2.0 g $NaHCO_3$, 2.5 g sodium-potassium tartarate and 20.0 g anhydrous Na_2SO_4 dissolved sequentially in 80.0 mL water and finally volume made up to 100 mL. Solution B - 15.0 g of $CuSO_4$ was dissolved in a small volume of distilled water having a drop of conc. H_2SO_4 and volume made up to 100 mL with water. 4.0 mL of solution B and 96 mL of solution A were mixed well before use.] All the tubes

were kept in boiling water bath for 10 min using marbles on top to prevent evaporation loss. Cooling of the tubes to room temperature, followed by addition of 1.0 mL arsenomolybdic acid reagent [Arsenomolybdate reagent: 2.5 g of ammonium molybdate was dissolved in 45.0 mL water. To it 2.5 mL conc. H_2SO_4 was then added. 0.3 g disodium hydrogen arsenate was dissolved in 25 mL water. Finally the above solutions were mixed and then incubated at 37ºC for more than 24 hrs]. The volume in each tube made up to 10 mL by adding water followed by incubation for 10 min at room temp. Absorbance was taken at 620 nm immediately after vortexing.

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

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

4.2.21 ***Isolation of Starch granules:*** Starch granules were isolated from developing (two month old) and freshly harvested (mature) potato tubers. A simple protocol was followed for the isolation of starch granules as described by Nielsen et al. (1994). Weighed amount of potato tubers (6.0 g - 10.0 g) were crushed using mortar and pestle to form a uniform paste using ice-cold water followed by filtration through double-layered muslin cloth. The above filtrates were incubated in ice for at least 4 hours to allow the starch granules to settle down. Supernatant was decanted carefully to avoid any loss of starch granules followed by washing thrice with ice-cold water. The starch granules were further washed twice with acetone to remove other organic impurities. The starch granules were dried at 37ºC overnight. Finally the samples were weighed and stored at -20ºC for starch-granule-bound phosphate estimation. The ascorbic acid method for Pi estimation is very sensitive, by which one can easily determine as low as 0.01 micromole of phosphate.

4.2.22 ***Iodine staining of starch granules:*** Iodine stains solution composition: 2.5 g KI and 250 mg of I_2 in 125 mL of water. This solution was used for staining of starch granules isolated from potato tubers of different cultivars.

4.2.23 ***X-ray diffraction analysis of starch powder:*** Monochromatic Cu-Kα radiation (wavelength =1.542A°) was produced by X'Pert PRO, PANalytical was used for diffraction analysis of starch powder. For this, the starch powders were placed in an rectangular aluminium cell. The samples were exposed to the X-ray beam with the X-ray generator running at 45 KV and 40 mA. The scanning regions of the diffraction angle 2θ were 5º-30º, which covers all the significant diffraction peaks of starch crystallites. The conditions were as follows: Step interval 0.02, scan rate 2º min^{-1}, Sollet and divergence slit 1º, receiving slit 1º, and scattering slit 0.15º.

4.2.24 ***Determination of total starch-bound phosphorus:*** Approx. 5 mg of each dried starch sample was used for determination of starch-bound total phosphorus according to the method as described by Morrison (1964) and Nielsen et al. (1994). The ascorbic acid method for Pi estimation is very sensitive, by which one can easily determine as low as 0.01 micromole of phosphate. Here complete ashing procedure of starch was adopted to determine starch-bound phosphorus. Complete ashing enables the conversion of organic phosphates to inorganic phosphates. Inorganic phosphate reacts with ammonium molybdate in an acid solution to form phosphomolybdate complex. Addition of a reducing agent like ascorbic acid reduces the molybdenum in the phosphomolybdate complex to give a blue color. The different steps involved for estimation of starch-bound phosphorus:

Approximately 5.0 mg of starch granule isolated from potato tubers was suspended in 1-2 drops of water and the material was completely burnt to ashes over a strong flame of gas burner. Then allowed to cool and 0.4 mL of 0.1 N H_2SO_4 was added. The tubes were tightly capped with plastic caps and heated in boiling water bath for 10 minutes, to ensure solubilization as well as conversion of any pyrophosphate formed during ashing to inorganic phosphate. The solution was allowed to cool and then transferred to fresh microfuge tubes. Quantitative transfer was ensured by rinsing with small volume of 0.1 N H_2SO_4 .The final volume of the phosphate solution was kept at 0.5 mL. To set up reaction mixture, sequential addition was done in the following way, 300 µL aliquots of the sample, 180 µL of 0.1N H_2SO_4, made up the volume to 900.0 µL by addition of water. In case of blank 0.1N H_2SO_4 and water were mixed in the ratio of 1:1. To each reaction tube including blank 2.1 mL of the reagent mix [Composition of Reagent Mix: 1.0 volume of reagent A (10 %Ascorbic acid) was mixed with 6.0 volume of reagent B (0.42 % Ammonium molybdate.$4H_2O$ in 1.0 N H_2SO_4) before use] was added, mixed well and incubated in the water bath at 45^0C for 20 minutes. Then the tubes were cooled to room temperature, and absorbance was read at 820nm.

Note: (i) The standard curve was made using KH_2PO_4 solution with 100 ppm of phosphorus. [Standard KH_2PO_4 (100 mg P in 1.0 liter solution i.e. 100 ppm of phosphorus); 87.9 mg of KH_2PO_4 was dissolved in 80 mL of distilled water, then 5.0 ml. of 7 N H_2SO_4 was added, then volume made up to 200 mL].

(ii) For each starch sample the procedure was repeated for three times with almost similar results.

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

4.2.26 ***Protein estimation by Folin Lowry method:*** Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue. Solution A (Alkaline sodium carbonate solution): 2% Sodium carbonate in 0.1 N NaOH; Solution B (Copper sulphate-sodium potassium tartarate solution) 2% of sodium potassium tartarate in 1% copper sulphate; BSA stock (0.2 mg mL^{-1}), Folin ciocalteu reagent (diluted with water in 1:1)

5.0 mL of alkaline solution (mixture of 50 mL solution A and 1.0 mL of solution B freshly prepared) was added to 1.0 mL of test solution (protein sample and volume made up with water) and mixed well and kept at room temperature for 10 minute, then 0.5 mL of diluted Folin-ciocalteu reagent was added and mixed immediately. Samples were kept at room

temperature for 30 min. and O.D. was taken at 750 nm. Standard curve was made using BSA stock (0.2 mg mL^{-1}) 20 µg, 40 µg, 60 µg, 80 µg and 100 µg.

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

Composition of resolving gel (10.0 mL):

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

Composition of stacking gel (5.0 mL):

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

The SDS-PAGE gel casting apparatus was arranged and all the components of resolving gel were mixed well in a test tube and gel was poured into the apparatus. A layer of water saturated Isobutanol was also poured over the gel immediately. Gel was allowed for polymerization for minimum 30 min. After polymerization, isobutanol was decanted and gel was washed with water. Stacking gel was poured over the resolving gel and comb was placed. Gel was allowed for polymerization for 30 minutes. Equal volume of 2X sample buffer was added to the protein samples and boiled for 3-5 minutes, cooled to room temperature and loaded in the gel. Gel electrophoresis was carried out at 100 volts for 2 hours in 1X PAGE running buffer (3.03 g Tris base, 14.4 g glycine, 1.0 g SDS and made up the volume to one litre). Gel was removed from the apparatus, stained in staining solution for one hr. followed by destaining; protein bands were visualized and photographed.

4.2.28 ***Western Blot:*** After electrophoresis, the gel was washed in water and a transfer sandwich was made consisting of two blotting papers on spongy support of transfer system saturated with transfer buffer (2.9 g of glycine, 5.8 g of Tris base, 0.37 g of SDS, 200 ml of methanol and volume made to one litre with water). Then PVDF membrane (pre-treated with methanol for 10 min) was placed and then gel was placed over the membrane. The membrane was covered with two blotting papers and second spongy support of transfer system and this transfer sandwich was placed in electro-blotter for transfer of proteins. Electroblotting was carried out at 70 volts for 2 hrs. The blot was semidried and blocked in the blocking buffer for 1 hr. R1 protein specific polyclonal antibody (50 µL) was added in 5.0 mL of blocking buffer and blot was incubated at 25ºC overnight with agitation. The blot was washed thrice with PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.2 g KH_2PO_4 and made volume to 1.0 litre) and once washed with phosphate free buffer (150 mM NaCl, 50mM Tris-HCl). Blot was incubated in blocking buffer (10 mL) containing secondary antibody IgG (10 µL) for one hr. at 25 ºC. The blot was washed thrice with phosphate free buffer. Then blot was incubated with DAB containing buffer with hydrogen peroxide for 5-10 min and reaction was stopped by washing the blot several times with sterile water and placed in the PBS buffer and photographed.

Sr. No.	Chemicals / Biochemicals solutions	Stock concentration	Working concentration	Solvent
1.	Ethidium bromide	5 mg mL^{-1}	0.5 µg mL^{-1}	Sterile water
2.	X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside)	20 mg mL^{-1}	20 µg mL^{-1} (30 µL $plate^{-1}$)	N, N-dimethyl formamide
3.	IPTG (Isopropyl thio-β-D-galactoside)	100 mg mL^{-1}	100 µg mL^{-1} (8 µL $plate^{-1}$)	Sterile water
4.	Ampicillin	50 mg mL^{-1}	50 µg mL^{-1}	Sterile water
5.	Kanamycin	50 mg mL^{-1}	50 µg mL^{-1}	Sterile water
6.	Rifampicin	15 mg mL^{-1}	15 µg mL^{-1}	Sterile water
7.	Streptomycin	50 mg mL^{-1}	50 µg mL^{-1}	Sterile water
8.	Cefotaxime	250 mg mL^{-1}	250 µg mL^{-1}	Sterile water
9.	Lysozyme	10 mg mL^{-1}	300 µg mL^{-1}	sterile water
10.	Ribonuclease A	10 mg mL^{-1}	10 µg mL^{-1}	Dissolved in 10 mM Tris (pH 8.0) and 15 mM NaCl buffer and kept in boiling water bath for 10 minutes followed by slow cooling to room temperature and stored at –20 ºC for subsequent use

Sr. No.	Buffers	Composition and preparation
1.	0.5 M Tris-HCl, pH 8.0	For 100ml stock, 6.05 g of Tris base was dissolved in 50 mL water and pH was adjusted with 6.0N HCl and made up volume with water and autoclaved
2.	0.5 M EDTA, pH 8.0	For 100 mL stock, 18.6 g of sodium salt of EDTA was dissolved in 50 mL water and pH was adjusted with concentrated NaOH and made up volume with water and autoclaved
3.	3.0 M Sodium acetate (pH 5.5)	For 50 mL stock, 12.3 g of Sodium acetate was dissolved in water and adjust the pH with glacial acetic acid and final volume 50 mL.
4.	5 M Potassium acetate	49 g of potassium acetate was dissolved in water and made final volume 100 mL and autoclave
5.	3 M Potassium acetate (pH4.8)	29.4 g of potassium acetate was dissolved in water and 11.5 mL of glacial acetate was added and made final volume 100 mL and autoclave
6.	0.2 M MOPS (pH 7.5)	4.2 g of MOPS was dissolved in water and adjust pH 7.5 using NaOH and made volume 100 mL
7.	0.5 M $MgCl_2$	10.2 g of $MgCl_2$ was dissolved in water and volume 100 mL
8.	Solutions used for alkali lysis method of plasmid isolation:	
	Solution I:	Glucose 50 mM; Tris.HCl 25 mM (pH 8.0); EDTA 10 mM (pH 8.0)
	Solution II:	NaOH 0.2 N; SDS 1.0%
	Solution III:	Potassium acetate (3M)
9.	STET Buffer for boiling prep method of plasmid isolation	8.0 % (w/v) Sucrose, 0.5 % (w/v)Triton X 100, 50.0 mM EDTA(pH 8.0), 10.0 mM Tris-HCl (pH 8.0) Volume was made up by double distilled water and autoclaved

10.	STE Buffer	0.3 M NaCl, 50mM TrisHCl (pH 8.0), 5 mM EDTA (pH 8.0)
11.	Saline EDTA	0.15 M Sodium chloride, 0.1 M EDTA (pH-8)
12.	TE Buffer (1X)	10.0 mM Tris HCl (pH 8.0), 1.0 mM EDTA (pH 8.0) Volume was made up by distilled water and autoclaved
13.	TBE Buffer (5X)	54 g L^{-1} Tris base, 28 g L^{-1} Boric acid, 3.8 g L^{-1} EDTA The pH of the buffer was set at 8.0 Volume was made up by distilled water and autoclaved
14.	TAE Buffer (5X)	24.2 g L^{-1} Tris-base, 5.7 mL L^{-1} Glacial acetic acid, 10 mL L^{-1} 0.5M EDTA (pH-8) Volume was made up by double distilled water and autoclaved.
15.	DNA Gel Loading Buffer (5X)	35 % (w/v) Sucrose or 40% glycerol, 20.0 mM EDTA pH 8.0, 0.1 % (w/v) Bromophenol blue Volume was made up by sterile water
16.	DNA extraction buffer	50 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 250 mM NaCl, 15% sucrose
17.	REX buffer for RNA extraction	100 mM LiCl, 100 mM Tris HCl (pH~8.0), 1.0 % SDS, 10 mM EDTA (pH~ 7.3), 0.2% ß- Mercaptoethanol
18.	Protein extraction buffer	50 mM MOPS- NaOH (pH 7.5), 10 mM $MgCl_2$, 1.0 mM EDTA, 5.0 mM DTT or 0.2% β Mercaptoethanol, 0.1%(v/v) TritonX 100
19.	Formaldehyde Gel Buffers	
	5X Formaldehyde Gel running buffer	0.1 M MOPS, 40 mM sodium acetate, 5 mM EDTA (pH-8)
	Formaldehyde Gel loading buffer	50% glycerol, 1 mM EDTA (pH-7.5), 0.25% bromophenol blue
20.	Solutions for Folin Lowry protein estimation:	Solution I : Alkaline Sodium carbonate(20 g of sodium carbonate was dissolved in final volume of 0.1 N NaOH) Solution II **:** Copper sulphate-sodium potassium tartarate solution (5g per litre CuSO4.5H2O was dissolved in 10 g per litre of Sodium potassium tartarate) Mix 50 mL solution I with 1 mL of solution II for use

21.	SDS-PAGE Buffers	
	10 X Electrophoresis buffer	For one litre: 30.3g Tris Base, 144 g glycine, 10g SDS
	30% Acrylamide	Acrylamide (29.2) : Bis Acrylamide (0.8)
	4X Separating gel buffer	1.5 M TrisHCl (pH 8.8)
	4X Stacking gel buffer	0.5 M TrisHCl (pH 6.8)
	Separating gel overlay solution	Water saturated butanol
	10 % SDS	10 g of SDS was dissolved in 70 mL sterile water and made volume to 100 mL
	2X Sample buffer (10ml)	0.5 M Tris HCl (pH 6.8)- 2.5 mL,10% SDS- 4.0 mL, glycerol-2.0 mL, β-Mercepta-ethanol-0.8 mL, 0.1% Bromophenol blue-0.3 mL and made the volume with water
	10% APS	10 mg of APS dissolved in 1 mL of sterile water
	TEMED	Commercially available
	Staining solution	0.15% Coomassie Blue-R250, 30% Methanol, 10% glacial acetic acid and made final volume with water
	Destaining solution	30% Methanol, 10% glacial acetic acid and made final volume with water
22.	Western blot buffers	
	Transfer Buffer	2.9 g L^{-1} Glycine, 5.8 g L^{-1} Tris Base, 0.37 g L^{-1} SDS, 200 mL methanol made in sterile water under sterile conditions
	PBS	8.0 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.44 g L^{-1} Na2HPO4, 0.24 g L^{-1} KH_2PO_4
	Phosphate removal buffer	150 mM NaCl, 50 mM TrisHCl

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

Composition and Stock Preparation of Murashige and Skoog (MS) Basal Medium

MS Major Salts:

Sr. No.	MS Major Salts	MS Basal Concentration (mg/L)	Stock Concentration 100 X (g/L)	Use of stock in ml/L	For 500 ml of stock
1.	KNO_3	1900.0	190.0 g	10.0 mL	95.0 g
2.	NH_4NO_3	1650.0	165.0 g	10.0 mL	82.5 g
3.	$MgSO_4.7H_20$	370.0	37.0 g	10.0 mL	18.5 g
4.	$CaCl_2.2H_2O$	440.0	44.0 g	10.0 mL	22.0 g
5.	KH_2PO_4	170.0	17.0 g	10.0 mL	8.5 g

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

MS Minor Salts:

Sr. No.	MS Minor Salts	MS Basal Concentration (mg/L)	Stock Concentration 1000 X (g/L)	Use of stock in ml/L	For 100 ml of stock
1.	H_3BO_4	6.20	6.20 g	1.0 mL	0.62 g
2.	$MnSO_4.4H_2O$	22.30	22.30 g	1.0 mL	2.23 g
3.	$ZnSO_4.7H_2O$	8.60	8.60 g	1.0 mL	0.860 g
4.	$Na_2MoO_4.2H_2O$	0.25	0.25 g	1.0 mL	0.025 g
5.	$CuSO_4.5H_2O$	0.025	0.025 g	1.0 mL	0.0025 g
6.	$CoCl_2.6H_2O$	0.025	0.025 g	1.0 mL	0.0025 g
7.	KI	0.83	0.83 g	1.0 mL	0.083 g

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

MS Vitamins:

Sr. No.	Name of Vitamins	MS Basal Concentration (mg/L)	Stock Concentration (mg/ ml)	Use of stock in ml/L	For 50 ml of stock
1.	Nicotinic Acid	0.5	0.5	1.0 mL	25.0 mg
2.	Pyridoxine HCl	0.5	0.5	1.0 mL	25.0 mg
3.	Thiamine HCl	0.1	0.1	1.0 mL	5.0 mg
4.	Glycine	2.0	2.0	1.0 mL	100.0 mg
5.	Myo-inositol	100.0	100.0	1.0 mL	5.0 g

Note: All the MS vitamins stock solutions to be prepared separately.

Sr. No.	Name of Chemical	MS Basal Concentration (mg/L)	Stock Concentration (mg/ ml)	Use of stock in ml/L	For 50 ml of stock
1.	$Fe_2EDTA.\ 2H_2O$ (sodium salt)	30.0	30.0	1.0 mL	1.5 g

Preparation of MS basal medium: Major salts + Minor salts + Vitamins + $Fe_2EDTA.\ 2H_2O$ + 3.0% Sugar + 0.7% to 0.8%. Agar agar, set the pH of medium to 5.8 using 0.01N HCl or 0.01N NaOH.

Results and Discussion

5. Results and Discussion

5.1 Studies on the status of starch phosphorylation and temperature dependence of sugar accumulation in potato tubers from various cultivars

There are more than 40 high-yielding Indian potato cultivars developed through conventional breeding during the last few decades. Some of them belong to the processing varieties. These cultivars vary with regard to genetic make up, maturation time, disease resistance and growth in different agro-climatic zones of the Indian subcontinent. In this study, a total of six Indian potato cultivars were chosen: Kufri Chipsona-1, Kufri Chipsona-2, Kufri Chandramukhi, Kufri Jyoti, Kufri Ashoka, and Kufri Pukhraj along with Desiree as a reference cultivar; for convenience they are also written as CS-1, CS-2, KCM, KJ, As, PR, and De, respectively in this thesis. Mature mini tubers were harvested from all these cultivars for the following experiments to determine a) the tuber starch-bound phosphorus contents in different potato cultivars, b) the accumulation of reducing and total soluble sugars in the cold-stored tubers from different potato cultivars. The purpose was to make a comparison between these potato cultivars (in other words to see the varietal differences) with regard to the above biochemical attributes.

5.1.1. *Status of starch phosphorylation*

Starch isolation from potato tubers: Starch granules were isolated from the mini tubers of all the above potato cultivars. The starches were essentially devoid of organic impurities and free inorganic phosphates. The yield of starch granules was found to be in the range of 7.0-9.0 % of the tuber weight. In case of Kufri Chipsona-1, the yield was found to be maximum followed by Kufri Jyoti, and minimum for the cultivar Kufri Pukhraj.

Microscopic study of the starch granules: The starch powder of different cultivars were stained with iodine stain and observed under the microscope. The starch granules of different cultivars were found to vary with regard to size and shape as shown in Fig. 1.

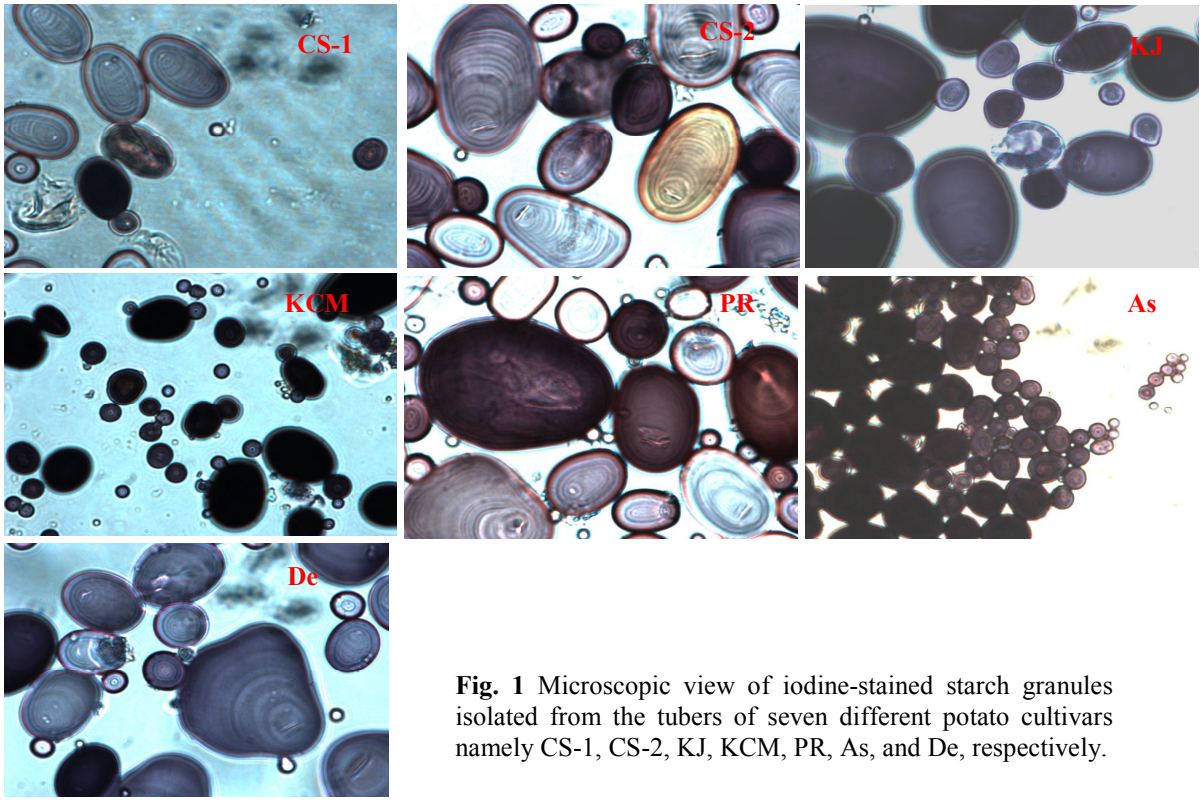

Fig. 1 Microscopic view of iodine-stained starch granules isolated from the tubers of seven different potato cultivars namely CS-1, CS-2, KJ, KCM, PR, As, and De, respectively.

X-ray diffraction Analyses of starch granules: Different studies on crystalline properties of starch granules using X-ray diffraction revealed that starch can be classified into A, B and C forms. Cereal starch shows A-type whereas B-type was characteristic feature of tuber starches, and pea and bean starches show C-type which is a mixture of A and B-types. A-type pattern shows a close-packed arrangement with water molecules between each double helical structure, while the B-type is more open, there being more water molecules, essentially all of which are located in a central cavity surrounded by six double helices. A-type crystallinity is

due to short chain length amylopectin and long chain length for B-type crystallinity whereas intermediate chain length for C-type crystallinity (Hejazi et al. 2009).

The XRD pattern of potato shows peak at 5.59, 14.4, 17.2, and 22.1 (2θ). Potato starch is of B type. The varietal differences were observed in the XRD pattern of the starch powders of different Indian cultivars. The XRD patterns of the potato cultivars CS-2, KJ, As, Pr and De show distinct peaks at 17, 21 and 22 (2θ). CS-1 and KCM tuber starches show similar XRD pattern, and weak peaks at 17, 22, 23 (2θ); but in case of CS-1 tuber starch, the peak was absent at 17 (2θ). These XRD data suggest that distinct variation in the degree of crystallinity exist in the tuber starches between the different Indian potato cultivars (Fig. 2). It is also known that degree of crystallinity is inversely proportional to the amylose content. The results suggest that the tuber starches from different cultivars are likely to vary with respect amylose to amylopectin ratio and also glucan chain lengths. Further biochemical studies are required for clear understanding in these aspects.

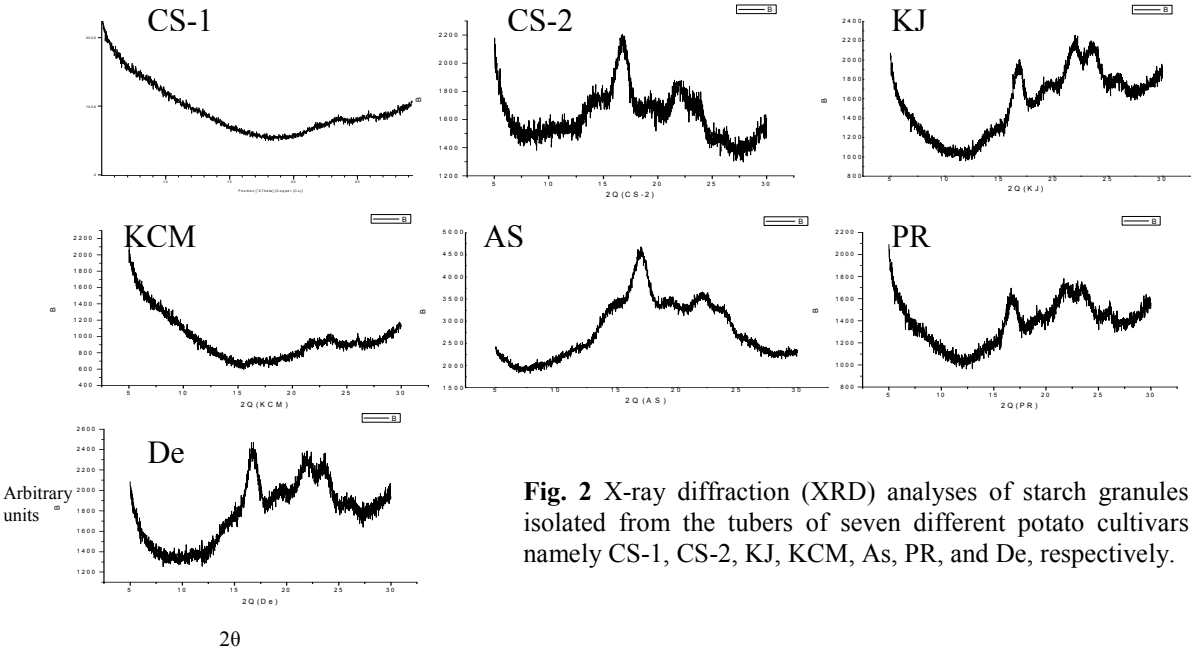

Fig. 2 X-ray diffraction (XRD) analyses of starch granules isolated from the tubers of seven different potato cultivars namely CS-1, CS-2, KJ, KCM, As, PR, and De, respectively.

Determination of Starch-bound total phosphorus content: The starch-bound total phosphorus of different cultivars was determined by the Ascorbic acid-molybdate method. The phosphorus content was in the range of 0.35-0.60 mg P g^{-1} tuber starch from different cultivars**.** The lowest value, 0.39 mg P g^{-1}starch was found in Kufri Chipsona-2 and highest value in case of Kufri Chandramukhi i.e., 0.55 mg P g^{-1}starch as shown in Fig. 3.

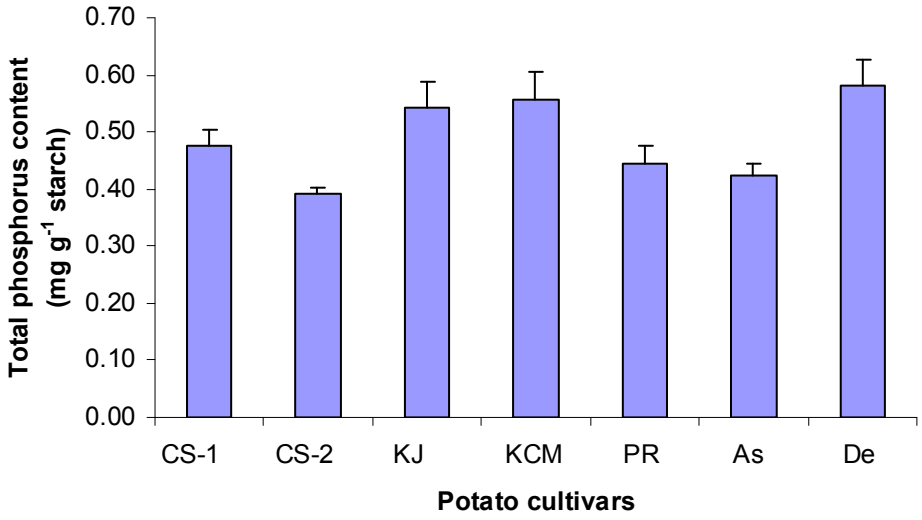

Fig. 3 Total starch-bound phosphorus content in the tuber starches from different potato cultivars

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5.1.2 *Temperature dependence of sugar accumulation in the potato tubers*

The freshly harvested tubers were kept at room temperature for around three weeks, and then transferred to different experimental temperatures 20ºC, 10ºC, 5ºC and 3ºC for one month and two months. Extraction of soluble sugars was carried out from weighed amount (~ 200 mg) of thin slices from the freshly harvested tubers as well as other tubers stored at different temperatures.

Estimation of reducing sugars: The level of reducing sugars was determined by Nelson-somogyi's method. Varietal differences were found with respect to sugar accumulation. For freshly harvested tubers from different cultivars, the reducing sugar level was found to be in

the range of 0.15 to 1.50 mg g^{-1}FW (Fig. 4A). But the storage of tubers at 3ºC for one month to two months led to very high accumulation (25 to 60-fold increase) of reducing sugars and found to be in the range of 12.0-27.0 mg g^{-1}FW. The level of reducing sugar was found to high in the cultivar Kufri Ashoka (26.78 mg g^{-1}FW), whereas relatively less accumulation in the cultivars Kufri Chipsona-2 (13.02 mg g^{-1}FW) and the exotic cultivar Desiree (12.55 mg g^{-1}FW) (Fig. 4B). More or less similar trend was noted if the tubers were stored at 5ºC for the same period of time (Fig. 4C). Even the tubers stored at 10ºC also showed considerable accumulation of reducing sugars (Fig. 4D).

Estimation of total soluble sugars: The level of total soluble sugars was also determined among the different Indian potato cultivars by Anthrone method. The total soluble sugar content of freshly harvested tubers was in the range of 1.9-6.2 mg g^{-1}FW, showing lowest value for Kufri Jyoti, 1.98 mg g^{-1}FW and highest in Kufri Chandramukhi i.e., 6.22 mg g^{-1}FW (Fig. 4A). The total soluble sugar content of tubers stored at 3ºC was found to be highest for Kufri Jyoti (34.06 mg g^{-1} FW) and lowest for Kufri Chipsona-2 (15.46 mg g^{-1} FW) (Fig. 4B). The total soluble sugar content of tubers stored at 5ºC was found to be highest in Kufri Ashoka (17.77 mg g^{-1} FW) whereas lowest value in case of Kufri Chipsona-2 (4.24 mg g^{-1} FW) (Fig. 4C). The total soluble sugar content of tubers stored at 10ºC was also found maximum 20.95 mg g^{-1} FW in Kufri Ashoka (Fig. 4D).

