NUCLEOTIDE DIVERSITY STUDY FOR STARCH SYNTHASE I AND STARCH SYNTHASE II GENES FOR PHYSICOCHEMICAL PROPERTIES IN RICE GENOTYPES

Under the Supervision of

Dr. HARVINDER SINGH

By

Ambika Sharma (091563) BTDD

Sukanya Verma (091575) BTDD

Submitted in partial fulfillment of the degree of

Bachelor of technology

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY – WAKNAGHAT (SOLAN)

2013

CERTIFICATE

This is to certify that the work entitled, **"Nucleotide diversity study for starch synthase I and starch synthase II genes for physicochemical properties in rice genotypes"** submitted by **Sukanya Verma** and **Ambika Sharma** in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology of **Jaypee University of Information Technology Waknaghat Solan** 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 the Supervisor:

Dr. Harvinder Singh

Assistant Professor

Date: 25/5/13

ACKNOWLEDGEMENT

Any assignment puts to litmus test an individual's knowledge, credibility and experience and thus, sole efforts of an individual are not sufficient to accomplish the desired work. Successful completion of a project involves interest and efforts of many people and so this becomes obligatory on my part to record thanks to them.

Therefore, first of all we would like to thank our guide and mentor **Dr. Harvinder Singh** for his guidance, help and constant encouragement throughout this project. Working under him was an enriching experience.

We express our heartfelt thanks to the Head of Department **Dr. R.S Chauhan** for providing us with the opportunity of doing this final year project.

Sukanya Verma

SUKANYA VERMA (091575)

AMBIKA SHARMA (091563)

DATE: 25/5/13

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

SSI	Starch synthase I
SSII	Starch synthase II
AAC	Apparent amylose content
GT	Gelatinization temperature
SNP	Single nucleotide polymorphism
CTAB	Hexadecyl trimethyl ammonium bromide
C:I	Chloroform isoamyl alcohol
EDTA	Ethylene diamine tetra acetic acid
Nacl	Sodium chloride
PVP	Polyvinyl pyrolidone
TE	Tris EDTA
TAE	Tris acetate EDTA
B-M	Beta-mercaptoethanol
EtBr	Ethidium bromide
dNTPs	Deoxyribonucleotide triphosphate
KI	Potassium iodide
NaOH	Sodium hydroxide
µl	Microliter
ml	Milli litre
PCR	Polymerase chain reaction

ABSTRACT

SSI and SSII are major genes involved in many grain quality properties such as amylose content and gelatinization temperature. SSII had a high association with pasting temperature, gelatinization temperature and peak time. These genes influence human health through its contribution to the glycemic index and levels of resistant starch. The incomplete digestion-absorption of resistant starch in the small intestine leads to non-digestible starch fractions with physiological functions similar to dietary fiber with significant beneficial impacts. The characteristics of starch, such as gelatinization temperature (GT), apparent amylose content (AAC), pasting temperature (PT) and other physicochemical properties, determine the quality of various products of rice such as eating, cooking and processing qualities e.g., low GT is preferred in manufacturing rice breads and beer.

Seventeen pair of primers was used for amplification of the two genes in sixteen varieties, out of seventeen primers only five primers gave consistent amplified product. The amplified PCR products are sent for sequencing so as to analyze them for SNP prediction. Amylose content is inversely related to the Glycemic index. If amylose content is high then Glycemic Index will be low and the rice grains will show high volume expansion (not necessarily elongation) and a high degree of flakiness. The rice grains cook dry, are less tender, and become hard upon cooling. In our study we estimated the amylose content for sixteen varieties. The lowest amylose content was observed in s3 variety i.e. basmati seed and intermediate amylose content was observed in s5 variety i.e. RR seed.

CHAPTER 1

INTRODUCTION

Rice is the seed of the monocot plants *Oryza sativa* (Asian rice) or *Oryza glaberrima* (African rice). As a cereal grain, it is the most widely consumed staple food for a large part of the world's human population, especially in Asia and the West Indies. It is the grain with the second-highest worldwide production, after maize (corn).

There are many varieties of rice and culinary preferences tend to vary regionally. In the Far East, there is a preference for softer and stickier varieties.

The many varieties of rice, for many purposes, are distinguished as long-, medium-, and short-grain rice. The grains of fragrant long-grain rice (high amylose) tend to remain intact after cooking; medium-grain rice (high amylopectin) becomes more sticky.

The awareness of the general public related to health foods has been on the rise recently and people are looking for the right variety of rice. Rice is a major human food composed largely of starch. (Kharabian-Masouleh et al 2012).

1.1 BACKGROUND

Starch or **amylum** is a carbohydrate consisting of a large number of glucose units joined by glycosidic bonds. It consists of two types of molecules: the linear and helical **amylose** and the branched **amylopectin.** Depending on the plant, starch generally contains 20 to 25% amylose and 75 to 80% amylopectin by weight. Glycogen, the glucose store of animals, is a more branched version of amylopectin.

AMYLOSE

Amylose is a linear polymer made up of D-glucose units.

Because of its tightly packed structure, amylose is more resistant to digestion than other starch molecules and is therefore an important form of resistant starch, which has been found to be an effective prebiotic.

Amylose is made up of α(1→4) bound glucose molecules. The carbon atoms on glucose are numbered, starting at the aldehyde (C=O) carbon, so, in amylose, the 1-carbon on one glucose molecule is linked to the 4-carbon on the next glucose molecule (α(1→4) bonds). **(**Ashish Jain *et al,* 'Effect of cooking on amylose content of rice", European Journal of Experimental Biology, 2012, 2 (2):385-388)

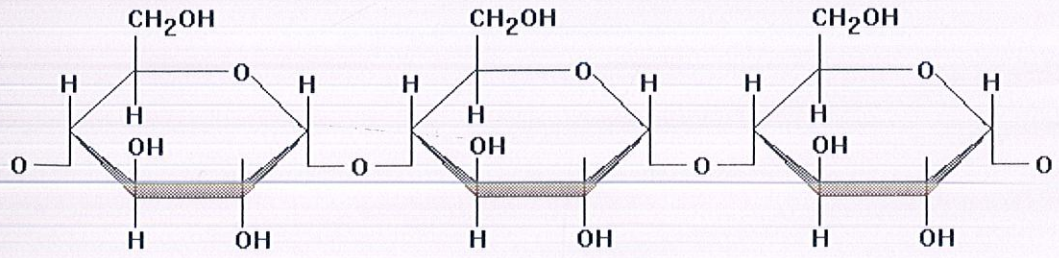


Fig.1 Amylose structure

AMYLOPECTIN

Amylopectin is a soluble polysaccharide and highly branched polymer of glucose found in plants. Glucose units are linked in a linear way with α(1→4) glycosidic bonds.

