ISOLATION AND CHARACTERIZATION OF ADP-GLUCOSE PYROPHOSPHORYLASE ENZYME IN HIGH AND LOW STARCH VARIETY OF CASSAVA

by

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "**Isolation and characterization of ADP-glucose pyrophosphorylase enzyme in high and low starch variety of cassava**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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Certified that this thesis entitled **"Isolation and characterization of ADP-glucose pyrophosphorylase enzyme in high and low starch variety of cassava"** is a record of research work done independently by Ms. Revathi, V. S. (2012-09-108) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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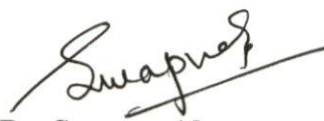Principal Scientist (Plant Breeding)

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LIST OF ABBREVATIONS

%	Percentage
A_{230}	Absorbance at 230 nm wavelength
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
AGPase	ADP-glucose pyrophosphorylase
ADPG	ADP-glucose
Bt2	Brittle2
bp	Base pair
cm	centimetre
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxy nucleotide tri phosphates
E	East
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed Sequence Tag
FAOSTAT	Food and Agriculture Organization Statistical Database
h	Hour
ha	Hectare
ICAR-CTCRI	ICAR- Central Tuber Crops Research Institute

KAU	Kerala Agricultural University
kb	Kilo bases
kg	Kilogram
L	Litre
m	Meter
M	Molar
mg	milligram
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitre
mm	Millimeter
mM	Millimolar
NaCl	Sodium chloride
NCBI	National centre for biotechnology information
ng	Nanogram
nm	Nanometer
^{o}C	Degree Celsius
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol

PVP	Polyvinylpyrrolidine
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
s	second
sp.	Species
spp.	Species (plural)
Taq	*Thermusaquaticus*
TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
T_m	Melting temperature
Tris HCl	Tris (Hydroxy methyl) aminomethane hydrochloride
U	Enzyme units
UV	Ultra violet
V	Volt
v/v	volume/volume

W	Watt
w/v	weight/volume
μg	Microgram
μl	Microlitre
μM	Micromolar
DW	Dry weight
FW	Fresh weight
G-1 –P	Glucose-1-phosphate
Sh2	Shrunken 2
WT	Wild type

INTRODUCTION

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) originated in the Amazon tropical area and is now being widely cultivated in more than 100 countries. Cassava is known as the granary of underground or king of starch (Balagopalan, 2002). Cassava is a monoecious perennial crop belonging to the family Euphorbiaceae and is grown in the low lands of Asia, Africa and South America. Globally, cassava is grown in an area of 20.7 M. ha producing 276 million tons with a productivity of 13.35 T/ha (FAO, 2015). It provides food to about 800 million people worldwide. Cassava ranks in the fifth place after maize, rice, wheat and potato (FAO, 2015). The roots contain starch up to 70-90% of the root dry weight (Baguma, 2004). Apart from the dietary aspect, cassava is also demanded by diverse industries including adhesives, textiles, biopolymer, cosmetics, medicines, bio-fuels.

Starch is the most common source of energy of a plant (Zeeman *et al.*, 2010). Cassava starch can be fractionated into two types of polymers viz; amylose and amylopectin. The amylose content of cassava ranges from 15-25% depending upon the cultivars and growth condition (Angraini, 2009). Amylose:amylopectin ratio greatly impacts the functional properties of starch. Formation of amylose and amylopectin in chloroplast is carried out by a series of biosynthetic enzymes including ADP- glucose pyrophosphorylase (AGPase), Starch Synthase (SS), Granule Bound Starch Synthase (GBSS), Starch Branching Enzyme (SBE) and Starch De-branching Enzyme (Buleon *et al.*, 2004). AGPase catalyses the synthesis of ADP-glucose from ATP and glucose-1-phosphate and is the rate limiting step in starch biosynthesis.