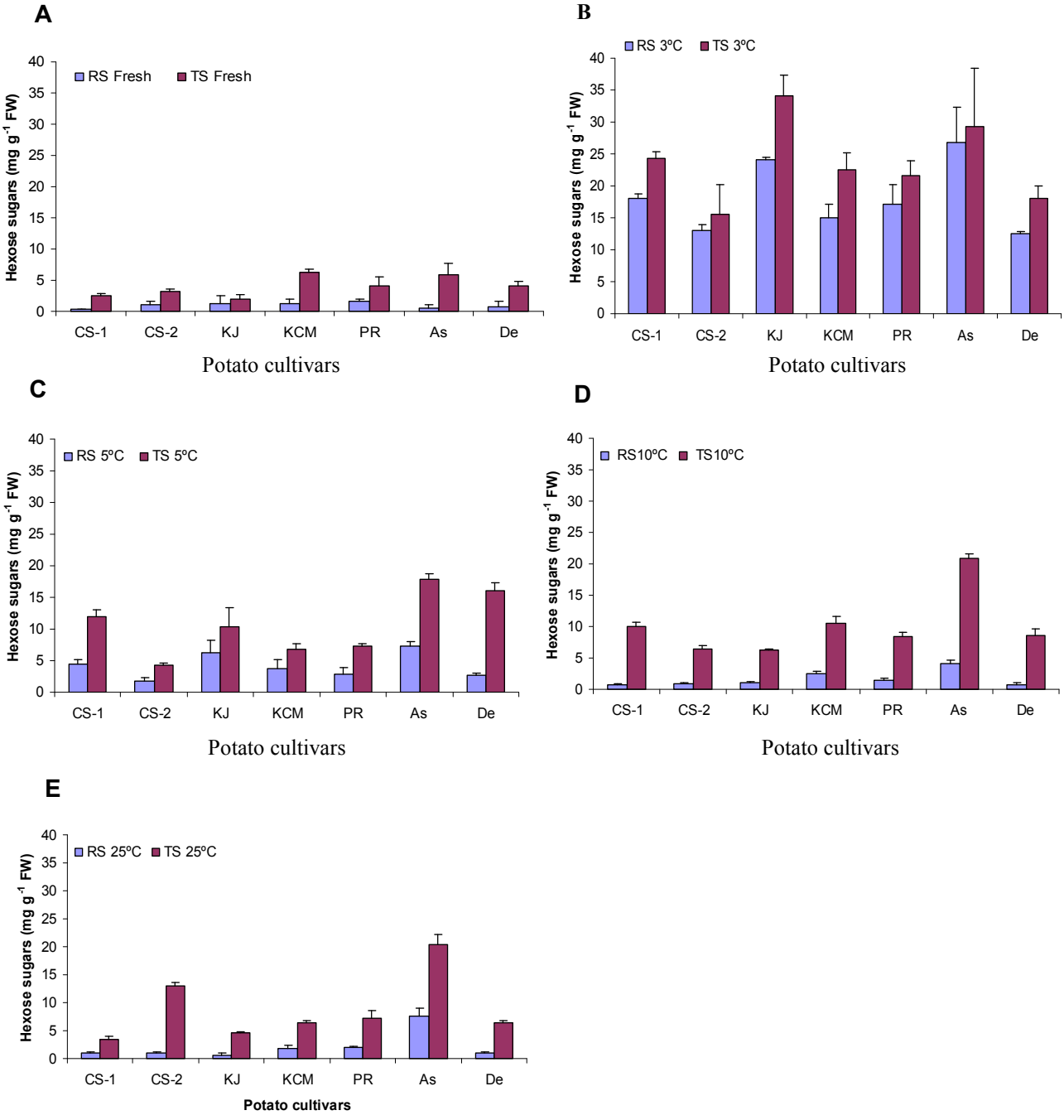

Fig. 4 Hexose sugar contents of freshly harvested potato tubers, and tubers stored at different temperatures 3ºC, 5ºC, 10ºC and 25ºC for one month. The results are the mean ± SD of four individual tubers. RS refers to reducing sugars, and TS refers to total soluble sugars. **A** Freshly harvested tubers; **B** tubers stored at 3ºC; **C** tubers stored at 5ºC; **D** tubers stored at 10ºC; **E** tubers stored at 25ºC

In earlier reports it has been well documented that the extent of hexose accumulation in potato tubers during cold storage is cultivar-dependent (Vliet and van Schriemer 1960; Burton 1969; Samotus et al. 1974). It is generally believed that the cold-induced hexose accumulation is caused by an imbalance between starch breakdown and glycolytic activities. A number of different gene functions are believed to be involved in this undesirable process (Zrenner et al. 1996). With regard to the Indian potato cultivars, no comprehensive reports are available for in-depth understanding of the cold-induced sweetening phenomenon. Keeping in view of this, the biochemical studies as carried out here would be useful. One of the objectives of this study to know the varietal differences with regard to tuber starch phosphorylation and the extent of accumulation of reducing and soluble sugars.

Starch-bound phosphorus content, XRD analyses, and microscopic studies revealed the variation with regard to size, shape, crystallinity, and phosphorus content of tuber starch granules isolated from different potato cultivars. Starch bound-phosphate content affects the different physico-chemical properties of starch and also conversion of starch into the hexose sugars during cold storage of potato tubers. It was believed that more phosphate content in starch leads to more degradability of starch during storage at low temperatures. Here efforts were made to see any correlation between starch-bound phosphate content and sugar accumulation during cold storage between the different Indian potato cultivars; since such type of studies are relevant for further crop improvement programmes. During storage at low temperatures from 3ºC-5ºC, all the potato cultivars showed very high accumulation of reducing and soluble sugars (25 to 60-fold), and extent of accumulation was found to vary between the cultivars. Overall variations of the starch structures, starch-bound phosphate contents are likely to be responsible for varying degree of starch degradability during low

temperature storage of the potato tubers. In case of Kufri Chipsona-2 the starch-bound phosphate content was found to be relatively low, also showed less accumulation of reducing sugars. But in case of Kufri Chandramukhi, such type of direct correlation was not found since the starch-bound phosphate content was high but reducing sugar accumulation was less in the cold-stored tubers. Likewise, in Kufri Ashoka also no such correlation was found. This is true for other cultivars also. Therefore, more in-depth biochemical studies are required for clear understanding of the cold-induced sweetening phenomenon.

5.2 Isolation and characterization of cDNA and/or genomic clones corresponding to starch-granule-bound protein R1 protein from potato cultivars

5.2.1 *Isolation and characterization of R1 (GWD) cDNA from potato*

RNA isolation, RT-PCR, restriction analysis, and cloning of GWD cDNA: DNA-free total RNA was isolated from the tubers and leaves of each of the seven field-grown potato cultivars as mentioned earlier. The intactness of the RNA preparations was checked by normal and formaldehyde agarose gel electrophoreses (Fig. 5), and the A_{260}/A_{280} ratio was found to be nearly 2.0.

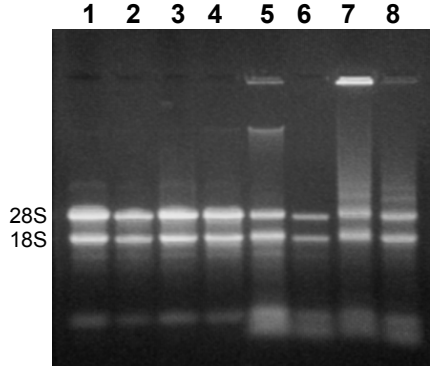

Fig. 5 Formaldehyde agarose gel electrophoresis of total RNA isolated from different potato cultivars, lane 1-6 tuber total RNA and lane 7-8 leaf total RNA, lane 1 Kufri Chipsona-1; lane 2 Kufri Chipsona-2; lane 3 Kufri Jyoti; lane 4 Kufri Chandramukhi; lane 5 Kufri Pukhraj; lane 6 Kufri Ashoka; lane 7 Kufri Chipsona-1; lane 8 Kufri Chipsona-2

In the first instance, tuber and leaf RNA samples from the three potato cultivars, Kufri Chipsona-1, Kufri Jyoti, and Kufri Chandramukhi were used as templates in the reverse transcription (RT) reactions using the reverse primer, R1-4810. The individual RT products were used to carry out PCR using the potato GWD cDNA-specific primer pair A20-R1 and R1-4810. The size of the amplified product was ~ 4.8 kb in each case (Fig. 6A). Moreover, all these RT-PCR products showed similar restriction patterns for a few restriction enzymes such as *Eco*RI, *Hind*III, and *Sac*I (for the enzyme *Eco*RI it was shown in Fig. 6B). Here, only the RT-PCR product corresponding to the tuber RNA of Kufri Chipsona-1 was cloned into the *Sma*I site of pUC19 vector, followed by sequencing in both directions by Bangalore Genei, Bangalore. The nucleotide sequence was analyzed by NCBI BLAST tool, and found to encode glucan, water dikinase (GWD). The sequence information was submitted in NCBI database (GenBank Acc No EU599037).

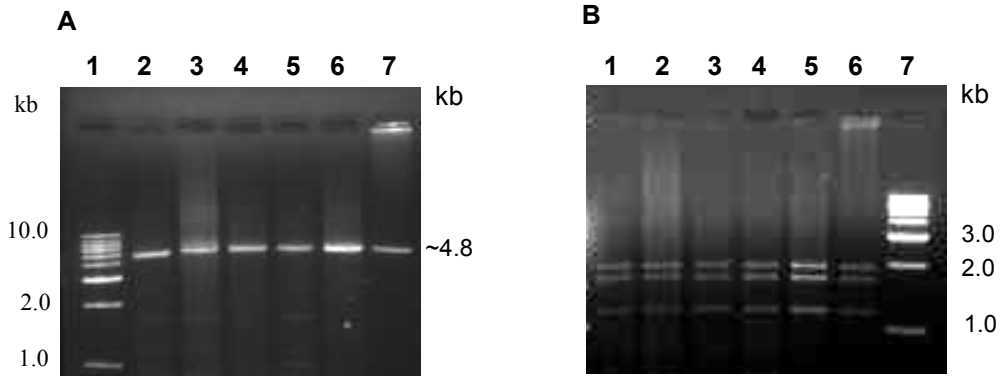

Fig. 6 A RT-PCR amplification products (~4.8 kb) using total RNA from the tubers and leaves of different potato cultivars, and the GWD cDNA specific primers A20-R1 and R1-4810. *Lane 1* 1.0 kb DNA ladder; *lanes 2, 4,* and *6* correspond to tuber RNA from the cultivars Kufri Chipsona-1, Kufri Jyoti, and Kufri Chandramukhi, respectively; whereas, *lanes 3, 5,* and *7* correspond to leaf RNA from the respective potato cultivars

B Restriction analyses of the RT-PCR products as in **A** with *Eco*R1. *Lanes 1, 3,* and *5* show the restriction pattern of the tuber RNA derived RT-PCR products; *lanes 2, 4,* and *6* show the restriction pattern of the leaf RNA derived RT-PCR products (with same order of the potato cultivars as in **A**); *lane 7* 1.0 kb DNA ladder

Sequence analyses of GWD cDNA, multiple sequence alignment, and phylogenetic tree: The 4789-bp GWD cDNA of this study contained a 4392-bp ORF (bases 100-4491). The predicted protein consisted of 1463 amino acids, designated as KC-GWD, having a plastid-specific N-terminal 77-amino acid transit peptide, and 1386-amino acid mature protein. The mature GWD appeared to be shorter by one amino acid as compared to the other mature potato GWDs documented earlier. At nucleotide level, the entire 4789-bp GWD cDNA shares 98% sequence identity with the full-length GWD cDNA from the potato cv. Desiree (Lorberth et al. 1998); but the 4392-bp coding region of the cDNA shares 99%, 99%, and 97% nucleotide identity with that of potato cv. Desiree (Y09533), potato cv. Prevalent (AY027522), and tomato cv. Micro-Tom (EU908574), respectively. More sequence divergence was noted for the other plant species as revealed by the considerable decrease of the query coverage length during NCBI BLAST search. In these cases, approx. two-third of the coding region of KC-GWD showed nucleotide identity in the range of 70-80%.

Based on the ProtParam tool (http://web.expasy.org/cgi-bin/protparam), the calculated molecular mass of the mature KC-GWD was found to be ~155.0 kDa with a predicted pI of 5.45. Out of a total 1386 amino acids, 172 are strongly basic (+) (K, R), 202 are strongly acidic (–) (D, E), 496 are hydrophobic (A, I, L, F, W, V), and 320 are polar (N, C, Q, S, T, Y). Moreover, the instability index (II) was computed as 40.66, which classified the protein as unstable. The amino acid composition data also revealed that some of the amino acids such as Asp (6.3%), Glu (8.3%), Leu (10.5%), Lys (7.6%), and Ser (7.7%) occurred more frequently as compared to their average occurrence; whereas, the amino acids, namely Cys (0.6%), Met (1.7%), Pro (4.3%), Gln (3.7%), His (1.5%), and Tyr (2.8%) occurred less frequently (Doolittle 1989). Latest NCBI protein-protein BLAST search (blastp), revealed that the

mature KC-GWD shared 98, 98, 96, 74, 73, 72, 71, 68, 69, 68, 63, 75 , 46, and 26% sequence identity with potato cv. Prevalent (R1 or GWD, AAK11735.1), potato cv. Desiree (R1 or GWD, Q9AWA5.2), tomato cv. Micro-Tom (GWD, NP_001234405.1), *Vitis vinifera* (Hypothetical protein, XP_002270485), *Ricinus communis* (GWD, XP_002527902), *Populus trichocarpa* (Predicted protein, XP_002315679), *Citrus reticulata* (GWD, Q8LPT9), *Oryza sativa* Indica Group (Hypothetical protein, EEC80673) *Oryza sativa* Japonica Group (Hypothetical protein, NP_001057699), *Sorghum bicolor* (Hypothetical protein, XP_002438419), *Arabidopsis thaliana* (GWD1/SEX1, NP_563877), *Triticum aestivum* (GWD, ADG27838, query coverage being 68%), *Arabidopsis thaliana* (AtGWD2, AAO42141.1), *Arabidopsis thaliana* (AtGWD3, AAU93516, query coverage being 36%), respectively.

Multiple sequence alignment was done using clustalW2, an EBI sequence analysis tool, using the GWD sequences from the three potato cultivars, Kufri Chipsona-1 (KC-GWD), Desiree (De-GWD), Prevalent (Pr-GWD), and the tomato cv. Micro-Tom (MT-GWD) shown in Fig.7. Alignments for the transit peptides (Fig. 7A) and the mature proteins (Fig. 7B) were shown separately to examine sequence similarities, nature and location of the amino acid substitutions as compared to KC-GWD. The transit peptides of KC-GWD and De-GWD were identical, whereas Pr-GWD transit peptide varies by two non-conservative substitutions, E66G and K68N. MT-GWD consisted of 78-amino acid transit peptide, longer by one more amino acid, Asn, occupying the position 61. The other five substitutions (three conservative and two non-conservative ones) were noted in this transit peptide: S27T, K57R, E66G, S73T, and S74Y. The mature protein of KC-GWD consisted of 1386 amino acids, whereas the GWDs from the other cultivars of potato and tomato consisted of 1387 amino acids. One extra

amino acid i.e., Asn occupied the position 305 in the other three GWDs. The mature proteins of KC-GWD and De-GWD were found to vary at 25 locations: E17G, R18G, A41V, Y114H, T163P, E177V, E215D, K269T, A279V, V283L, Q302H, P307T, R388P, D462G, K603N, E612Q, N679G, G736D, G756R, N965K, T1131M, I1149V, A1235T, T1279P, and S1350N. Out of this, a total of 19 positions represent non-conservative substitutions. Broadly, this overall variation is quite significant which needs to be further elucidated. Similarly, sequence comparison between the mature proteins of KC-GWD and Pr-GWD revealed the variations at 27 places (mostly non-conservative substitutions), including some common substitutions as noted previously between KC-GWD and De-GWD. More sequence divergence (variations at 47 places, including 19 non-conservative substitutions) was noted between the mature proteins of KC-GWD and MT-GWD.

The CBMs are present as tandem domains in the N-terminal part of the potato and tomato mature GWDs. In KC-GWD, the amino acid residues 32-133 represent CBM45-1, and the residues 359-467 represent CBM45-2. These two tandem domains are separated by a linker of 225 amino acid residues, for the other three GWDs, the linker consists of 226 amino acids. CBM45-2 domain appeared to be more conserved relative to CBM45-1 between the GWD isoforms. However, some conservative and non-conservative substitutions were found in either of these domains. Relatively more sequence divergence was noted in the linker regions. In CBM45-1 and CBM45-2 of KC-GWD the conserved aromatic amino acids were clearly indicated (Fig. 7B). The catalytic domain in the GWD isoforms (corresponding to the position 720 to 1050 of mature KC-GWD) is mostly conserved with some substitutions (both conservative and non-conservative ones). However, the region from 966 to 1031, containing the catalytic His residue appeared to be identical. The 38 kDa C-terminal fragment (approx

position 1051 to 1386), containing the ATP binding site, showed that most of the regions are conserved; but MT-GWD appeared to be relatively more divergent.

A phylogenetic tree (Fig. 8) was generated based on the 11 GWD sequences from different plant species (including both predicted and hypothetical proteins). First, multiple sequence alignment was done by the *MultAlin* software, followed by generation of the phylogenetic tree using MEGA 5.0 software by the Neighbor-Joining method. The KC-GWD, as isolated in this study, was found to be in a distinct branch in the phylogenetic tree.

A. Transit peptide

```
KC-GWD MSNSLGNNLLYQGFLTSTVLEHKSRISPPCVGGNSLFQQQVISKSPLSTEFRGNRLKVQK-KKIPMEKKRAFSSSPHA 77
De-GWD ..........................................................-.................. 77
Pr-GWD ..........................................................-.....G.N......... 77
MT-GWD ........................T..............................R...N.....G......TY... 78
       ************************* ****************************** *** ***** * ****  ***
```

B. Mature protein

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                                                          ↓         ↓
KC-GWD VLTTDTSSELAEKFSLERNIELQVDVRPPTSGDVSFVDFQATNGSDKLFLHWGAVKFGKETWSLPNDRPDGTKVYKNKAL   80
De-GWD ................GG.....................V.....................................   80
Pr-GWD ........Q........G.....................V.....................................   80
MT-GWD L................G.............D.A......V.........................C.............   80
        ******* *******  ************ * ****** ************************* ************
                                  ↓         ↓       ↓
KC-GWD RTPFVKSGSNSILRLEIRDTAIEAIEFLIYDEAYDKWIKNNGGNFRVKLSRKEIRGPDVSVPEELVQIQSYLRWERKGKQ  160
De-GWD ................................H.............................................  160
Pr-GWD ................................H......I....HI..............................  160
MT-GWD ..................L..............R..................R..........................  160
       ***************** *************** ****** ****  **** ****************************

KC-GWD NYTPEKEKEEYEAARTELQEEIARGASIQDIRARLTKTNDKSQSKEEPLHVTKSEIPDDLAQAQAYIRWEKAGKPNYPPE  240
De-GWD ..P.............V.....................................D.........................  240
Pr-GWD ..........................................................N.....................  240
MT-GWD ..................................................P...E.D.......V.................  240
       ** ************* ******************************** *** * ******* *****************

KC-GWD KQIEELEEARRELQLELEKGITLDELRKKITKGEIKTKAEKHVKRSSFAVERIQRKKRDFGQLI-KYPSSPAVQVQKVLE  319
De-GWD ............................T.........V...L..................H..N..T............  320
Pr-GWD ..................................E..V...L.....................N................  320
MT-GWD R.....................................V...L.....................N................  320
        *************************** ****** ** *** ****************** ** ** ************
                                                                    ↓         ↓
KC-GWD EPPALSKIKLYAKEKEEQIDDPILNKKIFKVDDGELLVLVAKSSGKTKVHLATDLNQPITLHWALSKSRGEWMVPPSSIL  399
De-GWD ..........................................................................P.....  400
Pr-GWD ..A.....................................S.........................P.....  400
MT-GWD ...................V.....................................................R.P.....  400
       ** *************** ******************** ************************ * ************
                                                  ↓         ↓         ↓
KC-GWD PPGSIILDKAAETPFSASSSDGLTSKVQSLDIVIEDGNFVGMPFVLLSGEKWIKNQGSDFYVDFSAASKLALKAAGDGSG  479
De-GWD ..................................................................G.............  480
Pr-GWD ..........................................................................S.....  480
MT-GWD .....V..........V.............................F.............................  480
       ***** ********** ***************************** *************** ****** **********
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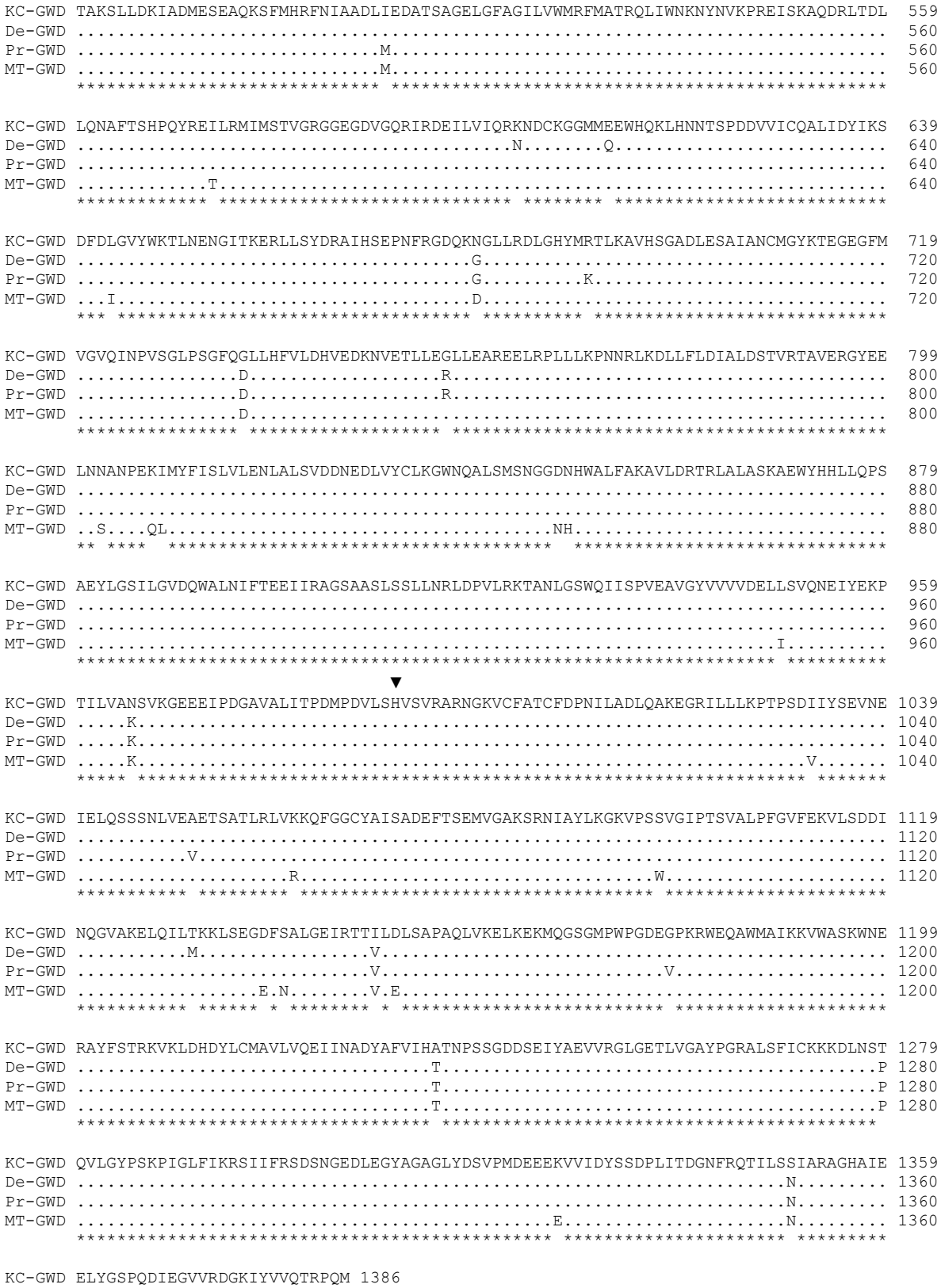

KC-GWD TAKSLLDKIADMESEAQKSFMHRFNIAADLIEDATSAGELGFAGILVWMRFMATRQLIWNKNYNVKPREISKAQDRLTDL 559
De-GWD 560
Pr-GWD .....M..... 560
MT-GWD .....M..... 560
KC-GWD LQNAFTSHPQYREILRMIMSTVGRGGEGDVGQRIRDEILVIQRKNDCKGGMMEEWHQKLHNNTSPDDVVICQALIDYIKS 639
De-GWD .....N.....Q..... 640
Pr-GWD 640
MT-GWD .....T..... 640
KC-GWD DFDLGVYWKTLNENGITKERLLSYDRAIHSEPNFRGDQKNGLLRDLGHYMRTLKAVHSGADLESAIANCMGYKTEGEGFM 719
De-GWD .....G..... 720
Pr-GWD .....G.....K..... 720
MT-GWD ...I.....D..... 720
KC-GWD VGVQINPVSGLPSGFQGLLHFVLDHVEDKNVETLLEGLLEAREELRPLLLKPNNRLKDLLFLDIALDSTVRTAVERGYEE 799
De-GWD .....D.....R..... 800
Pr-GWD .....D.....R..... 800
MT-GWD .....D..... 800
KC-GWD LNNANPEKIMYFISLVLENLALSVDDNEDLVYCLKGWNQALSMSNGGDNHWALFAKAVLDRTRLALASKAEWYHHLLQPS 879
De-GWD 880
Pr-GWD 880
MT-GWD ..S....QL.....NH..... 880
KC-GWD AEYLGSILGVDQWALNIFTEEIIRAGSAASLSSLLNRLDPVLRKTANLGSWQIISPVEAVGYVVVVDELLSVQNEIYEKP 959
De-GWD 960
Pr-GWD 960
MT-GWD .....I..... 960
KC-GWD TILVANSVKGEEEIPDGAVALITPDMPDVLSHVSVRARNGKVCFATCFDPNILADLQAKEGRILLLKPTPSDIIYSEVNE 1039
De-GWD .....K..... 1040
Pr-GWD .....K..... 1040
MT-GWD .....K.....V..... 1040
KC-GWD IELQSSSNLVEAETSATLRLVKKQFGGCYAISADEFTSEMVGAKSRNIAYLKGKVPSSVGIPTSVALPFGVFEKVLSDDI 1119
De-GWD 1120
Pr-GWD .....V..... 1120
MT-GWD .....R.....W..... 1120
KC-GWD NQGVAKELQILTKKLSEGDFSALGEIRTTILDLSAPAQLVKELKEKMQGSGMPWPGDEGPKRWEQAWMAIKKVWASKWNE 1199
De-GWD .....M.....V..... 1200
Pr-GWD .....V.....V..... 1200
MT-GWD .....E.N.....V.E..... 1200
KC-GWD RAYFSTRKVKLDHDYLCMAVLVQEIINADYAFVIHATNPSSGDDSEIYAEVVRGLGETLVGAYPGRALSFICKKKDLNST 1279
De-GWD .....T.....P 1280
Pr-GWD .....T.....P 1280
MT-GWD .....T.....P 1280
KC-GWD QVLGYPSKPIGLFIKRSIIFRSDSNGEDLEGYAGAGLYDSVPMDEEEKVVIDYSSDPLITDGNFRQTILSSIARAGHAIE 1359
De-GWD .....N..... 1360
Pr-GWD .....N..... 1360
MT-GWD .....E.....N..... 1360
KC-GWD ELYGSPQDIEGVVRDGKIYVVQTRPQM 1386
De-GWD 1387
Pr-GWD 1387
MT-GWD .....S.....L..... 1387

◄**Fig. 7** Comparison of the predicted amino acid sequences of four GWD homologues from the *Solanaceae* family: KC-GWD (*S. tuberosum* cv. Kufri Chipsona-1), De-GWD (*S. tuberosum* cv. Desiree), Pr-GWD (*S. tuberosum* cv. Prevalent), MT-GWD (*S. lycopersicum* cv. Micro-Tom) (see the text for the GenBank accession numbers). **A** refers to the comparison of the GWD transit peptides; whereas **B** refers to the comparison of the GWD mature proteins. Dots indicate the common amino acids with KC-GWD; dashes indicate gaps that arise during the alignment; asterisks indicate the conserved amino acids in all the sequences. The carbohydrate-binding modules, CBM45-1 (32-133) and CBM45-2 (359-467) are highlighted also showing the five conserved aromatic amino acids (across the plant species) in each module by downward arrows (↓). '▼' indicates the catalytic histidine residue at the position 991 of the KC-GWD mature protein

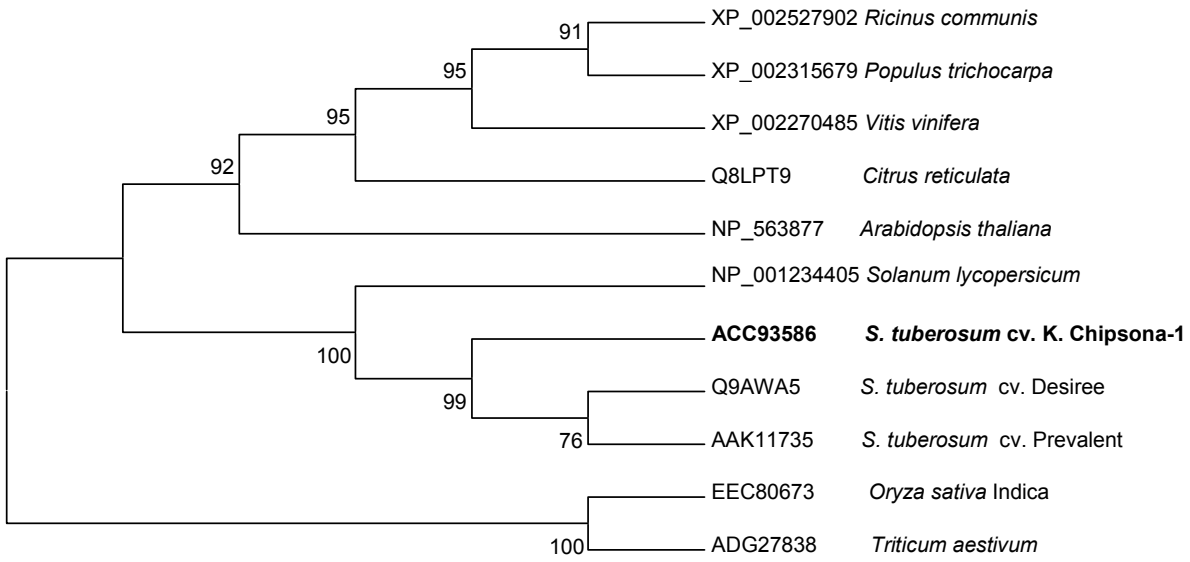


Fig. 8 The phylogenetic tree was generated by the MEGA 5.0 software using the Neighbor-Joining method based on the 11 GWD sequences from the different plant species (the name of the plant species and the GenBank accession numbers are indicated at each branch). The number at each node represented the bootstrap value, with 1000 replicates. KC-GWD (ACC93586), as isolated in this study, appeared to occupy a distinct position in the phylogenetic tree

Hydropathy plot, prediction of the secondary structures: Based on the ProtParam tool, the grand average of hydropathicity was computed to be –0.340 for the KC-GWD mature protein. With the help of ProtScale tool (http://web.expasy.org/cgi-bin/protscale) based on the Kyte-Doolittle scale (Kyte and Doolittle 1982), the hydrophobic characters of the mature protein of KC-GWD were predicted and shown in the Fig. 9A. Most of the regions of the carbohydrate-binding modules (both CBM45-1 and CBM45-2) are hydrophilic in character. However, a few segments in these regions were found to be hydrophobic. The 225-amino acid linker region between the CBM45s was mostly hydrophilic. The entire catalytic domain, containing 991H residue, consisted of both hydrophilic and hydrophobic segments. The ATP-binding domain consisted of some prominent hydrophilic segments along with some hydrophobic segments towards the C-terminus.

