Single Nucleotide Polymorphism Survey for Granule Bound Starch Synthase-1 Gene Involved in Starch Biosynthetic Metabolic Pathway in Selected Potato accessions

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DEPARTMENT OF BIOTECHNOLOGY

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CERTIFICATE

This is to certify that the work titled **Single Nucleotide Polymorphism Survey for Granule bound starch synthase-1 Gene in Starch Biosynthetic Pathway in Potato accession.**" submitted by Ravi Sharma and Utkarsh Khandelwal in partial fulfillment for the award of degree of B.Tech of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor .....

Name of Supervisor: **Dr. Harvinder Singh**

Designation: Asst. Professor

Date: 27/5/13

ACKNOWLEDGEMENT

Of all the people who have helped us complete this project, we are particularly thankful to our esteemed project supervisor Dr. (Mr.) Harvinder Singh, for his guidance, patience and encouragement throughout the project. This project would not have been possible without his guidance and active support. We deem it a privilege to have worked under Dr. (Mr.) Harvinder Singh, who has endeared himself to his students and scholars.

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We are especially grateful to all the lab assistants, our family and friends for their support, help and belief in us. For without their presence it would not have been possible for us to successfully complete our project.

Needless to say, errors and omissions are ours.

Signature of the student	.....	.....
Name of Student	Ravi Sharma	Utkarsh Khandelwal
Date	27/5/13	27/5/13

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SUMMARY

Single Nucleotide Polymorphism survey for Granule bound starch synthase-1 gene in Potato Accessions: Variable starch quantity and quality affects the eating and cooking quality of potato. The starch biosynthesis mainly involves 18 genes in its metabolic pathway. In this project we tried to tap the variability (SNPs) present in the genes which are responsible for high/low amylose content in Potato accession.

In order to do so, studies were performed on 6 potato varieties ,DNAs were extracted from the tubers and amplified with 5 different primers separately (designed using Primer 3 Software), using PCR. The amplified DNA samples were sent for sequencing. Once the sequences for all the DNA samples are obtained, they will be studied for all the putative SNPs present in both the intron and the exon sequences.

Identification of allelic series of variants of starch biosynthesis gene and their association with the starch quality will provide a potential tool for crop improvement.

Signature of Student	Signature of Student	Signature of Supervisor
Name: Ravi Sharma	Name: Utkarsh khandelwal	Name: Dr. Harvinder Singh
Date: 27/5/13	Date: 27/5/13	Date: 27/5/13

CHAPTER-1

Introduction

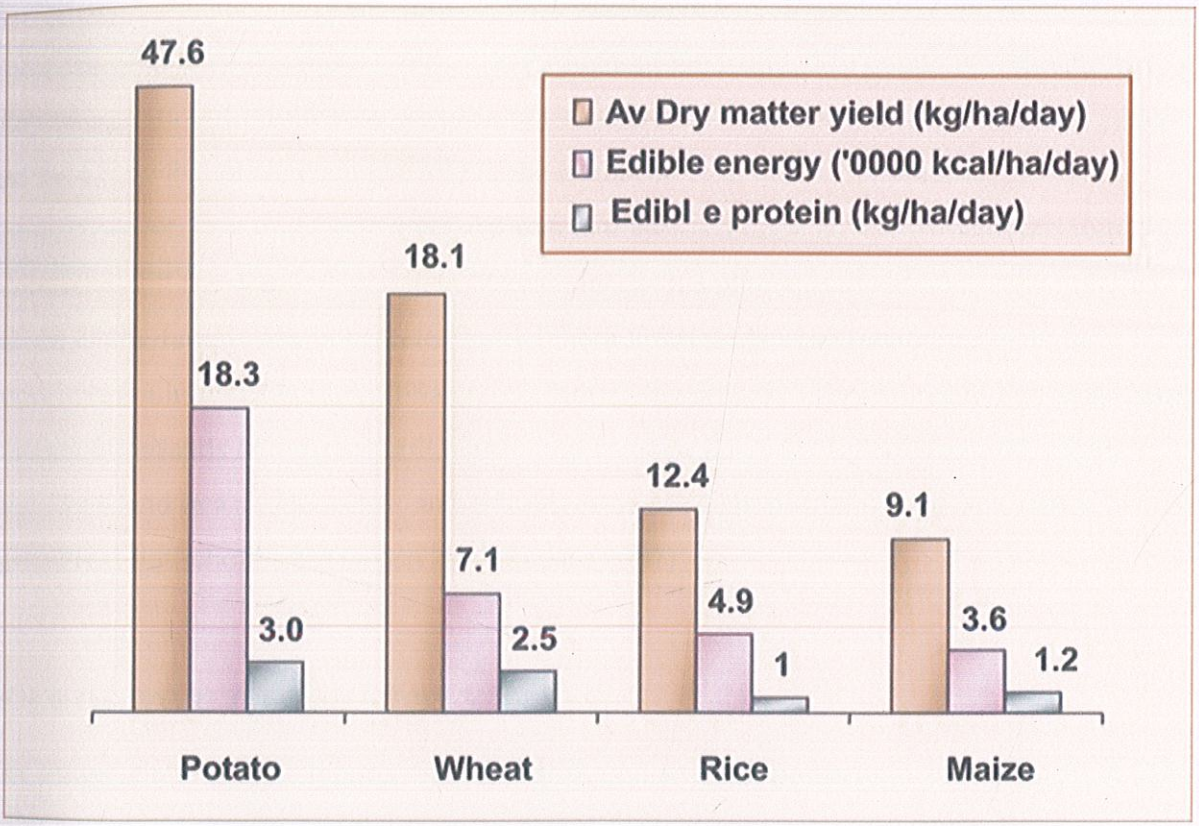
The potato is a starchy, tuberous crop from the perennial *Solanum tuberosum* of the Solanaceae family (also known as the nightshades). The word may refer to the plant itself as well as the edible tuber. In the region of the Andes, there are some other closely related cultivated potato species. Potatoes were introduced outside the Andes region four centuries ago, and have become an integral part of much of the world's cuisine. It is the world's fourth-largest food crop, following rice, wheat and maize. Long-term storage of potatoes requires specialised care in cold warehouses. (Quattrocchi *et al.*1980)

Wild potato species occur throughout the Americas, from the United States to southern Chile. The potato was originally believed to have been domesticated independently in multiple locations,but later genetic testing of the wide variety of cultivars and wild species proved a single origin for potatoes in the area of present-day southern Peru and extreme northwestern Bolivia (from a species in the Solanum brevicaule complex), where they were domesticated 7,000–10,000 years ago (RJ Hijmans *et al.* 1992)Following centuries of selective breeding, there are now over a thousand different types of potatoes. Of these subspecies, a variety that at one point grew in the Chiloé Archipelago (the potato's south-central Chilean sub-center of origin) left its germplasm on over 99% of the cultivated potatoes worldwide.

The annual diet of an average global citizen in the first decade of the 21st century included about 33 kg of potato. However, the local importance of potato is extremely variable and rapidly changing. It remains an essential crop in Europe (especially eastern and central Europe), where per capita production is still the highest in the world, but the most rapid expansion over the past few decades has occurred in southern and eastern Asia. China is now the world's largest potato-producing country, and nearly a third of the world's potatoes are harvested in China and India.(Spooner *et al.* 2005)

WHY POTATO ?

A short duration crop like potato, which produces more dry matter, edible energy and edible protein per unit land and time than many other major crops such as wheat, rice and maize is the most potential and nutritionally superior crop for fighting hunger and malnutrition.



Source: Gopalan, C, BV Ramashashtri and SC Balsubramanian. 1972. Nutritive Value of Indian Foods. National Institute of Nutrition, Hyderabad, India 8

wild potato is a relative of the cultivated potato, and is found in the alpine biome of the Andes Mountains. In Latin, the word alps means high mountains and the word alpine comes from the word alps. The weather conditions in the alpine biome are severe. Plants have to survive in extreme temperatures, heavy snowfall and strong winds. You can find the potato in places in the Andes where the the temperature ranges from 60 to 70 °F during the day, and frost at night. Most of the wild potato plants are hard to find, and grow in few places. Wild potatoes are found in thickets and waste areas.

HISTORY

The potato was first domesticated in the region of modern-day southern Peru and extreme northwestern Bolivia between 8000 and 5000 BC.It has since spread around the world and become a staple crop in many countries.