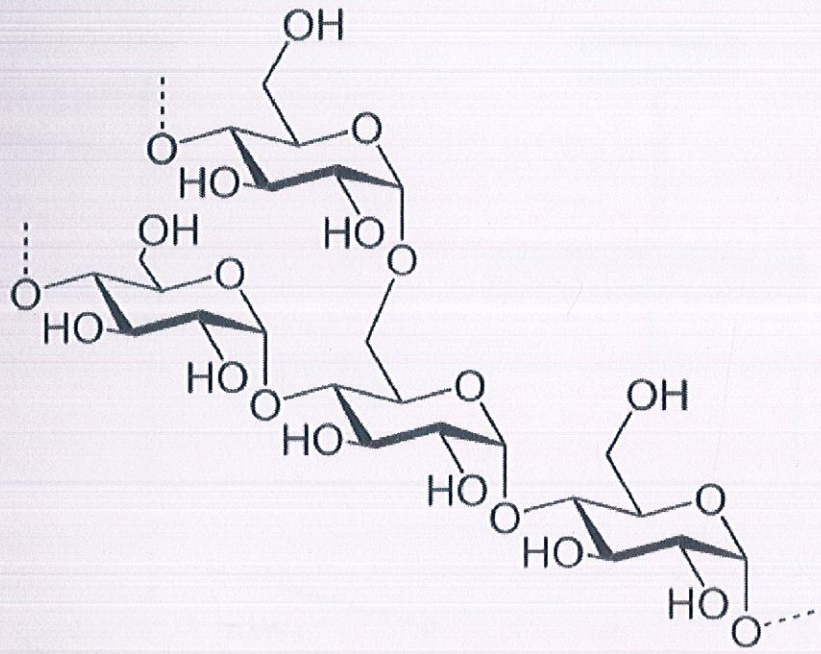


Fig.2 Amylopectin structure

STARCH METABOLISM PATHWAY

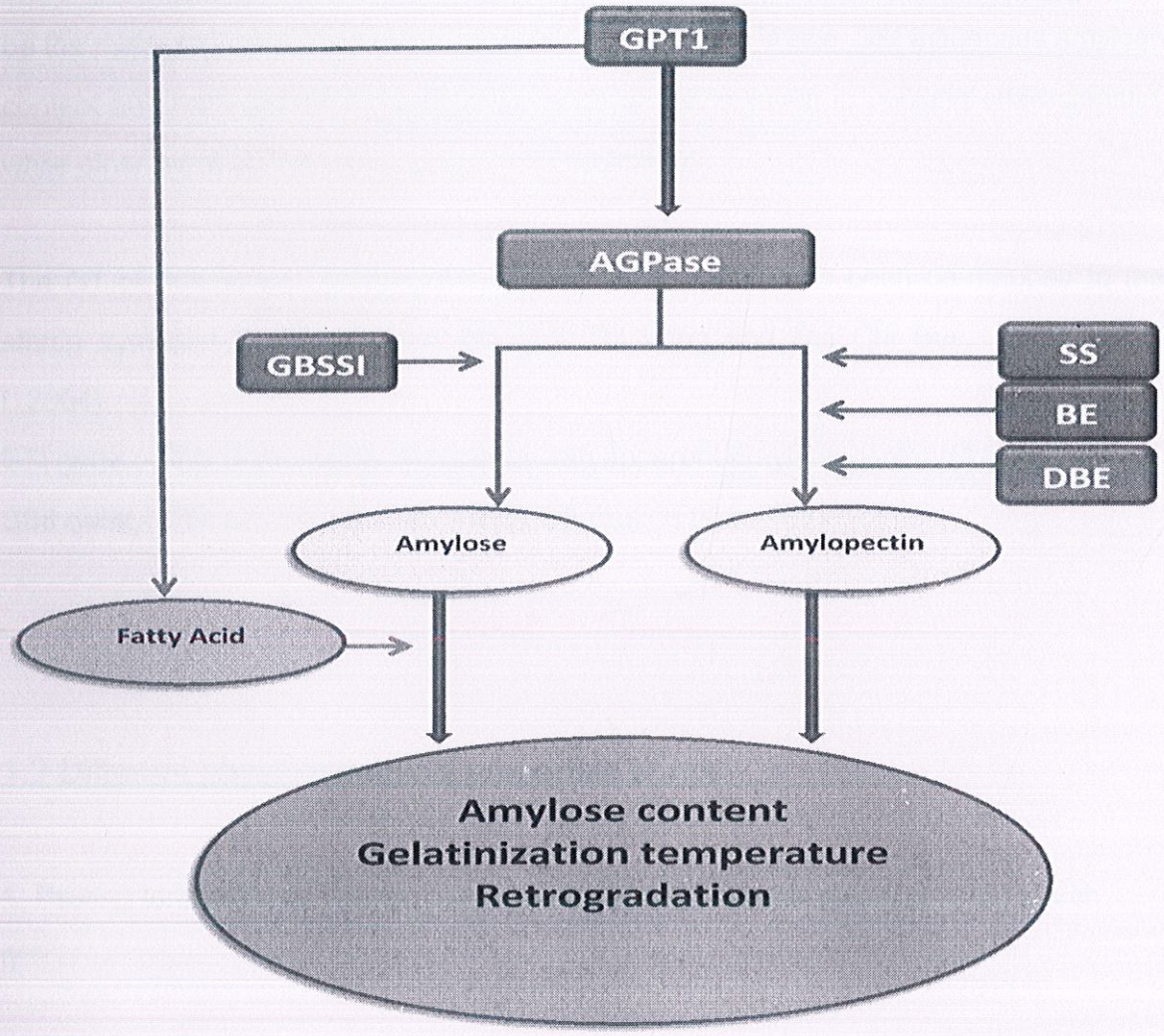


Fig.3 Starch metabolism pathway

There are 18 genes involved in the Starch biosynthetic pathway which are responsible for the control of physicochemical properties, out of which we are working on 2 genes **SSI (starch synthase I) and SSII (starch synthase II)** genes. These two genes appear to be the major loci controlling traits under human selection in rice, SSI influences amylose content and retro gradation, it explains much of the variation in cooking characteristic, while other gene SSII is contributing to retrogradation.

The GT of rice flour is controlled by the alk locus, which has been co-mapped to the starch synthase II (SSII) locus. (Nguyen Thi Lang and Bui Chi Buu. Oryza sativa L.2004).

SSI gene :- This gene mainly associates with apparent amylose content(AAC).

SSII gene:- This gene is involved with gelatinization temperature of rice.

1.2 Different physicochemical properties of rice

1. Pasting temperature: The temperature at which irreversible swelling of the starch granules occur.

2. Chalkiness: is determined by the clarity of the endosperm. If part of the milled rice kernel is not transparent (or opaque), it is often characterized as chalky. While chalkiness disappears upon cooking and has no effect on taste or aroma, it downgrades the quality of milled rice.

3. Gelatinization temperature: Gelatinization is the irreversible melting process of starch. It is an endothermic process, and the peak temperature at which starch absorbs heat is called gelatinization temperature (GT).There are several ways to measure GT. The most common method is alkali spreading value (ASV), for which the degree of disintegration of milled rice is graded after immersion in potassium hydroxide for 24 hours. A more modern assay is by differential scanning calorimetry (DSC). This detects the peak temperature absorbed by the flour-water mixture during gelatinization and is thus more precise than ASV.

4. Peak viscosity: - The peak viscosity is defined as the maximum viscosity that occurs prior to the initiation of sample cooling.

The highest viscosity reached during gelatinization of starch usually corresponding to the point where all the granules are swollen to occupy a high proportion of the available volume with each in contact with its immediate neighbors. Note that on cooling the starch paste, the viscosity may rise above this level, but only the initial shoulder or true peak values determined on the hot paste are termed peak viscosity. Hans-Dieter Belitz, Werner Grosch, Peter Schieberle, *Food chemistry*, Edition 3, Springer, page: 318-323, year: 2004.

5. Retrogradation: - Retrogradation is a reaction that takes place in gelatinized starch when the amylose and amylopectin chains realign themselves, causing the liquid to gel.

In this study we are trying to investigate the role of 2 starch-related genes and their SNPs by assessing their contribution to variation in starch properties in a rice breeding population.

1.3 RESEARCH GAP

With the advancement in time people are getting more aware about healthy food. However, in Indica rice consuming countries like India long grain rice with intermediate amylose and intermediate gelatinization temperature is preferred since it is soft, fluffy and non-sticky after cooking, improvement in rice starch quality, especially increasing the amylose level in high yielding rice with Amylose content (AC), is an important determinant. This will be the first study where association analysis will be carried out on Indica rice genotypes.

1.4 OBJECTIVE

a. Identification putative SNPs in sixteen rice genotypes.

b. Association analysis with starch quality (GT and AAC).

CHAPTER 2

REVIEW OF LITERATURE

Oryza sativa, commonly known as Asian rice, is the plant species most commonly referred in English as rice. Rice is an important crop in the world. *Oryza sativa* is the cereal with the smallest genome, consisting of just 430Mb across 12 chromosomes. It is renowned for being easy to genetically modify, and is a model organism for cereal biology.(CECAP, PhilRice and IIRR. 2000").

Oryza sativa was the cereal selected to be sequenced as a priority and has gained the status "model organism". It has the smallest genome of all the cereals: 430 million nucleotides and it can serve as a model genome for one of the two main groups of flowering plants, the monocotyledons. Because it has been the subject of studies on yield, hybrid vigor, genetic resistance to disease and adaptive responses, scientists have taken advantage of the existence of a multitude of varieties that have adapted to a very wide range of environmental conditions.

There are about 120,000 varieties known to exist. Two of the types sequenced are Indica and japonica.

Rice is well known for its divergence between the Indica and japonica subspecies.

1. **Japonica** varieties are usually cultivated in dry fields, in temperate East Asia, upland areas of Southeast Asia and high elevations in South Asia.

2. **Indica** varieties are mainly low land rice, grown mostly submerged, throughout tropical Asia. Rice is known to come in a variety of colors, including: white, brown, black, purple, and red.

The deep genetic structure in rice may be influenced by the natural history of ancestral populations prior to domestication, as well as by the autogamous breeding system and complexity of the breeding practices exercised by humans (Garris et al. 2005). Even in well designed studies, modest amounts of population stratification can still exist

(Freedman et al. 2004), indicating the difficulties in detecting and controlling population stratification as a source of false positive associations. To elucidate the mechanism of starch biosynthesis in amyloplast and chloroplast, plant starch-synthesizing enzymes have been identified, characterized, and cloned (reviewed by Preiss and Sivak.1995).