With the help of Chou & Fasman Secondary Structure Prediction Server (CFSSP) (http://www.biogem.net/cgi-bin/cho-fas.pl), and ProtScale tool (http://web.expasy.org/cgi-bin/protscale) α-helix (Chou & Fasman scale), β-sheet (Chou & Fasman scale), β-turn (Chou & Fasman scale), and random coil (Deleage & Roux scale) in KC-GWD were predicted. Only predicted α-helical structure of the KC-GWD mature protein was shown in Fig. 9B (other secondary structures not shown). The linker region between CBM45s, a stretch nearly 100 amino acids immediately after CBM45-2, the catalytic domain, and the N-terminal region of the ATP-binding domain were predicted to assume α-helical structures.

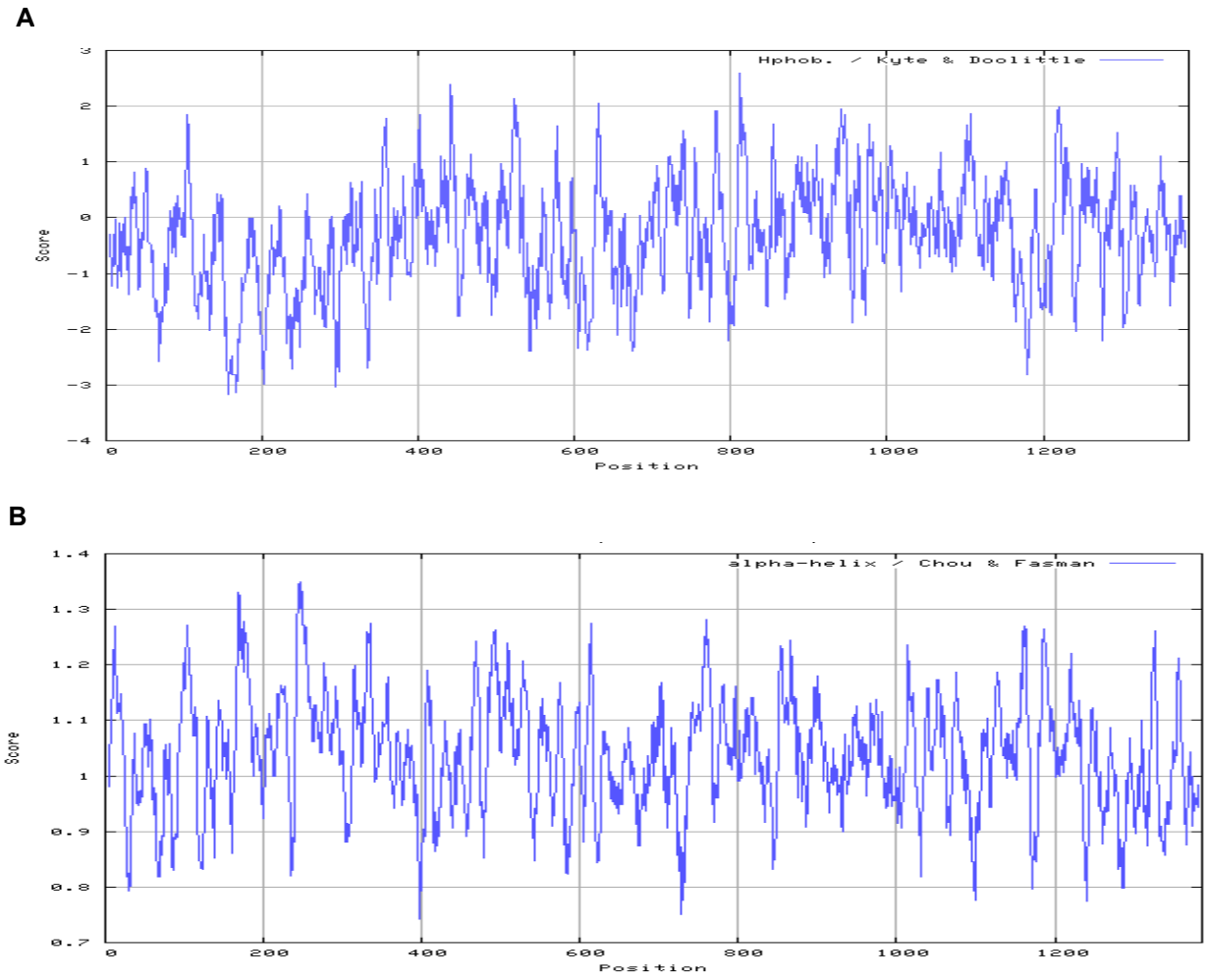


Fig. 9 Hydropathy plot and predicted secondary structure of the deduced mature protein of KC-GWD. **A** refers to the hydropathy plot based on the Kyte and Doolittle scale; **B** refers to the α-helical structures in the mature protein based on the Chou & Fasman scale

GWD expression in the potato tubers and leaves: GWD expression was studied at the levels of transcription and translation in both the tubers and leaves from the field-grown potato cultivars. By semi-quantitative RT-PCR, ~1.4 kb GWD cDNA could be amplified corresponding to the 5′-end of the transcript using both the tuber and leaf total RNA samples from the different potato cultivars. The GWD expression both in the tubers and leaves of different potato cultivars was shown in Fig. 10A. The 16-mer polypeptide

NIELQVDVRPPTSGDV, corresponding to the amino acids 19 to 34 of the mature KC-GWD protein proved to be immunogenic as evident from the protein gel blot analysis as shown in Fig. 10B. A band corresponding to ~ 155 kDa protein was detected only in the tuber protein extracts, not in the leaf protein extracts (data not shown).

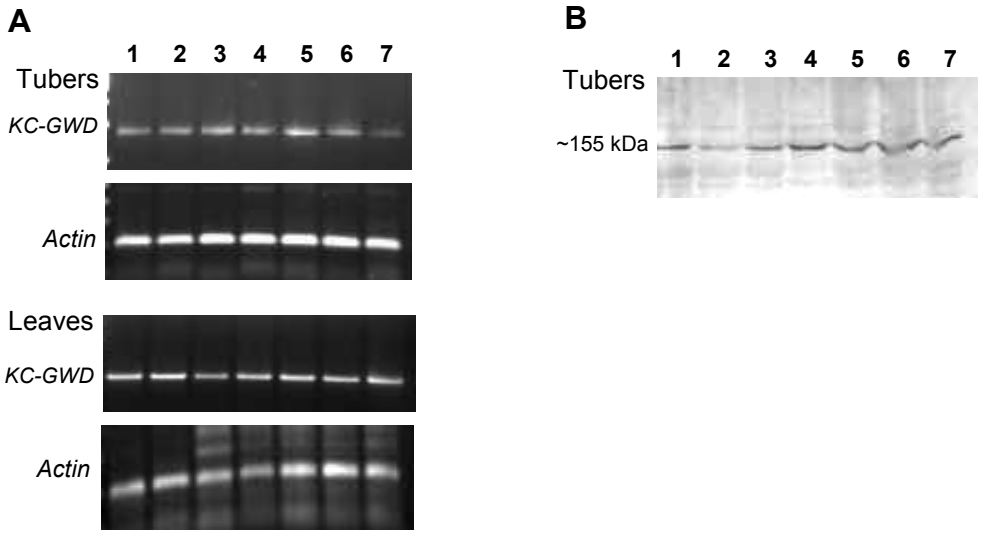


Fig. 10 A Semi-quantitative RT-PCR approaches for GWD expression analysis in the tubers and leaves of the field-grown potato cultivars (for details see the materials and methods). *Lanes 1-7* correspond to the potato cultivars Kufri Chipsona-1, Kufri Chipsona-2, Kufri Jyoti, Kufri Chandramukhi, Kufri Pukhraj, Kufri Ashoka, and the cv. Desiree, respectively. The size of the GWD-specific amplified product was found to be ~1.4 kb in each case. Actin-specific primers were used as control (the size of the amplified product ~0.25 kb)
B Protein gel blot analysis using soluble potato tuber proteins (for details see the materials and methods). The size of the immuno-detected band was ~ 155 kDa. *Lanes 1-7* correspond to the same order of the cultivars as referred in **A**

In this study, a full-length cDNA (4789 bp) encoding GWD was isolated, and characterized by sequencing from an Indian potato cultivar, Kufri Chipsona-1 by RT-PCR approach using tuber RNA. The predicted protein, designated as KC-GWD, consisted of 1463 amino acids (having 77-amino acid transit peptide and 1386-amino acid mature protein). Sequence comparison both at nucleotide and amino acid levels, phylogenetic analysis clearly indicated that KC-GWD represents a distinct GWD isoform (a product of distinct *GWD* gene or allele)

in potato. Hydrophobic characters, and α-helical structures were predicted in KC-GWD sequence. In terms of molecular cloning and characterization studies on GWD, this is the second research report in potato after Lorberth et al. (1998). By the same RT-PCR approach, the amplified DNA products of similar size were obtained using tuber and leaf RNA from other cultivars; having similar restriction patterns which suggest that the corresponding GWD genes are mostly conserved in the potato cultivars; however, microheterogeneity between them could be revealed once these remaining RT-PCR products are sequenced.

Sequence comparison between the available GWD isoforms in the *Solanaceae* family (three from the potato cultivars and one from tomato cultivar) revealed variation of the amino acids (both conservative and non-conservative) at a number of places both in the transit peptide and mature protein keeping most of the conserved regions. In addition to five conserved aromatic amino acids, a few more conserved aromatic amino acids could be found in both the CBM45 domains in the potato and tomato GWDs, their significance, if any, needs to be understood. The sequence comparison clearly revealed that the CBM45 domains present in the potato and tomato GWD isoforms have some degree of variations. Therefore, these individual noncatalytic CBM45s along with the linker regions need to be characterized biochemically to see any sort of difference between them; for example their *Km* values for the glucan substrates, the influence of the CBM45s on their catalytic efficiencies. It is likely that the variations what we noted both in the CBM45s, catalytic, and ATP-binding domains may have some influence on the overall catalytic efficiencies of the individual GWD isoforms during starch phosphorylation. Therefore, it may be speculated that the individual GWD isoforms what we know from the potato cultivars and tomato are likely to vary with regard to their organ-specific and spatio-temporal nature of expression, affinity towards various starches,

competitive advantages, and overall catalytic efficiency in terms of starch phosphorylation. All these questions remain to be elucidated categorically through molecular and biochemical approaches. It is known that there are a few types of stable secondary structures occur widely in both structural and globular proteins. The most prominent are the α-helix and β-sheet, the other types include β-turn and random coil. Usually, hydrophobic parts occupy the interior regions, and hydrophilic parts remain in the exterior regions of globular proteins (Kyte and Doolittle 1982). All the predicted data on hydropathy plot, secondary structures of the mature GWD need to be further validated by various corroborative experimental studies. As a preliminary study, GWD gene expression analyses were carried out in the different tissues of the potato cultivars only at a particular stage of potato plants by semi-quantitative RT-PCR approach. The level of GWD transcripts in the tubers was more or less comparable between the Indian potato cultivars; however, the level was slightly lower in the reference cultivar Desiree. Such type of variations could be due to potato genotypes and/or varying maturation level of the potato tubers. In the leaves, the level of GWD transcripts appeared to be nearly uniform. By immunodetection technique, a band corresponding to ~ 155 kDa protein, as expected, was detected only in the tuber protein extracts with nearly uniform intensity in all the potato cultivars except Kufri Chipsona 2. No such band was detected for the potato leaf protein extracts despite of repeated attempts. Based on the earlier reports, it is known that in both leaves and tubers potato GWD exists in three different states: enclosed inside starch granules, associated with the granule surface, and soluble in the plastid stroma. GWD was detected in the starch granules if isolated from the darkened leaves only but not from the illuminated leaves. Moreover, the leaves from the relatively younger potato plants (~3 weeks old) were used (Ritte et al. 2000a (Starch); Ritte et al. 2000b). In this study, protein extracts

were made from the leaves collected randomly from the nearly maturing field-grown potato plants (~9 weeks old). It is reasonable to assume that the intracellular GWD level could be low and/or because of compartmentation the amount of GWD was negligible in such leaf protein extracts. Gene expression analyses in different potato tissues at various stages of development may provide clues for understanding such variations.

The tuber starch-bound phosphorus content data showed only minor variations between the different potato cultivars; not correlated well with the GWD expression patterns as shown in this report. However, this result was more or less consistent with the observations made by Ritte et al. (2000b). They screened various starch containing tissues from different plant species, but could not reveal a positive correlation between the R1 (GWD) level and the starch-bound phosphate content. Further studies are required to know more about the structural attributes, physico-chemical properties, and degradability of the tuber starch in the individual Indian potato cultivars. In conclusion, isolation and characterization of this new GWD isoform will be useful and relevant for further biochemical and molecular studies on starch phosphorylation aspects in potato.

During the last more than one decade substantial progress has been made in *Arabidopsis* and cereal endosperm starch research as evident from biochemical, molecular and genetic studies, and enriched considerably our knowledge on the mechanisms of starch granule initiation, assembly, and more importantly in understanding the role of transient glucan phosphorylation in starch breakdown particularly with respect to leaf starch metabolism in *Arabidopsis*. More precisely, in plants the function of glucan phosphorylation is required to promote or maintain glucan accessibility for hydrolysis, with a requirement for the removal of phosphate groups for complete degradation. However, the current knowledge mainly on the *Arabidopsis* model

is not enough in understanding the similar aspects of starch metabolism which occur in heterotrophic plant organs, and plays a crucial role with respect to plant growth and development.

Based on the growing body of evidences it is now believed that the pathways of starch degradation in leaves and other starch storage organs have some common features, but also have major differences. In leaves, starch degradation could be studied at biochemical and molecular levels over a short and controllable period. But similar type of in-depth studies in other plant organs like roots, tubers are relatively difficult. One of the reasons being starch degradation may occur over long periods, during which the concerned organs undergo many conspicuous developmental changes. The nature of the starch granules and their appearances during degradation differ substantially between plant organs. A host of developmental, metabolic, and environmental factors influence starch degradation in various plant organs. Moreover, the biochemical roles of the individual isoforms of the concerned enzymes still remain vastly unknown, and the precise biochemical role of the individual enzymes needs to be assessed. Although GWD was discovered in the potato cultivar Desiree, still we lack in-depth knowledge on storage starch phosphorylation and its degradation aspects because of scarce experimental data. For example, still we do not know clearly the number of GWD isoforms involved in starch phosphorylation in potato. In the tetraploid heterozygous potato cultivar genomes, we can not rule out the multiplicity of the *GWD* genes and their allelic variations. In this context, molecular cloning and gene expression studies on GWD as presented in this thesis are quite relevant. Moreover, the different regions of the KC-GWD cDNA were used for making different antisense and sense genetic constructs for the purpose of inhibition of its corresponding gene function in a processing Indian potato cultivar.

5.2.2 *Isolation and characterization of GBSSI alleles and tuber-specific promoters*

One of the objectives of this work was to use the tuber-specific promoter in making different binary genetic constructs for potato transformation. For this purpose, efforts were made for isolation and characterization of GBSSI alleles from Indian potato cultivars, followed by cloning of a tuber-specific promoter. The various steps involved in this molecular cloning and characterization studies are briefly described below:

Isolation of potato genomic DNA: Total genomic DNA was isolated from aseptically grown micropropagated plantlets from the following potato cultivars: Kufri Chipsona-1, Kufri Chandramukhi, Kufri Jyoti, Kufri Chipsona-2, Kufri Ashoka, Kufri Pukhraj and Desiree. The potato DNA preparations, used as templates in polymerase chain reactions, were essentially devoid of inhibitory materials as checked spectrophotometrically, and by restriction analyses.

PCR amplification of GBSSI alleles from the potato cultivars: For amplification of partial GBSSI alleles having promoter regions, PCR was carried out using the primer pair, GB1-F01 and GB1-R01 keeping annealing temperature at 55^0C. Potato cultivar-specific PCR amplification products were analyzed by 1.0% agarose gel electrophoresis as shown in Fig. 11a. In case of Kufri Chipsona-1, only one DNA band, and for the remaining six cultivars i.e., two DNA bands were amplified. Close inspection revealed that an intense DNA band of ~1.3 kb is common for all the cultivars studied here. Apart from this, a less intense DNA band of ~1.4 kb was found for the cultivars Kufri Chandramukhi, Kufri Jyoti, Kufri Chipsona-2, Kufri Ashoka, and Desiree; whereas in case of Kufri Pukhraj, the second DNA band was found to be slightly smaller than 1.4 kb. Using the same primer pair, PCR was carried out at lower annealing temperature, i.e., at 50^0C which resulted in similar banding pattern of PCR amplification products in some of the cultivars (Fig. 11b). PCR was further

carried out at 55^0C annealing temperature using another set of primer pair, GB1-F02 and GB1-R01 which resulted in only one amplification product of ~ 0.85 kb for all the seven potato cultivars as shown in Fig. 11c.

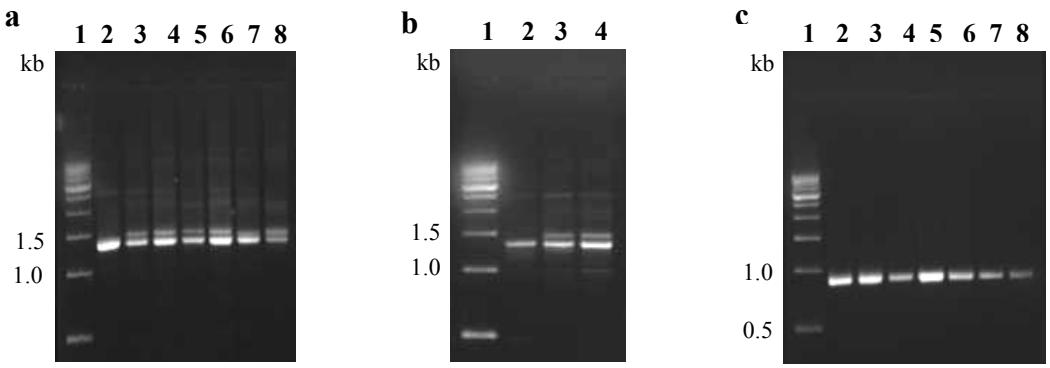


Fig. 11 DNA banding patterns of partial granule-bound starch synthase I (GBSSI) alleles amplified at varying annealing temperatures, and by different primers using genomic DNA as template from different potato cultivars as indicated lane-wise. **a** Annealing temperature-55^0C, primers used GB1-F01 & GB1-R01, *lane 1* 500 bp DNA ladder; *lanes 2-7* correspond to the Indian potato cultivars Kufri Chipsona-1, Kufri Chandramukhi, Kufri Jyoti, Kufri Chipsona-2, Kufri Ashoka, Kufri Pukhraj, respectively; *lane 8* cv. Desiree. In case of Kufri Chipsona-1, only one DNA band of ~ 1.3 kb, common to all cultivars, was amplified. Size of the second DNA band (~ 1.4 kb) appeared to be common in other cultivars except Kufri Pukhraj (the size appeared to smaller than 1.4 kb). **b** Annealing temperature-50^0C with the same primer pair; *lane 1* 500 bp DNA ladder; *lanes 2-4* correspond to the cultvars Kufri Chipsona-1, Kufri Chandramukhi, Kufri Jyoti, respectively. DNA banding pattern of the amplified products appeared to be same. **c** Annealing temperature-55^0C, primers used GB1-F02 & GB1-R01; all the lanes were in same order as shown in Fig. 11a. Here, only one DNA band (~ 0.85 kb) was amplified for each cultivar.

*Molecular cloning and sequence analyses of the GBSSI alleles from Kufri Chandramukhi***:** Out of seven potato cultivars, the two amplified DNA bands corresponding to Kufri Chandramukhi were only cloned into the *Sma*1 site of pUC19 vector. Each cloned fragment was sequenced in both directions by Bangalore Genei, Bangalore. The nucleotide sequences were analyzed by NCBI Blast tools, and found to be two partial GBSSI alleles. The size of one allele was 1320 bp (designated as GKC1), and the other one was 1461 bp (designated as GKC2), and the sequence information was submitted in NCBI GenBank database under the

Accession Numbers EU548081 and EU548082, respectively. Each sequence consists of 5′-flanking (promoter) region, first exon, first intron and the major part of the second exon which encodes the entire 77-amino acid transit peptide and a small portion of the mature peptide towards the N-terminus (Fig. 12). The major difference between GKC1 and GKC2 was that the former allele lacks a 140-bp fragment, nearly 0.65 kb upstream of the translation start codon, ATG. Upstream of the predicted transcription start site (TSS), the 758 bp promoter region of GKC1 showed ~ 96% sequence identity with the corresponding region of GKC2, and the available transcribed regions (corresponding to the bases 759-1320 of GKC1) of these two alleles having ~ 96% identity. Nucleotide sequence alignment of GKC1 and GKC2 as shown in Fig. 12 showed variations arising mainly due to the difference in CACT repeats in the promoters close to the TSS, and CTT repeats in their first introns. A gap of a short stretch of nucleotides was noted in the beginning of the second exon of GKC1. The available coding regions of these two alleles are nearly identical with only three nucleotide variants that lead to a single non conservative substitution (R/T at 51st position) in the 77-amino acid transit peptide, and two amino acid substitutions in the mature peptide, one is non conservative (M/K at 2nd position) and the other one is conservative (S/T at 11th position). The two GBSSI alleles, GKC1 and GKC2 from the potato cultivar Kufri Chandramukhi, therefore, represent two major classes of GBSSI alleles based on the absence or presence of 140 bp promoter region as reported previously by van de Wal et al. (2001). According to their classification, the GKC1 allele belongs to the *A1* class and GKC2 belongs to the *A3* class. A NCBI Blast search revealed that the available 1320 nucleotides of GKC1 allele showed 97% and 98% sequence identity with the GBSSI alleles reported by van der Leij et al. (1991) and Dai et al. (1996), respectively. But the sequence identity appeared to be 99% with the G1

allele from the cultivar Granola (Rohde et al. 1990), the query coverage being 87%. The GKC1 allele was found to be very close to the G1 allele as compared to others. The GKC2 allele of this study appeared to be more distinct. The 1461 nucleotides of this allele showed 97% sequence identity, query coverage being 87%, with the G28 allele from Granola (Rohde et al. 1990). The GKC2 allele appeared to be more close to the *pra3a* allele (van de Wal et al. 2001) showing 98% identity, query coverage being 55%. Therefore, none of the GBSSI alleles, as isolated and characterized in this study, was found to be identical with the other alleles reported to date.

Sequence comparison between the GBSSI alleles in potato and multiple sequence alignment: Out of the total GBSSI alleles from different potato breeding lines and cultivars as reported to date, only a few have complete nucleotide sequences, whereas the remaining are partial ones with varying lengths of promoter sequences. For the purpose of multiple sequence alignment, a total of seven potato GBSSI sequences having 5′-flanking (promoter) regions were chosen that included two GBSSI alleles, GKC1 and GKC2, of this study, and the other five GBSSI alleles were a) *St*AM: Monoploid *S. tuberosum* (AM79.7322), Acc. No. X58453 (van der Leij et al 1991); b) *St*DN: *S. tuberosum* Dongnong 303, Acc. No. X83220 (Dai et al. 1996); c) *St*G1: *S. tuberosum* Granola (G1WxSt), Acc. No. X52416 (Rohde et al. 1990); d) StG28: *S. tuberosum* Granola (G28WxSt), Acc. No. X52417 (Rohde et al. 1990); and e) *pra3a*: *S. tuberosum* Kanjer, Acc. No. AF274513, (van de Wal et al. 2001). *St*AM and *St*DN represent complete GBSSI alleles, whereas *St*G1, *St*G28 and *pra3a* are partial ones like GKC1 and GKC2 alleles (Fig. 12).

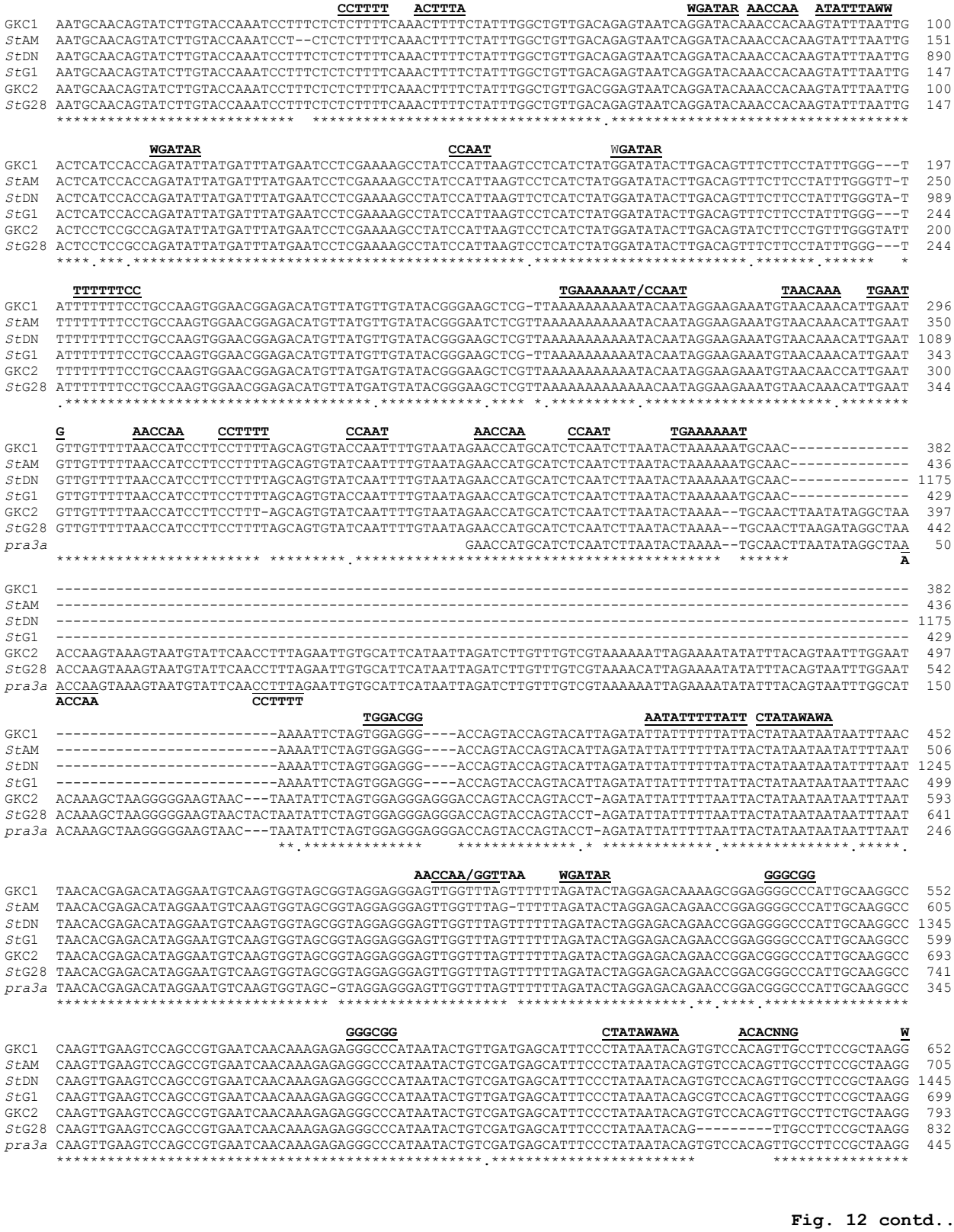
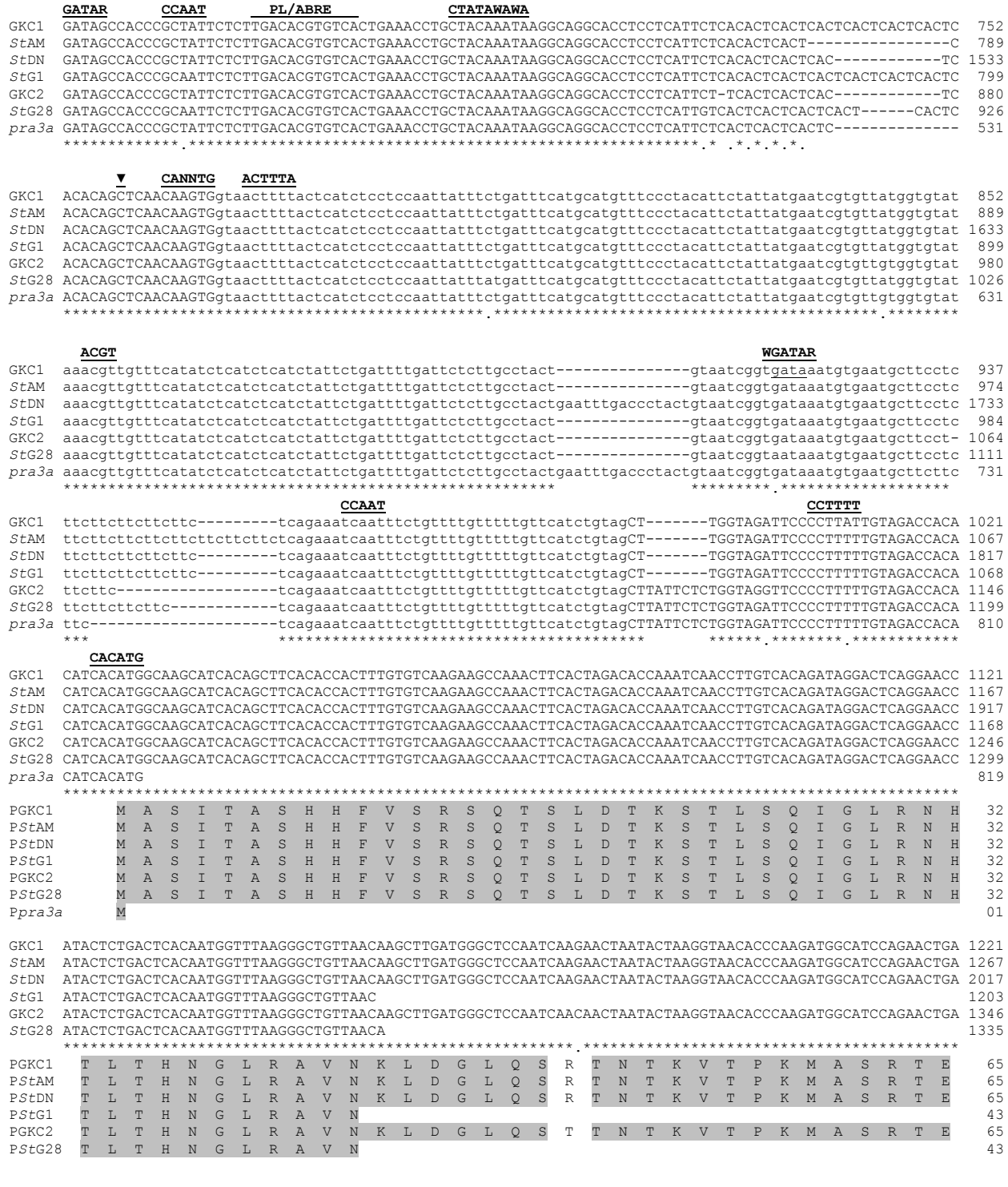


Fig. 12 contd..