According to conservative estimates, the introduction of the potato was responsible for a quarter of the growth in Old World population and urbanization between 1700 and 1900. Following the Spanish conquest of the Inca Empire, the Spanish introduced the potato to Europe in the second half of the 16th century. The staple was subsequently conveyed by European mariners to territories and ports throughout the world. The potato was slow to be adopted by distrustful European farmers, but soon enough it became an important food staple and field crop that played a major role in the European 19th century population boom.However, lack of genetic diversity, due to the very limited number of varieties initially introduced, left the crop vulnerable to disease. In 1845, a plant disease known as late blight, caused by the fungus-like oomycete Phytophthora infestans, spread rapidly through the poorer communities of western Ireland, resulting in the crop failures that led to the Great Irish Famine. Thousands of varieties still persist in the Andes however, where over 100 cultivars might be found in a single valley, and a dozen or more might be maintained by a single agricultural household. (K Theisen *et al* January 2007)

ROLE OF WORLD FOOD SUPPLY

The United Nations FAO reports that the world production of potatoes in 2010 was about 324 million tonnes. Just over two thirds of the global production is eaten directly by humans with the rest being fed to animals or used to produce starch. This means that the annual diet of an average global citizen in the first decade of the 21st century included about 33 kg of potato. However, the local importance of potato is extremely variable and rapidly changing. It remains an essential crop in Europe (especially eastern and central Europe), where per capita production is still the highest in the world, but the most rapid expansion over the past few decades has occurred in southern and eastern Asia. China is now the world's largest potato-producing country, and nearly a third of the world's potatoes are harvested in China and India (Robert Hijmans *et.al.* 2001). The geographic shift of potato production has been away from wealthier countries toward lower-income areas of the world, although the degree of this trend is ambiguous .

In 2008, several international organizations highlighted the potato's role in world food production, in the face of developing economic problems. They cited its potential derived from its status as a cheap and plentiful crop that grows in a wide variety of climates and locales.Due to perishability, only about 5% of the world's potato crop is traded internationally; its minimal presence in world financial markets contributed to its stable pricing during the 2007–2008 world food price crisis(Rosenthal *et.al.* 2008). Thus, the United Nations officially declared 2008 as the International Year of the Potato, to raise its profile in developing nations, calling the crop a "hidden treasure". This followed the International Rice Year in 2004.

Worldwide potato production in 2000

Top Potato Producers
in *2010*
(million metric tons)

China	74.8
India	36.6
Russia	21.1
Ukraine	18.7
United States	18.3
Germany	10.2
Poland	8.8
Bangladesh	7.9
France	7.2
Netherlands	6.8
World Total	**324.4**

Source:
UN Food & Agriculture Organisation

VARIETIES

While there are close to 4000 different varieties of potato, it has been bred into many standard or well-known varieties, each of which has particular agricultural or culinary attributes. In general, varieties are categorized into a few main groups, such as russets, reds,

whites, yellows (also called Yukons) and purples—based on common characteristics. Around 80 varieties are commercially available in the UK.For culinary purposes, varieties are often differentiated by their waxiness. Floury, or mealy (baking) potatoes have more starch (20–22%) than waxy (boiling) potatoes (16–18%). The distinction may also arise from variation in the comparative ratio of two potato starch compounds: amylose and amylopectin. Amylose, a long-chain molecule, diffuses from the starch granule when cooked in water, and lends itself to dishes where the potato is mashed (Roach *et. Al.* June, 2002). Varieties that contain a slightly higher amylopectin content, a highly branched molecule, help the potato retain its shape when boiled.

The European Cultivated Potato Database (ECPD) is an online collaborative database of potato variety descriptions, updated and maintained by the Scottish Agricultural Science Agency within the framework of the European Cooperative Programme for Crop Genetic Resources Networks (ECP/GR)—which is organised by the International Plant Genetic Resources Institute (IPGRI).

Waxy potato varieties produce two main kinds of potato starch, amylose and amylopectin, the latter of which is most industrially useful. The German chemical company BASF created the Amflora potato, which has been modified to contain antisense against the enzyme that drives synthesis of amylose, namely granule bound starch synthase .This resulting potato almost exclusively produces amylopectin, and thus is more useful for the starch industry. In 2010, the European Commission cleared the way for 'Amflora' to be grown in the European Union for industrial purposes only --not for food. Nevertheless, under EU rules, individual countries have the right to decide whether they will allow this potato to be grown on their territory. Commercial planting of 'Amflora' was expected in the Czech Republic and Germany in the spring of 2010, and Sweden and the Netherlands in subsequent years. Another GM potato variety developed by BASF is 'Fortuna' which was made resistant to late blight by adding two resistance genes, blb1 and blb2, which originate from the Mexican wild potato Solanum bulbocastanum.In October 2011 BASF requested cultivation and marketing approval as a feed and food from the EFSA. In 2012 GMO development in Europe was stopped by BASF. (Burger, Ludwig *et.al.* 31 October 2011)

In 2010, a team of Indian scientists announced they had developed a genetically modified potato with 35 to 60% more protein than non-modified potatoes. Protein content was boosted by adding the gene AmA1 from the grain amaranth. They also found 15 to 25% greater crop

yields with these potatoes.The researchers expected that a key market for the GM potato would be the developing world, where more than a billion people are chronically undernourished. (Subhra Chakrabortya, Niranjan Chakrabortya,Lalit Agrawala ,Sudip Ghosha, Kanika Narulaa;Subhendu Shekhara,B Naik,S Prakash, P.C. Pandec *et al.* 20 September 2010)

RESEARCH GAP:

No such insilico analysis has been performed till date regarding amylose content of potato species . The discovery of single nucleotide polymorphisms (SNPs) in genome-scale sequencing initiatives opens new avenues to the study of the genome-wide distribution of diversity and gene-based study (and its significance).

The GBSS genes of potato to be studied for their economical importance and suspected impact on starch quality

Objectives

1. **Insilico analysis and amplification of GBSSI gene using de novo primers**

2. **Study of sequence polymorphism (putative SNPs) in the amplified segment among different accessions**

CHAPTER-2

REVIEW OF LITERATURE

ES

nain source of income in many countries It is now the fourth most
od crop, surpassed only by wheat, rice, and maize. In five centuries, this
ble tuber has spread from its original South American heartland in the high
ion zones in temperate regions of all the continents, and, lately, its
n increasing most rapidly in the warm, humid, tropical Asian lowlands
son. Bolivia, Peru, Argentina, and Mexico are where 90% of the wild
. There are about 199 species of wild potato. In the northern Andes,
toes in the lower Paramos. The Paramos are at 3000 and 4000 meters above
ral Andes have a climate that is semi-arid. Potatoes are grown on the Puña
are high altitude plains. The southern Andes has a dry Mediterranean like
mers can grow potatoes easily.

tato we eat are called tubers. These tubers grow underground on their roots.
called the white potato, to tell it apart from the sweet potato. The wild
ltivated by Indians near Lake Titicaca, who grew the earliest potatoes
00 years ago. Wild potato tubers have been found in the mountains and
here the climate was too cold for wheat or corn. The tubers were easy to
Frost resistant varieties were developed by the the Mochia, Chimu, and
ound ways to freeze-dry the potatoes (Monte Hayes *et al* June ,2007). The
ped by the Inca people. They prayed to potato gods to ensure the success of

2.1 PO

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The potato is best known for its carbohydrate content (approximately 26 grams in a medium potato). The predominant form of this carbohydrate is starch. A small but significant portion of this starch is resistant to digestion by enzymes in the stomach and small intestine, and so reaches the large intestine essentially intact. This resistant starch is considered to have similar physiological effects and health benefits as fiber: It provides bulk, offers protection against colon cancer, improves glucose tolerance and insulin sensitivity, lowers plasma cholesterol and triglyceride concentrations, increases satiety, and possibly even reduces fat storage.The amount of resistant starch in potatoes depends much on preparation methods. Cooking and then cooling potatoes significantly increases resistant starch. For example, cooked potato starch contains about 7% resistant starch, which increases to about 13% upon cooling.

Due to carbohydrate content, potatoes are considered to make a person obese if used in excess i.e. more than RDA of carbohydrates and fats. However, research by the University of California, Davis and the National Center for Food Safety and Technology, Illinois Institute of Technology demonstrates that people can include potatoes in their diet and still lose weight.