Starch properties determine the key functional properties of rice such as cooking temperature and influence human health through its contribution to the glycemic index and levels of resistant starch. The incomplete digestion-absorption of resistant starch in the small intestine leads to non-digestible starch fractions with physiological functions similar to dietary fiber with significant beneficial impacts.

Retrogradation describes the hardening of cooked starch after cooling due to re-crystallization of gelatinized starch components during storage. It is believed there is a significant correlation between the tendency of any one starch sample to retrograde and its levels of resistant starch. High-amylose rice cultivars usually have more resistant starch (RS) and lower estimated glycemic index (EGS), suggesting highly-retrograded cooked rice cultivars tend to a reduction of hydrolysis index (HI) and glycemic index (GI). Conversely, starch of low-amylose rice which have higher HI are more quickly hydrolyzed than intermediate and high-amylose rice (high HI). Characteristics of high amylose rice cultivars are normally determined by RVA (Rapid Visco Analysis) which are described by parameters such as peak viscosity (PKV), hot paste viscosity (HPV) and cool paste viscosity (CPV).

There are seven starch synthesis enzyme classes including ADP-glucose pyrophosphorylase (AGPase), granule bound starch synthase (GBSS), starch synthase (SS), branching enzyme (BE), debranching enzyme (DBE), starch phosphorylase (PHO) and glucose 6-phosphate translocator (GPT). These genes/enzymes contribute directly or indirectly to the production of starch granules.

Gene

ADP glucose pyrophosphorylase (AGPase)

Granule bound starch synthases (GBSSI and GBSSII)

Starch synthases (SSI, SSIIa, SSIIb, SSIIIa, SSIIIb, SSIVa, SSIVb)

Branching enzymes (BEI, BEIIa, BEIIb)

Debranching enzymes (ISA1, ISA2, Pullulanase)

Starch phosphorylase (SPHOL)

Glucose 6-phosphate translocator (GPT1)

The genes in red are those most correlated with starch properties, those in green do not explain variation in starch properties while genes in black have low to medium effects on rice starch quality.

Seven genes (AGPS2b, SPHOL, SSIIb, SSIVb and ISA1,BEIIa and BEIIb) of 18 do not contribute to starch physiochemical properties.

Starch synthase, which catalyzes the elongation of α-1,4-glucosidic bonds on amylose and amylopectin molecules by transfer of ADP-Glc, is present in two forms: SSS and GBSS enzymes (reviewed by Preiss, 1991). Two genes appear to be the major loci controlling traits under human selection in rice, GBSSI (waxy gene) and SSIIa. GBSSI influenced amylose content and retrogradation. Other genes contributing to retrogradation were GPT1, SSI, BEI and SSIIIa. SSIIa explained much of the variation in cooking characteristics. Other genes had relatively small effects.

The genes encoding SSI and GBSS are closely located on rice chromosome 6.

It is generally thought that GBSS is responsible for the synthesis of amylose in starch granules (Sprague et al., 1943; Nelson and Rines, 1962; Echt and Schwarz, 1981; Shure et al., 1983). The role of SSS in starch synthesis still remains unclear, although

this enzyme appears to play an important role(s) in the amylopectin synthesis by the cooperative reaction with branching enzyme and/or to participate in the amylose synthesis together with GBSS (Hagenblad et al. 2004).

Three apparent isoforms of SSS with molecular sizes of 55 and 57 kD in developing rice (Oryza *sativa* L.) seeds were identified (Baba et al., 1993). Analysis of the amino terminal amino acid sequence reveals that the three isoforms of SSS are identical except that the 55-kD isoform lacks the first eight amino acids at the amino terminus.

Thus, it appears that these three isoforms are products of the same gene, although additional evidence is necessary. The cDNA cloning of the rice SSS form (Baba et al., 1993), termed SSS1, demonstrates that this enzyme shares only a limited degree of sequence identity with rice GBSS (Wang et al., 1990) and *E.coli* glycogen synthase (Kumar et al., 1986). The gene expression pattern is also different between SSSI and GBSS; the SSSI gene is expressed at the same level in both leaves and developing seeds (Baba et al.,1993), whereas the transcript of the GBSS gene is present only in the developing seeds (Hirano and Sano, 1991). These facts may imply the distinct role(s) of SSSI and GBSS in starch synthesis. At any rate, because of the extreme differences of the exon/intron organizations between two genes encoding rice SSSI and GBSS and the significant differences between their primary structures

(Baba et al., 1993), it is possible that the two genes have evolved from an ancestral gene in a different way or that these genes are products of different ancestral genes that have converged during evolution.(Tanaka et al. Plant Physiol. Vol. 108, 1995).

The gene encoding starch synthase IIa (SSIIa), alk, is exclusively expressed in the rice endosperm and has been extensively studied in the context of its effect on cooking quality and starch texture. Two SNPs within exon 8, [A/G] and [GC/TT] are significantly associated with rice alkali disintegration and eating quality and starch gelatinization temperature.

Single nucleotide polymorphisms (SNP) are the most abundant type of genetic variation found within all species and many important plant traits and human diseases are attributed to these sequence variations. Identifying SNP and associating them with grain starch quality advances our understanding of the starch biosynthesis pathway and highlights ways to improve crops that are higher yielding and of better quality, directly impacting food security and human nutrition and health. Massively parallel sequencing (MPS) technology is a highthroughput platform for genetic analysis based on ultra deep DNA sequencing. Kharabian-Masouleh et al. (2011) discovered more than 501 SNPs and 113 In/dels in 17 starch synthesis genes in an Australian rice breeding population using a combination of a target pooled long range PCR and MPS. By combining MPS with high throughput genotyping technologies such as multiplexed-MALDI-TOF (Sequenom), rapid polymorphism discovery followed by association analysis is now possible.

Only one ´T/C´ nsSNP at position 5153 of this gene showed minor associations with FV, SB and Martin test (MT), with R2 values of 0.16, 0.11, 0.16, respectively SSIIa. Highly significant associations were found between SNP of SSIIa and PT, peak time (PKT),GT and breakdown viscosity. The highest Ftest value of 199.65 was observed for the [GC at position 4827–4828 of SSIIa and PT. This SNP is associated with PT, PKT and BDV with R2 values of 0.642, 0.323 and 0.168, respectively. This SNP has one of the strongest associations among the physiochemical properties studied in this rice population.

GBSSI and SSIIa are major genes involved in many grain quality properties such as amylose content and gelatinization temperature SSIIa had a high association with pasting temperature, gelatinization temperature and peak time.

Gelatinization is the irreversible melting process of starch. It is an endothermic process, and the peak temperature at which starch absorbs heat is called **gelatinization**

temperature (GT). GT is one of the key traits measured in breeding programs in rice. It is important because it affects the texture of cooked rice and it is said to be related to the cooking time of rice. Given the number of times that rice is cooked in the world every day, selecting rice varieties with low GT represents huge potential savings in fuel consumption. The effect of this gene on cooking quality and starch texture has been extensively studied by many authors. Highly significant associations were found between SSIIa SNPs and important physiochemical properties such as PT, PKT, GT and BDV. Melting of starch crystalline regions is measured by pasting and gelatinization temperature and peak time (Bao, et al.2006).

The characteristics of starch, such as gelatinization temperature (GT), apparent amylose content (AAC), pasting temperature (PT) and other physicochemical properties, determine the quality of various products of rice, e.g., eating, cooking and processing qualities. The GT of rice flour is controlled by the alk locus, which has been co-mapped to the starch synthase IIa (SSIIa) locus. Differences in starch properties of rice grains determine their eating, cooking and processing qualities.