GATAR CCAAT PL/ABRE CTATAWAWA
GKC1 GATAGCCACCCGCTATTCTCTTGACACGTGTCACTGAAACCTGCTACAAATAAGGCAGGCACCTCCTCATTCTCACACTCACTCACTCACTCACTCACTC 752
StAM GATAGCCACCCGCTATTCTCTTGACACGTGTCACTGAAACCTGCTACAAATAAGGCAGGCACCTCCTCATTCTCACACTCACT----------C 789
StDN GATAGCCACCCGCTATTCTCTTGACACGTGTCACTGAAACCTGCTACAAATAAGGCAGGCACCTCCTCATTCTCACACTCACTCAC----------TC 1533
StG1 GATAGCCACCCGCAATTCTCTTGACACGTGTCACTGAAACCTGCTACAAATAAGGCAGGCACCTCCTCATTCTCACACTCACTCACTCACTCACTC 799
GKC2 GATAGCCACCCGCTATTCTCTTGACACGTGTCACTGAAACCTGCTACAAATAAGGCAGGCACCTCCTCATTCT-TCACTCACTCAC----------TC 880
StG28 GATAGCCACCCGCAATTCTCTTGACACGTGTCACTGAAACCTGCTACAAATAAGGCAGGCACCTCCTCATTGTCACTCACTCACTCACT------CACTC 926
pra3a GATAGCCACCCGCTATTCTCTTGACACGTGTCACTGAAACCTGCTACAAATAAGGCAGGCACCTCCTCATTCTCACTCACTCACTC---------- 531
▼ CANNTG ACTTTA
GKC1 ACACAGCTCAACAAGTGgtaacttttactcatctcctccaattatttctgatttcatgcatgtttccctacattctattatgaatcgtgttatggtgtat 852
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ACGT WGATAR
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CACATG
GKC1 CATCACATGGCAAGCATCACAGCTTCACACCACTTTGTGTCAAGAAGCCAAACTTCACTAGACACCAAATCAACCTTGTCACAGATAGGACTCAGGAACC 1121
StAM CATCACATGGCAAGCATCACAGCTTCACACCACTTTGTGTCAAGAAGCCAAACTTCACTAGACACCAAATCAACCTTGTCACAGATAGGACTCAGGAACC 1167
StDN CATCACATGGCAAGCATCACAGCTTCACACCACTTTGTGTCAAGAAGCCAAACTTCACTAGACACCAAATCAACCTTGTCACAGATAGGACTCAGGAACC 1917
StG1 CATCACATGGCAAGCATCACAGCTTCACACCACTTTGTGTCAAGAAGCCAAACTTCACTAGACACCAAATCAACCTTGTCACAGATAGGACTCAGGAACC 1168
GKC2 CATCACATGGCAAGCATCACAGCTTCACACCACTTTGTGTCAAGAAGCCAAACTTCACTAGACACCAAATCAACCTTGTCACAGATAGGACTCAGGAACC 1246
StG28 CATCACATGGCAAGCATCACAGCTTCACACCACTTTGTGTCAAGAAGCCAAACTTCACTAGACACCAAATCAACCTTGTCACAGATAGGACTCAGGAACC 1299
pra3a CATCACATG 819
PGKC1 M A S I T A S H H F V S R S Q T S L D T K S T L S Q I G L R N H 32
PStAM M A S I T A S H H F V S R S Q T S L D T K S T L S Q I G L R N H 32
PStDN M A S I T A S H H F V S R S Q T S L D T K S T L S Q I G L R N H 32
PStG1 M A S I T A S H H F V S R S Q T S L D T K S T L S Q I G L R N H 32
PGKC2 M A S I T A S H H F V S R S Q T S L D T K S T L S Q I G L R N H 32
PStG28 M A S I T A S H H F V S R S Q T S L D T K S T L S Q I G L R N H 32
Ppra3a M 01
GKC1 ATACTCTGACTCACAATGGTTTAAGGGCTGTTAACAAGCTTGATGGGCTCCAATCAAGAACTAATACTAAGGTAACACCCAAGATGGCATCCAGAACTGA 1221
StAM ATACTCTGACTCACAATGGTTTAAGGGCTGTTAACAAGCTTGATGGGCTCCAATCAAGAACTAATACTAAGGTAACACCCAAGATGGCATCCAGAACTGA 1267
StDN ATACTCTGACTCACAATGGTTTAAGGGCTGTTAACAAGCTTGATGGGCTCCAATCAAGAACTAATACTAAGGTAACACCCAAGATGGCATCCAGAACTGA 2017
StG1 ATACTCTGACTCACAATGGTTTAAGGGCTGTTAAC 1203
GKC2 ATACTCTGACTCACAATGGTTTAAGGGCTGTTAACAAGCTTGATGGGCTCCAATCAACAACTAATACTAAGGTAACACCCAAGATGGCATCCAGAACTGA 1346
StG28 ATACTCTGACTCACAATGGTTTAAGGGCTGTTAACA 1335
PGKC1 T L T H N G L R A V N K L D G L Q S R T N T K V T P K M A S R T E 65
PStAM T L T H N G L R A V N K L D G L Q S R T N T K V T P K M A S R T E 65
PStDN T L T H N G L R A V N K L D G L Q S R T N T K V T P K M A S R T E 65
PStG1 T L T H N G L R A V N 43
PGKC2 T L T H N G L R A V N K L D G L Q S T T N T K V T P K M A S R T E 65
PStG28 T L T H N G L R A V N 43

Fig. 12 contd..

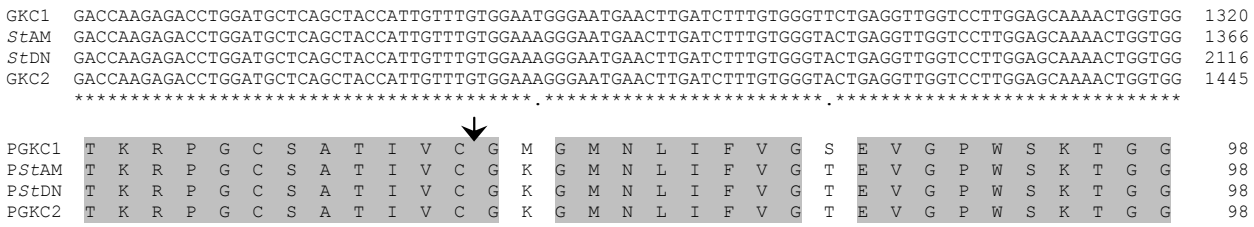

Fig. 12 Multiple sequence alignment of the nucleotide and deduced amino acid sequences of seven GBSS alleles from potato (for the GenBank Accession numbers see the text; the numbering of bases is kept unaltered as they appear in the GenBank database). Capital letters denote promoter and exon sequences, small letters intron sequences, and single letter code for the amino acids. This alignment is based on ClustalW2 tool. Dashes in the nucleotide sequences indicate gaps arising during alignment, dots are for mismatches and asterisk for common nucleotides, and highlighted for common amino acids. '▼' denotes putative transcription start site (TSS). Downward arrow indicates the cleavage site of the transit peptide. Common basal promoter elements and other *cis*-regulatory sequence motifs are overlined in GKC1 sequence, and underlined in *pra3a* sequence for the 140 bp unique region. The consensus sequence of the *cis*-regulatory motifs are shown in bold types in the respective positions.

Promoter isolation and construction of binary vector: The binary vector pBI121, having the constitutive CaMV 35S promoter located upstream of the GUS gene, was used as a control for potato transformation (for schematic view, refer to Fig. 13A)**.** The same binary vector was also used for making the GBSSI-GUS gene fusion. The steps adopted were: i) The 767 bp promoter region of the GKC1 allele having predicted transcription start site at position 759 was amplified using its corresponding clone as template and the primer pair GB1-F01 and GB1-R02; ii) The amplified DNA was then cloned into the *Sma*I site of pUC19; iii) the GKC1 promoter was released as *Ecl*136II-*Bam*H1 fragment and then cloned into the upstream of GUS gene of pBI121 binary vector by replacing the constitutive CaMV 35S promoter including its own TSS. Prior to this ligation, in order to remove the CaMV 35S promoter the pBI121 was digested first with *Hind*III, filled with Klenow followed by digestion with *Bam*HI. The resulting binary vector having GBSSI-GUS gene fusion was designated as **pAN-GB01** (Fig. 13B). The orientation and intactness of GKC1 promoter relative to GUS

gene in pAN-GB01 was further checked through PCR using the GKC1-specific forward primer and GUS-specific reverse primer (data not shown).

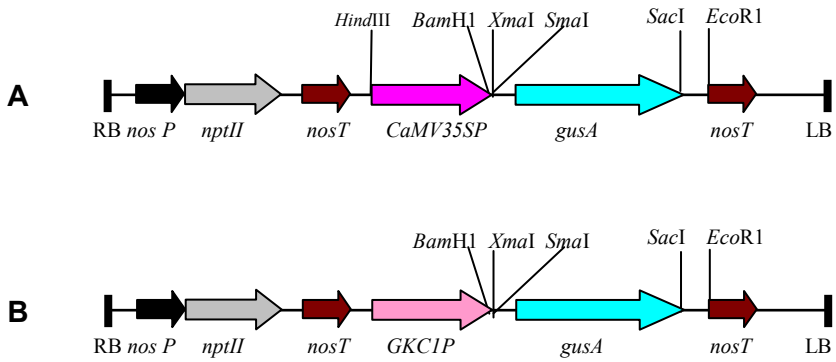

Fig. 13 Schematic diagrams of the T-DNA regions of the binary vectors; **A** pBI121 and **B** pAN-GB01 (not to scale). RB right border; LB left border; *nos P* the *nos* promoter; *nos T* the *nos* terminator; *nptII* neomycin phosphotransferase; *gusA* β-glucuronidase; *CaMV35SP* the CaMV 35S promoter; *GKC1P* the promoter of GKC1 allele; different restriction sites are indicated in the diagrams.

Analysis of GKC1 promoter-GUS and CaMV35S-GUS expression: The binary vectors pBI121 (having CaMV 35S-GUS fusion, see Fig. 13A) and pAN-GB01 (having GBSSI-GUS fusion, where GBSSI promoter was from the GKC1 allele, see Fig. 13B) were used in this study to carry out *Agrobacterium*-mediated transformation of the cultivar Kufri Chipsona-1. The details of transformation procedure, subsequent regeneration of the transformed plantlets and their screening are given in Materials and methods. It is to be noted here that multiple shoot regeneration occurred from the internodal stem segments through minimum intervening callus phases followed by rooting using selective regeneration medium; secondly, after proper hardening and acclimatization, the independent transgenic potato lines were allowed to grow for 70-80 days under field conditions. The necessary explants including mini tubers were collected from nearly mature potato plants. The purpose was to assess the strength and to know tissue specificity of GKC1 promoter with its own transcription start site.

Histochemical studies clearly indicated that GKC1 promoter-driven GUS expression occurred mainly in tubers (Fig. 14l) but not in leaves, stem sections or roots (Fig. 14i, j, k). In the tuber tissue GUS staining was predominantly confined to the medulla but not evident in the epidermis. In the case of the constitutive CaMV 35S promoter, GUS expression was observed in different cell types of leaves, stem sections, roots and tuber sections (Fig. 14e, f, g, h). The CaMV 35S-driven GUS expression was found to be more prominent in the medulla of tubers and vascular bundles of stem as compared to leaves and roots. No GUS staining was noted in the control non transgenic potato explants (Fig. 14a, b, c, d). Close inspection of histochemical data clearly suggests that the GBSSI promoter as isolated and characterized from the GKC1 allele appeared to be strong and tuber-specific. Moreover, it also showed a qualitative difference with the constitutive CaMV 35S promoter in terms of tissue specificity. Varying lengths of GBSSI promoters were used previously to drive GUS expression in potato (Visser et al. 1991a; van der Steege et al. 1992). It was also shown that the 0.4 kb GBSSI promoter was sufficient to drive tuber-specific GUS expression (van der Steege et al. 1992). It is likely that apart from this 0.4 kb promoter region, the *cis*-regulatory sequence motifs located in the further upstream region of the GBSSI promoter could influence overall efficiency of transcription in different potato tissues. This question can only be addressed by thorough functional characterization with the help of site-directed mutagenesis. However, the present data allow us to conclude that the ~ 0.75 kb GKC1 promoter in this study is quite effective for tuber-specific expression in the heterozygous genetic background of potato under field conditions.

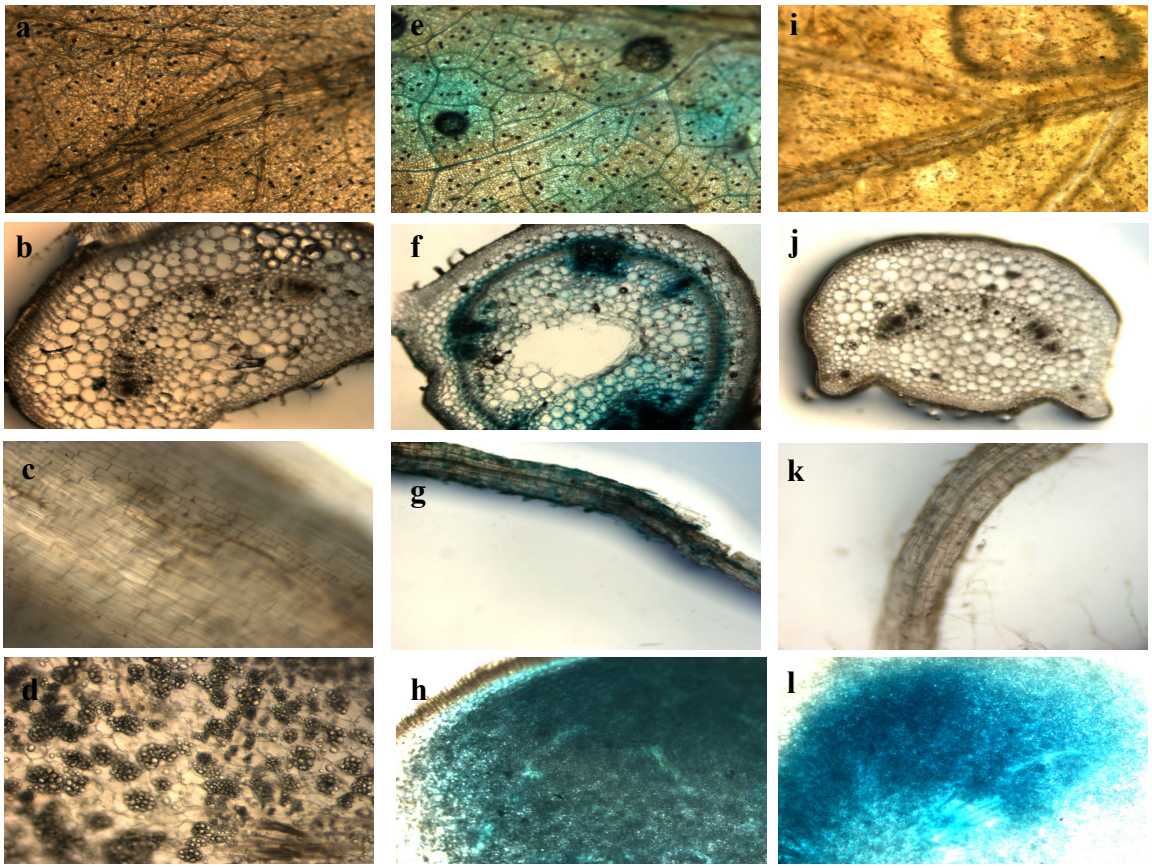

Fig. 14 GUS expression patterns in different tissues of transgenic and non transgenic potato plants corresponding to the potato cultivar Kufri Chipsona-1. The order was kept same for each column of this figure from top to bottom, i.e. leaf, stem section, root and tuber section. a, b, c & d correspond to non transgenic (control) potato plant; e, f, g & h correspond to pBI121 (having CaMV 35S-GUS fusion) derived transgenic plant; likewise, i, j, k & l are for pAN-GB01 (having GKC1 promoter-GUS fusion) derived transgenic plant.

Molecular cloning and functional characterization of the GBSSI alleles, GKC1 and GKC2, constitute the first report with respect to the Indian potato cultivars. Various *cis*-regulatory motifs in the GBSSI promoters will be helpful for further in-depth functional characterization at molecular level. The GBSSI promoter from the GKC1 allele was found to be strong and tuber-specific under field conditions. In terms of tissue-specific expression, it showed clear difference with the constitutive CaMV35S promoter. The promoter of GKC1 allele was employed in making the binary genetic constructs as described in the next section.

5.3 Use of different fragments from R1 cDNA and/or genomic clones for making various genetic constructs for inhibition of R1 gene function

Based on R1 (GWD) cDNA transgenic potato plants were first generated by Lorberth et al. (1998). For this, they used only antisense binary genetic construct employing the 1.9 kb region of the R1 cDNA towards its 5' end under the constitutive CaMV 35S promoter. The potato cultivar 'Desiree', regarded as reference cultivar for research purpose, was used for *Agrobacterium*-mediated genetic transformation. Based on similar R1-cDNA fragment towards the 5′ end, Viksø-Nielsen et al. 2001 also generated transgenic potato lines using the cultivar 'Dianella' employing both antisense and sense genetic constructs.

In this study, for the purpose of inhibition of R1 (GWD) gene function in the Indian potato cultivar, various strategic approaches were adopted which differ considerably from the above. With respect to the Indian potato cultivars, no such types of reports are available till date. A number of antisense and sense binary genetic constructs were used for generation of transgenic potato lines. Both constitutive and tuber-specific promoters were used for making the binary genetic constructs; moreover, different regions of the KC-GWD cDNA (as isolated in this study) were used in the genetic constructs to see the effects. Instead of cv. Desiree, Kufri Chipsona-1 (a commercially important Indian potato cultivar) was used during *Agrobacterium*-mediated genetic transformation.

Construction of the antisense and sense binary genetic constructs: Different binary genetic constructs were made using different regions of cDNA encoding KC-GWD. Nearly 1.38 kb region towards 5′-terminus was amplified using the primer pair A20R1 and C20R1; and ~1.2 kb region towards 3′-terminus was amplified using another primer pair R1-F3559 and R1-4810 using full length KC-GWD cDNA as shown in Fig. 15. The DNA fragments were

first cloned in *Sma*I site of pUC19 vector. For directional cloning, the cloned inserts in pUC19 were isolated by restriction digestion(s) using *Ecl136* II and *Bam*H1 restriction enzymes, and

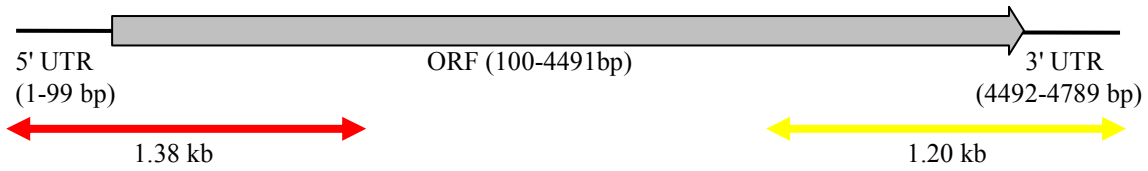

Fig. 15 Schematic view of the full length KC-GWD cDNA (4789 bp), 100-4491 bp corresponds to the open reading frame (ORF); 1-99 bp represents 5' UTR; 4492-4789 bp represents 3' UTR. The 1.38 kb region towards 5′ end and the 1.20 kb towards 3′ end were used for making different binary genetic constructs as shown by red arrow and yellow arrow, respectively.

placed under the constitutive CaMV35S promoter in pBI121 and the tuber-specific GKC1 (GBSSI) promoter in pAN-GB01 by replacing the existing GUS gene. The orientation and intactness of the inserts in the individual genetic constructs were further checked through restriction digestion, and PCR using specific primers. In this study, a total of eight binary genetic constructs were made as listed below:

A. List of antisense and sense binary genetic constructs using ~1.38 kb KC-GWD cDNA towards its 5′ end

- **i.** **RA** (antisense construct under the *CaMV35S* promoter, Fig. 16A)
- **ii.** **RB** (sense construct under the *CaMV35S* promoter, Fig. 16B)
- **iii.** **RC** (antisense construct under the *GBSSI* promoter, Fig. 16C)
- **iv.** **RD** (sense construct under the *GBSSI* promoter, Fig. 16D)

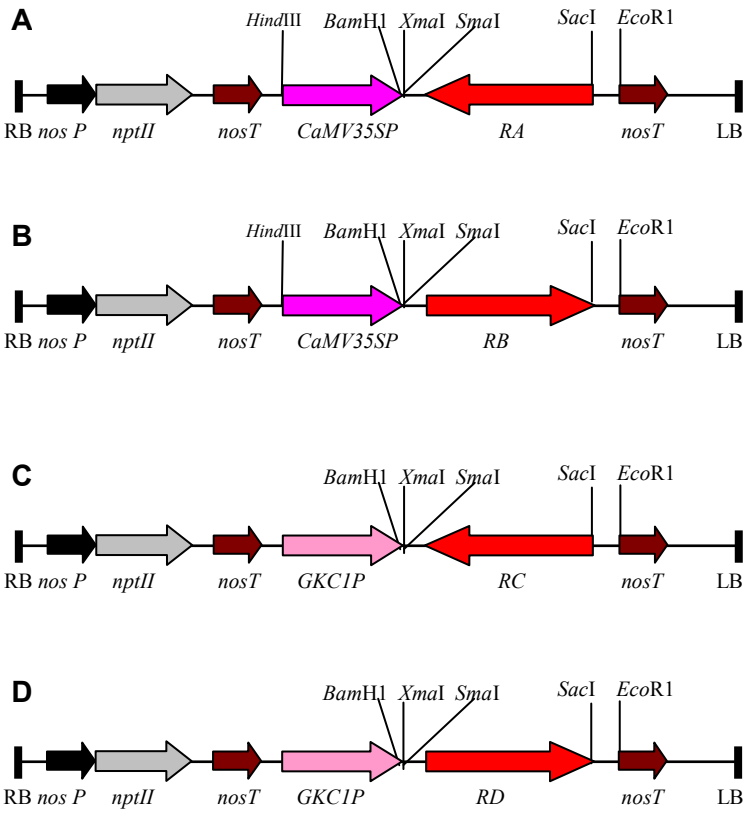

Fig. 16 Schematic diagrams of the T-DNA regions of the binary genetic constructs using ~1.38 kb KC-GWD cDNA towards its 5′ end (not to scale) RB right border; LB left border; *nos P* the *nos* promoter; *nos T* the *nos* terminator; *nptII* neomycin phosphotransferase; *CaMV35S P* the CaMV 35S promoter; *GKC1P* the promoter of GKC1 allele; different restriction sites are indicated in the diagrams.

B. List of antisense and sense binary genetic constructs using ~1.2 kb KC-GWD cDNA towards its 3′ end

v. **RE** (antisense construct under the *CaMV35S* promoter, Fig. 17A)

vi. **RF** (sense construct under the *CaMV35S* promoter, Fig. 17B)

vii. **RG** (antisense construct under the *GBSSI* promoter, Fig. 17C)

viii. **RH** (sense construct under the *GBSSI* promoter, Fig. 17D)

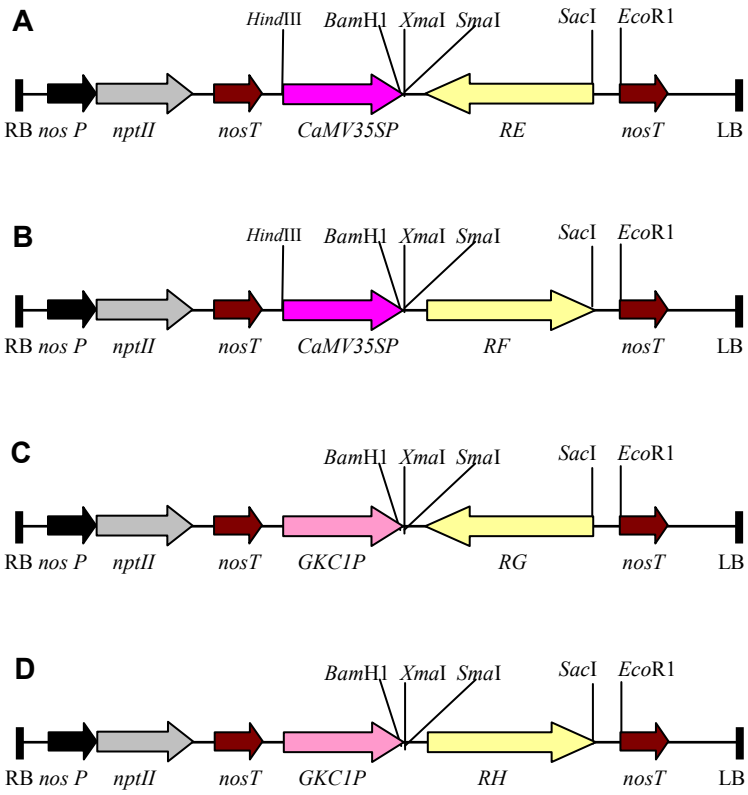

Fig. 17 Schematic diagrams of the T-DNA regions of the binary genetic constructs using ~1.20 kb KC-GWD cDNA towards its 3′ end (not to scale) RB right border; LB left border; *nos P* the *nos* promoter; *nos T* the *nos* terminator; *nptII* neomycin phosphotransferase; *CaMV35S P* the CaMV 35S promoter; *GKC1P* the promoter of GKC1 allele; different restriction sites are indicated in the diagrams.

5.4 Transformation of potato plants using the above antisense & sense genetic constructs followed by screening of transgenic potato lines

Genetic transformation of Agrobacterium tumefaciens (LBA4404): The well characterized different binary genetic constructs in antisense and sense orientations under both the constitutive (CaMV35S) and tuber-specific (GBSSI) promoters were used to transform *Agrobacterium tumefaciens* strain (LBA4404) either through electroporation or tri-parental mating techniques. The transformant *Agrobacterium* were selected on selective YEM media containing rifampcin (15 μg mL^{-1}) and Kanamycin (50 μg mL^{-1}).

Generation of transgenic potato lines through Agrobacterium mediated co-cultivation: The inter-nodal stem segments of the cultivar Kufri Chipsona-1 were co-cultivated with the above *Agrobacterium* transformants using the protocol as reported by Beaujean et al. (1998) with some modifications. The composition of shoot regeneration medium was MS basal containing zeatin 2.5 mg L^{-1}, GA_3 3.0 mg L^{-1}, IAA 0.01 mg L^{-1} along with kanamycin 80 mg L^{-1} and cefotaxime 250 mg L^{-1} for primary selection of transgenic potato lines. The initial shoots were further transferred to the rooting media to obtain plantlets followed by screening of around ten independent transgenic potato lines for each genetic construct through PCR approach using promoter and KC-GWD cDNA specific primers. On an average, six to seven independent regenerated potato lines corresponding to the individual binary constructs appeared to be positive in terms of PCR amplification suggesting stable integration of the transgene(s) into potato genome (data not shown).

Production of transgenic potato mini tubers: After proper hardening and acclimatization, the transgenic potato plantlets were grown in polybags for mini-tuber production in the restricted experimental plots. Mature mini tubers were harvested. The visual examination of transgenic plants and the tubers revealed no visible changes with regard to the plant morphology overall, tuber size and yield as compared to the controlled ones. Starting from regeneration to the

production of transgenic potato mini tubers corresponding to only one binary construct i.e., RC are shown in the Fig. 18 (A-I) (the transgenic potato lines for other binary genetic constructs are not shown).

Fig. 18 Production of transgenic potato mini tubers starting from regeneration corresponding to the binary genetic construct RC. **A** & **B** Multiple shoot regeneration from intermodal stem segments after *Agrobacterium*-mediated cocultivation; **C** Micropropagation of the transgenic potato lines; **D** Hardening and acclimatization in in potting mix of soil and coco-peat; **E** Transgenic potato plants planted in the polybag filled with soil; **F** Growing transgenic potato plants after one month, **G** after two months; **H** Harvesting of the transgenic mini tubers; **I** Transgenic mini tubers

PCR analyses using genomic DNA from transgenic potato tubers: The genomic DNA was isolated from mini-tubers harvested from the individual independent transgenic potato lines corresponding to each binary genetic construct. PCR approach was adopted for checking the presence and orientation of the transgene (s). For this purpose, promoter-specific forward and gene-specific reverse primers were used. For the transgenic potato lines, RA-2, RA-5, RA-6, RA-8 and RA-13 as generated by the binary construct **RA**, ~1.9 kb DNA was amplified (as expected) using AF-3501 and A20-R1 primer pair (Fig. 19A). For the transgenic potato lines, RC-2, RC-3, RC-4, RC-7 and RC-8 as generated by the binary construct **RC**, ~2.1 kb DNA was amplified (as expected) using GB-F01 and A20-R1 primer pair (Fig. 19B). For the transgenic potato lines, RD-1, RD-2, RD-3, RD-4, RD-5, RD-6, RD-8, RD-9, RD-10 and RD-15 as generated by the binary construct **RD**, ~2.1 kb DNA was amplified (as expected) using GB-F01 and C20-R1 primer pair (Fig. 19C).

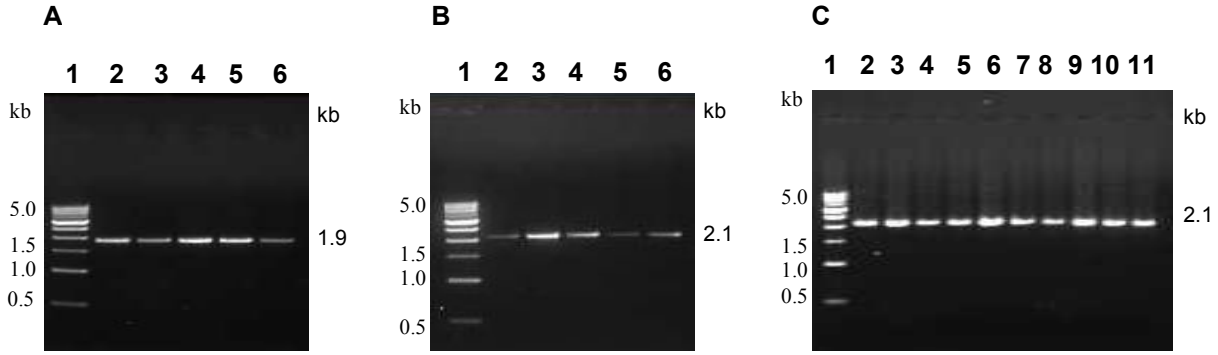

Fig. 19 PCR analyses using genomic DNA from transgenic potato mini tubers **A** RA-based transgenic lines, Lane 1 500 bp DNA ladder; lanes 2 to 6 refer to the transgenic potato lines RA-2, RA-5, RA-6, RA-8 and RA-13, respectively; **B** RC-based transgenic lines, Lane 1 500 bp ladder; lanes 2 to 6 refer to the transgenic lines RC-2, RC-3, RC-4, RC-7 and RC-8, respectively; **C** RD-based transgenic lines, Lane 1 500 bp ladder; Lanes 2 to11 refer to the transgenic lines RD-1, RD-2, RD-3, RD-4, RD-5, RD-6, RD-8, RD-9, RD-10 and RD-15, respectively.