Potato, raw, with skin Nutritional value per 100 g (3.5 oz) Energy 321 kJ (77 kcal) Carbohydrates 19 g - Starch 15 g - Dietary fiber 2.2 g Fat 0.1 g Protein 2 g Water 75 g Vitamin A equiv. 0 µg (0%) Thiamine (vit. B_1) 0.08 mg (7%) Riboflavin (vit. B_2) 0.03 mg (3%) Niacin (vit. B_3) 1.05 mg (7%) Vitamin B_6 0.295 mg (23%) Folate (vit. B_9) 16 µg (4%) Vitamin C 19.7 mg (24%) Vitamin E 0.01 mg (0%) Vitamin K 1.9 µg (2%) Calcium 12 mg (1%) Iron 0.78 mg (6%) Magnesium 23 mg (6%) Phosphorus 57 mg (8%) Potassium 421 mg (9%) Sodium 6 mg (0%) Zinc 0.29 mg (3%)
Percentages are relative to US recommendations for adults.
Source: USDA Nutrient Database

Potatoes are generally grown from seed potatoes – these are tubers specifically grown to be disease free and provide consistent and healthy plants. To be disease free, the areas where seed potatoes are grown are selected with care. In the USA this restricts production of seed potatoes to only 15 states out of the 50 states that grow potatoes.These locations are selected for their cold hard winters that kill pests and long sunshine hours in the summer for optimum growth. In the UK, most seed potatoes originate in Scotland in areas where westerly winds prevent aphid attack and thus prevent spread of potato virus pathogens. Potato growth has been divided into five phases. During the first phase, sprouts emerge from the seed potatoes

and root growth begins. During the second, photosynthesis begins as the plant develops leaves and branches. In the third phase stolons develop from lower leaf axils on the stem and grow downwards into the ground and on these stolons new tubers develop as swellings of the stolon. This phase is often (but not always) associated with flowering. Tuber formation halts when soil temperatures reach 80 °F (26.7 °C); hence potatoes are considered a cool-season crop.Tuber bulking occurs during the fourth phase, when the plant begins investing the majority of its resources in its newly formed tubers. At this stage, several factors are critical to yield: optimal soil moisture and temperature, soil nutrient availability and balance, and resistance to pest attacks. The final phase is maturation: The plant canopy dies back, the tuber skins harden, and their sugars convert to starches. (Kingman , Cummings *et. Al.* 1992)

2.3 MOLECULAR MARKERS

A molecular marker is a fragment of DNA that is associated with a certain location within the genome. Molecular markers are used in molecular biology and biotechnology to identify a particular sequence of DNA in a pool of unknown DNA.

Genetic improvement of crop species is required to enhance their economic traits such as yield, resistance to abiotic and biotic stresses, etc. and thus forms the ultimate goal of plant breeding. The most fundamental and important step in plant breeding programme is the selection of plants/individuals with desirable characters. The conventional method used by plant breeders for selection is the phenotypic selection where morphological/phenotypic agronomic traits such as plant height; grain yields, etc are taken into account. These can be called as phenotypic markers or morphological markers. They are visible manifestations of genes and therefore, provide some idea about the genotype. However, most of them are controlled by many genes and follow quantitative inheritance and thus are highly influenced by environment. They are also subjected to allelic interactions like epistasis or pleiotropy. Since too plastic in nature and subjected to many factors, they sometimes do not give correct picture of genetic make-up of the plants.

In phenotypic markers, the extent of variation available is also limited. The dominant-recessive interactions between the alleles make it dominant marker system. Moreover, use of morphological markers excludes the analysis of non- coding sequences of genomes, which in higher plants often account for more than 95% of the total genome Further they are field requiring, that is, for phenotypic selection the accessions are first raised in field and then scored at appropriate growth stages, e.g. for scoring yield, fruiting time is to be attained. Thus, raising large populations in field up to appropriate stage makes it time, effort and labour requiring. In the field they are subjected to environmental hazards also. In some cases, a trait may not express if suitable environment. They are also subjected to allelic environment/condition is not available particularly in the case of stress related genes (Gupta P K *et. Al.* 1999) Moreover, scoring of these markers is subjective the results may differ when scored by different breeders. These constraints make the use of phenotypic markers limited.

Another set of markers called biochemical markers can be used which overcome most of the limitations of phenotypic markers. They include proteins, isozyme/allozyme and secondary metabolites, etc. Proteins/Allozymes are quite stable and are minimally influenced by environment. They give closest insight into the genetic makeup. These markers can be analyzed at early stages, e.g. isozymes can be accessed from seeds, and that too with small amount of tissue as against the morphological markers. Electrophoretic variations in proteins can be detected by staining and directly related to allelic variation they are codominant markers, an attribute useful for the detection of recessive alleles in heterozygotes. However, as in the case of morphological markers they depict functional polymorphisms thus only giving polymorphism in coding regions selectively that too only the enzyme coding sequences. Furthermore, only those variations are detected that affect the electrophoretic mobility of the proteins. As with classical phenotypic markers, the extent of variation is also somewhat limited. In case of secondary metabolites, their use is restricted to only such plants that produce a suitable range of those metabolites. Moreover, secondary metabolites are products of long and complex pathways and therefore, require study of many genes.

Over the last three decades a new class of markers, namely, molecular markers or DNA markers have been introduced, which have totally revolutionized the entire field of

molecular biology. Though, due to simplicity and low cost, biochemical markers especially isozymes are still frequently used in various laboratories, the high information content and high quality of the DNA markers have made them preferred over biochemical markers. DNA markers can broadly be defined as 'DNA profiles that give information about the genotype'. They possess unique advantages over the phenotypic and biochemical markers. They look directly at the basic level of variation, i.e. DNA level giving direct insight into the genetic makeup, screen the whole genome and reveal variations in both coding and non-coding regions and hence offer large extent of polymorphism. Since the only marker systems to assess organelle genome diversity, make it more important in plants, as many traits are controlled or influenced by the organelle (mitochondria and chloroplast) genome. Analysis of chloroplast genome is used for phylogenetic studies at taxonomic level as these genomes are conserved as compared to nuclear and mitochondrial genome. DNA markers are highly amenable to automation and once automated, they can be used as efficient selection tools by the plant breeders and contribute in Marker Assisted Selection (MAS). Different DNA markers can be classified into three classes: (i) the hybridi- zation based markers, which include RFLPs and their modifications. RFLPs have their origin due to sequence insertions or deletions that create or delete restriction site/s. Polymorphism is observed by treating the DNA with restriction enzymes followed by electrophoresis and hybridization by a labeled probe. However, RFLPs suffer from some drawbacks. They require large amount of DNA, are labour intensive, time consuming, mostly require radio-actively labeled probes, hence safety factors must be considered (O'Neill *et. al.* 2003). (ii) PCR based markers that include RAPDs and their modifications. In RAPD, ten-mer, arbitrary primers are used to amplify the genomic DNA and the products are separated by agarose gel electrophoresis and visualized by staining. RAPD has its modifications like DAF and AP-PCR that differ in the length of the primer used. They are relatively low cost markers and prior sequence information of the target genome is not required. However, RAPD markers are dominant markers and considered less reproducible. They also show fragment allelism i.e. RAPD bands of same molecular weight may not have same nucleotide sequence. (iii) Markers that combine principles of both RFLP and PCR include SSRs, AFLPs and their modifications. These markers have high information content and have high resolution of genetic variations than the first generation markers. SSRs, also known as microsatellites, are ubiquitous short tendem repeat motifs of 1-6 bp in genome. They show high mutation rate, which has now been estimated to be between 10^{-2} - 10^{-}

[6]making them highly polymorphic markers (O'Neill *et. al.* 2003). They are codominant markers. SSR assays are, however, quite costly and time consuming as primers are to be synthesized from the flanking sequences of microsatellite, which need to be isolated, cloned and sequenced. Since the polymorphism can be the result of addition or deletion of single copy of repeat motif, it requires high- resolution agarose/polyacrylamide gel electrophoresis or the use of automated sequencers. AFLP marker is based on selective amplification of double digested restriction products using adaptors linked to restriction fragment ends acting as specific primer binding site for PCR amplification. For more specific amplification one to three extra nucleotides arbitrarily chosen) are added to adapter sequence. AFLP markers are highly reproducible and number of markers that can be produced is unlimited as single restriction digest can be used for amplification with different primer combinations. However, they are of high cost and are dominant markers. As in the case of SSRs they also need high-resolution electrophoresis or automated sequencers.In recent years, a novel class of markers namely SNPs has emerged as an important tool in genomics and are increasingly being used as molecular markers in various laboratories for diverse applications. They possess unique merits that make them preferred over the above classes of markers. They have high information content and depict extremely high level of polymorphisms. Initial cost involved is quite high for these markers. However, they are highly amenable to automation, thus eventually can become cost-effective. Since most of them are non-gel based, they are less time consuming against rest of the markers.