Apparent amylose content (AAC) and gelatinization temperature (GT) are two of the well-established parameters used to evaluate these qualities (Juliano 1998; Bergman et al. 2004). For example, low-GT rice have a softer texture than high-GT rice among freshly cooked waxy and low-AAC rice; and among intermediate-and high-AAC rice, intermediate-GT rice are softer than low-GT rice when freshly cooked, but they have similar hardness values on accelerated staling (Perez et al. 1993). Different rice products require different types of rice with different starch properties, e.g., low GT is preferred in manufacturing rice breads and beer (Juliano 1998). Knowledge of the relationship between physicochemical properties and end-use qualities can direct breeding activities in selecting desired rice with unique qualities. However, lack of information on the genetic basis and related techniques for selection retards the breeding progress. The GT of starch can be measured indirectly by alkali spreading

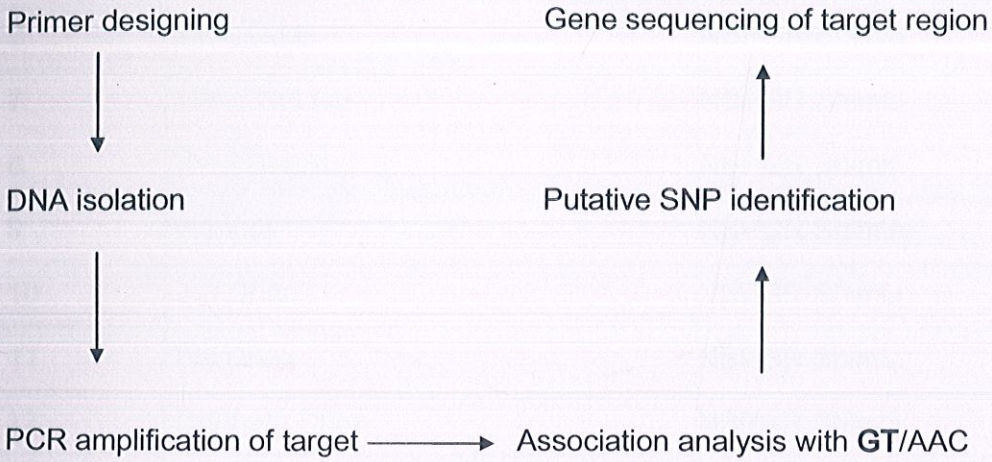
value or directly by differential scanning calorimetry (DSC) as peak temperature (Tp) from the endotherm. The pasting temperature (PT) measured by visco amylography such as using Rapid Visco (McKenzie and Rutger 1983).

Umemoto et al. (2002) reported that the starch synthase IIa (SSIIa) gene is located at the alk locus on chromosome 6 in the rice genome. Association analyses have also been conducted for the SSIIa gene in relation to GT, but only two or three SNPs were used in such studies (Chen et al. 2003; Umemoto et al. 2004), resulting in the same haplotypes consisting of rices with both high and low GT. Furthermore, previous studies began with alignment of SSIIa sequences to find SNPs among a few rice (Chen et al. 2003; Umemoto et al.2004), e.g., the two genome-sequenced rice cultivars, 93-11 and Nipponbare (Chen et al. 2003). The few sequences studied unlikely represent all naturally occurring variations in this gene. Therefore, it is necessary to search for more SNPs from a different set of germplasm in order to find those that can differentiate high- and low-GT rice varieties. Furthermore, nucleotide diversity in SSIIa has not been investigated. The SSIIa plays an important role in the elongation of short chains of DP < 10 that leads to the formation of intermediate chains of amylopectin, which is especially responsible for gelatinization of starch (Umemoto et al. 2002; Gao et al. 2003; Jiang et al. 2004; Nakamura 2002). The present association analysis also indicated that the SSIIa GC/TT explained more than 60% of the total variation of the phenotypes. The present association study indicates that both Wx and SSIIa SNP loci are important in controlling these traits, each explaining > 50% of the total variation for both traits (Theor Appl Genet .2006).

CHAPTER 3

MATERIALS AND METHODS

Strategy flowchart:-



Seeds of twenty varieties of rice were procured from NBPGR Shimla and NBPGR Delhi (Table 1). For good germination dormancy breakdown treatment was given to the genotypes by soaking them in 500 ppm gibberelic acid overnight at temperature 37°C.

TABLE NO.1 List of genotypes used in the study

S/No	Variety Name	
1	Navri Nut (white)	NBPGR Shimla
2	Rhodu Red	NBPGR Shimla
3	Jatoo Dhan	NBPGR Shimla
4	Basmati	NBPGR Shimla
5	Ram Jawain	NBPGR Shimla
6	Kala Dhan	NBPGR Shimla
7	Navri Nut Lal	NBPGR Shimla
8	Phul Patash	NBPGR Shimla
9	Rangoli	NBPGR Shimla
10	Ziri Dhan	NBPGR Shimla
11	Lal Dhan	NBPGR Shimla
12	Chuhetu Dhan	NBPGR Shimla
13	Jaulia Dhan	NBPGR Shimla
14	Rhodu White Dhan	NBPGR Shimla
15	Kard Dhan	NBPGR Shimla
16	Sukara Dhan	NBPGR delhi
17	S2 Sugandh seed	NBPGR delhi
18	S3 Basmati Seed	NBPGR delhi
19	S4 Duplicate Basmati Seed	NBPGR delhi
20	S5 RR Seed	NBPGR delhi

3.1. PRIMER DESIGNING:-

A primer is a strand of nucleic acid that serves as a starting point for DNA synthesis. They are required for DNA replication because the enzymes that catalyze this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA. The polymerase starts replication at the 3'-end of the primer, and copies the opposite strand.

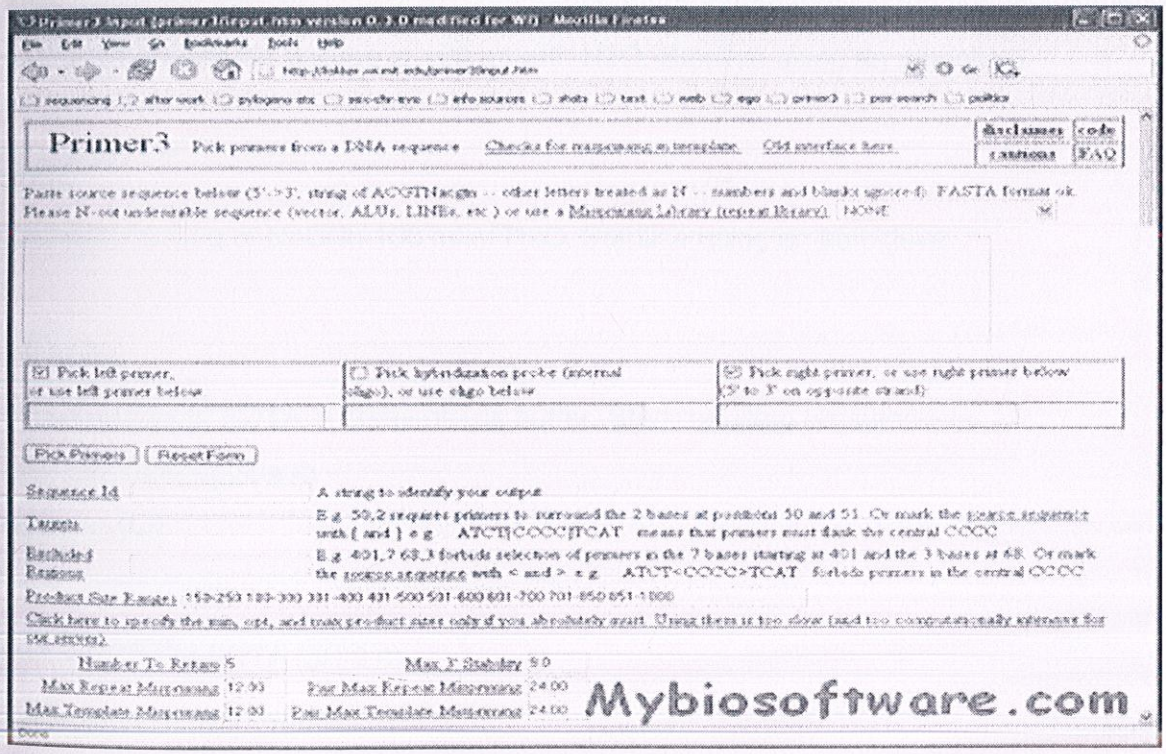


Fig.4: Screenshot of primer 3: a primer designing tool

3.2 DNA ISOLATION

Chemicals preparation:-

1 M Tris (pH 8.0)

Dissolve 12.11 g of Tris base in 80 ml of H2O. Adjust pH to 8.0 by adding concentrated HCL. Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume to 100 ml with H2O. Sterilize using an autoclave.

0.5 M EDTA (pH 8.0)

Dissolve 18.612 g of EDTA in 80 ml of dH2O. Adjust pH to 8.0 by adding concentrated HCL. Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume to 100 ml with H2O. Sterilize using an autoclave.

NaCl

Dissolve 29.22 g of NaCl in 100 ml of H2O. Sterilize using an autoclave.

TE buffer

Dissolve 2 ml of EDTA in 10 ml of Tris buffer. Sterilize using an autoclave.