Similarly, for the transgenic potato lines, RE-2, RE-5, RE-6, RE-7, RE-8, RE-15 and RE-16 as generated by the binary construct **RE**, ~1.8 kb DNA was amplified (as expected) using AF-3501 and R1-F3539 primer pair (Fig. 20A). For the transgenic potato lines, RF-1, RF-2, RF-3, RF-4, RF-6, RF-8 and RF-9 as generated by the binary construct **RF**, ~1.8 kb DNA was amplified (as expected) using AF-3501 and R1-4810 primer pair (Fig. 20B). For the transgenic potato lines, RG-1, RG-2, RG-3, RG-4, RG-5, RG-6, RG-7, RG-8, RG-9, RG-10 and RG-11 as generated by the binary construct **RG**, ~2.0 kb DNA was amplified (as expected) using GB-F01 and R1-F3539 primer pair (Fig. 20C). For the transgenic potato lines, RH-2, RH-3, RH-5, RH-6 and RH-7 as generated by the binary construct **RH**, ~2.0 kb DNA was amplified (as expected) using GB-F01 and R1-4810 primer pair (Fig. 20D)**.**

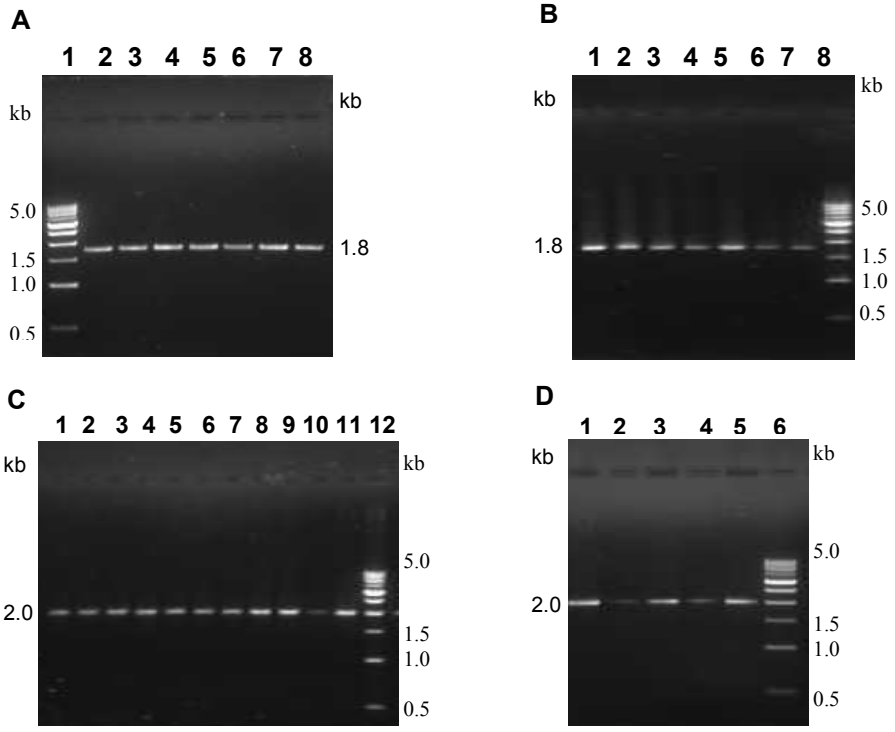

Fig. 20 PCR analyses using genomic DNA from transgenic potato mini tubers. **A** RE-based transgenic lines, Lane 1 500 bp DNA ladder; Lanes 2 to 8 refer to the transgenic lines RE-2, RE-5, RE-6, RE-7, RE-8, RE-15 and RE-16, respectively**; B** RF-based transgenic lines, Lanes 1 to 7 refer to the transgenic lines RF-1, RF-2, RF-3, RF-4, RF-6, RF-8 and RF-9, respectively; Lane 8 500 bp ladder; **C** RG-based transgenic lines, Lanes 1 to 11 refer to the transgenic lines RG-1, RG-2, RG-3, RG-4, RG-5, RG-6, RG-7, RG-8, RG-9, RG-10 and RG-11, respectively; Lane 12 500 bp DNA ladder; **D** RH-based transgenic lines, Lanes 1 to 5 refer to the transgenic lines RH-2, RH-3, RH-5, RH-6 and RH-7, respectively; lane 6 500 bp DNA ladder.

Protein gel blot analysis: Protein gel blot analyses were carried out to know the expression patterns of GWD in the protein extracts of tubers and leaves from different potato cultivars using KC-GWD specific polyclonal antibody (a 16-mer polypeptide NIELQVDVRPPTSGDV corresponding to the amino acids 19 to 34 of the mature KC-GWD protein was used for raising antibody in rabbit) and IgG-HRP conjugated secondary antibody using DAB system. Approx. 155 kDa was detected in the proteins isolated from tuber tissue in six Indian cultivars and cv. Desiree with minor variations between the cultivars. But in case of leaf protein, GWD could not be detected which needs to be further investigated. Similarly, protein extracts were made from the tubers of the selected transgenic potato lines corresponding to the individual binary constructs. The expression level of GWD was found to be very low or negligible almost in all the selected transgenic potato tubers as compared to the control ones suggesting down regulation of R1 (GWD) in the storage tissues (data not shown).

Biochemical analyses of the transgenic potato tubers: Mature mini tubers from Kufri Chipsona-1 based independent transgenic potato plants were harvested. Similarly, mini tubers were harvested from Kufri Chipsona-1 control plants (non-transgenic). The harvested mini-tubers were kept at room temperature (~ 25^0C) for 3 weeks, and then transferred to different temperatures for one to two months to carry out further biochemical studies. Soluble sugars were extracted from mini-tubers stored at different temperatures, mainly 4ºC, 10ºC and 25ºC for a period of one month and two months to determine the accumulation of reducing sugars as well as total soluble sugars. The transgenic potato mini tubers from the independent transgenic lines corresponding to each genetic construct showed variations with regard to the level of sugar accumulation during storage at low temperatures as supported by the following experimental data (Fig. 21-28; also see Tables 1-16 in R&D:Appendix-1)

For RA genetic construct-based transgenic potato mini tubers, out of ten transgenic lines, only three lines RA-1, RA-13 and RA-14 showed less sugar accumulation. The reducing sugar level was found in the range of 1.4 to 4.5 mg g^{-1} FW; whereas the total soluble sugar level was found in the range of 3.63 to 9.7 mg g^{-1} FW (Fig. 21; also see Table 1 & 2).

For RB genetic construct-based transgenic mini tubers, the reducing sugar level for the transgenic lines RB-1, RB-3, RB-8 and RB-13 was found below 1.58 mg g^{-1} FW, and the total soluble sugar level was in the range of 2.15 to 5.26 mg g^{-1}FW (Fig. 22; also see Table 3 & 4).

For RC genetic construct-based transgenic mini tubers, the reducing sugar level was found below 1.64 mg g^{-1} FW for transgenic lines RC-1, RC-2 and RC-7, whereas the total soluble sugar level was found in the range of 2.32 to 9.90 mg g^{-1} FW (Fig. 23; also see Table 5 & 6).

For RD genetic construct-based transgenic mini tubers, the reducing sugar level was found below 2.58 mg g^{-1} FW for the transgenic lines RD-1, RD-6, RD-9, RD-10 and RD-15, and the total soluble sugar level was found in the range of 2.98 to 7.67 mg g^{-1} FW (Fig. 24; also see Table 7 & 8).

For RE genetic construct-based transgenic mini tubers, the reducing sugar level was found below 4.6 mg g^{-1} FW for the transgenic lines RE-2, RE-5, RE-15 and RE-16, and the total soluble sugar level was in the range of 4.66 to 9.77 mg g^{-1} FW (Fig. 25; see Table 9 & 10).

For RF genetic construct-based transgenic mini tubers, the reducing sugar level was found below 2.4 mg g^{-1} FW for the transgenic lines RF-1, RF-2, RF-4, RF-6 and RF-8; and the total soluble sugar level was found in the range of 3.11 to 6.83 mg g^{-1} FW (Fig.26; Table 11 & 12).

For RG genetic construct-based transgenic mini tubers, the reducing sugar level was found below 1.76 mg g^{-1} FW for the transgenic lines RG-3, RG-4, RG-6 and RG-9, the total soluble sugar level was found in the range of 4.31 to 8.70 mg g^{-1} FW (Fig. 27; see Table 13 & 14).

For RH genetic construct-based transgenic mini tubers, the reducing sugar level was found below 2.44 mg g^{-1} FW for the transgenic lines RH-2, RH-3, RH-4, RH-5 and RH-6, and the total soluble sugar level was in the range of 1.38 to 4.94 mg g^{-1} FW (Fig. 28; Table 15 & 16).

Level of reducing sugar in the control potato tubers: In freshly harvested control (non-transgenic) potato tubers (cv. Kufri Chipsona-1), the reducing sugar was found to be 0.09-0.15 mg g^{-1}FW; when stored at 4°C for one month, the value became 3.80 mg g^{-1}FW, and 4.9 mg g^{-1} FW if stored for two months; at 10°C for one month, it was 2.5 mg g^{-1} FW; and 0.24 mg g^{-1} FW at 25°C for one month. After one month storage at 4^0C the total soluble was 12.05 mg g^{-1} FW. The data clearly indicates that considerably high level of reducing and soluble sugar accumulations occur at 4°C even after storage for only one month.

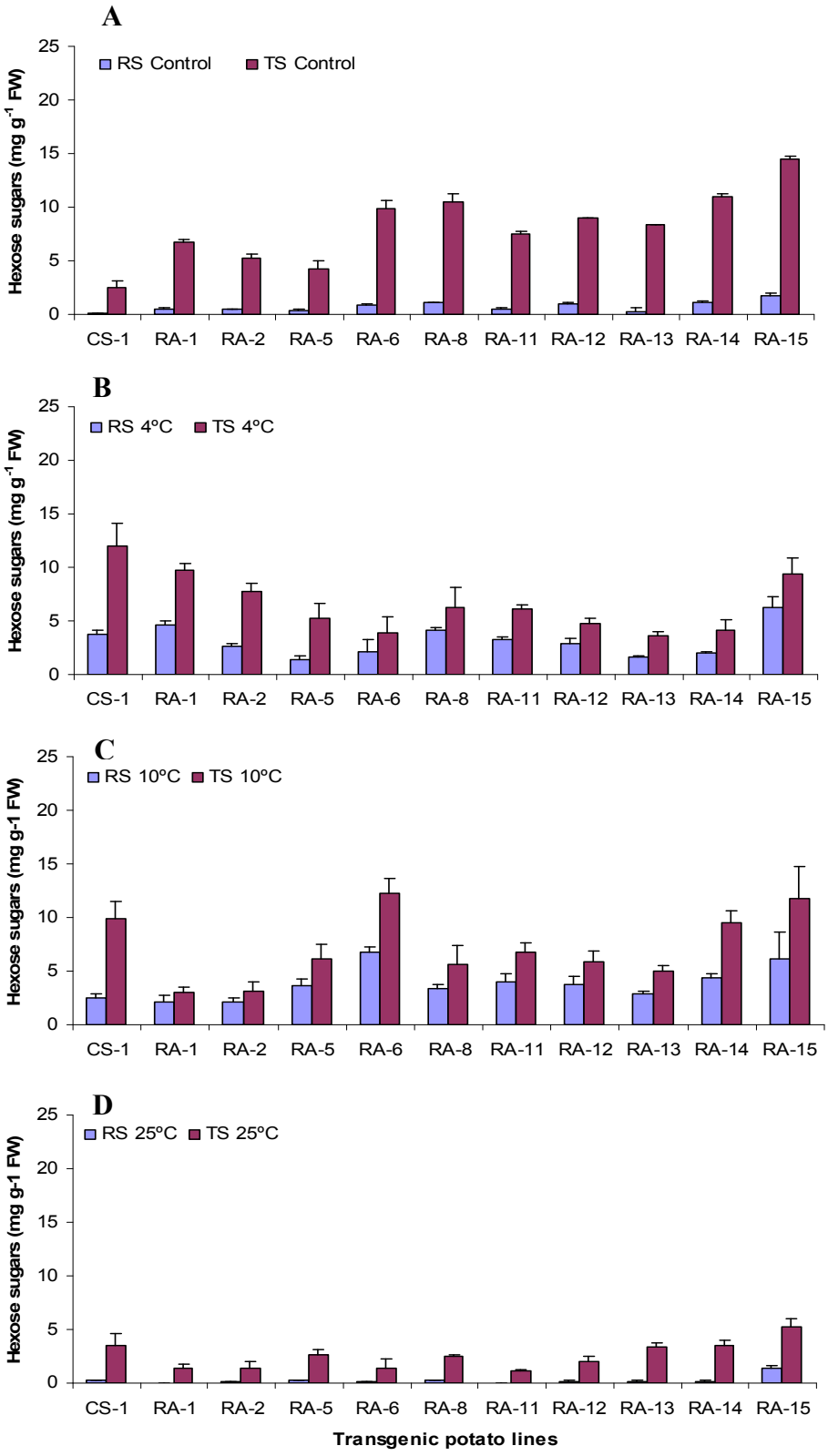

Fig. 21 Hexose sugar contents in the potato tubers (freshly harvested and stored at different temperatures 4ºC, 10ºC and 25ºC for one month) corresponding to the RA-based transgenic lines. The results are the mean ± SD of four individual tubers. RS refers to reducing sugars; TS refers to total soluble sugars. **A** Freshly harvested tubers; **B** tubers stored at 4ºC; **C** tubers stored at 10ºC; **D** tubers stored at 25ºC

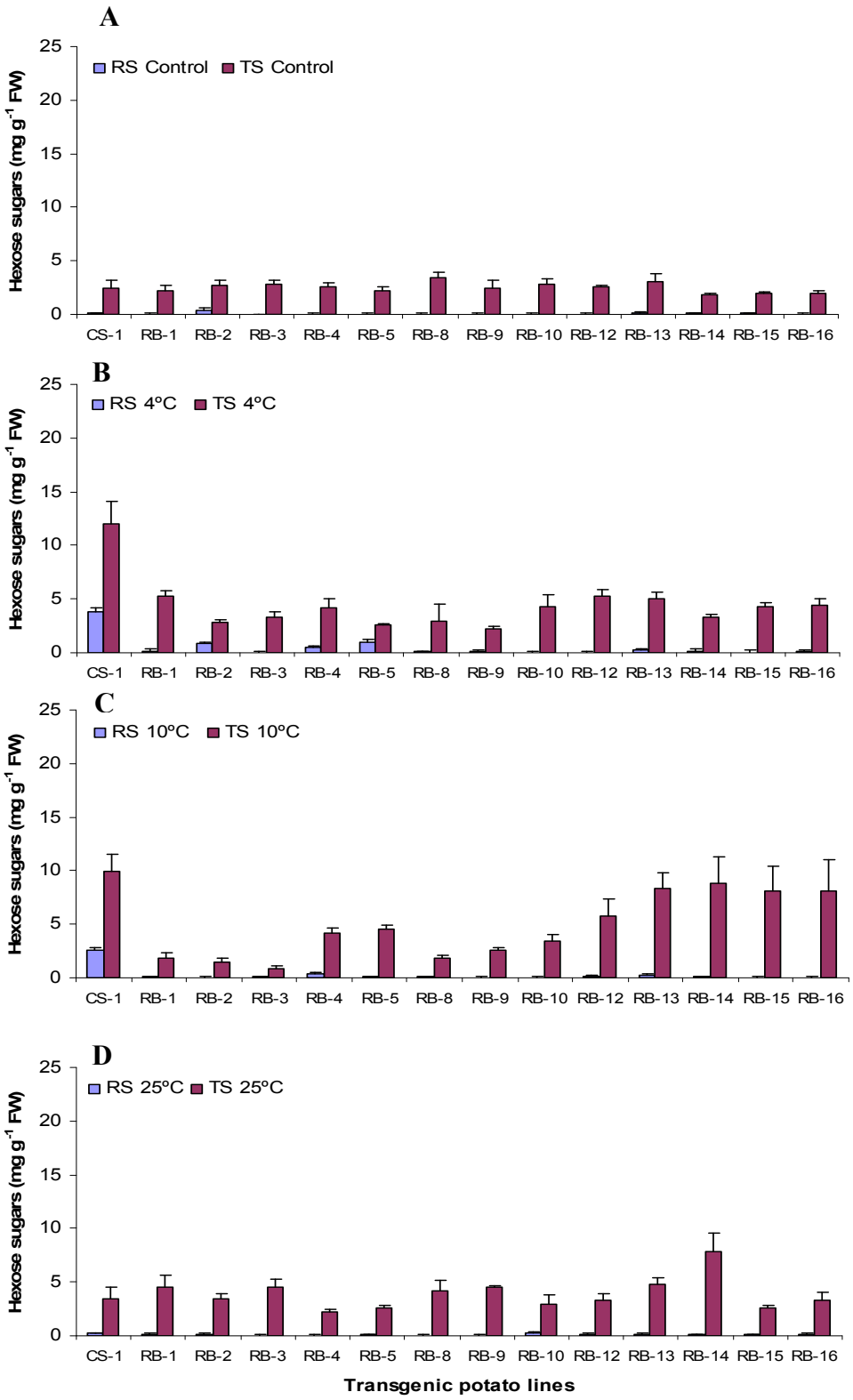

Fig. 22 Hexose sugar contents in the potato tubers (freshly harvested and stored at different temperatures 4ºC, 10ºC and 25ºC for one month) corresponding to the RB-based transgenic lines. The results are the mean ± SD of four individual tubers. RS refers to reducing sugars; TS refers to total soluble sugars. **A** Freshly harvested tubers; **B** tubers stored at 4ºC; **C** tubers stored at 10ºC; **D** tubers stored at 25ºC

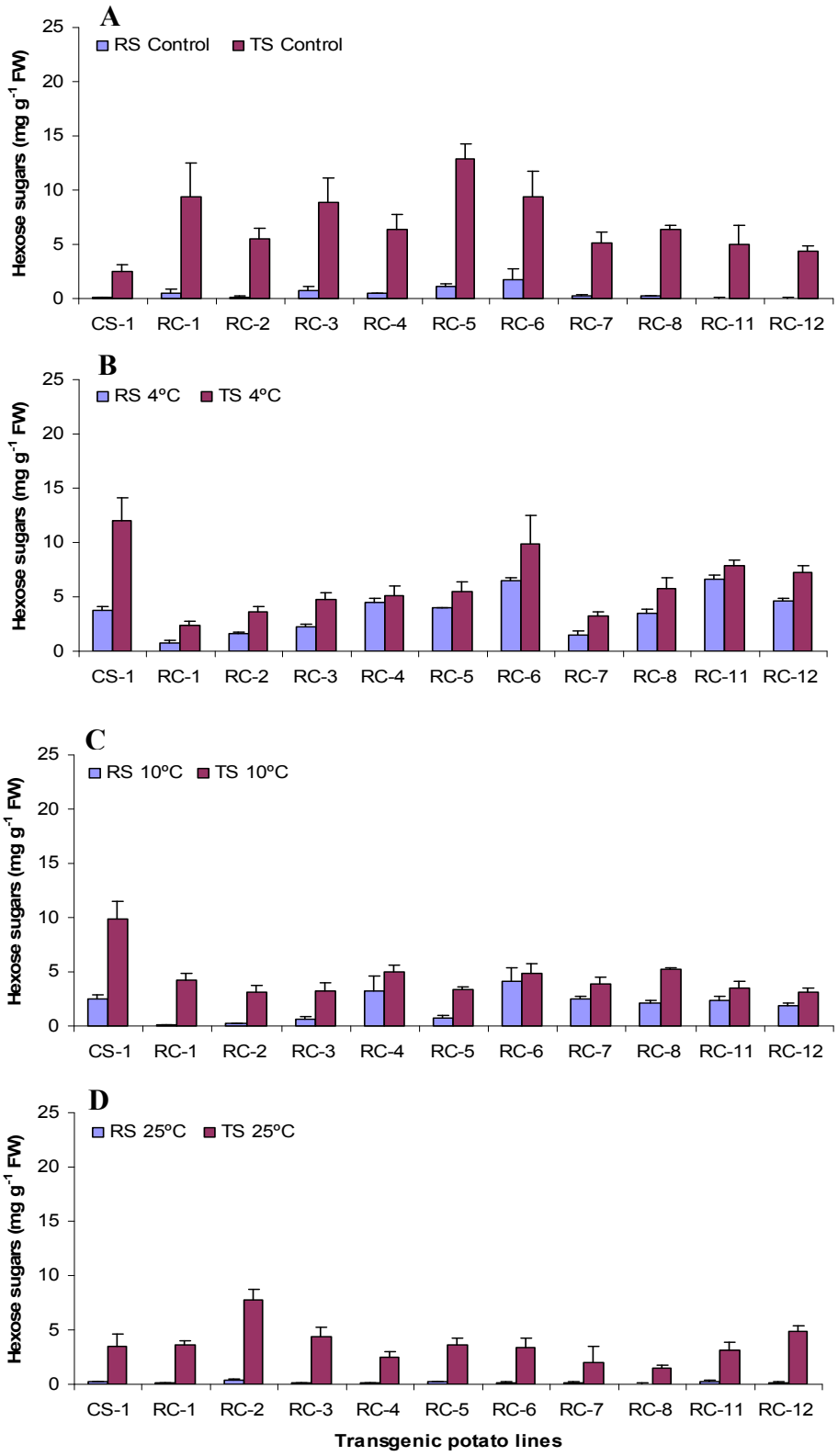

Fig. 23 Hexose sugar contents in the potato tubers (freshly harvested and stored at different temperatures 4ºC, 10ºC and 25ºC for one month) corresponding to the RC-based transgenic lines. The results are the mean ± SD of four individual tubers. RS refers to reducing sugars; TS refers to total soluble sugars. **A** Freshly harvested tubers; **B** tubers stored at 4ºC; **C** tubers stored at 10ºC; **D** tubers stored at 25ºC

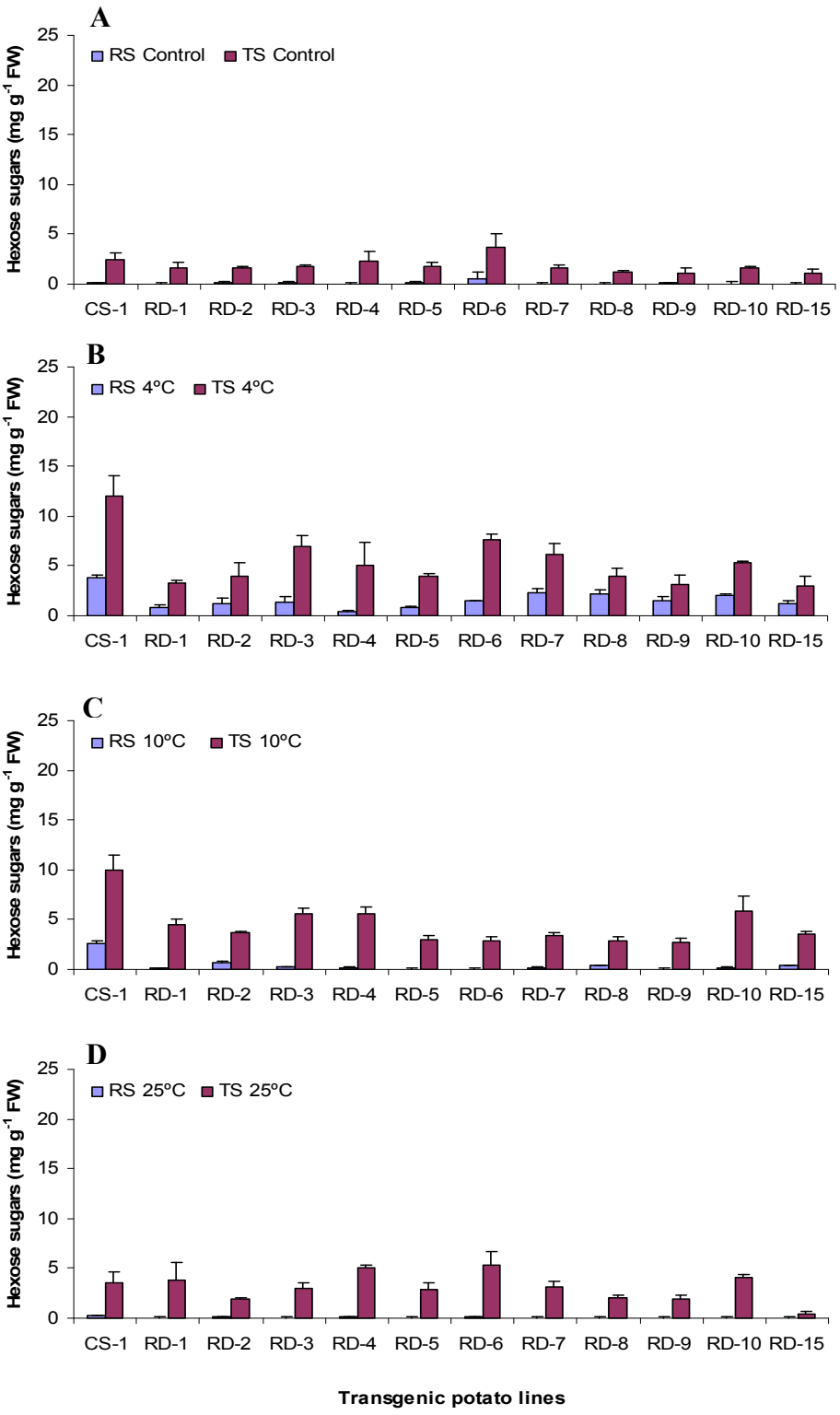

Fig. 24 Hexose sugar contents in the potato tubers (freshly harvested and stored at different temperatures 4ºC, 10ºC and 25ºC for one month) corresponding to the RD-based transgenic lines. The results are the mean ± SD of four individual tubers. RS refers to reducing sugars; TS refers to total soluble sugars. **A** Freshly harvested tubers; **B** tubers stored at 4ºC; **C** tubers stored at 10ºC; **D** tubers stored at 25ºC

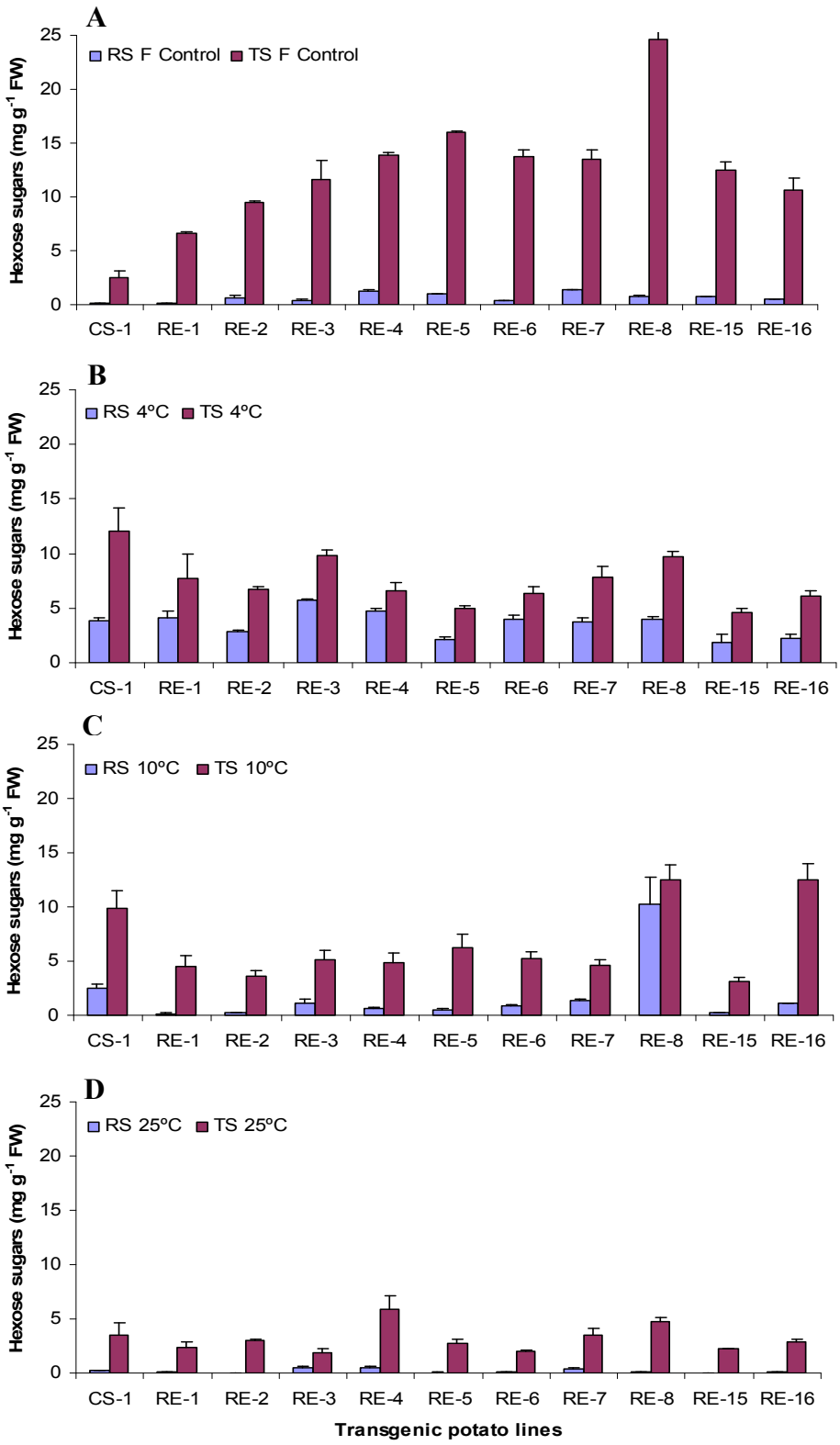

Fig. 25 Hexose sugar contents in the potato tubers (freshly harvested and stored at different temperatures 4ºC, 10ºC and 25ºC for one month) corresponding to the RE-based transgenic lines. The results are the mean ± SD of four individual tubers. RS refers to reducing sugars; TS refers to total soluble sugars. **A** Freshly harvested tubers; **B** tubers stored at 4ºC; **C** tubers stored at 10ºC; **D** tubers stored at 25ºC