2.4 SINGLE NUCLEOTIDE POLYMORPHISM

A single-nucleotide polymorphism (SNP, pronounced snip; plural snips) is a DNA sequence variation occurring when a single nucleotide — A, T, C or G — in the genome (or other shared sequence) differs between members of a biological species or paired chromosomes in a human. For example, two sequenced DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide. In this case we say that there are two alleles: C and T. Almost all common SNPs have only two alleles. The

genomic distribution of SNPs is not homogenous, SNPs usually occur in non-coding regions more frequently than in coding regions or, in general, where natural selection is acting and fixating the allele of the SNP that constitutes the most favorable genetic adaptation.
Besides natural selection other factors like genetic recombination and mutation rate can also determine SNP density. SNP density can be predicted by the presence of microsatellites as regions of thousands of nucleotides flanking microsatellites have an increased or decreased density of SNPs depending on the microsatellite sequence.

Within a population, SNPs can be assigned a minor allele frequency — the lowest allele frequency at a locus that is observed in a particular population. This is simply the lesser of the two allele frequencies for single-nucleotide polymorphisms. There are variations between human populations, so a SNP allele that is common in one geographical or ethnic group may be much rarer in another.
These genetic variations between individuals (particularly in non-coding parts of the genome) are exploited in DNA fingerprinting, which is used in forensic science . Also, these genetic variations underlie differences in our susceptibility to, or protection from all kinds of diseases. The severity of illness and the way our body responds to treatments are also manifestations of genetic variations. For example, a single base mutation in the Apolipoprotein E gene is associated with a higher risk for Alzheimer's disease.

USE AND IMPORTANCE

Variations in the DNA sequences of humans can affect how humans develop diseases and respond to pathogens, chemicals, drugs, vaccines, and other agents. SNPs are also thought to be key enablers in realizing the concept of personalized medicine. However, their greatest importance in biomedical research is for comparing regions of the genome between cohorts (such as with matched cohorts with and without a disease) in genome-wide association studies.

The study of SNPs is also important in crop and livestock breeding programs (see genotyping). See SNP genotyping for details on the various methods used to identify SNPs.

They are usually biallelic and thus easily assayed. A single SNP may cause a Mendelian disease. For complex diseases, SNPs do not usually function individually, rather, they work in coordination with other SNPs to manifest a disease condition as has been seen in osteoporosis.

2.6 AMYLOSE AND AMYLOPECTIN

There is considerable potential to develop the potato as a functional food with health-promoting properties beyond the basic function of supplying nutrients.Approximately 20% of fresh tuber weight is starch and the remainder is water. Potato starch is typically low in amylose (~20-25%) Most of the starch (around 70%) in the tuber is amylopectin High amylose starch has superior nutritional qualities.

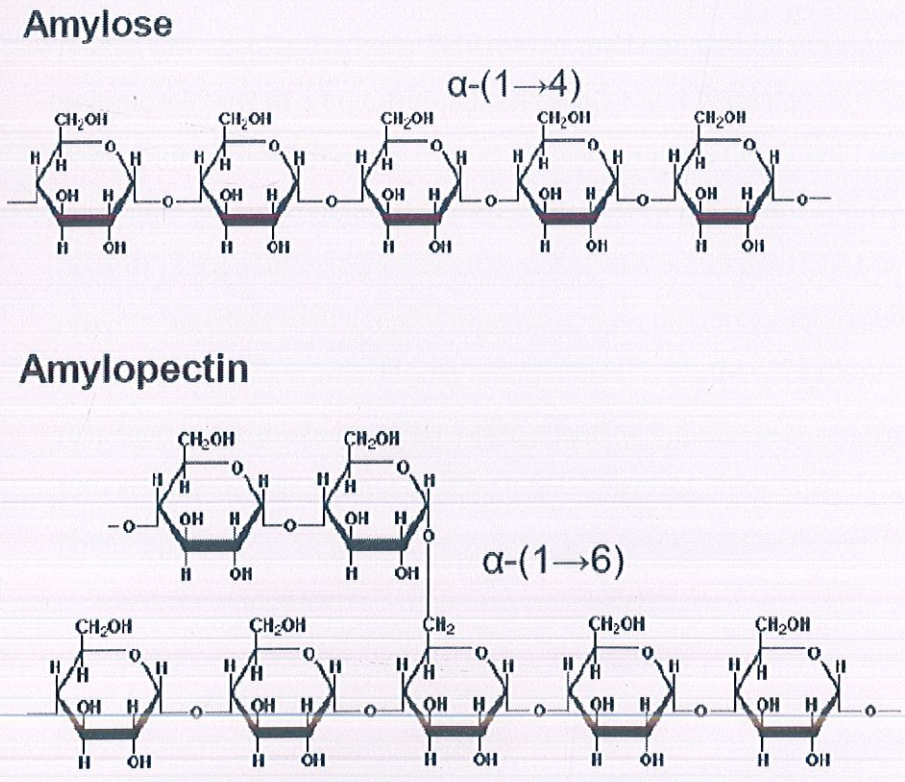

Fig 2: structures of Amylose and Amylopectin

- An increase in the amylose content of potato is highly desirable to improve the nutritional quality. The development of nutritionally improved potatoes would benefit potato growers, as it would likely increase consumer interest and consumption. Genetic variation for AC in the starch of potato tubers is narrow in existing cultivars, which warrants the utilization of accessions of wild Solanum species with higher amylose content in a predictable in future breeding programs. Before starting such breeding program it will be prudent to characterize the wild genetic resources with respect to amylose contentand to identify **molecular markers linked with high amylose content** that potato breeders could use to select for high amylose potato clones during breeding process.This will provide breeders a high throughput, cost-effective tool to select clones with high AC in early generations and will be a big step forward in the development of more nutritious potatoes and is likely to stimulate potato consumption. Comparison of sequences from high and low amylose wild potato clones will identify polymorphisms that will be considered as putative molecular markers. PCR-based methods will be developed to detect these polymorphisms in a high-throughput manner and validated on a segregating population. These markers can be be tested in additional wild species and in the cultivated potato to determine the extent to which they are applicable in a broad gene pool. If these markers can be extrapolated to a broad gene pool in potato, then we will provide breeders with a high throughput, cost-effective tool to select clones with high AC in early generations. Granule-bound starch synthase I (GBSS-I) is solely responsible for the biosynthesis of amylose in storage tissues (Smith *et al.* 1995, Edwards *et al.* 2002). So it will be a good starting point to study GBSS-I in wild potato species.

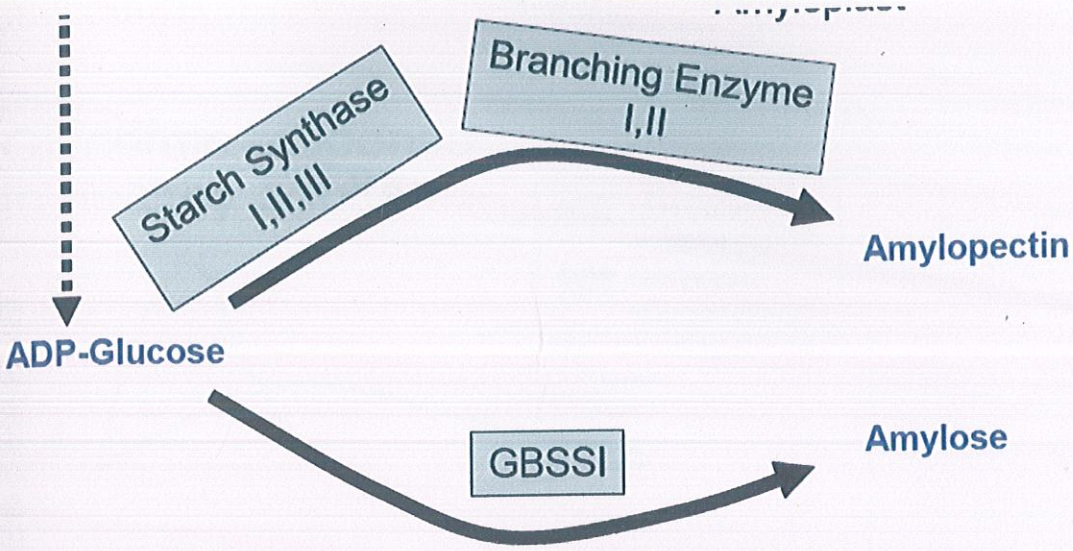

Fig 3: starch metabolic pathway

- Cooking increases the digestibility of standard potato starch. However, following cooking, a portion of high amylose starch recrystallizes to form resistant starch, which acts as dietary fiber.
- In humans, amylose is more slowly digested than amylopectin, so blood glucose and insulin levels are lower after a meal high in amylose, is maintained longer, and the next meal is likely to be smaller.
- An increase in the AC of potato starch could improve the nutritional quality of potato by increasing fiber content and reducing the glycemic index. This would positively impact some of the most serious health problems including type II diabetes, hypertension, cardiovascular disease, and obesity