50x TAE buffer (pH 8.5)

242 g Tris cl

57.1 g acetic acid

100 ml of 0.5M EDTA (pH 8.0)

Make up to 1L with water.

To make a 1x working solution, dissolve 2ml of 50x TAE buffer in 98 ml of dH2O.

0.8% Agarose gel

0.8g Agarose dissolved in 100 ml TAE

Protocol (CTAB method)

1. Grind 200 mg of plant tissue to a fine powder in liquid nitrogen.
2. Take the sample in microfuge tubes (2ml).
3. Add 700µl prewarmed cTAB buffer/extraction buffer in microfuge tubes.
4. Incubate the cTAB/plant extract mixture for about 1-2 hr at 60°C in a recirculating water bath.
5. After incubation add 700µl of Chloroform : Isoamyl Alcohol (24:1) and mix the solution by inversion.
6. Spin/centrifuge the CTAB/plant extract mixture @10000 rpm for 10-15 minutes
7. Transfer the supernatant to clean microfuge tubes(1.5ml).
8. Add 500 µl of chilled isopropanol(-20°C). Mix well.
9. Keep it at -20°C for 1 hr.
10. Centrifuge @10000 rpm for 10 minutes.
11. Discard the supernatant.
12. Wash the pellet with 200µl of 70% ethanol(chilled).
13. Centrifuge @10000 for 10 minutes.
14. Discard the supernatant and repeat 70% ethanol washing step for 1-2 minutes.
15. Dry the pallet. There should not be any smell of ethanol left.
16. Dissolve the pellet in 50µl of TE buffer.
17. Keep it at 4°C overnight.

DNA quantification: Agarose gel electrophoresis

1. Prepare 0.8% solution of agarose by melting 0.8 g of agarose in 100ml of 1X TAE buffer/ running buffer in a microwave for approximately 2 min.
2. Allow to cool for a couple of minutes then add 5µl ethidium bromide, stir to mix.
3. Cast a gel using a supplied tray and comb. Allow the gel to set for a minimum of 20 min at room temperature on a flat surface.
4. After proper solidification of gel remove the comb and keep gel in gel running tank. Pour running buffer gently.
5. Load samples with DNA loading dye in separate wells.
6. Run the gel for 45-1hr at 100 V.
7. Expose the gel to UV light and photograph.
8. Confirm DNA quality, presence of a highly resolved high molecular weight band indicates good quality DNA, presence of a smeared band indicates DNA degradation.

3.3 PCR (polymerase chain reaction)

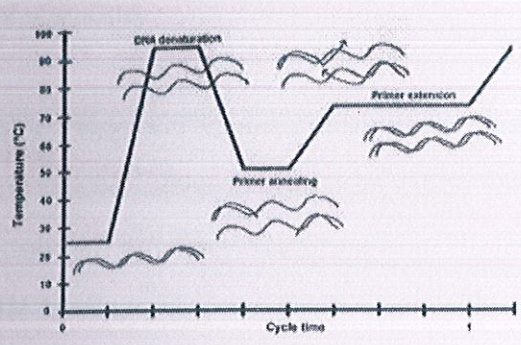


Fig.5 Strategy for Polymerase chain reaction

PCR Programme Conditions

Stage 1		
Initial denaturation	94°C	4 min
Stage 2		
Denaturation	94°C	1 min
Annealing	Tm	1 min
Extension	72°C	2 min
Stage 3		
Final extension	72°C	7 min

Number of Cycles - 35 cycles

3.4 AMYLOSE ESTIMATION (potassium iodide method):-

The following reagents were 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.

Standard Amylose Solution- Take 40mg of pure potato starch (amylose) in a 100 ml volumetric flask and add 1 ml of 95% ethanol and 9.0 ml of 1N NaOH. Shake well and boil over water bath for 10 minutes and make up the solution to 100 ml using distilled water.

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 blank, 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

4.1 Insilico analysis:

SSI GENE was downloaded from gramene database with following detail:

Gramene result:

Identification no. LOC_os06g06560

Sequence- SSI GENE

Location-MSU6:6:3077460:308640

Chromosome: 6

SSII GENE was downloaded from gramene database with following detail:

Gramene result:

Identification no. LOC_os06g12450

Sequence- SSII GENE

Location-MSU6:6746759:6752939

Chromosome: 6

Table No 2: List of primers with guanine/cytosine content (GC%)and annealing temperature (TM)

List of primers	Sequences of primers	GC%	TM	LENGTH
SS-I_1F	ACAAGGGGTGGAAAAATGTG	45.00	59.69	20
SS-I_1R	TCAGGGCACAAGCATTACAC	50.00	59.72	20
SS-I_2F	ACCCTCCCAAGAGGACTTGT	55.00	59.97	20
SS-I_2R	GCATTCTCGCCACTAACCAT	50.00	60.10	20
SS-I_3F	TTGATGTTAAGAGGGCCAAT	40.00	57.15	20
SS-I_3R	GGTTTCTGGCTTTCAACTGG	50.00	59.71	20
SS-I_4F	TCGATTTCAGTTGTTGGCTA	36.36	59.76	22
SS-I_4R	CTTTGCACCTCGTCGTTTCT	50.00	60.43	20
SS-I_5F	TCCTCCTGCAATTCAGCT	45.00	59.96	20
SS-I_5R	GCACATCAGGTTTGAGCCTA	50.00	58.88	20
SS-I_6F	GGCTCCACACCCTATCACAT	55.00	59.81	20
SS-I_6R	TGCTTTGTGTTTGTGTGTGTCTA	39.13	59.35	23
SS-I_7F	AGTTCTCCTGCGCTTTTGAG	50.00	59.76	20
SS-I_7R	CTTCTGTTTGCAACAAGCACT	42.86	58.23	21
SS-I_8F	CCCACATGTTCACTTTCAAGA	42.86	58.61	21
SS-I_8R	AAGGGAGCAATGACAACCTG	50.00	60.11	20
SS-I_9F	CATCTTGGCATGGCAGTACA	50.00	60.69	20
SS-I_9R	GTCCTGCCGACCTTCCTC	66.67	60.77	18

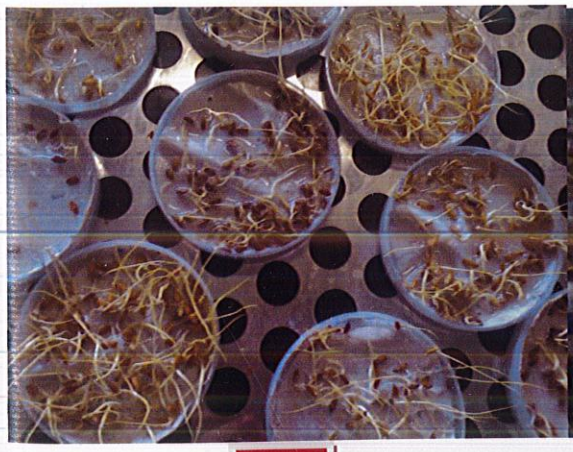
SS-II_1F	AAGAGAGACCCATAGACACAGTA	38.46	58.8	26
SS-II_1R	GGTTTAAGCGCTTCATCA	38.10	59.38	21
SS-II_2F	GTTGGTTTTGGGGTTTTGG	47.37	60.05	19
SS-II_2R	GTGAGGCAGGTGTGTCGTC	63.16	60.30	19
SS-II_3F	ACGCCGCCTCAAGTAAGAA	52.63	60.92	19
SS-II_3R	GCCTTTTTAATTTGCCATCA	35.00	57.7	20
SS-II_4F	TCCTTCATGGTTGGATGTTTC	42.86	59.78	21
SS-II_4R	TGACGGTGACGGAAGAGAG	57.89	59.96	19
SS-II_5F	TGCAGGATCTGGAAGTGAAA	45.00	59.37	20
SS-II_5R	AACAGCAGGAGATGCATACA	40.91	59.77	22
SS-II_6F	CACTCGCTCTGTCCTTGTGA	55.00	60.18	20
SS-II_6R	ACCTTTCTGCCCGTCGAG	61.11	61.36	18
SS-II_7F	GACGGCTACGCCAACTACAC	60.00	60.72	20
SS-II_7R	ATGCCTCGCCATGAACAC	55.56	60.66	18
SS-II_8F	GTTTGTGCTGACCGAGCTGT	55.00	61.46	20
SS-II_8R	GAGGGACATCCCCTTGTTATT	47.62	59.26	21

4.2 Seed germination:-

Seeds of twenty varieties of rice were procured from NBPGR Shimla and NBPGR Delhi (Table 1). For good germination dormancy breakdown treatment was given to the genotypes by soaking them in 500 ppm gibberelic acid overnight at temperature 37 °C.