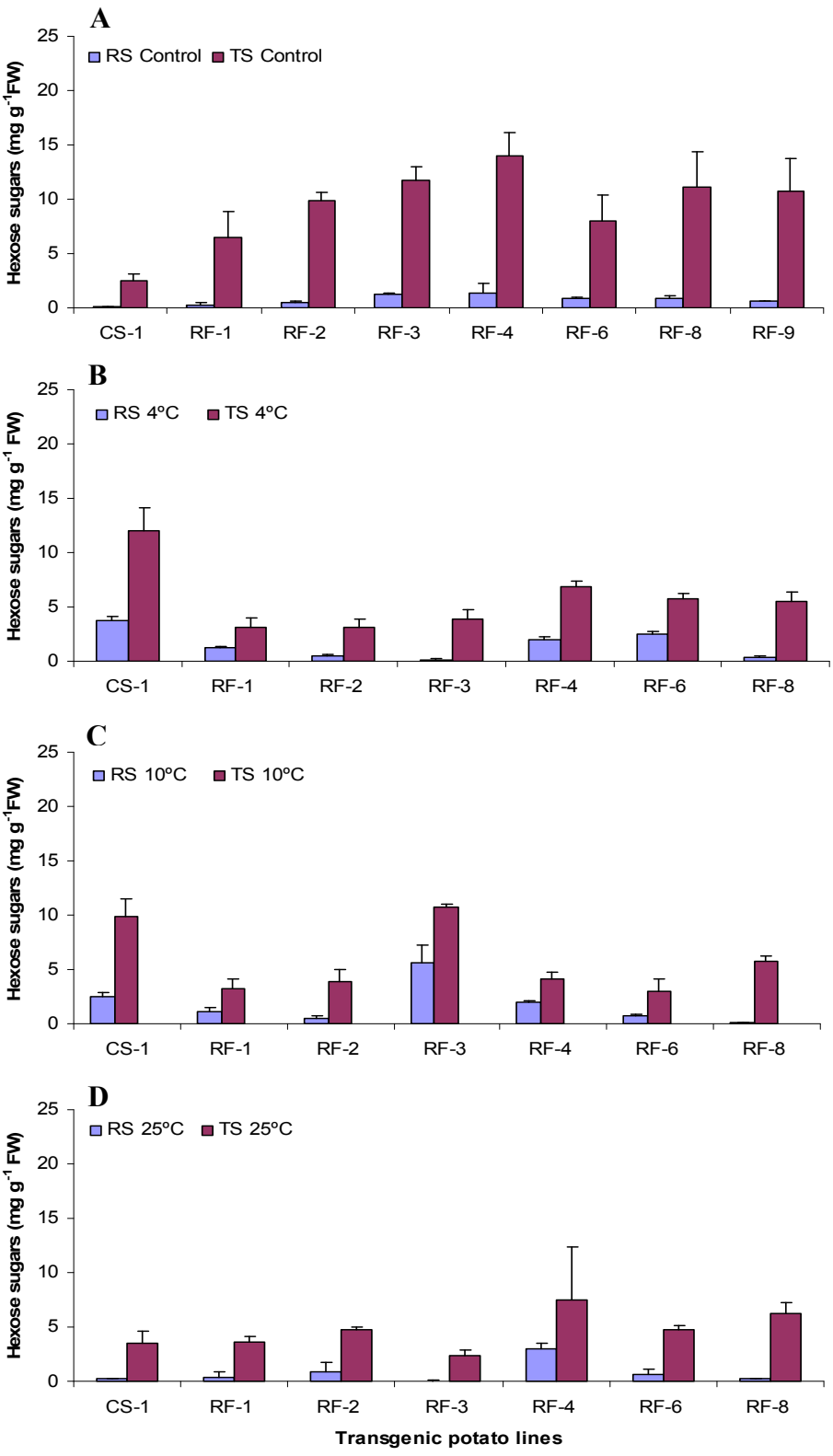

Fig. 26 Hexose sugar contents in the potato tubers (freshly harvested and stored at different temperatures 4ºC, 10ºC and 25ºC for one month) corresponding to the RF-based transgenic lines. The results are the mean ± SD of four individual tubers. RS refers to reducing sugars; TS refers to total soluble sugars. **A** Freshly harvested tubers; **B** tubers stored at 4ºC; **C** tubers stored at 10ºC; **D** tubers stored at 25ºC

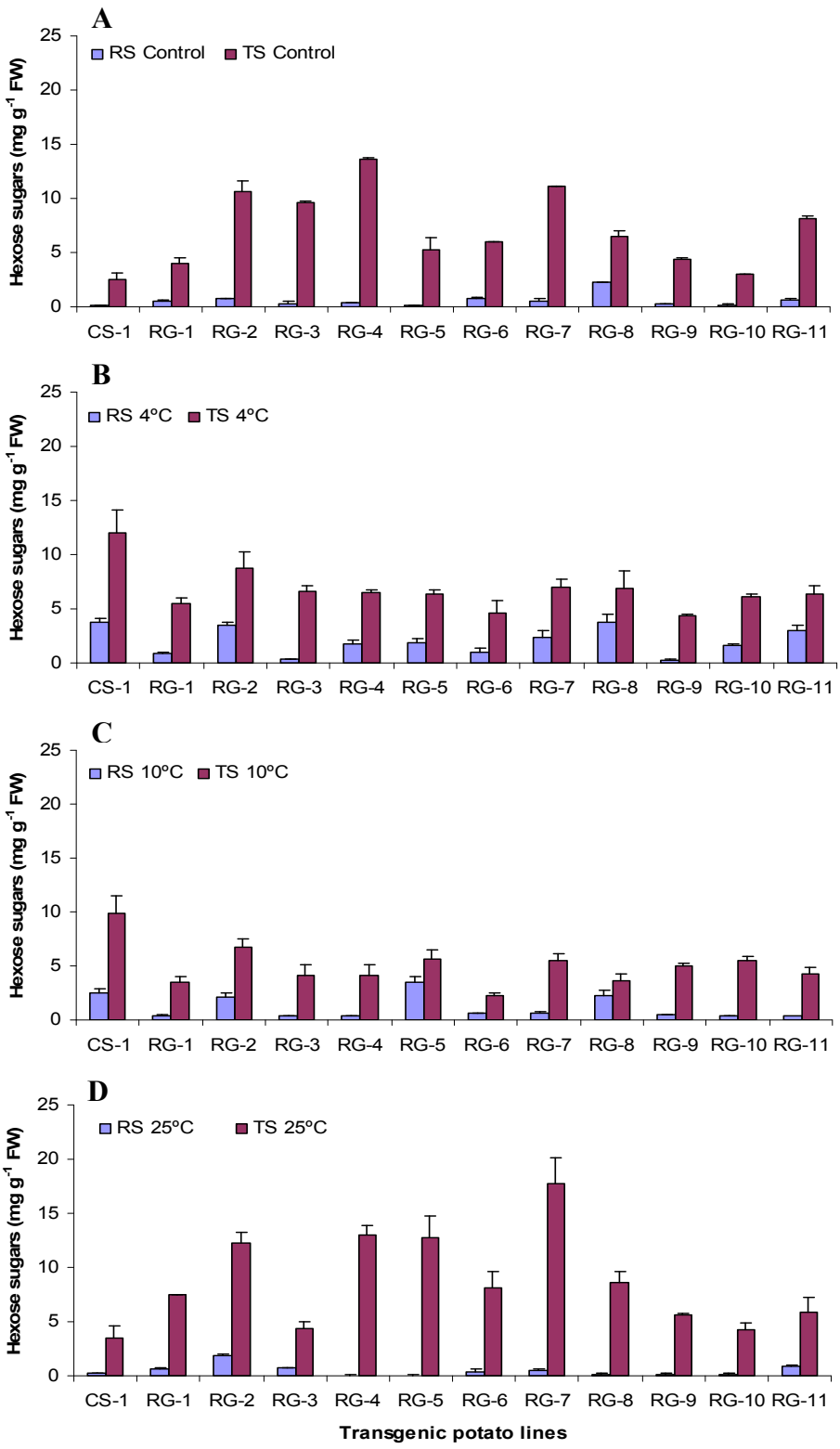

Fig. 27 Hexose sugar contents in the potato tubers (freshly harvested and stored at different temperatures 4ºC, 10ºC and 25ºC for one month) corresponding to the RG-based transgenic lines. The results are the mean ± SD of four individual tubers. RS refers to reducing sugars; TS refers to total soluble sugars. **A** Freshly harvested tubers; **B** tubers stored at 4ºC; **C** tubers stored at 10ºC; **D** tubers stored at 25ºC

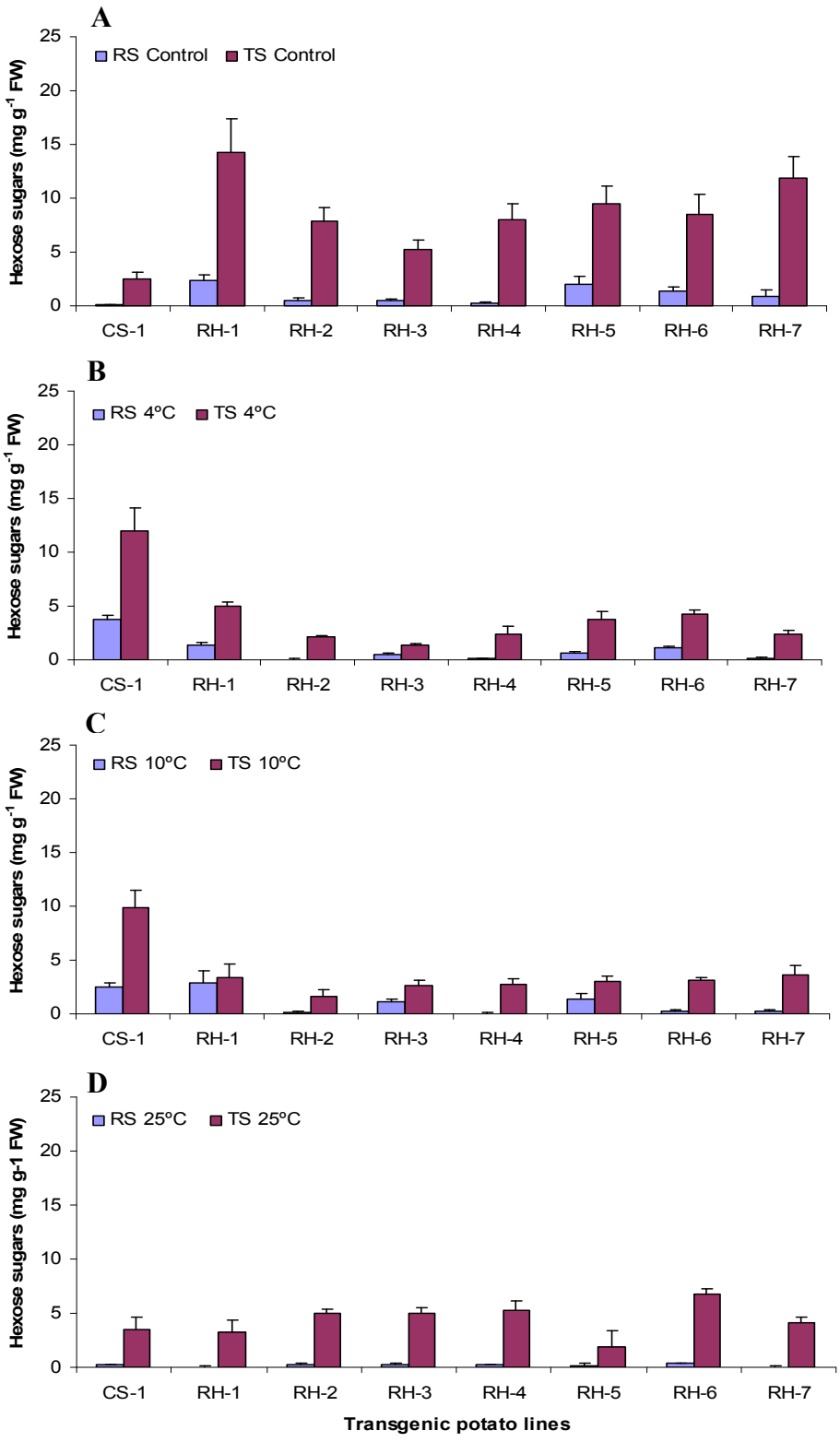

Fig. 28 Hexose sugar contents in the potato tubers (freshly harvested and stored at different temperatures 4ºC, 10ºC and 25ºC for one month) corresponding to the RH-based transgenic lines. The results are the mean ± SD of four individual tubers. RS refers to reducing sugars; TS refers to total soluble sugars. **A** Freshly harvested tubers; **B** tubers stored at 4ºC; **C** tubers stored at 10ºC; **D** tubers stored at 25ºC

In this study, collectively a large number of independent transgenic potato lines were generated using different antisense and sense binary genetic constructs. However, some of the transgenic lines appeared to be promising in terms of repression of cold-induced sweetening; since a number of transgenic mini tubers showed considerably lower accumulation of reducing sugars during storage at 4^0C in comparison to the non-transgenic control tubers. More specifically, the overall reducing sugar levels of the transgenic tubers corresponding to the genetic constructs RB, RC, RD, RF, RG and RH after storage at 4^0C up to two months were found to be in the range of 1.5-2.5 mg g^{-1} FW, which was less as compared to the control potato tubers (3.80-4.90 mg g^{-1} FW). The reducing sugar contents in the transgenic tubers corresponding to the RA and RE genetic constructs was found to be in the range of 2.5-4.6 mg g^{-1} FW if stored at 4^0C up to two months.

The tuber-specific GKC1 (GBSSI) promoter based both antisense and sense genetic constructs appeared to be effective in terms of repression of the cold-induced sweetening in the transgenic mini tubers. The CaMV 35S promoter based sense genetic constructs appeared to be more effective as compared to corresponding antisense genetic constructs in the transgenic potato lines generated in this study. However, down-regulation of R1 (GWD) was noted in these transgenic potato tubers since immunological method failed to detect 155 kD protein in the respective tuber protein extracts.

Starch-bound phosphorus content in the transgenic potato tubers: Starch granules were isolated from different transgenic potato tubers. As a control, starch was also isolated from Kufri Chipsona-1 tubers. The starch-bound phosphorus content was reduced only up to 10% (in comparison to control) in the transgenic tubers corresponding to the genetic constructs RA, RB, RC, RD, RE, and RG (Tables 17 & 18). But RF and RH sense constructs based transgenic lines showed up to 50% reduction in the tuber starch-bound phosphorus content (0.12-0.25 mg P g^{-1} starch) as compared to control (0.28-0.40 mg P g^{-1} starch) .

Table 17: Starch bound total phosphorus content (mg P g^{-1} starch) in the freshly harvested transgenic mini tubers

Antisense transgenic potato mini tubers				**Sense transgenic potato mini tubers**			
RA	**P(Mean ±SD)**	**RC**	**P(Mean ±SD)**	**RB**	**P(Mean ±SD)**	**RD**	**P(Mean ±SD)**
Control	0.40 ±0.04ab	Control	0.40 ±0.04b	Control	0.40 ±0.04cd	Control	0.40 ±0.04bc
RA-1	0.40 ± 0.03ab	RC-1	0.40 ±0.12b	RB-1	0.57 ±0.04abc	RD-1	0.59 ±0.01ab
RA-2	0.36 ±0.01b	RC-2	0.46 ±0.07b	RB-2	0.62 ±0.04a	RD-2	0.38 ±0.06c
RA-5	0.46 ±0.04ab	RC-3	0.42 ±0.07b	RB-3	0.61 ±0.02ab	RD-3	0.62 ±0.03ab
RA-6	0.51 ±0.02ab	RC-4	0.44 ±0.03b	RB-4	0.45 ±0.06bcd	RD-4	0.43 ±0.04abc
RA-8	0.39 ±0.01ab	RC-5	0.43 ±0.00b	RB-5	0.32 ±0.01d	RD-5	0.48 ±0.00abc
RA-11	0.59 ±0.08a	RC-6	0.35 ±0.02bc	RB-8	0.52 ±0.07abc	RD-6	0.35 ±0.04c
RA-12	0.51 ±0.08ab	RC-7	0.73 ±0.35a	RB-9	0.63 ±0.01a	RD-7	0.37 ±0.02c
RA-13	0.49 ±0.10ab	RC-8	0.50 ±0.12ab	RB-10	0.44 ±0.02bcd	RD-8	0.50 ±0.00abc
RA-14	0.43 ±0.03ab	RC-11	0.56 ±0.08ab	RB-12	0.43 ±0.02cd	RD-9	0.45 ±0.00abc
RA-15	0.41 ±0.08ab	RC-12	0.40 ±0.03b	RB-13	0.44 ±0.08bcd	RD-10	0.45 ±0.03abc
				RB-14	0.50 ±0.07abc	RD-15	0.42 ±0.14bc
				RB-15	0.59 ±0.02abc		
				RB-16	0.42 ±0.02cd		

The values sharing a common letter with in the column are not significant at $P \leq 0.05$.

Table 18: Starch bound total phosphorus content (mg P g^{-1} starch) in the freshly harvested transgenic mini tubers

Antisense transgenic potato mini tubers				Sense transgenic potato mini tubers			
RE	**P(Mean ±SD)**	**RG**	**P(Mean ±SD)**	**RF**	**P(Mean ±SD)**	**RH**	**P(Mean ±SD)**
Control	0.40 ±0.04a	Control	0.40 ±0.04b	Control	0.40 ±0.04a	Control	0.40 ±0.04a
RE-1	0.46 ±0.15a	RG-1	0.50 ±0.04a	RF-1	0.37 ±0.05a	RH-1	0.27 ±0.07b
RE-2	0.25 ± 0.01c	RG-2	0.37 ±0.00b	RF-2	0.15 ±0.00bc	RH2	0.29 ±0.01b
RE-3	0.26 ±0.03c	RG-3	0.33 ±0.04bc	RF-3	0.16 ±0.01bc	RH-3	0.12 ±0.00c
RE-4	0.24 ±0.03c	RG-4	0.25 ±0.03cd	RF-4	0.17 ±0.01bc	RH-4	0.25 ±0.01b
RE-5	0.29 ±0.03b	RG-5	0.23 ±0.04cd	RF-6	0.25 ±0.09abc	RH-5	0.26 ±0.01b
RE-6	0.36 ±0.10ab	RG-6	0.34 ±0.01bc	RF-8	0.27 ±0.01ab	RH-6	0.30 ±0.00b
RE-7	0.29 ±0.01b	RG-7	0.33 ±0.02bc	RF-9	0.12 ±0.00c	RH-7	0.29 ±0.01b
RE-8	0.30 ±0.00b	RG-8	0.32 ±0.04bcd				
RE-15	0.28 ±0.02b	RG-9	0.28 ±0.04bcd				
RE-16	0.27 ±0.04b	RG-10	0.20 ±0.00d				
		RG-11	0.32 ±0.01bcd				

The values sharing a common letter with in the column are not significant at $P \leq 0.05$.

The reducing sugar level was considerably reduced in a number of transgenic potato tubers corresponding to the different genetic constructs as used in this study; but tuber starch-bound phosphorus content remained more or less unaltered in these cases. However, tuber starch-bound phosphorus content was reduced significantly only in the transgenic lines generated by the sense genetic constructs namely RF and RH. Based on these data, at present it is difficult to find an exact correlation between the starch-bound phosphorus content and the extent of reducing sugar level in the cold-stored tubers. But the transgenic potato tubers showing lower level of reducing sugar at low temperatures also showed lower R1 (GWD) level as evident from protein gel blot analysis. In other words, there was down-regulation of R1 (GWD) in the

sense and antisense constructs based transgenic mini tubers. The earlier conceived notion was that more phosphate content in the starch leads to its more degradability. It is likely that apart from starch phosphorylation, GWD may have influence in stimulating the activities of different starch hydrolytic and/or phosphorolytic enzymes involved in starch degradation through protein-protein interactions. In fact, more investigation is required for in-depth understanding of R1 (GWD) functionality.

Concluding remarks: Based on the biochemical studies, varietal differences were observed with regard to the extent of tuber starch-bound phosphorus in the freshly harvested tubers, and accumulation of reducing and soluble sugars in cold-stored tubers between the Indian potato cultivars. Molecular cloning and characterization studies led to the isolation of a full-length cDNA encoding R1 protein (GWD) for the first time from a processing Indian potato cultivar namely, Kufri Chipsona 1. Thorough sequence analyses clearly revealed that the above cDNA encodes a distinct R1 (GWD) isoform from potato which was not reported earlier. As a preliminary study, GWD gene expression analyses were carried out in the different tissues of the potato cultivars only at a particular stage of potato plants by semi-quantitative RT-PCR approach and protein blot analysis. In addition to R1(GWD) cDNA, two new GBSSI alleles were also isolated for the first time from an Indian potato cultivar, Kufri Chandramukhi. Functional characterization of one of the GBSSI alleles revealed its strong tuber specificity as evident from histochemical GUS assay. A number of antisense and sense binary genetic constructs were made using the constitutive promoter (the CaMV 35S promoter) and the tuber-specific GBSSI promoter (as isolated and characterized in this study). Transgenic potato tubers corresponding to a number of different transgenic lines showed considerably less reducing sugar levels as compared to the control ones during storage at low temperatures.It

may be presumed that the mechanism of inhibition of R1 gene function in some of the sense transgenic potato lines could be similar to RNAi-mediated gene silencing which needs to be further understood. For future prospects of research, direct RNAi approach could be adopted for inhibition of R1 gene function in potato. Tuber starch-bound phosphorus contents were determined both in the normal and transgenic potato tubers in order to find correlation between the starch-bound phosphorus content and the extent of reducing sugar level in the cold-stored tubers. Interestingly, in some transgenic potato tubers, there was no significant change in the starch-bound phosphorus content although they showed repressed cold sweetening phenotype which needs to be further addressed by molecular and biochemical approaches. More investigation is required for in-depth understanding of GWD functionality. The thesis work is quite relevant with regard to the basic aspects of starch research, and also a step forward in terms of redesigning of a commercially important Indian potato cultivar.

Summary

6. Summary

- The tuber starch-bound phosphate contents of different Indian potato cultivars were found to be in the range of 0.40-0.60 mg P g^{-1}starch. The reducing sugar levels of the stored potato tubers of different Indian potato cultivars at different temperatures were studied; at 3ºC the values were in the range of 12.0-26.0 mg g^{-1}FW; whereas the reducing sugar levels in the freshly harvested tubers were found in the range of 0.5-1.5 mg g^{-1}FW. In terms of starch-bound phosphate contents and sugar accumulation, varietal differences were noted between the potato cultivars.
- Based on molecular cloning and characterization studies, a ~4789 bp full-length cDNA encoding R1 (GWD) was isolated from the potato cultivar Kufri Chipsona-1, and the sequence information was submitted to the NCBI database (GenBank Accession no. is EU599037). The predicted protein consists of 1463 amino acids, designated as KC-GWD. Sequence and phylogenetic tree analyses clearly indicate that KC-GWD is a distinct GWD isoform, being reported for first time from Indian potato cultivar.
- Similarly, two partial GBSSI alleles with promoter regions were isolated and characterized from the potato cultivar Kufri Chandramukhi, designated as GKC1 and GKC2. The sequence information was submitted to the NCBI under the GenBank Acc Nos EU548081 and EU548082, respectively. This is also first report from the Indian potato cultivar. The promoter of GKC1 allele was tuber-specific as evident from functional characterization. This promoter used for the construction of a binary vector, designated as pAN-GB01 and used in this study.

- A total of eight Ti-plasmid based antisense and sense genetic constructs were made using different GWD cDNA fragments under both constitutive CaMV35S and tuber-specific GKC1 (GBSSI) promoter.
- The biochemical analyses of the selected transgenic potato mini tubers revealed that the level of reducing sugars were in the range of 1.0-3.0 mg g^{-1} FW after storage at low temperature i.e., 4ºC up to two months which was considerably lower in comparison to the non-transgenic potato tubers as control. Tuber-starch bound phosphorus content was found to be in the range of 0.15-0.40 mg P g^{-1}starch in some of the transgenic lines which was 10-50 % less in comparison to the control tuber starch.
- Some of the transgenic potato lines as generated in this study appeared to be promising ones as the potato tubers showed less accumulation of reducing and soluble sugars during storage at low temperatures.

References

7. References

Alexander RJ (1995) Potato Starch: New prospects of an old product**.** Cereal Foods World-40:763-764

Allen RD, Bernier F, Lessard PA, Beachy RN (1989) Nuclear factors interact with a soybean beta-conglycinin enhancer. Plant Cell 1:623-631

Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8:115-118.

Ames BN, Chen PS Jr, Toribara TY, Warner H (1956) Assay of phosphate and Phosphatases. Anal Chem 28:1756-1758

Asatsuma S, Sawada C, Itoh K, Okito M, Kitajima A, Mitsui T (2005) Involvement of α-amylase I-1 in starch degradation in rice chloroplasts. Plant Cell Physiol 46:858-869

Baunsgaard L, Lutken H, Mikkelsen R, Glaring MA, Pham TT, Blennow A (2005) A novel isoform of Glucan, water dikinase phosphorylates pre-phosphorylated α-glucans and is involved in starch degradation in *Arabidopsis*. Plant J 41:595-605

Bay-Smidt A, Wischmann B, Olsen CE, Nielsen TH (1994) Starch bound phosphate in potato as studied by a simple method for determination of organic phosphate and ^{31}P-NMR. Starch/Stärke 46:167-172

Beaujean A, Ducrocq-Assaf C, Sangwan RS, Lilius G, Bulow L, Sangwan-Norreel BS (2000) Engineering Direct Fructose production in processed potato tubers by expressing a bifunctional alpha-amylase/glucose isomerase gene complex. Biotechnol Bioengg 70:9-16

Beaujean A, Sangwan RS, Lecardonne A, and Sangwan-Norreel BS (1998) *Agrobacterium*-mediated transformation of three economically important potato cultivars using sliced internodal explants:an efficient protocol of transformation. J Exp Bot 49:1589-1595

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 24:1513-1523

Blennow A, Bay-Smidt AM, Olsen CE, Møller BL (2000a) The distribution of covalently bound phosphate in the starch granule in relation to starch crystallinity. Int J Biol Macromol 27:211-218

Blennow A, Engelsen SB, Munck L, Møller BL (2000b) Starch molecular structure and phosphorylation investigated by a combined chromatographic and chemometric approach. Cabohydr Polym 41:163-174

Blenow A, Engelsen SB, Nielson TH, Baunsgaard and Mikkelsen R (2002) Starch Phosphorylation: a new front line in starch research. Trends in Plant Sci 7:445-450

Burton WG (1969) The sugar balance in some british potato varieties during storage. II. The effects of tuber age, previous storage temperature, and intermittent refrigeration upon low-temperature sweetening. Eur Potato 12:81-95

Cercos M, Gomez-Cadenas A, Ho T-HD (1999) Hormonal regulation of a cysteine proteinase gene, EPB-1, in barley aleurone layers: cis-and trans-acting elements involved in the co-ordinated gene expression regulated by gibberellins and abscisic acid. Plant J 19:107–118

Chen PY, Wang CK, Soong SC, To KY (2003) Complete sequence of the binary vector pBI121 and its application in T-DNA insertion from transgenic plants. Mol Breed 11:287-293

Chou PY and Fasman GD (1974a) Prediction of protein conformation. Biochem 13:222–245

Chou PY and Fasman GD (1974b) Conformational parameters for amino acids in helical, β-sheet, and random coil regions calculated from proteins. Biochem 13:211–222

Chou PY and Fasman GD (1977) Beta-turns in proteins. J Mol Biol 115:135-175

Chou PY and Fasman GD(1978) Empirical predictions of protein conformation. Annu Rev Biochem 47:251-276

Chou PY and Fasman GD(1978) Prediction of the secondary structure of proteins from their amino acid sequence. Adv Enzymol 47:45-148

Christopher JP, Tomap R (1975) Activities of enzymes of sugar metabolism in cold-stored tubers of *Solanum tuberosum*. Phytochem 14:613-617

Colot V, Robert LS, Kavanagh TA, Bevan MW, Thompson RD (1987) Localization of sequences in wheat endosperm protein genes which confer tissue-specific expression in tobacco. EMBO J 6:3559-3564

Corpet F (1998) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16:10881-10890

D'Aoust M-A, Nguyen-Quoc B, Le V-Q, Yelle S (1999) Upstream regulatory regions from the maize Sh1 promoter confer tissue-specific expression of the β-glucuronidase gene in tomato. Plant Cell Rep 18:803-808

Dai WL, Deng W, Cui WY, Zhao SY, Wang XM (1996) Molecular cloning and sequence of potato granule-bound starch synthase gene. Acta Bot Sinica 38:777-784

Degenhardt J, Tobin EM (1996) A DNA binding activity for one of two closely defined phytochrome regulatory elements in an Lhcb promoter is more abundant in etiolated than in green plants. Plant Cell 8:31-41

Deiting U, Zrenner, R, Stitt, M (1998) Similar temperature requirement for sucrose accumulation for the induction of new forms of sucrose phosphate synthase and amylase in cold-stored potato tubers. Plant Cell Environ 21:127-138

Delatte T, Umhang M, Trevisan M, Eicke S, Thorneycroft D, Smith SM, Zeeman SC (2006) Evidence for distinct mechanisms of starch granule breakdown in plants. J Biol Chem 281:12050-12059

Deleage G and Roux B (1987) Amino acid scale: Conformational parameter for coil. Protein Engg 1:289-294

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19-21

Denyer K, Johnson P, Zeeman S, Smith AM (2001) The control of amylose synthesis. J Plant Physiol 158:479-487

Dodds KS (1962) Classification of cultivated potatoes, In The Potato and its Wild Relatives: Section Tuberarium of the Genus Solanum (Correll DS ed.), pp 517-539, Texas Res Foundation, Renner, TX

Dohmann EMN, Kuhnle C, Schwechheimer C (2005) Loss of the Constitutive photomorphogenic9 sigalosome subunit 5 is sufficient to cause the *cop/det/fus* mutant phenotype in Arabidopsis. Plant Cell 17:1967-1978

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

Dry I, Smith A, Edwards A, Bhattacharrya M, Dunn P, Martin C (1992) Characterization of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs of pea and potato. Plant J 2:193-202

Edner C, Li J, Albrecht T, Mahlow S, Hejazi M, Hussain H, Kaplan F, Guy C, Smith SM, Steup M, Ritte G (2007) Glucan, Water Dikinase Activity Stimulates Breakdown of Starch Granules by Plastidial β-Amylases. Plant Physiol 145:17-28

Edwards A, Vincken JP, Suurs LCJM, Visser RGF, Zeeman S, Smith A, Martin C (2002) Discrete forms of amylose are synthesized by isoforms of GBSSI in Pea. Plant Cell 14:1767-1785

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evol 39:783-791

Fettke J, Hejazi M, Smirnova J, Hochel E, Stage M, Steup M. (2009) Eukaryotic starch degradation: integration of plastidial and cytosolic pathways. J Exp Bot 60:2907-2922

Fincher GB (1989) Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. Annu Rev Plant Physiol Plant Mol Biol 40:305-346

Flipse E, Straatman-Engelen I, Kuipers AGJ, Jacobsen E, Visser RGF (1996) GBSS T-DNA inserts giving partial complementation of the *amylose-free* potato mutant can also cause co-suppression of the endogenous GBSS gene in a wild-type background. Plant Mol Biol 31:731-739

Gamborg OL, Philips GC (1998) Plant, Cell and organ culture, 21-30

Gaur PC, Naik PC, Kaushik SK, Chakrabati SK (1999) Indian Potato Varieties, CPRI Technical Bulletin 51:5-10

Gilman, M (1987) Phenol/SDS method for plant RNA preparation. In Current Protocols in Molecular Biology, Ausubel FM et al (eds), John Wiley and Sons, New York, 431-434

Gilmartin PM, Sarokin L, Memelink J, Chua N-H (1990) Molecular Light Switches for Plant Genes. Plant Cell 2:369-378

Glaring MA, Baumann MJ, Hachem MA, Nakai H, Nakai N, Santelia D, Sigurskjold BW, Zeeman SC, Blennow A, Svensson B (2011) Starch-binding domains in the CBM45 family-low-affinity domains from glucan, water dikinase and α-amylase involved in plastidial starch metabolism. FEBS J 278: 1175–1185

Glaring MA, Zygadlo A, Thorneycroft D, Schulz A, Smith SM, Blennow A, Baunsgaard L (2007) An extra-plastidial α-glucan, water dikinase from Arabidopsis phosphorylates amylopectin in vitro and is not necessary for transient starch degradation. J Expt Bot 58:3949-3960.