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2.6 GLYCEMIC INDEX

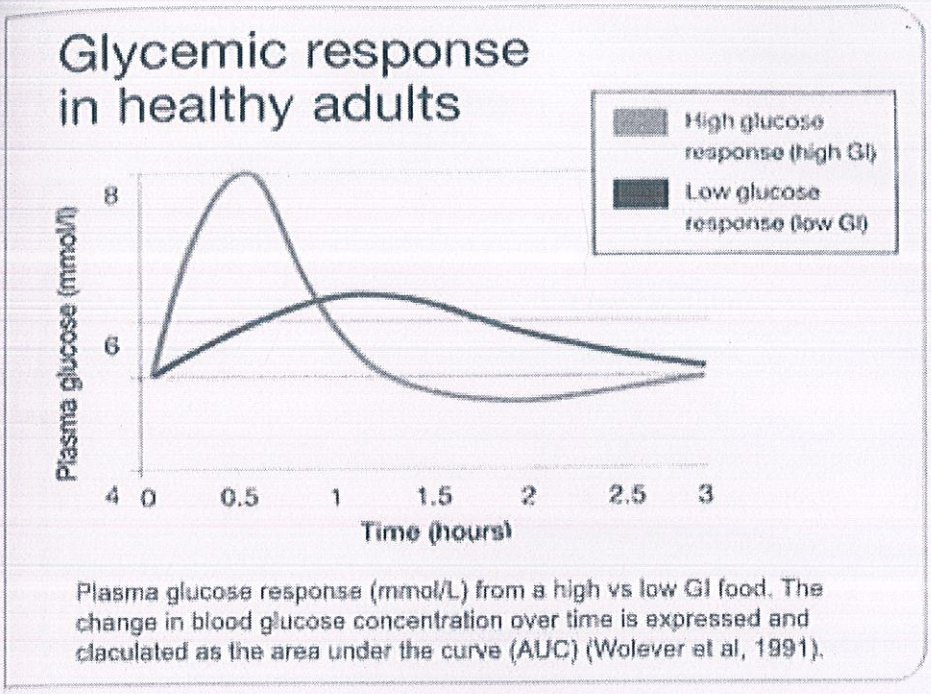


Fig 4 : glycemic responce in healthy adult with time .

Is a ranking of carbohydrates on a scale from 0 to 100 (or more) according to the extent to which they raise blood sugar levels after eating.

Low (0-55) Med (56-69) High (70 or more)

Foods with a high GI are those which are rapidly digested and absorbed and result in marked fluctuations in blood sugar levels.

Food Item	Rating Food	Glycemic Index (Glucose=100)
Carrots, raw (Romania)	Low	16
Carrots, peeled, boiled (Australia)	Low	32-49
Carrots NS (Canada)	High	92
Potato, boiled	Low-High	24-101
New potato	Low-High	47-78
Chips, plain, salted	Low-Medium	51-57
Steamed potato	Low-Medium	52-65
Potato, baked	Medium-High	56-111
Canned potatoes, microwave	Medium	61-65
Mashed potato	Medium-High	67-91
Instant mashed potato	High	74-88
French fries, frozen, microwave	High	75
Microwaved potato (peeled)	High	79
Rice, white high-amylose	Low-Medium	37-66
Rice, white low-amylose	High	83-139

Low (0-55) **Med (56-69)** **High (70 or more)**

Adapted from International Table of GI and GL, 2002

Table 1 : Glycemic index

CHAPTER-3

MATERIALS AND METHODS

3.1 Plant Materials:

A Sets of 6 potato accessions were procured from CPRI Shimla and used for the study (Table 2) .

3.2 *In silico* analysis of GBSS-I gene and Primer designing

DNA sequences of potato GBSS-I Starch synthesis related gene was searched and retrieved from relevant DNA databases. Primers for each target region (Forward/reverse) were designed using Primer3.

3.3 Potato accessions

Table 2: list of potato accessions used for SNP survey

S. No	Name of potato accession
1	K Chipsona 1
2	K Jyoti
3	K Chipsona 3
4	K Pubhraj
5	K Himsona
6	K Frysona

The accessions were lyophilized before use so that DNA extraction can be performed efficiently . DNA was extracted from peel of accession as carbohydrate contamination becomes major problem associated with potato tuber.

DNA Isolation

Initially the DNA was isolated from lyophilized tuber samples using modified CTAB method of DNA isolation(Doyal and Doyal)

Amplification of GBSS-I Gene fragments

The gene segment was amplified using Polymarase Chain reaction using the designed primers .

Mining of Single Nucleotide Polymorphism within the GBSS-I

DNA sequencing had to be performed using the Sanger dideoxy-chain terminator method on an automated sequencer using Dye-Terminator chemistry.

The identification of putative SNPs among *potato clones* would have been done by Auto SNP Analyzer, SNP genotyping Profiler and Polyphred software (Nickerson et al., 1997) that automatically detects the presence of heterozygous single nucleotide substitutions by fluorescence based sequencing of PCR products.

SAMPLING AND DNA EXTRACTION OF POTATO:-

EQUIPMENT/REAGENTS/PLASTICWARE

1.EQUIPMENT

The following equipments were used in the DNA extraction procedure described (equivalents may be substituted):

Table 3: list of Equipments

Equipment
Pipettes with adjustable volume
Incubator with shaker or shaking water bath
Balances for the preparation of buffers and solutions and for sample weigh in
Centrifuge with rotors for 50 ml centrifuge tubes and microcentrifuge tubes
Thermoblock for 1.5/2.0 ml microcentrifuge tubes
Vortex

2.REAGENTS

The following reagents were used in the DNA extraction procedure described(equivalents may be substituted):

Table 4: List of Reagents used in experiment

Reagent	Specification
Nacl	p.a quality or Molecular biology grade
CTAB	p.a quality or Molecular biology grade
Tris	p.a quality or Molecular biology grade
EDTA,Na2-salt	p.a quality or Molecular biology grade
HCL	p.a quality
Proteinase K	from Tritirachium album,DNAses,Rnases,Exonucleases not detectable,molecular biology grade
RNase A	From bovine pancreas,salt free,protease free and chromatographically homogeneous,ca. 90K unitz/mg
Sodium acetate	p.a quality or Molecular biology grade
Isopropanol	p.a quality
Glycogen	From oyster,research grade
Ethanol	p.a quality
S-300 HR Microspin Columns(50)	Amersham Pharmacia
Chloroform	p.a quality

The following buffers and solutions were used in the DNA extraction procedure described:

CTAB buffer

1.4 M Nacl

2%(w/v) CTAB

0.1 M Tris-Base

0.015 M EDTA

For 1 litre CTAB buffer weigh out 81.8g Nacl, 20g CTAB, 12.1g Tris-Base and5.84g EDTA in an appropriate beaker and add about 800ml water.Adjust ph with HCL to ph 8.0,stir until all reagents were dissolved.Adjust volume to 1litre with water.Do not autoclave.Store at room temperature for upto 2 years.

Proteinase K

20mg/ml water

For 10 ml Proteinase K solution dissolve 200 mg Proteinase K in 10ml water.

Store at -20 degree C for upto 2 years.

RNase A

91mg/ml

Dissolve 0.5g RNase A in 5ml 0.01 M sodium acetate(ph 5.2).Aliquot in 1ml portions,boil for 15 minutes to inactivate DNases,cool slowly to room temperature and added 100ul 1M Tris-Hcl(ph 7.4) to each aliquot.Store at -20 degree C for upto 2 years.

Glycogen

20 mg/ml of water

For 5ml glycogen solution, dissolve 100 mg glycogen in 5ml water.

Store at – 20 degree C for upto 5 years.