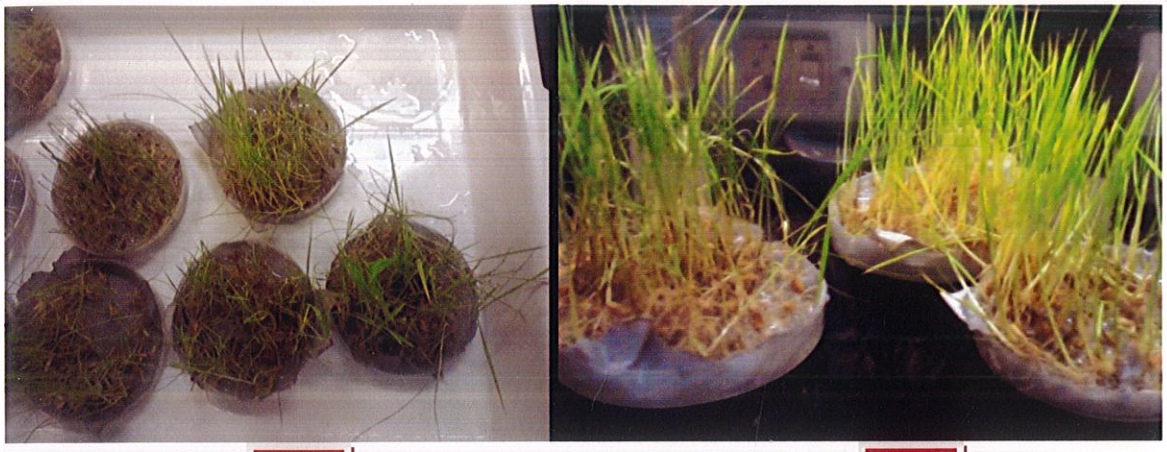
A

B



C

D



E F

Fig.6 **Different rice varieties growing in Petri plates containing bloating paper.**

a. Day 1 seeds covered with bloating paper.

b. Day 5 seeds started to germinate and upper layer of bloating paper has been removed.

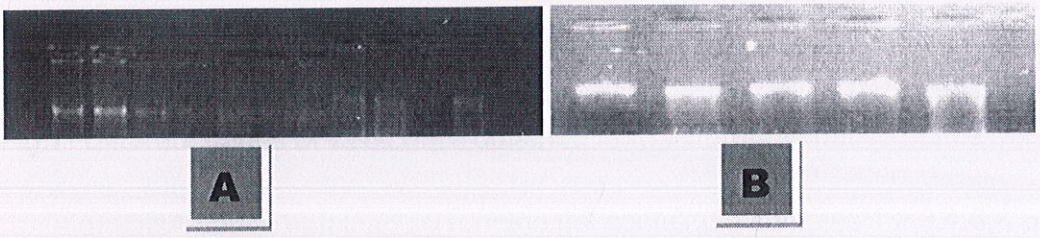
c. Seedlings of day 8.

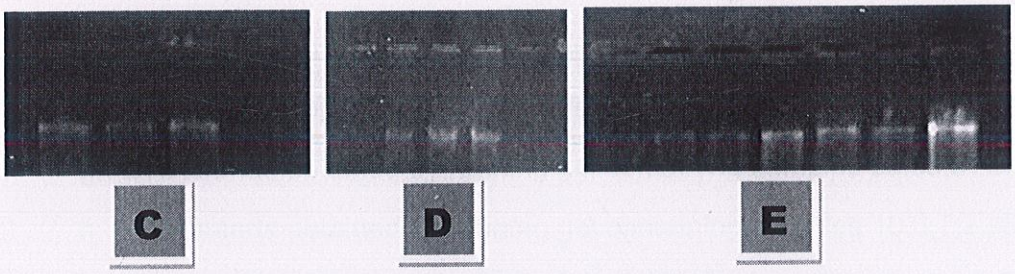
d. Seedlings of day 14.

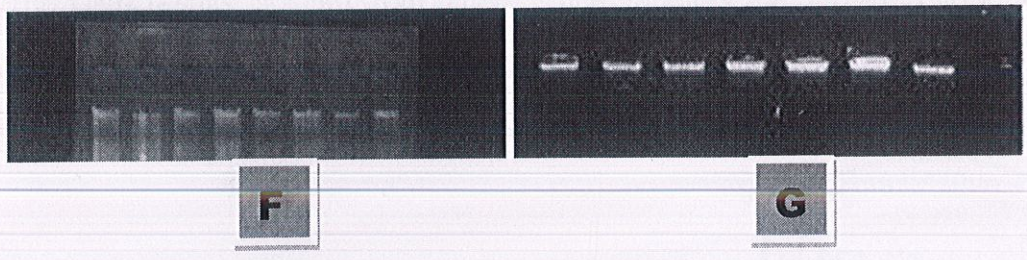
e. Seedlings of day 18.

f. Seedlings of day 24.

4.3 DNA isolation result







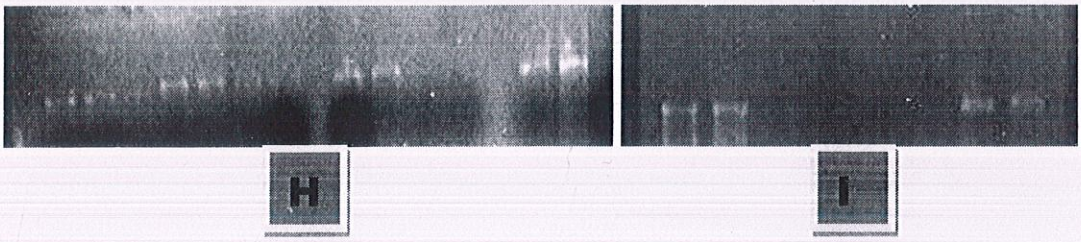


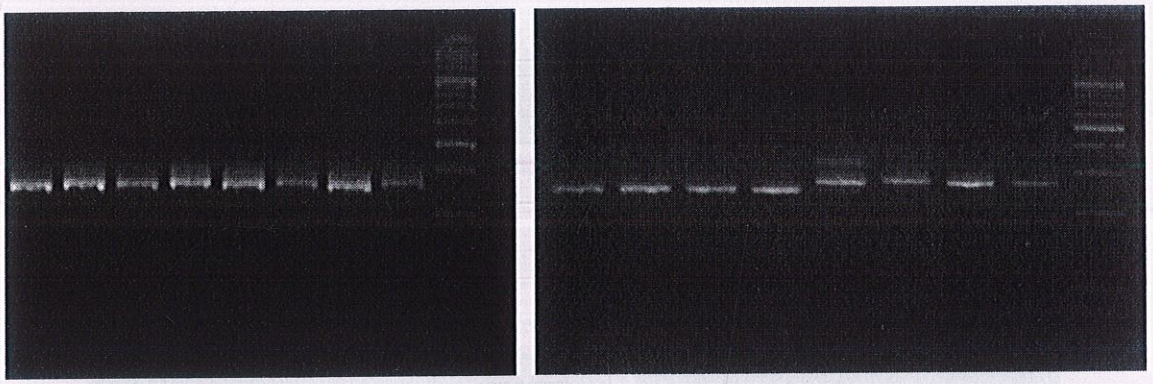
Fig.7 Different bands of DNA were obtained from different varieties of rice seedlings.

a. DNA bands obtained by running gel containing samples 1,2,3,4,5,& 6.
b. DNA bands obtained by running gel containing samples 3,4 & 5.
c. DNA bands obtained by running gel containing samples 6 & 7.
d. DNA bands obtained by running gel containing samples 8,9 & 10 .
e. DNA bands obtained by running gel containing samples 10,11 & 12.
f. DNA bands obtained by running gel containing samples 13,14,15 & 16.
g. DNA bands obtained by running gel containing samples s2,s3,s4 & s5.
h. DNA bands obtained by running gel containing samples 10,6,7,2,s3,s4 & 15.
i. DNA bands obtained by running gel containing samples 11,12,s5 &s2.