Griener Steffen et al. (1999) Ecotopic expression of a tobacco invertase inhibitor homolog prevents cold-induced sweetening of potato tubers. Nature Biotechnology 17: 708-711

Griffiths DW, Bain H, Dale MFB (1997) The effect of low-temperature storage on the glycoalkaloid content of potato (*Solanum tuberosum*) tubers. J Sci Food Agric 74:301-307

Gubler F, Kalla R, Roberts JK, Jacobsen JV (1995) Gibberellin-regulated expression of the *myb* gene in barley aleurone cells: evidence for Myb transactivation of a high-pI α-amylase gene promoter. Plant Cell 7:1879-1891

Guillen D, Sanchez S, Rodriguez-Sanoja R (2010) Carbohydrate-binding domains: multiplicity of biological roles. Appl Microbiol Biotechnol 85:1241-1249

Heilersig BHJB, Loonen AEHM, Janssen EM, Wolters AM, Visser RGF (2006a) Efficiency of transcriptional gene silencing of GBSSI in potato depends on the promoter region that is used in an inverted repeat. Mol Gen Genomics 275:437-449.

Heilersig HJB, Loonen A, Bergervoet M, Wolters AMA, Visser RGF (2006a) Post-transcriptional gene silencing of GBSSI in potato:effects of size and sequence of the inverted repeats. Plant Mol Biol 60:647-662

Heilersig HJB, Loonen A, Janssen EM, Wolters AM, Visser RGF (2006b) Efficiency of transcriptional gene silencing of GBSSI in potato depends on the promoter region that is used in an inverted repeat. Mol Gen Genom 275:437-449.

Hejazi H, Fettke J, Kötting O, Zeeman SC, Steup M (2010) The laforin-like dual-specificity phosphataseSEX4 from *Arabidopsis thaliana* hydrolyses both C6- and C3-phosphate esters introduced by starch-related dikinases and thereby affects phase transition of α-glucans. Plant Physiol 152:711-722

Hejazi M, Fettke J, Haebel S, Edner C, Paris O, Frohberg C, Steup M, Ritte G (2008) Glucan, water dikinase phosphorylates crystalline Maltodextrins and thereby initiates solubilization. Plant J 55:323-334

Hejazi M, Fettke J, Paris O, Steup M (2009) The two plastidial starch-related dikinases sequentially phosphorylate glucosyl residues at the surface of both the A- and B-type allomorphs of crystallized maltodextrins but the mode of action differs. Plant Physiol 150:962–976

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. Nucl Acids Res 27:297-300

Hill L, Smith AM (1991) Evidence that glucose 6-phosphate is imported as the substrate for starch synthesis by the plastids of developing pea embryos. Planta 185:91-96

Hizukuri S, Tabata S and Nikuni Z (1970) Studies on starch phosphate. Part 1. Estimation of glucose-6-phosphate residues in starch and the presence of other bound phosphate(s). Starch/Stärke 22:338-343

Holmes DS, Quigley (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem 114:193-197

Hovenkamp-Hermelink JHM, Jacobsen E, Ponstein AS, Visser RGF, Vos-Scheperkeuter GH, Bijmolt EW, de Vries JN, de Witholt B, Feenstra WJ (1987) Isolation of an amylose-free starch mutant of the potato (*Solanum tuberosum* L). Theor Appl Genet 75:217-221

Hussain H, Mant A, Seale R, Zeeman SC, Hinchliffe E, et al. (2003) Three isoforms of isoamylase contribute different catalytic properties for the debranching of potato glucans. Plant Cell 15:133-49

Jacobsen E, Hovenkamp-Hermelink JHM, Krijgsheld HT, Pijnacker LP, Withold B, Feenstra WJ (1989) Phenotypic and genotypic characterisation of an amylose-free starch mutant of potato. Euphytica 44:43-48

Jane J, Kasemsuwan T, Chen JF, Juliano BO (1996) Phosphorous in rice and other starches. Cereal Foods 41:827-832

Jane J, Shen JJ (1992) Internal structure of the potato starch granule revealed by chemical gelatinisation. Carbohydr Res 247:279-290

Jansen G, Flamme W, Schuler K, Vandrey M (2001) Tuber & Starch quality of wild and cultivated potato species and cultivars. Potato Research 44:137-146

Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Rep 5:387-405

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, EMBO J 6:3901-3907

Jobling S (2004) Improving starch for food and industrial applications. Curr Opin Plant Biol 7:210-218.

Joshi CP (1987) An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucleic Acids Res 15:6643-6653

Kaneko T, Kihara M, Ito K, Takeda K (2000) Molecular and chemical analysis of β-amylase-less mutant barley in Tibet. Plant Breeding 119:383-87

Kerk D, Conley TR, Rodriguez FA, Tran HT, Nimick M, Muench DG, Moorhead GBG (2006) A chloroplast-localized dual-specificity protein phosphatase in *Arabidopsis* contains a phylogenetically dispersed and ancient carbohydrate-binding domain, which binds the polysaccharide starch. Plant J 46:400-413

Kolaskar AS, Tongaonkar PC (1990) A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett 276:172-174

Kossmann J, Lloyd J (2000) Understanding and influencing starch biochemistry. Crit Rev Plant Sci 19:171-226.

Kötting O, Kossman J, Zeeman SC, Lloyd J (2010) Regulation of starch metabolism: the age of enlightenment? Curr Opin Plant Biol 13:321-329

Kötting O, Pusch K, Tiessen A, Geigenberger P, Steup M, Ritte G (2005) Identification of a novel enzyme required for starch metabolism in Arabidopsis leaves. The Phosphoglucan, Water Dikinase. Plant Physiol 137:242-252

Kötting O, Santelia D, Edner C, Eicke S, Marthaler T, Gentry MS, Comparot-Moss S, Chen J, Smith AM, Steup M, Ritte G, Zeeman SC (2009) STACH-EXCESS4 is a laforin-like phosphoglucan phosphatase required for starch degradation in *Arabidopsis thaliana*. Plant Cell 21:334-346

Kuipers GJ, Vreem JTM, Meyer H, Jacobsen E, Feenstra WJ, Visser RGF (1992) Field evaluation of antisense RNA mediated inhibition of GBSS gene expression in potato. Euphytica 59:83-91

Kyte J and Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105-132

Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouze P, Rombauts S (2002) PlantCARE, database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucl Acids Res 30:325-327

Li CY, Weiss D, Goldschmidt EE (2003) Effects of carbohydrate starvation on gene expression in citrus root. Planta 217:11-20

Lloyd JR, Blennow A, Burhenne K, Kossmann J (2004) Repression of a novel isoform of disproportionating enzyme (*St*DPE2) in potato leads to inhibition of starch degradation in leaves but not tubers stored at low temperature. Plant Physiol 134:1347-1354

Lomako J, Lomako WM, Whelan WJ, Marchase RB (1993) Glycogen contains phosphodiester groups that can be introduced by UDPglucose:glycogen glucose 1-phosphotransferase. FEBS Lett 329:263-267

Lorberth R, Ritte G, Willmitzer L, Kossmann J (1998) Inhibition of a starch-granule-bound protein leads to modified starch and repression of cold sweetening. Nature Biotech 16:473-477

Mandel M and Higa A (1970) Calcium-dependent bacteriophage DNA infection. J Mol Biol 53:159-162

Martin C and Smith AM (1995) Starch Biosynthesis. Plant Cell 7:971-985

Mikkelsen R and Blennow A (2005) Functional domain organization of the potato α-glucan, water dikinase (GWD): evidence for separate site catalysis as revealed by limited proteolysis and deletion mutants. Biochem J 385:355-361

Mikkelsen R, Baungaard L, Blennow A (2004) Functional characterization of α-glucan, water dikinase, the starch phosphorylating enzyme. Biochem J 377:525-532

Mikkelsen R, Suszkiewicz K, Blennow A (2006) A novel type carbohydrate-binding module identified in alpha-glucan, water dikinases is specific for regulated plastidial starch metabolism. Biochem 45:4674-4682

Miura M, Sugawara A (1996) Dosage effect of the three *Wx* genes on amylose synthesis in wheat endosperm. Theor Appl Genet 93:1066-1070

Mørrell S and ap Rèes T (1986) Control of the hexose content of potato tubers. Phytochemistry 25:1073-1076

Morrison WR (1964) A fast, simple and reliable method for the microdetermination of phosphorus in biological materials. Anal Biochem 7:218-224

Nashilevitz S, Bessudo CM, Aharoni A, Kossmann J, Wolf S, and Levy AA (2009) The legwd mutant uncovers the role of starch Phosphorylation in pollen development and germination in tomato. Plant J 57:1-13

Nielsen AV, Blennow A, Nielsen TH, Møller BL (1998) Phosphorylated α (1-4)Glucans as substrate for potato starch-branching enzyme1. Plant Physiol 117:869-875

Nielsen TH, Wischmann B, Enevoldsen K, Moller BL (1994) Starch phosphorylation in potato tubers proceeds concurrently with de novo biosynthesis of starch. Plant Physiol 105:111-117

Niittyla T, Comparot-Moss S, Lue W-L, Messerlie G, Trevisan M, et al. (2006) Similar protein phosphatases control starch metabolism in plants and glycogen metabolism in mammals. J Biol Chem 281:11815-11818

Niittyla T, Messerli G, Trevisan M, Chen J, Smith AM, Zeeman SC (2004) A novel maltose transporter essential for starch degradation in leaves. Science 303:87-89

Nikuni Z, Hizukuri S, Kamagai K, Hasegawa H, Moriwaki T, Doi K, Tutul T, Nara.S, Maeda I (1969) The effect of temperature during maturation period on the physico-chemical properties of potato and rice starches. Memoirs of the scientific and Industrial Research, Osaka University, 26:27

Otani M, Hamada T, Katayama K, Kitahara K, Kim SH, Takahata Y, Suganuma T, Shimada T (2007) Inhibition of the gene expression for granule-bound starch synthase I by RNA interference in sweet potato plants. Plant Cell Rep 26:1801-1807

Preiss J (1988) Biosynthesis of starch and its regulation. The Biochemistry of plants, vol. 14 carbohydrates, (eds). Stumph PK and Conn EE, Academic press, San Diego, CA

Quick WA, Li PL (1976) Phosphate balance in potato tubers. Potato Res 19:305-312

Ramanna MS, Hermsen JG (1979) Genome relationships in tuber-bearing solanums. Biology and Taxonomy of the solanaceae ed. (Hawkes JG, Lester RN, Skelding AD) Academic Press, London

Reimann R, Ritte G, Steup M, Appenroth KJ (2002) Association of α-amylase and the R1 protein with starch granules precedes the initiation of net starch degradation in turions of Spirodela polyrhiza. Physiol Planta 114:2-12

Ritte G, Eckermann N, Haebel S, Lorberth R, Steup M (2000) Compartmentation of the starch-related R1 protein in higher plants. Starch/Stärke 52:145-149

Ritte G, Heydenreich M, Mahlow S, Haebel S, Kötting O, Steup M (2006) Phosphorylation of C6 and C3-positions of Glucosyl residues in starch is catalysed by distinct dikinases. FEBS Lett 580:4872-4876

Ritte G, Lloyd JR, Ekermann N, Rottmann A, Kossmann J, Steup M (2002) The starch-related R1 protein is an α-glucan, water dikinase. Proc Natl Acad Sci USA 99:7166-7171

Ritte G, Lorberth R, Steup M (2000) Reversible binding of the starch-related R1 protein to the surface of transitory starch granules. Plant J 21:387-391

Ritte G, Scharf A, Eckermann N, Haebel S, Steup M (2004) Phosphorylation of transitory starch is increased during degradation. Plant Physiol 135:2068-2077

Ritte G, Steup M, Kossmann J, Lloyd JR (2003) Determination of the starch-phosphorylating enzyme activity in plant extracts. Planta 216:798-801

Rohde W, Becker D, Kull B, Salamini F (1990) Structural and functional analysis of two *waxy* gene promoters from potato. J Gen Breeding 44:311-315

Saitou N and Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A Laboratory Manual (2nd edn) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Samotus B, Niedzwiedz M, Kolodziej Z, Leja M, Czajkowska B (1974) Storage and reconditioning of tubers of polish potato varieties and strains. I. Influence of storage temperature on sugar level in potato tubers of different varieties and strains. Potato Res 17:64-81

Santelia D and Zeeman SC (2011) Progress in *Arabidopsis* starch research and potential biotechnological applications. Curr Opin Biotechnol 22:271-280

Smith AM (2008) Prospects for increasing starch and sucrose yields for bioethanol production. Plant J 54:546-558

Smith AM and Stitt M (2007) Coordination of carbon supply and plant growth. Plant Cell Environ 30:1126-1149

Smith AM, Denyer K, Martin C (1997) The synthesis of starch granule. Annu Rev Plant Physiol Plant Mol Biol 48:67-87

Smith AM, Zeeman SC, Smith SM (2005) Starch degradation. Annu Rev Plant Biol 56:73-97

Sokolov LN, Dominguez-Solis JR, Allary AL, Buchanan BB, Luan S (2006) A redox-regulated chloroplast protein phosphatase binds to starch diurnally and functions in its accumulation. Proc Natl Acad Sci USA 103:9732-9737

Somogyi M (1952) Estimation of sugars by colorimetric method. J Biol Chem 200:245

Sowokinos JR (1981) Pyrophosphorylases in *Solanum tuberosum* L. II Catalytic properties and regulation of ADP-Glucose and UDP-Glucose pyrophosphorylase activities in potatoes. Plant Physiol 68:924-929

Sowokinos JR, Thomas C, Burrell MM (1997) Pyrophosphorylases in potato. V. Allelic polymorphism of UDP-Glucose pyrophosphorylase in potato cultivars and its association with tuber resistance to sweetening in the cold. Plant Physiol 113:511-517

Tabata S, Nagata K, Hizukuri S (1975) Studies on starch phosphates. Part 3. On the esterified phosphates in some cereal starches. Staerke 27:333-335

Tagliabracci VS, Turnbull J, Wang W, Girard JM, Zhao X, Skurat AV, Delgado-Escueta AV, Minassian BA, Depaoli-Roach AA, Roach PJ (2007) Laforin is a glycogen phosphatase, deficiency of which leads to elevated phosphorylation of glycogen in vivo. Proc Natl Acad Sci USA 104:19262-19266

Takashima Y, Senoura T, Yoshizaki T, Haniada S, Ito H, Matsui H (2007) Differential chain-length specificities of two isoamylase-type starch-debranching enzymes from developing seeds of kidney bean. Biosci Biotechnol Biochem 71:2308-2312

Takeda Y, Hizukuri S (1971) Studies on starch phosphate: Part 2. Isolation of glucose 3-phosphate and maltose phosphate by acid hydrolysis of potato starch. Starch/Stärke 23:267-272

Takeda Y, Hizukuri S (1981) Re-examination of the action of sweetpotato beta-amylase on phosphorylated (1–4)-alpha-d-glucan. Carbohydr Res 89:174-178

Takeda Y, Hizukuri S (1982) Location of phosphate groups in potato amylopectin. Carbohydr Res 102:321-327

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci USA 101:11030-11035

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol 28:2731-2739

Tetlow IJ, Mørell MK, Emes MJ (2004) Recent developments in under-standing the regulation of starch metabolism in higher plants. J Exp Bot 55:2131-2145

Tyson RH, ap Rees T (1988) Starch synthesis by isolated amyloplasts from wheat endosperm. Planta 175:33-38

van de Wal MHBJ, Jacobsen E, Visser RGF (2001) Multiple allelism as a control mechanism in metabolic pathways: GBSSI allelic composition affects the activity of granule-bound starch synthase I and starch composition in potato. Mol Genet Genom 265:1011-1021

van der Leij FR, Visser RGF, Ponstein AS, Jacobsen E, Feenstra WJ (1991) Sequence of the structural gene for granule-bound starch synthase of potato *(Solanum tuberosum* L.) and evidence for a single point deletion in the *amf* allele. Mol Gen Genet 228:240-248

van der Steege G, Nieboer M, Swaving J, Tempelaar MJ (1992) Potato granule-bound starch synthase promoter-controlled GUS expression: regulation of expression after transient and stable transformation. Plant Mol Biol 20:19-30

Viksø-Nielsen A, Blennow A, Jørgensen K, Kristensen KH, Jensen A, Møller BL (2001) Structural, physicochemical, and pasting properties of starches from potato plants with repressed r1-gene. Biomacro 2:836-843

Visser RGF, Somhorst I, Kuipers GJ, Ruys NJ, Feenstra WJ, Jacobsen E (1991a) Inhibition of expression of the gene for granule-bound starch synthase in potato by anti-sense constructs. Mol Gen Genet 225:289-296

Visser RGF, Stolte A, Jacobsen E (1991b) Expression of a chimaeric granule-bound starch synthase-GUS gene in transgenic potato plants. Plant Mol Biol 17:691-699

Vliet WF, van Schriemer WH (1960) The sugar accumulation of potatoes kept at low temperature, as studied in a small selection of Dutch varieties. Eur Potato J 3:263-271

Vriet C, Welham T, Brachmann A, Pike M, Pike J, Perry J, Parniske M, Sato S, Tabata S, Smith AM, Wang TL (2010) A suite of *Lotus japonicus* starch mutants reveals both conserved and novel features of starch metabolism. Plant Physiol 154: 643-655

Vrinten PL, Nakamura T (2000) Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. Plant Physiol 122:255-263

Wang T, Bogracheva TY, Hedley CL (1998) Starch: As simple as A, B, C? J Exp Bot 49:481-502

Weber A, Servaites JC, Geiger DR, Kofler H, Hille D et al. (2000) Identification, purification, and molecular cloning of a putative plastidic glucose transporter. Plant Cell 12:787-801

Weise SE, Weber APM, Sharkey TD (2004) Maltose is the major form of carbon exported from the chloroplast at night. Planta 218:474-482

Wischmann B, Nielson TH, Møller BL (1999) In vitro biosynthesis of phosphorylated starch in intact potato amyloplasts. Plant Physiol 119:455-462

Yu TS, Kofler H, Häusler RE, Hille D, Flügge U, Zeeman SC, Smith AM, Kossmann J, Lloyd J, Ritte G, Steup M, Lue W, Chen J, Weber A (2001) The Arabidopsis sex1 mutant is defective in the R1 Protein, a General Regulator of Starch Degradation in plants, and not in the chloroplast hexose transporter. Plant Cell 13:1907-1918

Yu TS, Zeeman SC, Thorneycroft D, Fulton DC, Dunstan H, Lue WL, Hegemann B, Tung SY, Umemoto T, Chapple A, Tsai DL, Wang SM, Smith AM, Chen J, Smith SM (2005) α-Amylase is not required for breakdown of transitory starch in Arabidopsis leaves. J Biol Chem 280:9773-9779

Yun SH, Matheson NK (1992) Structural changes during development in the amylose and amylopectin fractions (separated by precipitation with concanavalin A) of starches from maize genotypes Carbohydr Res 270:85-101

Zeeman SC, Delatte T, Messerli G, Umhang M, Stettler M, Mettler T, Streb S, Reinheld H, Kötting O (2007) Starch breakdown: recent discoveries suggest distinct pathways and novel mechanisms. Funct Plant Biol 34:465-473

Zeeman SC, Kossmann J, Smith AM (2010) Starch: Its Metabolism, Evolution, and Biotechnological Modification in Plants. Annu Rev Plant Biol 61:209-234

Zeeman SC, Northrop F, Smith AM, ap Rees T (1998) A starch-accumulating mutant of *Arabidopsis thaliana* deficient in a starch-hydrolysing enzyme. Plant J 15:357-365

Zeeman SC, Smith SM, Smith AM (2002) The priming of amylose synthesis in *Arabidopsis* leaves. Plant Physiol 128:1069-1076

Zeeman SC, Smith SM, Smith AM (2004) The breakdown of starch in leaves. New Phytol. 163:247-261

Zeeman SC, Smith SM, Smith AM (2007) The diurnal metabolism of leaf starch. Biochem J 401:13-28

Zeeman SC, Thorneycroft D, Schupp N, Chapple A, Weck M et al (2004) The role of plastidial α-glucan phosphorylase in starch degradation and tolerance of abiotic stress in *Arabidopsis* leaves. Plant Physiol.135:849-858

Zeeman SC, Tiessen A, Pilling E, Kato KL, Donald AM, Smith AM (2002) Starch synthesis in *Arabidopsis*: granule synthesis, composition and structure. Plant Physiol 129:516-529

Zeeman SC, Umemoto T, Lue WL, Au-Yeung P, Martin C, Smith AM, Chen J (1998) A mutant of *Arabidopsis* lacking a chloroplastic isoamylase accumulates both starch and phytoglycogen. Plant Cell 10:1699-1711

Zrenner R, Schular K, Sonnewald U (1996) Soluble acid invertase determines the hexose-to-sucrose ratio in cold-stored potato tubers. Planta 198:246-252

Appendix related to the Results

Results and Discussion: Appendix-1

Table 1- Reducing sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RA genetic construct (R1 cDNA fragment towards 5' terminus in antisense orientation under the CaMV 35S promoter) based transgenic lines

Reducing sugar content in mg g^{-1} FW							
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	0.09 ±0.06f	3.80 ±0.31bcd	2.53 ±0.32cd	0.24 ±0.06b	4.91 ±2.85cde	1.50 ±0.41e	0.13 ±0.06c
RA-1	0.50 ±0.14d	4.57 ±0.37b	2.11 ±0.61d	0.01 ±0.01c	1.46 ±0.45e	3.09 ±0.56de	0.04 ±0.12c
RA-2	0.44 ±0.05de	2.56 ±0.25def	2.18 ±0.31d	0.09 ±0.08c	16.68 ±3.13a	4.49 ±0.12cd	0.55 ±0.04b
RA-5	0.35 ±0.15de	1.36 ±0.33f	3.60 ±0.67cd	0.19 ±0.03b	8.93 ±1.50bc	5.90 ±0.47bc	0.06 ±0.08c
RA-6	0.87 ±0.09c	2.17 ±1.07def	6.78 ±0.41a	0.07 ±0.02c	8.01±0.20cd	6.43 ±0.60b	0.27 ±0.06c
RA-8	1.12 ±0.01bc	4.06 ±0.34bc	3.41 ±0.36cd	0.22 ±0.08b	5.27 ±1.06cde	7.66 ±1.22b	0.02 ±0.09c
RA-11	0.54 ±0.04d	3.25 ±0.21bcd	3.99 ±0.70bcd	0.01 ±0.04c	6.00 ±0.66cde	2.58 ±0.11e	0.25 ±0.05bc
RA-12	1.05 ±0.07bc	2.85 ±0.58cde	3.68 ±0.86cd	0.18 ±0.04b	4.77 ±0.70cde	5.88 ±0.95bc	0.04 ±0.15c
RA-13	0.27 ±0.29e	1.60 ±0.17ef	2.93 ±0.14cd	0.16 ±0.05b	3.86 ±0.28de	2.33 ±0.14e	0.01 ±0.06c
RA-14	1.14 ±0.05b	1.96 ±0.11def	4.39 ±0.39bc	0.15 ±0.04b	2.73 ±0.31e	2.61 ±1.06e	0.19 ±0.18bc
RA-15	1.80 ±0.18a	6.18 ±1.08a	6.13 ±2.53ab	1.34 ±0.29a	12.83 ±5.07ab	10.58 ±1.17a	1.55 ±0.36a

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

Table 2- Total soluble sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RA genetic construct (R1 cDNA fragment towards 5' terminus in antisense orientation under the CaMV 35S promoter) based transgenic lines

Total soluble sugar content in mg g^{-1} FW							
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	2.47 ±0.67i	12.05 ±2.08a	9.92 ±1.57ab	3.48 ±1.11abc	24.37 ±0.97a	11.83 ±1.43a	4.87 ±0.90bcde
RA-1	6.73 ±0.30fg	9.74 ±0.63ab	3.02 ±0.50c	1.35 ±0.36cd	3.56 ±0.45g	3.73 ±0.10cd	5.04 ±0.65bcd
RA-2	5.22 ± 0.45gh	7.73 ±0.76bcd	3.13 ±0.86c	1.43 ±0.63bcd	14.72 ±3.36b	3.86 ±0.55cd	4.44 ±0.46cde
RA-5	4.26 ±0.76hi	5.27 ±1.36bcd	6.07 ±1.37abc	2.66 ±0.48bcd	9.30 ±1.76cde	5.24 ±1.13cd	4.71 ±0.33bcde
RA-6	9.82 ±0.77bcd	3.82 ±1.59d	12.28 ±1.28a	1.37 ±0.92bcd	9.92 ±0.25cd	5.75 ±0.80bc	6.34 ±0.79b
RA-8	10.48 ±0.81bc	6.28 ±1.90abcd	5.65 ±1.77abc	2.48 ±0.14bcd	6.39 ±1.26defg	7.40 ±1.67b	3.41 ±0.35de
RA-11	7.48 ±0.30ef	6.11 ±0.39bcd	6.75 ±0.89abc	1.10 ±0.21d	8.70 ±0.83def	3.96 ±0.30cd	5.07 ±0.53bcde
RA-12	9.02 ±0.02cde	4.69 ±0.52cd	5.82 ±1.11abc	2.06 ±0.40bcd	5.22 ±1.33efg	5.72 ±0.75bc	3.25 ±0.68de
RA-13	8.37 ±0.02def	3.63 ±0.34d	5.03 ±0.53bc	3.37 ±0.37abcd	5.09 ±0.91efg	3.54 ±0.57d	2.64 ±0.95e
RA-14	10.95 ±0.29b	4.18 ±0.97cd	9.48 ±1.17abc	3.48 ± 0.52ab	4.68 ±1.40fg	4.10 ±1.22cd	5.92 ±0.56bc
RA-15	14.50 ±0.22a	9.41 ±1.51abc	11.78 ±2.98a	5.19 ±0.76a	13.13 ±3.73bc	10.53 ±0.22a	19.54 ±2.04a

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

Table 3- Reducing sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RB genetic construct (R1 cDNA fragment towards 5' terminus in sense orientation under the CaMV 35S promoter) based transgenic lines

	Reducing sugar content in mg g^{-1} FW						
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	0.09 ±0.06b	3.80 ±0.31a	2.53 ±0.32a	0.24 ±0.06a	4.91 ±2.85ab	1.50 ±0.41a	0.13 ±0.06ab
RB-1	0.06 ±0.08b	0.16 ±0.16c	0.14 ±0.03cde	0.10 ±0.09ab	0.20 ±0.17f	0.11 ±0.07c	0.14 ±0.02ab
RB-2	0.43 ±0.14a	0.81 ±0.17b	0.03 ±0.04e	0.15 ±0.07ab	4.92 ±1.65ab	0.17 ±0.06c	0.16 ±0.06ab
RB-3	0.01±0.04b	0.01 ±0.06d	0.06 ±0.01de	0.05 ±0.06b	1.44 ±0.43def	0.21 ±0.06c	0.14 ±0.03ab
RB-4	0.05 ±0.07b	0.53 ±0.11c	0.39 ±0.08b	0.02 ±0.13b	3.10 ±0.18bcd	0.19 ±0.09c	0.18 ±0.06a
RB-5	0.06 ±0.10b	1.01 ±0.17b	0.10 ±0.03de	0.08 ±0.06ab	4.94 ±1.38ab	0.24 ± .12c	0.14 ±0.05ab
RB-8	0.02 ±0.05b	0.08 ±0.05d	0.12 ±0.05de	0.03 ±0.05b	0.67 ±0.32ef	0.14 ±0.06c	0.17±0.03a
RB-9	0.03 ±0.07b	0.09 ±0.10d	0.05 ±0.04e	0.06 ±0.10ab	4.80 ±1.55abc	0.20 ±0.07c	0.20 ±0.03a
RB-10	0.04 ±0.05b	0.03 ±0.13d	0.05 ±0.04e	0.21 ±0.11ab	2.46 ±1.01cdef	0.46 ±0.17b	0.19 ±0.05a
RB-12	0.03 ±0.09b	0.05 ±0.02d	0.16 ±0.04bc	0.11 ±0.11ab	2.83 ±0.93bcde	0.07 ±0.14c	0.18 ±0.08a
RB-13	0.09 ±0.11b	0.27 ±0.13cd	0.24 ±0.09c	0.14 ±0.09ab	1.58 ±0.27def	0.13 ±0.16c	0.01 ±0.02c
RB-14	0.06 ±0.08b	0.18 ±0.14d	0.10 ±0.01de	0.10 ±0.08ab	6.91 ±1.15a	0.05 ±0.10c	0.10 ±0.05c
RB-15	0.06 ±0.04b	0.06 ±0.16d	0.04 ±0.04e	0.08 ±0.08ab	5.57 ±1.24a	0.15 ±0.08c	0.01 ±0.04c
RB-16	0.03 ±0.08b	0.15 ±0.08d	0.05 ±0.05de	0.13 ±0.07ab	2.92 ±0.89bcde	0.19 ±0.12c	0.14 ±0.02ab

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

Table 4- Total soluble sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RB genetic construct (R1 cDNA fragment towards 5' terminus in sense orientation under the CaMV 35S promoter) based transgenic lines

Total soluble sugar content in mg g^{-1} FW							
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	2.47 ±0.67abc	12.05 ±2.08a	9.92 ±1.57a	3.48 ±1.11bcde	24.37 ±0.97a	11.83 ±1.43a	4.87 ±0.90bc
RB-1	2.15 ±0.59bc	5.24 ±0.55b	1.85 ±0.46de	4.60 ±1.08b	5.57 ±0.65cd	3.50 ±0.90c	4.19 ±0.94c
RB-2	2.74 ±0.50abc	2.86 ±0.23def	1.46 ±0.35de	3.47 ±0.46bcde	6.59 ±0.75cd	3.14 ±0.79c	4.14 ±0.46c
RB-3	2.86 ±0.38abc	3.36 ±0.37cdef	0.89 ±0.17e	4.55 ±0.70bc	7.23 ±1.87cd	4.20 ±0.55c	4.72 ±0.89bc
RB-4	2.52 ±0.40abc	4.14 ±0.83bcde	4.13 ±0.51cde	2.17 ±0.29e	6.85 ±1.12cd	4.87 ±0.65bc	6.83 ±0.77ab
RB-5	2.22 ±0.39bc	2.52 ±0.17ef	4.55 ±0.32cd	2.59 ±0.24cde	8.05 ±1.25c	3.53 ±1.28c	4.16 ±1.17c
RB-8	3.44 ±0.42a	2.93 ±1.62def	1.79 ±0.29de	4.15 ±0.96bcd	6.23 ±0.48cd	3.55 ±0.59c	5.60 ±0.49bc
RB-9	2.49 ±0.71abc	2.15 ±0.25f	2.63 ±0.14cde	4.49 ±0.14bcd	7.76 ±1.73c	3.79 ±0.28c	5.46 ±1.05bc
RB-10	2.85 ±0.46abc	4.30 ±1.07bcd	3.47 ±0.63cde	2.93 ±0.84bcd	6.73 ±1.17cd	8.71 ±4.51ab	5.68 ±0.66bc
RB-12	2.57 ±0.13abc	5.26 ±0.59b	5.79 ±1.55bc	3.26 ±0.72bcd	11.86 ±0.78b	3.61 ±0.44c	8.41 ±2.22a
RB-13	3.10 ±0.69ab	4.97 ±0.70bc	8.28 ±1.50ab	4.75 ±0.59b	5.12 ±0.56d	5.67 ±3.35bc	4.06 ±0.29c
RB-14	1.79 ±0.13c	3.31 ±0.29cdef	8.87 ±2.37ab	7.81 ±1.78a	11.88 ±1.11b	3.15 ±1.37c	6.23 ±0.64abc
RB-15	1.90 ±0.13c	4.32 ±0.36bcd	8.04 ±2.43ab	2.53 ±0.34de	6.80 ±0.47cd	3.26 ±0.50c	4.00 ±0.68c
RB-16	1.96 ±0.23c	4.36 ±0.70bcd	8.03 ±3.02ab	3.37 ±0.63bcde	5.04 ±0.69d	2.71 ±0.72c	5.86 ±0.10bc

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

Table 5- Reducing sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RC genetic construct (R1 cDNA fragment towards 5' terminus in antisense orientation under the GBSSI promoter) based transgenic lines