75%(v/v) Ethanol

For 200 ml we combined 150 ml 100% ethanol with 50 ml water.

Store at room temperarure for upto 5 years.

1X TE buffer

10mM Tris ph 8.3

1mM EDTA

For 100 ml 1X TE buffer combine 1ml 1M Tris(ph 8.3) and 200 ul 0.5 M EDTA (ph 8.0)

And adjust the volume to 100 ml with water.Autoclave.

Store at room temperature for upto 2 years.

0.2 X TE buffer

2mM Tris,ph 8.3-

0.2 Mm EDTA

For 100ml 0.2X TE buffer dilute 20 ml 1X TE buffer with 80 ml water.Autoclave.Store at room temperature for upto 2 years.

Table 4 : List of Plastic ware

Item
50 ml conical tubes
1.5 ml microcentrifuge tube
2ml microcentrifuge tube
Filter tips

Removal of contaminants such as lipophilic molecules and proteins by extraction with chloroform.Afterwards a crude DNA extract is generated by precipitation with ispropanol.

Note:

Milling/grinding of tubers not only facilitates the lysis by mechanically disrupting cellular structures and increasing the surface area,but is also indispersable for the generation of representative test portions by reducing the particle size.

Lysis/isopropanol precipitation of DNA

1. Transfer 15ml of CTAB buffer and 60ul Proteinase K into 50ml conical tube.
2. Weigh out 3g of freeze-dried homogenized potato tubers into the tube containing CTAB buffer and Proteinase K and mix thoroughly.
3. Incubate for 2-4 hours at 65 degree celcius with agitation.
4. Spin down at room temperature for 10 minutes at 2700-3000*g(approx.1200-1500 r.p.m
5. Transfer 1ml of the supernatant to a 2ml microcentrifuge tube containing 5ul RNase A.
6 Incubate for 15 minutes at 60 degree celcius.
7. Centrifuge at room temperature for 1 minute at approximately 10000 r.p.m.
8. Transfer 900ul of the supernatant to a 2ml microcentrifuge tube containing 600ul chloroform.
9. Vortex,centrifuge at room temperature for 10 minutes at approx.10000 r.p.m.
10. Transfer 625ul of the upper phase to a 1.5ml microcentrifuge tube containing 500ul isopropanol and 2ul glycogen(glycogen is pipetted into the lid,do not combine the glucogen and the isopropanol.
11. Mix completely(invert tubes several times) and let stand at room temperature for 30 minutes to allow precipitation to form.

12. Centrifuge at room temperature for 10 minutes at approximately 10000 r.p.m.

13. Discard supernatant. Add 500ul 75% ethanol and pipette carefully up and down until pellet is detached from the wall of the microcentrifuge tube.

14. Centrifuge at room temperature for 5 minutes at 10000 r.p.m.

15. Carefully remove and discard the supernatant.Centrifuge again and remove remaining ethanol. If any fluid remains,allow the pellet to dry at room temperature.

16. Resuspend the pellet in 50ul 0.2 TE buffer.Let stand for 15 minutes at room temperature with occasional mixing.Make sure that the pellet is dissolved,then centrifuge for 2 minutes at 10000 r.p.m and transfer the supernatant into fresh 1.5 ml microcentrifuge tube.

3.4.3 GEL ELECTROPHORESIS

Gel electrophoresis was done after DNA extraction ,to check the presence of DNA .

Agarose

TAE 50X BUFFER

DISTILLED water

Ethidium bromide

DNA sample

Lambda DNA

Gel electrophoresis setup.

Procedure :

1. Dilute 50x tris acetate buffer for a final concentration of 1x.
2. Prepare the gel such that it is 0.8% agarose .Weigh out approximately 0.8 gms of agarose powder.
3. Add 2 ul 1x TAE buffer and raise the volume 200ml by adding distilled water .
4. Heat the agarose – TAE buffer mixture in intervals to ensure that the agarose is completely dissolved.
5. While the mixture is cool enough, add 5ul ethidium bromide .

6. Pour the mixture in the casting tray and is kept for around 30 min to solidify,with comb in it .
7. After the gel has cooled completely and solidified the comb,the comb is removed and the tray is inserted properly into the gel chamber.
8. Pour enough TAE buffer into the chamber to cover the gel and fill the wells.
9. Load 5 ul DNA with 1.5 ul loading dye.
10. Load 1ul lambda DNA .(200ng/ul)
11. The gel is then run at 100 volts for around 30 min.
12. The gel is then studied under UV light.

3.4.4 PCR-POLYMERASE CHAIN REACTION

Material Required

Components require per PCR vial :

Table 5. Components used (along with their volumes) in PCR

COMPONENTS	VOLUME (ul)
PCR buffer	2.5
dNTPS	0.5
DNA	1.0
Taq polymerase	0.25
Forward primer	1.5
Reverse primer	1.5
dH2O	17.75
Total	25

Method

Gene amplification of all 6 varieties of potato accessions were carried out under following PCR condition:

Initial Denaturing	94°C	3 min	
Denaturing	94°C	30 sec	30 cycles
Annealing	53°C	1 min	
Extension	72°C	2 min	
Final extension	72°C	7 min	

PCR following amplification of DNA was quantified on a gel with a 100 b.p. ladder .

- **Amylose Quantification Protocol:**

The following reagents are used for estimation of amylose content and for preparation of standard:

- 95% Ethanol: Prepared from 100% Ethanol
- 1N NaOH, Iodine - Potassium iodide solution
- Standard amylose: Obtained from HIMEDIA
- 1N Acetic acid: From NICE Chemicals
- Glassware: Borosil
- Water bath: High Precision water bath from Acmas was used
- Spectrophotometer: From Amersham Biosciences to measure OD
- Cuvette: Quartz Cuvette to measure OD
- Software: HandyGraph Software to draw graph
- 1N NaOH solution: Dissolve 40g of NaOH in 1000ml distilled water
- 1N Acetic acid solution: Dilute 57.5 ml glacial acetic acid to 1000ml using distilled water
- Iodine - Potassium iodide solution: Dissolve 0.26 g of Iodine in 10 ml of Potassium iodide solution containing 2.6 g of KI

Protocol

1. Weigh 100 mg well powdered milled rice into 100 ml volumetric flask.

2. Add 1 ml 95% ethanol and 9 ml 1 N NaOH.
3. Heat the sample for 10 minutes in boiling water bath, cool it and make up the volume to 100 ml.
4. Pipette 5 ml from the 100 ml into another 100 ml volumetric flask.
5. Add 1 ml I N acetic acid and then 2 ml iodide solution and make up the volume to 100 ml.
6. Shake, stand for 20 minutes and determine the per cent Transmittance at 620 nm using a colorimeter.
7. Prepare a series of standard starch solution containing 0, 20, 40, 60, 80 and 100% amylose as in the steps 1 to 5
8. Read the transmittance of the standards at 620nm and plot a standard graph.
9. Amylose content of the sample is determined in reference to the standard curve and expressed on percent basis.
10. Making of amylose standards:

a. Pipette out 1, 2, 3, 4 and 5 ml of the standard amylose into 100 ml volumetric flasks in three replications.
b. Keep one flask as blank without adding anything.
c. Add 1.0 ml 1N acetic acid and 2.0 ml I-KI solution to all the flasks including blank.
d. Make up all the flasks to 100ml using distilled water and cover all the flasks with a black cloth or aluminum foil to prevent direct light exposure. I-KI disintegrates on exposure to light.
e. Keep for 20 minutes and take reading at 620nm in a spectrophotometer.
f. The standards including balnk, correspond to 0%, 4%, 8%, 12%, 16% and 20% of amylose.
g. Draw a standard curve using the absorbance reading.

CHAPTER 4

RESULTS AND DISCUSSION

With the recent background information available, the experiments were carried out to search the Granule bound starch synthase-I gene in potato and to identify the Single Nucleotide Polymorphisms (SNPs) within the gene of interest to study the natural variations at a single nucleotide level which may influence to ameliorate the quality of the product .The minor differences in starch amylose content resulting from a mutation at the GBSS loci are thought to influence the quality of potato

4.1 DNA sequences of potato starch synthesis related genes after *In silico* analysis.

Table 6. DNA sequences of potato starch synthesis related gene GBSS1

GBSS1	LOC_Os06g04200	MSU6:6	1764623:1769657

. After selecting these sequences, the possible primers for these genes were searched by performing a Global Sequence Alignment of sequences from two potato accession. After trying the list of thus obtained primers *in-silico* on the respective genes through BLAST program, the respective primers were designed.