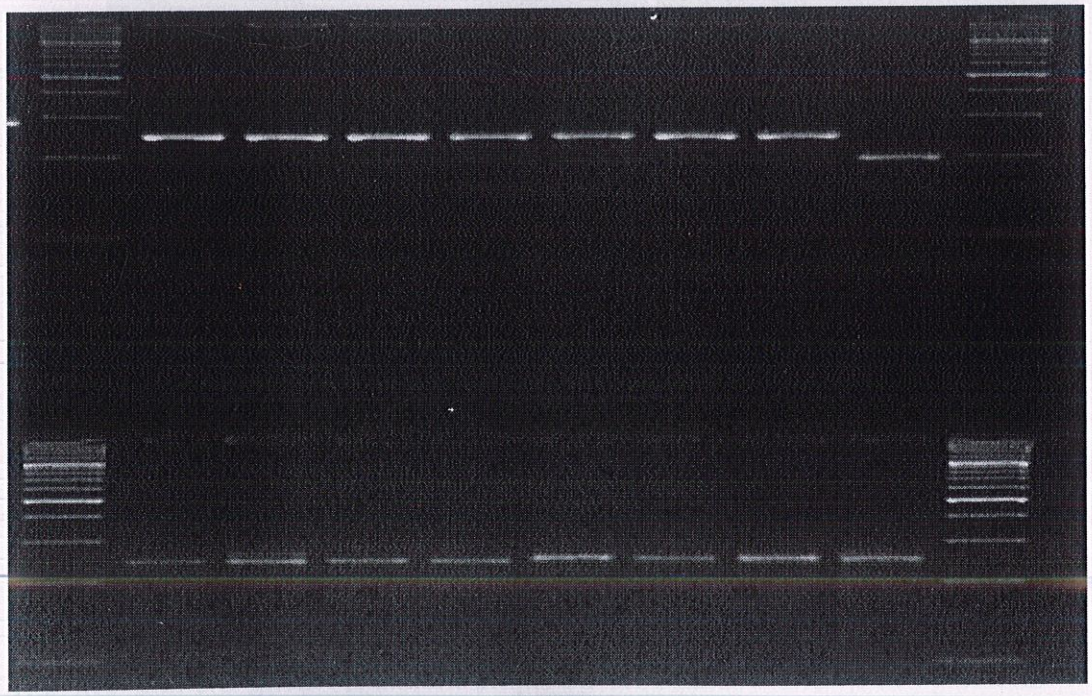
The DNA results isolated from different rice varieties are shown in fig.7. These were isolated by using CTAB method.

4.4 PCR amplified product

PCR Amplification for SSI and SSII genes and Temperature Optimization

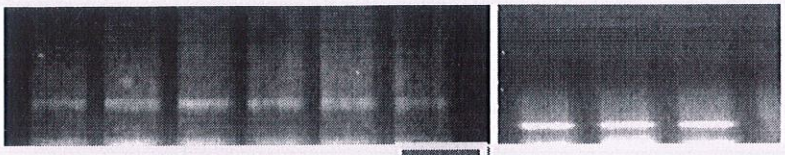


A

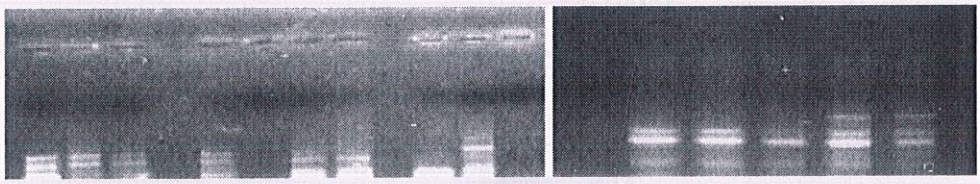


B

C



D



E

Fig: 8 Optimized Primers

A. Primer ss1(6F/6R) Tm-58°C

B. Primer ss2(7F/7R) Tm-56°C

C. Primer ss1(3F/3R) Tm-55°C

D. Primer ss1(4F/4R) Tm-48°C

E. Primer ss2 (5F/5R) Tm-55°C

The five primers gave consistent amplified product are shown in fig.8. The amplified PCR products are sent for sequencing so as to analyze them for SNP prediction.

4.5 Amylose estimation results

Quantification Of Amylose:

Standard	A1 (Abs 620nm)	A2 (Abs 620nm)	A3 (Abs 620nm)	Avg(Absorbance)
Blank	0	0	0	0
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

Table no 3: This 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 triplicated values.

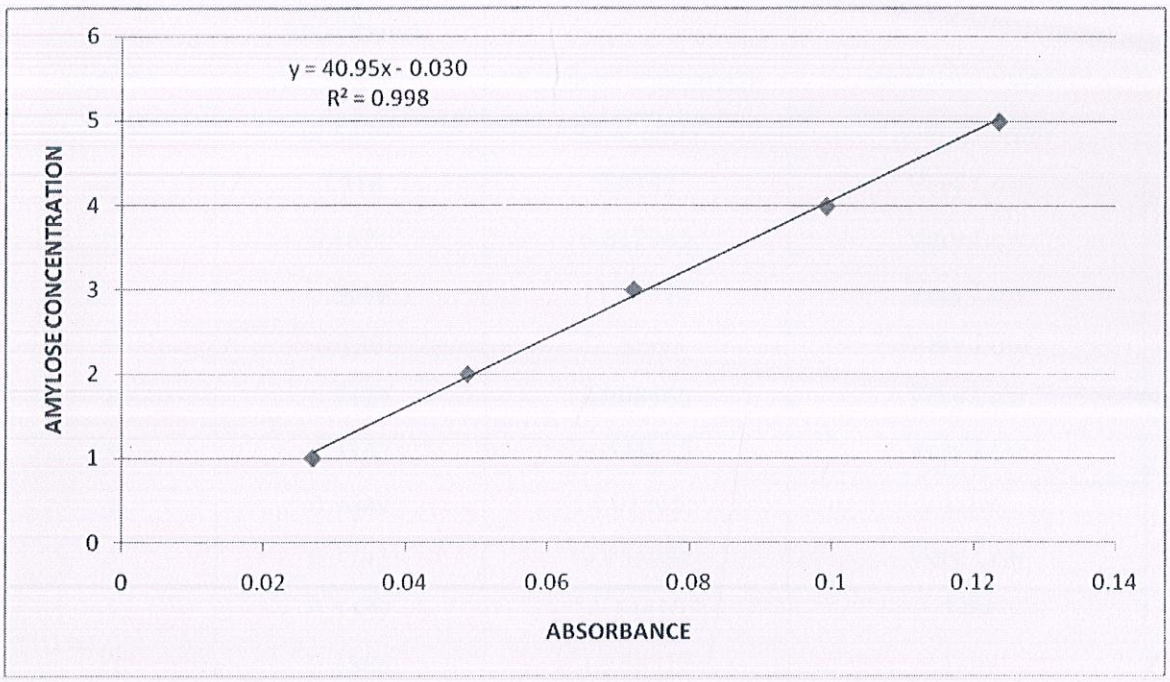


Fig.9 The above graphs are obtained by taking Concentration on the Y-axis and Absorbance at 620nm on the X-axis for each of the set of values.

Amylose Quantification on 20 Indica Varieties

Sample	Absorbance (Abs 620nm)	Value of Y (mg/ml)	Characterization
S5	0.5479	22.406505	Intermediate
s2	0.216	8.8152	Very Low
S3	0.1671	6.812745	Very Low
S4	0.2696	11.01012	Very Low
7	0.189	7.70955	Very Low
1	0.2427	9.908565	Very Low
2	0.2357	9.621915	Very Low
3	0.3649	14.912655	Low
4	0.2243	9.155085	Very Low
5	0.3559	14.544105	Low
11	0.3886	15.88317	Low
12	0.322	13.1559	Low
13	0.346	14.1387	Low
14	0.2897	11.833215	Very Low
15	0.3756	15.35082	Low
6	0.2546	10.39587	Very Low
8	0.3444	14.07318	Low
9	0.2336	9.53592	Very Low
10	0.2419	9.875805	Very Low
16	0.3384	13.82748	Low

Table no 4: This table lists the amylose content of 20 varieties of rice. The values are obtained from extrapolating from the standard amylose curve and based on which characterization is done.

If	Then
Amylose content is high	Low Glycemic Index and the rice grains will show high volume expansion (not necessarily elongation) and a high degree of flakiness. The rice grains cook dry, are less tender, and become hard upon cooling.
Amylose content is low	High glycemic Index and the rice grains will cook moist and sticky

Table no 5: Co-relation between amylose content and Glycemic index of rice . It is seen that amylose content is inversely related to the Glycemic index.

CHAPTER 5

CONCLUSION

SSI and SSII are major genes involved in many grain quality properties such as amylose content and gelatinization temperature. SSII had a high association with pasting temperature, gelatinization temperature and peak time.