	Reducing sugar content in mg g^{-1} FW						
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	0.09 ±0.06c	3.80 ±0.31d	2.53 ±0.32b	0.24 ±0.06abc	4.91 ±2.85bc	1.50 ±0.41bc	0.13 ±0.06cd
RC-1	0.56 ±0.35bc	0.81 ±0.14g	0.10 ±0.07e	0.10 ±0.08bc	0.40 ±0.12f	0.05 ±0.15f	0.03 ±0.06d
RC-2	0.11 ±0.14c	1.64 ±0.17ef	0.20 ±0.10e	0.39 ±0.14a	1.19 ±0.13ef	1.10 ±0.13cd	0.02 ±0.12cd
RC-3	0.72 ±0.45bc	2.20 ±0.24e	0.59 ±0.27de	0.08 ±0.03bc	6.40 ±0.48ab	0.33 ±0.11ef	0.15 ±0.05cd
RC-4	0.46 ±0.09bc	4.49 ±0.37bc	3.19 ±1.47ab	0.09 ±0.04bc	2.82 ±0.45de	0.68 ±0.10de	0.19 ±0.07cd
RC-5	1.09 ±0.32ab	3.94 ±0.11d	0.70 ±0.26cde	0.22 ±0.07abc	6.78 ±0.62a	0.04 ±0.09f	1.34 ±0.12a
RC-6	1.74 ±0.96a	6.47 ±0.33a	4.12 ±1.31a	0.13 ±0.06bc	7.82 ±0.09a	2.57 ±0.46a	0.24 ±0.14c
RC-7	0.27 ±0.08bc	1.52 ±0.33f	2.53 ±0.26b	0.15 ±0.04b	0.98 ±0.34f	0.14 ±0.13f	0.49 ±0.02b
RC-8	0.20 ±0.04bc	3.46 ±0.39d	2.16 ±0.16bc	0.05 ±0.11bc	6.41 ±1.72ab	1.78 ±0.43b	0.16 ±0.05cd
RC-11	0.05 ±0.09c	6.58 ±0.36a	2.36 ±0.44b	0.25 ±0.08ab	4.01 ±0.92cd	0.01 ±0.07f	0.17 ±0.02cd
RC-12	0.02 ±0.08c	4.62 ±0.25b	1.91 ±0.21bcd	0.15 ±0.09bc	4.12 ±0.26cd	0.85 ±0.28de	0.06 ±0.04d

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

Table 6- Total soluble sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RC genetic construct (R1 cDNA fragment towards 5' terminus in antisense orientation under the GBSSI promoter) based transgenic lines

	Total soluble sugar content in mg g^{-1} FW						
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	2.47 ±0.67d	12.05 ±2.08a	9.92 ±1.57a	3.48 ±1.11bcd	24.37 ±0.97a	11.83 ±1.43a	4.87 ±0.90d
RC-1	9.32 ±3.21ab	2.32 ±0.48g	4.29 ±0.61b	3.65 ±0.37bcd	1.81 ±0.35e	3.19 ±0.40cdef	3.41 ±0.26e
RC-2	5.53 ±0.98bcd	3.67 ±0.49efg	3.18 ±0.55b	7.78 ±1.02a	2.56 ±0.43e	4.12 ±0.39bcd	3.07 ±0.44e
RC-3	8.84 ±2.28bc	4.72 ±0.66efg	3.27 ±0.77b	4.43 ±0.82bc	7.45 ±1.21bcd	2.82 ±0.40ef	8.85 ±0.74a
RC-4	6.41 ±1.31bcd	5.11 ±0.89def	5.03 ±0.56b	2.47 ±0.53de	5.09 ±0.71d	2.50 ±0.66f	6.20 ±0.73bc
RC-5	12.90 ±1.32a	5.45 ±0.93cdef	3.37 ±0.32b	3.59 ±0.71bcd	7.11 ±0.74cd	3.74 ±0.69bcde	5.88 ±0.56bcd
RC-6	9.34 ±2.42ab	9.90 ±2.62ab	4.88 ±0.85b	3.37 ±0.90bcd	9.64 ±0.72b	4.58 ±0.49b	5.21 ±0.55cd
RC-7	5.15 ±0.98cd	3.27 ±0.36de	3.90 ±0.56b	2.04 ±1.46de	2.08 ±0.22e	2.92 ±0.27def	6.63 ±0.28b
RC-8	6.32 ±0.39bcd	5.79 ±0.95cde	5.28 ±0.10b	1.54 ±0.17e	8.45 ±2.55bc	4.31 ±0.85bc	5.03 ±0.60cd
RC-11	4.99 ±1.71cd	7.87 ±0.56bc	3.54 ±0.57b	3.09 ±0.78cde	5.11 ±0.66d	4.05 ±0.41bcd	9.67 ±0.62a
RC-12	4.33 ±0.52d	7.26 ±0.57cd	3.08 ±0.48b	4.94 ±0.46b	5.99 ±0.87 d	3.89 ±0.43bcde	5.93 ±0.27bcd

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

Table 7- Reducing sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RD genetic construct (R1 cDNA fragment towards 5' terminus in sense orientation under the GBSSI promoter) based transgenic lines

Reducing sugar content in mg g^{-1} FW							
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	0.09 ±0.06ab	3.80 ±0.31a	2.53 ±0.32a	0.24 ±0.06a	4.91 ±2.85ab	1.50 ±0.41a	0.13 ±0.06de
RD-1	0.03 ±0.05b	0.85 ±0.20de	0.13 ±0.05efg	0.04 ±0.07b	2.56 ±0.70cd	0.43 ±0.13d	0.37 ±0.07a
RD-2	0.09 ±0.13ab	1. 16 ±0.66d	0.74 ±0.05b	0.07 ±0.08b	3.77 ±0.23bc	0.88 ±0.14b	0.18 ±0.05cde
RD-3	0.14 ±0.07ab	1.43 ±0.47cd	0.23 ±0.02de	0.03 ±0.05b	6.04 ±1.05a	0.01 ±0.09e	0.19 ±0.06bcde
RD-4	0.05 ±0.05b	0.36 ±0.13e	0.20 ±0.07e	0.10 ±0.06b	3.93 ±0.43bc	0.11 ±0.13e	0.31 ±0.06ab
RD-5	0.14 ±0.09ab	0.88 ±0.12de	0.06 ±0.06fg	0.04 ±0.06b	3.47 ±0.19bc	0.01 ±0.22e	0.19 ±0.07bcd
RD-6	0.54 ±0.63a	1.46 ±0.09cd	0.07 ±0.08fg	0.07 ±0.09b	1.89 ±0.29d	0.05 ±0.18e	0.17 ±0.08de
RD-7	0.01 ±0.11ab	2.39 ±0.34b	0.19 ±0.04ef	0.02 ±0.10b	4.64 ±0.84ab	0.04 ±0.12e	0.13 ±0.05de
RD-8	0.06 ±0.11ab	2.21 ±0.39bc	0.38 ±0.04c	0.05 ±0.08b	4.07 ±0.77b	0.58 ±0.15cd	0.14 ±0.04de
RD-9	0.08 ±0.12ab	1.51 ±0.40cd	0.06 ±0.05fg	0.04 ±0.05b	1.44 ±0.19d	0.01 ±0.09e	0.01 ±0.08e
RD-10	0.03 ±0.19ab	2.08 ±0.05bc	0.10 ±0.10efg	0.06 ±0.02b	1.46 ±0.47d	0.66 ±0.13bcd	0.13 ±0.04de
RD-15	0.04 ±0.15ab	1.21 ±0.30d	0.35 ±0.09cd	0.03 ±0.04b	2.58 ±0.21cd	0.75 ±0.12bc	0.27 ±0.06abc

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

Table 8- Total soluble sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RD genetic construct (R1 cDNA fragment towards 5' terminus in sense orientation under the GBSSI promoter) based transgenic lines

Total soluble sugar content in mg g^{-1} FW							
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	2.47 ±0.67ab	12.05 ±2.08a	9.92 ±1.57a	3.48 ±1.11abc	24.37 ±0.97a	11.83 ±1.43a	4.87 ±0.90de
RD-1	1.61 ±0.55b	3.28 ±0.27e	4.55 ±0.44bc	3.85 ±1.79ab	8.11 ±1.96cde	13.64 ±1.91a	15.49 ±1.57a
RD-2	1.60 ±0.15b	4.03 ±1.27de	3.64 ±0.19cd	1.88 ±0.22cd	9.81 ±1.25c	4.54 ±0.86de	5.07 ±0.52de
RD-3	1.72 ±0.24b	7.02 ±1.11bc	5.59 ±0.59b	3.00 ±0.55bc	15.94 ±1.69b	3.88 ±0.85e	5.96 ±1.02cde
RD-4	2.37 ±0.89ab	5.05 ±2.30cde	5.59 ±0.64b	5.07 ±0.19a	14.64 ±1.51b	11.19 ±1.62ab	12.30 ±1.74b
RD-5	1.76 ±0.50b	3.91 ±0.38de	2.98 ±0.44d	2.88 ±0.61bc	5.83 ±0.57e	5.10 ±0.36de	6.49 ±0.52cd
RD-6	3.71 ±1.30a	7.67 ±0.56b	2.87 ±0.47d	5.27 ±1.45a	-	-	-
RD-7	1.60 ±0.35b	6.20 ±1.09bcd	3.44 ±0.29cd	3.14 ±0.55bc	10.06 ±1.53c	6.68 ±0.50cd	4.15 ±0.41e
RD-8	1.21 ±0.19b	4.01 ±0.81de	2.92 ±0.40d	2.04 ±0.25cd	14.70 ±1.60b	5.53 ±0.20de	4.70 ±0.88de
RD-9	1.12 ±0.48b	3.11 ±1.02e	2.73 ±0.47d	1.90 ±0.37cd	5.65±1.11e	6.14 ±2.02de	4.18 ±0.30e
RD-10	1.61 ±0.13b	5.33 ±0.11bcde	5.82 ±1.55b	4.09 ±0.27ab	6.45 ±1.10de	9.09 ±0.45bc	6.37 ±1.10cde
RD-15	1.14 ±0.33b	2.98 ±1.01e	3.51 ±0.38cd	0.39 ±0.35d	9.31 ±1.38cd	6.52 ±0.48cde	7.69 ±0.54c

Values sharing a common letter within the column are not significant at $P \leq 0.05$. " –" sample not available.

Table 9- Reducing sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RE genetic construct (R1 cDNA fragment towards 3' terminus in antisense orientation under the CaMV 35S promoter) based transgenic lines

	Reducing sugar content in mg g^{-1} FW						
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	0.09 ±0.06g	3.80 ±0.31c	2.53 ±0.32b	0.24 ±0.06b	4.91 ±2.85de	1.50 ±0.41a	0.13 ±0.06bc
RE-1	0.09 ±0.08g	4.05 ±0.73bc	0.17 ±0.07c	0.08 ±0.05c	7.39 ±0.55bcd	0.60 ±0.09b	0.01 ±0.05c
RE-2	0.62 ±0.20de	2.86 ±0.14d	0.22 ±0.05c	0.02 ±0.02c	3.11 ±0.40e	0.37 ±0.11c	0.11 ±0.07bc
RE-3	0.43 ±0.02f	5.76 ±0.09a	1.17 ± 0.32bc	0.51 ±0.07a	6.62 ±0.82cde	0.40 ±0.06c	0.25 ±0.06abc
RE-4	1.26 ±0.06a	4.67 ±0.33b	0.65 ±0.13c	0.51 ±0.06a	12.10 ±4.11a	0.68 ±0.06b	0.41 ±0.11ab
RE-5	1.03 ±0.01b	2.07 ±0.23d	0.52 ±0.15c	0.01 ±0.07c	3.66 ±0.15de	0.02 ±0.05d	0.17 ±0.18bc
RE-6	0.35 ±0.01de	3.99 ±0.36bc	0.92 ±0.09bc	0.12 ±0.06c	10.01 ±0.99abc	0.59 ±0.07b	0.21 ±0.13abc
RE-7	1.37 ±0.01a	3.69 ±0.37c	1.36 ±0.11bc	0.43 ±0.08a	10.71 ±2.64ab	0.42 ±0.05c	0.51 ±0.28a
RE-8	0.77 ±0.05c	4.00 ±0.28bc	10.22 ±2.56a	0.06 ±0.10c	5.41 ±0.27de	-	-
RE-15	0.72 ±0.01cd	1.80 ±0.86e	0.22 ±0.09c	0.01 ±0.01c	2.86 ±0.45e	-	-
RE-16	0.49 ±0.01ef	2.20 ±0.41d	1.07 ±0.10bc	0.12 ±0.01bc	4.68 ±1.16de	-	0.24 ±0.15abc

Values sharing a common letter within the column are not significant at $P \leq 0.05$. " –" sample not available.

Table 10- Total soluble sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RE genetic construct (R1 cDNA fragment towards 3' terminus in antisense orientation under the CaMV 35S promoter) based transgenic lines

Total soluble sugar content in mg g^{-1} FW							
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	2.47 ±0.67h	12.05 ±2.08a	9.92 ±1.57b	3.48 ±1.11b	24.37 ±0.97a	11.83 ±1.43a	4.87 ±0.90c
RE-1	6.63 ±0.14g	7.66 ±2.27c	4.50 ±0.94cd	2.40 ±0.51bc	9.35 ±0.59c	4.44 ±0.55cd	3.35 ±0.38cd
RE-2	9.46 ±0.19f	6.70 ±0.25cd	3.68 ±0.44d	3.02 ±0.10bc	5.16 ±0.68d	3.55 ±0.47de	2.79 ±0.83cd
RE-3	11.60 ±1.76de	9.77 ±0.51b	5.14 ±0.82cd	1.90 ±0.35c	8.74 ±0.60cd	4.37 ±1.15cd	2.09 ±0.37de
RE-4	13.88 ±0.29c	6.54 ±0.85cd	4.92 ±0.85cd	5.88 ±1.21a	13.81 ±3.56b	6.48 ±1.15b	8.11 ±1.60a
RE-5	15.96 ±0.14b	4.97 ±0.31d	6.25 ±1.27c	2.81 ±0.38bc	6.62 ±1.36cd	2.38 ±0.29e	7.90 ±2.14ab
RE-6	13.79 ±0.59c	6.35 ±0.55cd	5.25 ±0.62cd	2.03 ±0.07c	14.57 ±1.59b	6.29 ±0.06b	5.21 ±0.84bc
RE-7	13.45 ±0.94c	7.83 ±0.95bc	4.65 ±0.44cd	3.49 ±0.68b	14.93 ±1.95b	5.39 ±0.24bc	3.71 ±1.38cd
RE-8	24.58 ±1.32a	9.76 ±0.43b	12.45 ±1.48a	4.75 ±0.43a	8.84 ±1.10cd	-	-
RE-15	12.56 ±0.75cd	4.66 ±0.37d	3 .08 ±0.49d	2.24 ±0.06c	8.28 ±2.09cd	-	-
RE-16	10.58 ±1.19ef	6.04 ±0.50cd	12.55 ±1.39a	2.91 ±0.24bc	8.97 ±1.54cd	-	9.35 ±1.86a

Values sharing a common letter within the column are not significant at $P \leq 0.05$. " –" sample not available.

Table 11- Reducing sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RF genetic construct (R1 cDNA fragment towards 3' terminus in sense orientation under the CaMV 35S promoter) based transgenic lines

Reducing sugar content in mg g^{-1} FW							
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	0.09 ±0.06c	3.80 ±0.31a	2.53 ±0.32b	0.24 ±0.06b	4.91 ±2.85a	1.50 ±0.41a	0.13 ±0.06bc
RF-1	0.28 ±0.27c	1.24 ±0.19d	1.08 ±0.42cd	0.38 ±0.47b	1.88 ±0.11c	1.05 ±0.09b	0.27 ±0.04ab
RF-2	0.49 ±0.10bc	0.52 ±0.11e	0.56 ±0.18d	0.87 ±0.87b	2.47 ±0.33bc	0.90± 0.10b	0.33 ±0.11a
RF-3	1.24 ± 0.10ab	0.19 ±0.03ef	5.66 ±1.54a	0.04 ±0.06b	3.12 ±0.34b	0.59 ±0.11c	0.05± 0.13c
RF-4	1.35 ±0.88a	1.98 ±0.32c	1.94 ±0.21bc	2.99 ±0.57a	2.35 ±0.57c	1.03 ±0.13b	0.09 ±0.11c
RF-6	0.85 ±0.14abc	2.46 ±0.26b	0.77 ±0.10cd	0.59 ±0.60b	-	-	-
RF-8	0.86 ±0.29abc	0.37 ±0.11ef	0.12 ±0.07d	0.21 ±0.02b	-	-	-
RF-9	0.60 ±0.06abc	-	-	-	-	-	-

Values sharing a common letter within the column are not significant at $P \leq 0.05$. " –" sample not available.

Table 12- Total soluble sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RF genetic construct (R1 cDNA fragment towards 3' terminus in sense orientation under the CaMV 35S promoter) based transgenic lines

Total soluble sugar content in mg g^{-1} FW							
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	2.47 ±0.67d	12.05 ±2.08a	9.92 ±1.57a	3.48 ±1.11abc	24.37 ±0.97a	11.83 ±1.43a	4.87 ±0.90c
RF-1	6.47 ±2.38cd	3.14 ±0.83c	3.23 ±0.88c	3.67 ±0.42abc	2.85 ±0.87c	3.65 ±0.29b	3.97 ±0.40c
RF-2	9.93 ±0.65abc	3.11 ±0.82c	3.87 ±1.11c	4.71 ±0.25ab	3.55 ±0.85bc	3.78 ±0.21b	5.48 ±1.04c
RF-3	11.71 ±1.31ab	3.89 ±0.87c	10.75 ±0.29a	2.44 ±0.47bc	5.38 ±1.28b	3.78 ±0.67b	11.72 ±1.30a
RF-4	14.02 ±2.06a	6.83 ±0.50b	4 .08 ±0.63bc	7.55 ±4.78a	3.55 ±1.35bc	3.48 ±0.51b	8.01± 0.73b
RF-6	7.99 ±2.38bc	5.72 ±0.59b	3.01 ±1.13c	4.80 ±0.31ab	-	-	-
RF-8	11.14 ±3.21abc	5.47 ±0.92b	5.69 ±0.57b	6.29 ±0.92ab	-	-	-
RF-9	10.71 ±3.04abc	-	-	-	-	-	-

Values sharing a common letter within the column are not significant at $P \leq 0.05$. " –" sample not available.

Table 13- Reducing sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RG genetic construct (R1 cDNA fragment towards 3' terminus in antisense orientation under the GBSS1 promoter) based transgenic lines

	Reducing sugar content in mg g^{-1} FW						
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	0.09 ±0.06h	3.80 ±0.31a	2.53 ±0.32a	0.24 ±0.06ef	4.91 ±2.85a	1.50 ±0.41b	0.13 ±0.06bcd
RG-1	0.56 ±0.13cd	0.91 ±0.06de	0.39 ±0.10b	0.69 ±0.09bcd	0.76 ±0.12de	5.58 ±1.48a	0.31 ±0.07abc
RG-2	0.72 ±0.06bc	3.50 ±0.28a	2.10 ±0.40ab	1.90 ±0.15a	4.15 ±1.08a	1.47 ±0.16b	0.35 ±0.13ab
RG-3	0.30 ±0.16ef	0.39 ±0.01e	0.33 ±0.03b	0.69 ±0.12bc	1.60 ±0.25cd	1.42 ±0.14b	0.25 ±0.08bc
RG-4	0.38 ±0.02de	1.76 ±0.35cd	0.33 ±0.11b	0.02 ±0.07def	0.10 ±0.09e	0.20 ±0.07c	0.14 ±0.08bcd
RG-5	0.10 ±0.04gh	1.89 ±0.40c	3.55 ±0.41ab	0.04 ±0.12f	2.22 ±0.34bc	0.22 ±0.04c	0.51 ±0.20a
RG-6	0.77 ±0.07b	1.00 ±0.39de	0.57 ±0.06b	0.39 ±0.19cdef	0.80 ±0.40de	0.52 ±0.16bc	0.34 ±0.07abc
RG-7	0.55 ±0.15cd	2.32 ±0.64bc	0.65 ±0.08b	0.53 ±0.13cde	0.17 ±0.07e	0.81 ±0.37bc	0.24 ±0.09bc
RG-8	2.21 ±0.10a	3.79 ±0.67a	2.23 ±0.47ab	0.16 ±0.06f	0.12 ±0.03e	0.05 ±0.05c	0.15 ±0.08bcd
RG-9	0.28 ±0.02efg	0.29 ±0.05e	0.44 ±0.09b	0.15 ±0.15f	0.19 ±0.08e	0.10 ±0.13c	-
RG-10	0.17 ±0.06fgh	1.63 ±0.13cd	0.33 ±0.08b	0.09 ±0.10f	2.99 ±0.80b	0.53 ±0.19bc	0.10 ±0.10cd
RG-11	0.61 ±0.15bc	3.02 ±0.42ab	0.41 ±0.14b	0.92 ±0.14b	1.42 ±0.55cd	0.06 ±0.09c	-

Values sharing a common letter within the column are not significant at $P \leq 0.05$. " –" sample not available.

Table 14- Total soluble sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RG genetic construct (R1 cDNA fragment towards 3' terminus in antisense orientation under the GBSS1 promoter) based transgenic lines

Total soluble sugar content in mg g^{-1} FW							
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	2.47 ±0.67i	12.05 ±2.08a	9.92 ±1.57a	3.48 ±1.11g	24.37 ±0.97a	11.83 ±1.43a	4.87 ±0.90de
RG-1	4.02 ±0.45h	5.55 ±0.44cde	3.56 ±0.49c	7.44 ±0.12defg	3.35 ±0.21efg	6.08 ±3.13b	5.83 ±1.02cd
RG-2	10.58 ±1.06bc	8.70 ±1.54b	6.76 ±0.80ab	12.30 ±0.91bc	9.56 ±1.59b	5.01 ±0.50bc	3.27 ±0.41e
RG-3	9.65 ±0.14c	6.68 ±0.44c	4.13 ±0.97bc	4.33 ±0.64fg	4.84 ±0.59def	6.63 ±0.39b	6.12 ±0.83cd
RG-4	13.63 ±0.16a	6.53 ±0.21cd	4.15 ±1.00bc	12.95 ±0.89bcd	1.53 ±0.54g	2.50 ±0.36c	9.07 ±1.39ab
RG-5	5.25 ±1.16fg	6.41 ±0.33cd	5.57 ±0.92b	12.79 ±1.93b	5.02 ±0.89cdef	2.39 ±0.57c	8.61 ±0.75b
RG-6	5.97 ±0.02ef	4.63 ±1.16de	2.26 ±0.27d	8.09 ±1.59cdef	2.78 ±1.09fg	2.18 ±0.50c	7.19 ±0.40bc
RG-7	11.11 ±0.01b	7.01 ±0.80bc	5.46 ±0.61b	17.72 ±2.38a	5.60 ±0.66cde	4.77 ±1.60bc	7.20 ±0.32bc
RG-8	6.48 ±0.51e	6.93 ±1.53bc	3.58 ±0.62bc	8.67 ±0.92bcde	4.97 ±0.70cdef	5.13 ±1.48bc	6.48 ±0.95cd
RG-9	4.39 ±0.09gh	4.31 ±0.15e	5.04 ±0.20b	5.69 ±0.02defg	6.39 ±1.60cd	5.98 ±2.30b	-
RG-10	2.96 ±0.08i	6.10 ±0.29cde	5.45 ±0.43b	4.25 ±0.59efg	6.99 ±0.92cd	3.65 ±0.62bc	11.00 ±1.48a
RG-11	8.09 ±0.25d	6.41 ±0.68cd	4.20 ±0.70bc	5.83 ±1.39defg	7.41 ±1.72bc	4.88 ±0.36bc	-

Values sharing a common letter within the column are not significant at $P \leq 0.05$. " –" sample not available.

Table 15- Reducing sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RH genetic construct (R1 cDNA fragment towards 3' terminus in sense orientation under the GBSS1 promoter) based transgenic lines

	Reducing sugar content in mg g^{-1} FW						
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	0.09 ±0.06d	3.80 ±0.31a	2.53 ±0.32a	0.24 ±0.06ab	4.91 ±2.85a	1.50 ±0.41b	0.13 ±0.06b
RH-1	2.35 ±0.47a	1.40 ±0.21b	2.86 ±1.13a	0.01 ±0.08b	3.88 ±0.06b	3.02 ±0.54a	0.06 ±0.04b
RH-2	0.44 ±0.27cd	0.05 ±0.08d	0.09 ±0.11d	0.23 ±0.12ab	2.44 ±0.46c	0.18 ±0.21c	0.86 ±0.06a
RH-3	0.49 ±0.18cd	0.47 ±0.10c	1.15 ±0.19bc	0.20 ±0.15ab	2.21 ±0.15c	0.23 ±0.05c	0.59 ±0.30a
RH-4	0.31 ±0.11cd	0.13 ±0.04d	0.04 ±0.06d	0.19 ±0.05ab	0.26 ±0.10d	0.18 ±0.05c	0.22 ±0.11b
RH-5	2.05 ±0.74ab	0.68 ±0.12c	1.34 ±0.50b	0.14 ±0.19ab	0.36 ±0.09d	1.80 ±0.69b	0.57 ±0.11a
RH-6	1.33 ±0.47bc	1.16 ±0.10b	0.26 ±0.11cd	0.36 ±0.07a	0.84 ±0.10d	0.32 ±0.16c	0.15 ±0.07b
RH-7	0.89 ±0.62cd	0.16 ±0.07d	0.27 ±0.11cd	0.04 ±0.11b	3.84 ±0.61b	0.14 ±0.13c	0.11 ±0.04b

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

Table 16- Total soluble sugar content in the potato tubers (freshly harvested and stored at different temperatures for one and two months) corresponding to the RH genetic construct (R1 cDNA fragment towards 3' terminus in sense orientation under the GBSS1 promoter) based transgenic lines

Total soluble sugar content in mg g^{-1} FW							
		One month storage			Two months storage		
Transgenic potato lines	**Fresh± SD**	**4ºC ± SD**	**10ºC ±SD**	**25ºC ±SD**	**4º C ±SD**	**10ºC ±SD**	**25ºC ±SD**
Control	2.47 ±0.67d	12.05 ±2.08a	9.92 ±1.57a	3.48 ±1.11bcd	24.37 ±0.97a	11.83 ±1.43a	4.87 ±0.90d
RH-1	14.20 ±3.15a	4.94 ±0.47b	3.38 ±1.22b	3.31 ±1.03cd	8.58 ±0.89b	6.23 ±1.04c	6.77 ±1.79cd
RH-2	7.87 ±1.27bc	2.10 ±0.18de	1.59 ±0.70c	4.95 ±0.40abc	4.95 ±1.64cd	5.16 ±0.71c	6.48 ±2.03cd
RH-3	5.19 ±0.99c	1.38 ±0.06e	2.60 ±0.51bc	4.98 ±0.52abc	5.96 ±0.10c	6.75 ±3.09c	11.67 ±1.66a
RH-4	8.03 ±1.45bc	2.44 ±0.66d	2.79 ±0.44bc	5.21 ±0.88ab	3.41 ±0.47d	11.29 ±2.55ab	11.02 ±1.42ab
RH-5	9.51 ±1.67bc	3.70 ±0.75c	2.97 ±0.58bc	1.86 ±1.55d	4.95 ±0.86cd	7.95 ±1.74abc	7.38 ±1.31bcd
RH-6	8.47 ±1.96bc	4.21 ±0.43bc	3.10 ±0.33bc	6.79 ±0.41a	4.16 ±0.29cd	7.28 ±0.90bc	9.52 ±2.37abc
RH-7	11.83 ±2.07ab	2.37 ±0.37de	3.63 ±0.82b	4.18 ±0.41bc	8.19 ±0.94b	8.94 ±1.93abc	9.02 ±0.58abc

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

Status of Publications

Publications in the peer-reviewed journals

1. **Anshu Bansal,** Vijay Kumari, Dhakshi Taneja, Rupinder Sayal, Niranjan Das (2012) Molecular cloning and characterization of granule-bound starch synthase I (GBSSI) alleles from potato and sequence analysis for detection of *cis*-regulatory motifs. ***Plant Cell, Tissue and Organ Culture*** **(PCTOC)** 109: 247-261 IMPACT FACTOR: 3.09 (2011)

2. **Anshu Bansal** and Niranjan Das (2012) Molecular cloning and sequence comparison of a cDNA encoding α-glucan, water dikinase (GWD) from potato (*Solanum tuberosum* L.), and analysis of gene expression. ***Journal of Plant Biochemistry and Biotechnology (JPBB)*** **Accepted (**DOI: 10.1007/s13562-012-0174-6) IMPACT FACTOR: 0.523 (2011)

3. Vijay Kumari, **Anshu Bansal,** Raghavendra Aminedi, Dhakshi Taneja, Niranjan Das (2012) Simplified extraction of good quality genomic DNA from a variety of plant materials. ***African Journal of Biotechnology*** 11 (24): 6420-6427 IMPACT FACTOR: 0.565 (2011)

4. **Anshu Bansal** and Niranjan Das: Repression of cold-induced sweetening in transgenic potato (*Solanum tuberosum* L.) tubers generated by α-glucan, water dikinase (GWD) cDNA-based different binary genetic constructs **(Manuscript under preparation)**.

Nucleotide sequences submitted to the NCBI database:

1. GenBank Accession No. **EU599037**: 4789 bp cDNA, *Solanum tuberosum* Starch-granule- Bound R1 protein (R1) mRNA complete cds. Date of release in the database: 03-May- 2008 **Bansal A** and Das N

2. GenBank Accession No. **EU548081**: 1320 bp linear DNA, *Solanum tuberosum* clone GKCM-TU01 granule-bound starch synthase (waxy) gene, partial cds. Date of release in the database: 19-March-2008 **Bansal A**, Kumari V, Sayal R and Das N

3. GenBank Accession No. **EU548082**: 1461 bp linear DNA, *Solanum tuberosum* clone GKCM-TU02 granule-bound starch synthase (waxy) gene, partial cds. Date of release in the database: 19-March-2008 **Bansal A,** Kumari V, Sayal R and Das N

Full Papers published in the Conference Proceedings:

1. **Bansal A**, Kumari V, Taneja D, Das N (2011) Molecular cloning and Functional Characterization of 5' Flanking Regions of Granule-bound Starch Synthase I (GBSSI) genes from the Indian Potato Cultivars. AChemE 2011 Thapar University, Patiala 21-27

2. Kumari V, **Bansal A**, Aminedi R, Taneja D, Das N (2011) Isolation and quality Checking of DNA from Different Plant Materials by Employing Simple Methods. AChemE 2011, Thapar University, Patiala 89-96

Abstracts published in the Conference Proceedings:

1. **Bansal A**, Bansal S, Kumari V, Das N (2004) A simple and efficient method for the isolation of the RNA from potato tubers. Bioconvergence-2004, Thapar Institute of Engineering and Technology, Patiala pp 42

2. Shukla T, **Bansal A**, Mathur A, Das N (2004) Studies on the starch-bound phosphorus content in the potato tubers at various stages of development. Bioconvergence-2004, Thapar Institute of Engineering and Technology, Patiala pp 87

3. **Bansal A**, Kumari V, Kumara K, Das N (2008) Regeneration studies from internodal stem explants of important Indian potato cultivars during Agrobacterium-mediated transformation. 11th Punjab Science Congress-2008, Thapar University, Patiala

4. **Bansal A**, Kapoor S, Taneja D, Das N (2010) Molecular cloning studies on tuber-expressed genes from Indian potato cultivars. Emerging Trends in Biopharmaceuticals-2010, Thapar University, Patiala