Table 7. Primers used in the sequencing of genes related to potato amylopectin biosynthesis.

Gene	Primer	Forward/ Reverse primers	Primer sequences
GBSS	GBSS1-1	GBSS1-F1	GAAAATTATAATACTCCGTTTTGTTCA
		GBSS1-R1	CTCACACAGCTCAACAAGTGG
	GBSS1-2	GBSS1-F2	AGTGGAGGGACCAGTACCAG
		GBSS1-R2	GGTAACACCCAAGATGGCAT
	GBSS1-3	GBSS1-F3	TCGGTGATAAATGTGAATGCTT
		GBSS1-R3	ACCACCCAATGTTCTTGGAG
	GBSS1-4	GBSS1-F4	CCCCCGTTATGACCAATACA
		GBSS1-R4	CACTTGATTGCGCTTTACCC
	GBSS1-5	GBSS1-F5	GCCCCAAAGCTGGACTAGAT
		GBSS1-R5	GCTGTTGACAAGGGTGTTGA

- After designing, these primers were sent for synthesis (IDT pvt ltd.)

- **4.3 RESULTS OF DNA EXTRACTION**

- DNA extraction from starch can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow

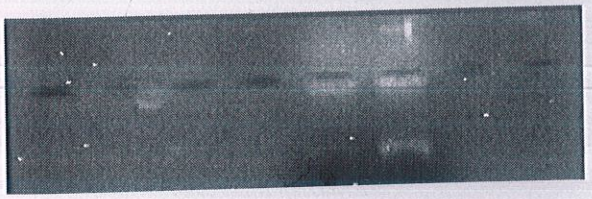
access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the potato accession is lyophilized, it can then be resuspended CTAB buffer. In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA must then be precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then resuspended and stored in TE buffer or sterile distilled water. To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualised under UV light.

Earlier we followed CTAB buffer methodology (Doyal and Doyal) to extract DNA from Potatoes:

Results Obtained

1)DNA Isolated from potato:

Fig 4: Potato DNA bands in UV before lyophilized tuber



2)DNA Isolation from Lypholized potato samples:

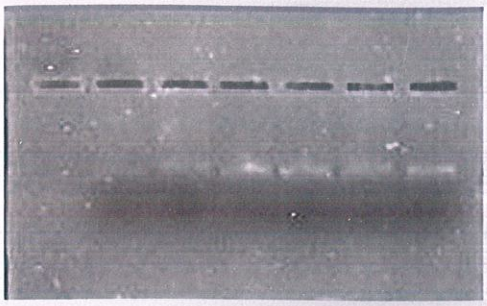


Fig 5: Potato DNA in UV after lyophilized tuber

- Results were good still we were not able to get the required DNA. We optimised the protocol an good quality DNA was observed to be better around peel. So, we lypholized the peel of Potatoes using CTAB buffer method to extract DNA but unfortunately did not obtained the desired results due to high percentage of carbohydrate contamination. We proceeded with other method for DNA extraction.

Extraction of DNA of Potato accessions –:

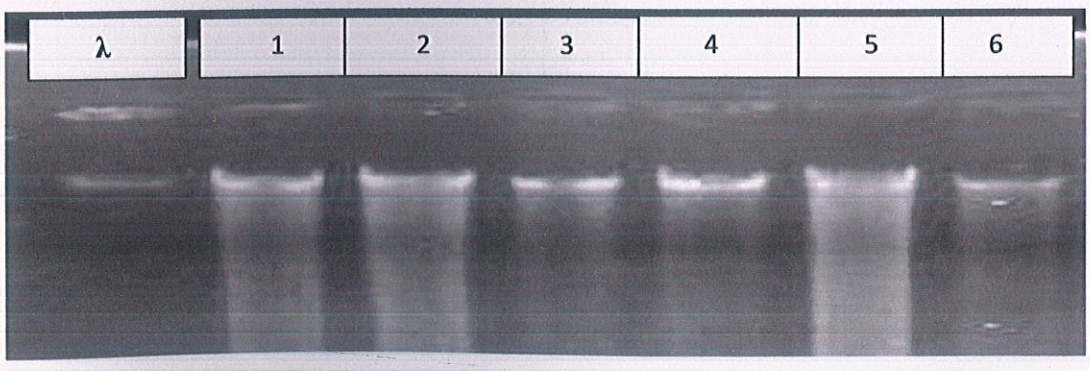


Fig 6: 1st well- K. Chipsona 1 DNA; 2nd well- K chipsona 3 DNA; 3rd well- K jyoti DNA; 4th well- K pubhraj DNA; 5th well- K Himsona DNA; 6th well- K frysona DNA

4. QUANTIFICATION OF AMYLOSE :-

Table 8: The table lists the amylose content of 6 varieties of Potato. The values are obtained from extrapolating from the standard amylose curve and based on which characterization is done.

Sample	Absorbance (Abs 620nm)	Value of Y (mg/ml)	Characterization
K Chipsona 1	1.4508	59.38026	Very High
K Chipsona 3	0.9703	39.703785	High
K Jyoti	0.6505	22.865975	Average
K Himsona	0.2696	11.01012	Very Low
K Frysona	0.8102	33.14769	High
K Pubhraj	0.2427	9.908565	Very Low

Table 9: Co-relation between amylose content and Glycemic index of potato

If	Then

Amylose content is high	Low Glycemic Index better nutritional value
Amylose content is low	High glycemic Index

Table 10: The table is used in making the graph shown below. The procedure followed to obtain these readings is described in the Materials and Methods section. A1, A2, and A3 are the triplicate values.

Standard	A1 (Abs 620nm)	A2 (Abs 620nm)	A3 (Abs 620nm)	Avg(Absorbance)
Blank	0	0	0	0
Standard	A1 (Abs 620nm)	A2 (Abs 620nm)	A3 (Abs 620nm)	Avg(Absorbance)
1	0.0245	0.0265	0.0293	0.026766667
2	0.05	0.046	0.0499	0.048633333
3	0.0776	0.0691	0.0693	0.072
4	0.0989	0.0989	0.0996	0.099133333
5	0.1239	0.1288	0.1176	0.123433333

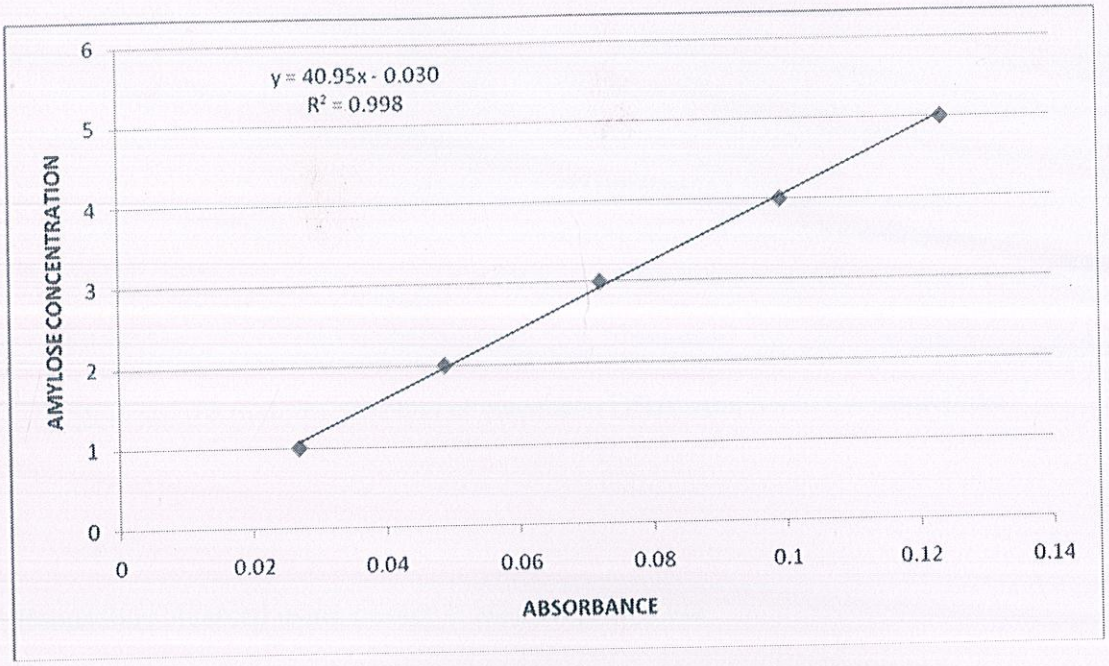


Fig 7: Graph for amylose concentration in six potato accessions

The graph was obtained by plotting the concentration on the Y-axis and Absorbance at 620nm on the X-axis for each of the set of values. It was observed that absorbance increased with the increase in the concentration

4.4 PRIMER STANDARIZATION

After the DNA was extracted, all the five primers had to be standardized at their respective tempratures for the appropriate amplification of our DNAz samples. This was done using PCR gradient for each primer at 6 different tempratures depending on their respective Tm (melting tempratures) as given in the primer list by G-biosciences Pvt. Ltd.