These genes influence human health through its contribution to the glycemic index and levels of resistant starch. The incomplete digestion-absorption of resistant starch in the small intestine leads to non-digestible starch fractions with physiological functions similar to dietary fiber with significant beneficial impacts.

The characteristics of starch, such as gelatinization temperature (GT), apparent amylose content (AAC), pasting temperature (PT) and other physicochemical properties, determine the quality of various products of rice such as eating, cooking and processing qualities e.g., low GT is preferred in manufacturing rice breads and beer.

Seventeen pair of primers was used for amplification of the two genes in sixteen varieties, out of seventeen primers only five primers gave consistent amplified product. The amplified PCR products are sent for sequencing so as to analyze them for SNP prediction.

Amylose content is inversely related to the Glycemic index. If amylose content is high then Glycemic Index will be low and the rice grains will show high volume expansion (not necessarily elongation) and a high degree of flakiness. The rice grains cook dry, are less tender, and become hard upon cooling. If amylose content is low then glycemic Index will be high and the rice grains will cook moist and sticky.

The lowest amylose content of 6.812745 mg/ml was observed in s3 variety i.e. basmati seed and intermediate amylose content of 22.406505 mg/ml was observed in s5 variety i.e. RR seed.

Band amplified from the primers are sent for sequencing. After the results are received the sequences will be subjected to insilico analysis for prediction of putative SNP's and these SNP's will be further validated to rule out the sequencing errors. Once the SNP's are validated they will be associated with gelatinization component.

CHAPTER 6

REFERENCES

1. Asp, N. G. & Bjo¨rck, I. Resistant starch. Trends Food Sci Tech 3, 111–114 (1992).

2. Fan, J. &Marks, B. Retrogradation kinetics of rice flours as influenced by cultivar. Cereal Chem 75, 153–155 (1998).

3. Faisant, N. et al. Structural features of resistant starch at the end of the human small intestine. Eur J Clin Nutr 47, 285 (1993).

4. Ring, S.G., Gee, J. M.,Whittam,M., Orford, P.&Johnson, I. T. Resistant starch: its chemical form in foodstuffs and effect on digestibility in vitro. Food Chem 28, 97–109 (1988).

5. Philpot, K., Martin, M., Butardo Jr, V., Willoughby, D. & Fitzgerald, M.Environmental factors that affect the ability of amylose to contribute to retrogradation in gels made from rice flour. J Agric Food Chem 54, 5182–5190 (2006).

6. Hu, P., Zhao, H., Duan, Z., Linlin, Z. & Wu, D. Starch digestibility and the estimated glycemic score of different types of rice differing in amylose contents. J Cereal Sci 40, 231–237 (2004).

7. Chung, H. J., Lim, H. S. & Lim, S. T. Effect of partial gelatinization and retrogradation on the enzymatic digestion of waxy rice starch. J Cereal Sci 43, 353–359 (2006).

8. Chen, M. H., Bergman, C., Pinson, S. & Fjellstrom, R. Waxy gene haplotypes: Associations with apparent amylose content and the effect by the environment in an international rice germplasm collection. J Cereal Sci 47, 536–545 (2008).

9. Cai, X. L.,Wang, Z. Y., Xing, Y. Y., Zhang, J. L. & Hong, M. M. Aberrant splicing

of intron 1 leads to the heterogeneous 5 UTR and decreased expression of waxy gene in rice cultivars of intermediate amylose content. Plant J 14, 459–465 (1998).

10. Larkin, P. D. & Park, W. D. Association of waxy gene single nucleotide polymorphisms with starch characteristics in rice (Oryza sativa L.). Mol Breed 12, 335–339 (2003).

11. Umemoto, T. et al. Effects of Variations in Starch Synthase on Starch Properties and Eating Quality of Rice. Plant Prod Sci 11, 472–480 (2008).

12. Umemoto, T. et al.Natural variation in rice starch synthase IIa affects enzyme and starch properties. Funct Plant Biolo 31, 671–684 (2004).

13. Waters, D. L. E., Henry, R. J., Reinke, R. F. & Fitzgerald, M. A. Gelatinization temperature of rice explained by polymorphisms in starch synthase. Plant Biotechnol J 4, 115–122 (2006).

14. Yan, C. J. et al. Genetic analysis of starch paste viscosity parameters in glutinous rice (Oryza sativa L.). Theor Appl Genet 1–14 (2010).

15. Fujita, N. et al. Function and characterization of starch synthase I using mutants in rice. Plant Physiol 140, 1070 (2006).

16. Shastry, B. S. SNP alleles in human disease and evolution. J Hum Genet 47, 561–566 (2002).

17. Imelfort, M., Duran, C., Batley, J. & Edwards, D. Discovering genetic polymorphisms in next generation sequencing data. Plant Biotechnol J 7, 312–317 (2009).

18. Masouleh, A. K.,Waters, D. L. E., Reinke, R. F. & Henry, R. J. A high throughput assay for rapid and simultaneous analysis of perfect markers for important quality and agronomic traits in rice using multiplexed MALDI TOF mass spectrometry. Plant Biotechnology J 7, 355–363 (2009).

19. Ohdan, T. et al. Expression profiling of genes involved in starch synthesis in sink

and source organs of rice. J Exp Bot 56, 3229 (2005).

20. Dian, W., Jiang, H., Chen, Q., Liu, F. & Wu, P. Cloning and characterization of the granule-bound starch synthase II gene in rice: gene expression is regulated by the nitrogen level, sugar and circadian rhythm. Planta 218, 261–268 (2003).

21. Kharabian Masouleh,A., Waters, D. L. E., Reinke, R. F. &Henry, R. J.Discovery of polymorphisms in starch related genes in rice germplasm by amplification of pooled DNA and deeply parallel sequencing{. Plant Biotechnol J 9(9),1074–1085 (2011).

22. Umemoto, T.& Aoki, N. Single-nucleotide polymorphisms in rice starch synthase IIa that alter starch gelatinisation and starch association of the enzyme. Funct Plant Biol 32, 763–768 (2005).

23. Waters, D. L. E.&Henry, R. J. Genetic manipulation of starch properties in plants:Patents 2001–2006. Recent Pat Biotechnol 1, 252–259 (2007).

24. Hirose, T., Ohdan, T., Nakamura, Y. & Terao, T. Expression profiling of genes related to starch synthesis in rice leaf sheaths during the heading period. Physiol Plantarum 128, 425–435 (2006).

25. Cao, H. et al. Identification of the soluble starch synthase activities of maize endosperm. Plant Physiol 120, 205–216 (1999).

26. Kawagoe, Y., Kubo, A., Satoh,H., Takaiwa, F. &Nakamura, Y. Roles of isoamylase and ADP-glucose pyrophosphorylase in starch granule synthesis in rice endosperm. Plant J 42, 164–174 (2005).

27. Tetlow, I. J. et al. Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein–protein interactions. Plant Cell Online 16, 694–708 (2004).

28. Rolletschek, H. et al. Antisense inhibition of the plastidial glucose 6 phosphate/ phosphate translocator in Vicia seeds shifts cellular differentiation and promotes protein storage. Plant J 51, 468–484 (2007).

29. Hirose, T. & Terao, T. A comprehensive expression analysis of the starch synthase gene family in rice (Oryza sativa L.). Planta 220, 9–16 (2004).

30. Sun, C., Sathish, P., Ahlandsberg, S., Deiber, A.& Jansson, C. Identification of four starch-branching enzymes in barley endosperm: partial purification of forms I, IIa and IIb. New Phytol 137, 215–222 (1997).

31. Yamakawa, H., Ebitani, T. & Terao, T. Comparison between locations of QTLs for grain chalkiness and genes responsive to high temperature during grain filling on the rice chromosome map. Breed Sci 58, 337–343 (2008).

32. Nishi, A., Nakamura, Y., Tanaka, N. & Satoh, H. Biochemical and genetic analysis of the effects of amylose-extender mutation in rice endosperm. Plant Physiol 127,459 (2001).

33. Fujita, N. et al. Antisense inhibition of isoamylase alters the structure of amylopectin and the physicochemical properties of starch in rice endosperm. Plant Cell Physiol 44, 607 (2003)

34. Bao, et al.Nucleotide diversity in starch synthase IIa and validation of single nucleotide polymorphisms in relation to starch gelatinization temperature and other physicochemical properties in rice (Oryza sativa L.)

35. Tanaka,et al.Structure, Organization, and Chromosomal Location of the Gene Encoding a Form of Rice Soluble Starch Synthase' Plant Physiol. (1995).

36. Ashish Jain *et al,* 'Effect of cooking on amylose content of rice", European Journal of Experimental Biology, 2012, 2 (2):385-388