Table 11. The primers used along with their standardized temperatures for amplification.

Primer	Temperature(C_0)
First	58.5

Second	59
Third	59

4.5 DNA AMPLIFICATION

Below are the pictures attached of amplified DNA samples in the same order.

Bands thus obtained using primer 1, after amplification

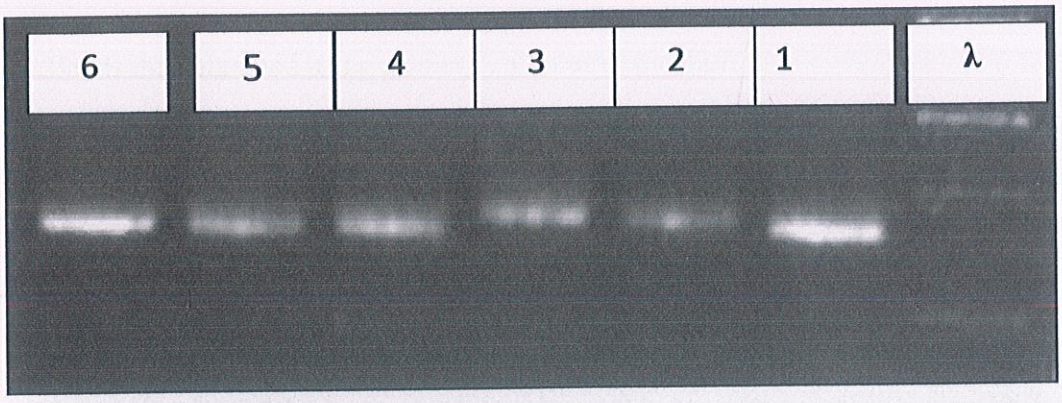


Fig 8. DNA amplification using Primer 1 at degree Celsius.

Bands thus obtained using primer 2, after amplification.

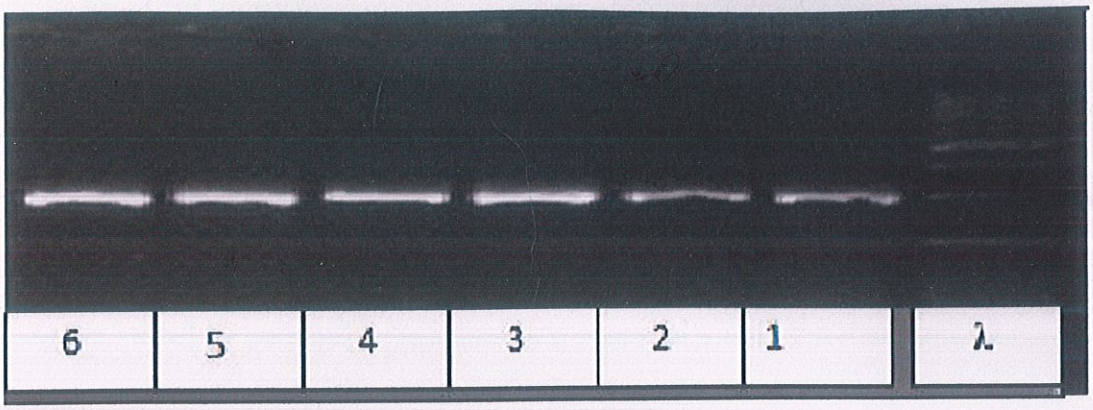


Fig 9. DNA amplification using Primer 2 at degree Celsius.

Bands thus obtained using primer 3, after amplification.

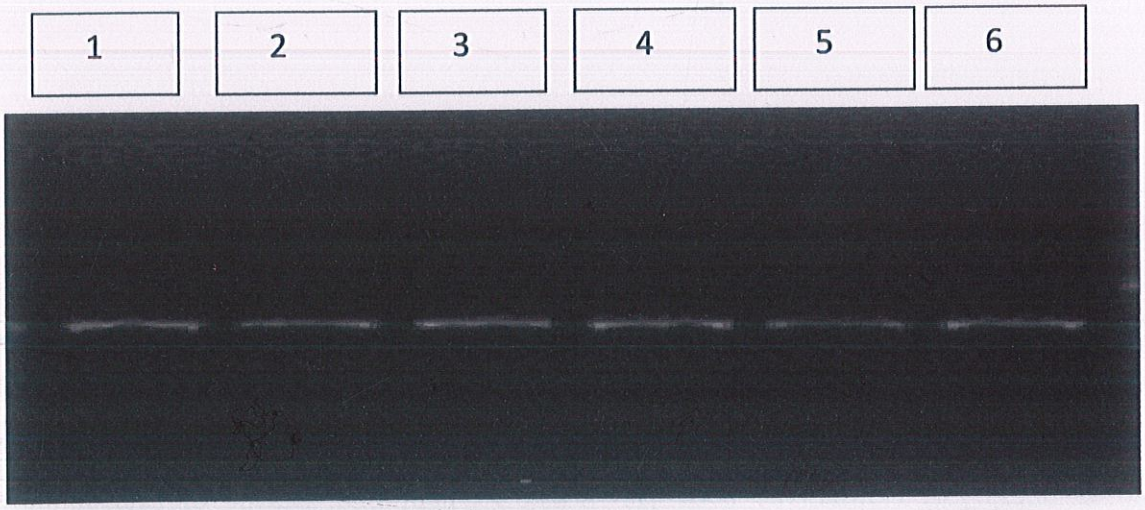


Fig 10. DNA amplification using Primer 3 at degree Celsius.

Table 12: List of Potato accession

S.No.	Name of the Potato Accessions
1.	Kufri Chipsona 1
2.	Kufri Chipsona 3
3.	Kufri Jyoti
4.	Kufri Pubhraj
5.	Kufri Himsona
6.	Kufri Frysona

4.7 SNP ANALYSIS

The discovery of large numbers of single nucleotide polymorphisms (SNPs) in genome-scale sequencing initiatives opens new avenues to the study of the genome-wide distribution of diversity and gene-based study (and it significance). Generally, SNPs are highly abundant but their density differs substantially in different regions of a genome and from genome to genome in many species, and even more so from species to species and may be effectively used as markers (Raymond *et al.*, 1999).With the recent background information available, the experiments were carried out to search the Granule bound starch synthase-I gene in potato and to identify the Single Nucleotide Polymorphisms (SNPs) within the gene of interest to study the natural variations at a single nucleotide level which may influence to ameliorate the quality of the product. The GBSS genes of potato were studied for their economical

importance and suspected impact on starch quality. The minor differences in starch amylose content resulting from a mutation at the GBSS loci are thought to influence the quality of potato. The GBSS-I gene of the potato genome sequence will be used for the identification of a single nucleotide polymorphism (SNP) in 6 genotypes of potato. The re-sequencing of the GBSS-I gene from different Indian genotypes will be done and comparisons will be made for identifying the variations in nucleotides. The multiple alignment feature which is known to be the simplest and the best way to detect will be employed using the ClustalW software for all the single contigs of GBSS-I gene including the *Solanum Tumersome* control sequence which was used for the gene amplifications as a template. All the nucleotide of the control DNA will be numbered starting from the $A^1T^2G^3$ and if there is any base deletion in the reference sequence, it will be left blank (-) by the ClustalW software itself. The SNP with reference sequence will be extracted using a simple Visual Basic script called 'SNP Extractor' .

CHAPTER-5

CONCLUSION

Once all the sequences for the amplified DNA samples will be obtained, they will be studied for any possible SNPs present in both the intron and exon regions of the entire sequences.

Identification of allelic series of variants of starch biosynthesis gene and their association with the starch quality will provide a potential tool for crop improvement.

FUTURE WORK

1. PCR amplification of left primers .
2. Identification of putative SNPs present in GBSS1.
3. The association findings will generate a possibility of developing elite accession through modification with selected major and/or minor starch synthesis-related genes.

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