AGPase is the key regulatory enzyme in the starch biosynthesis pathway. Molecular analysis of AGPase gene reveals a higher homology in amino acid sequence with other AGPase genes from different species (Munyikwa *et al.*, 1994). AGPase-B, AGPase-S2, AGPase-S3 are three c-DNA subunits from cDNA library

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that show higher sequence similarity with other species. AGPase enzyme is made up of different subunits encoded by different genes (Munyikwa *et al.*, 1997). AGPase regulation depends on the ratio of 3-phosphoglycerate (3-PGA) and inorganic phosphate (3PGA/Pi). It means that 3-PGA is directly proportional to starch accumulation and inversely proportional to inorganic phosphate (Press, 1984). Mutant analyses of AGPase gene in starch deficiency provide an evidence for importance of AGPase in starch synthesis (Tsai and Nelson, 1966). Transgenic plants with over and under expressed AGPase activity give more support for AGPase activity.

Successful transgenic modification was reported in tuber crops with an increased rate of starch gene expression by over expressing bacterial AGPase gene (David *et al.*, 1992). Based on the study an increased starch content of 135 % was observed in potato when compared with their wild relatives and 791% increase in starch content was observed in tobacco than its wild counterparts. From these studies, in conclusion AGPase gene has a very important role in starch biosynthesis. Hence, the characterization and study of AGPase gene is the need of hour. Also the demand for Cassava in commercial starch synthesis is high. So, molecular characterization of the AGPase gene opens up a world of genetic modification in cassava thereby providing a valuable idea on starch improvement.

The objectives of the study include:

- To design AGPase gene specific primers and cDNA amplification of AGPase gene in cassava.
- To sequence of AGPase gene and assess the variations in AGPase genes from low and high starch cultivars of cassava.

REVIEW OF LITRATURE

2. REVIEW OF LITERATURE

Tuber Crops

Cassava (*Manihot esculenta* Crantz), yam (*Dioscorea* spp.), sweet potato (*Ipomoea batatas* L.), potato (*Solanum* spp.) and edible aroids (*Colocasia* spp. and *Xanthosomas agittifolium*) are the main tropical tuber crops. They are widely cultivated and are the staple food crops in many parts of Africa, the Pacific Islands, Latin America and Asia. In 1993, about 300 million tonnes of tuber crops are produced in developing countries. Among these, cassava and potato accounts to 83% of the total tuber crop production. After cereals, the second most important crop as global source of carbohydrates is the tuber crop. Tubercrops are nutritionally rich foods that contain protein, Zinc, Iron, Vitamin-C and Vitamin-A. However, the nutrients are lost during processing of cassava. The amount of protein present in starch tubers is low compared with other protein sources. Hence, along with tubers, legumes and fish are supplemented. Tuber crops contribute to food and nutritional security in the country. Potato and sweet potato are suitable for double cropping system due to their short life cycle. Due to the broader agro-ecological adaptation, diverse maturity period and in-ground storage capacity, yam and cassava are vital for annual food availability (Bareja, 2010).

2.2. Cassava

Cassava is a very important dietary source with high a carbohydrate level, high industrial value and is used as food and feed for humans and animals (Sanchez *et al.*,2009). More than 600 million people from Africa, America, Asia, and in Latin America are dependent on cassava. In cassava, most of the starch is stored within the amyloplast in a thickened root called tuber. 73 to 84% of starch is present on a dry weight basis (Rickard, 1991). It is ranked fourth after rice, sugarcane and maize. Cassava leaves and roots are used as vegetable and stem is used for cooking food. It helps to eradicate extreme

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poverty, hunger and enhance food security. Cassava is used for the commercial production of starch along with potato, maize and wheat (Moorthy, 2002). Cassava occupies first position among the tropical crops in terms of area and production globally (Tavva, 2006). Cassava is tolerant to drought, soil acidity and can grow very well under low nutrient conditions. Cassava is cultivated in an area of 20.7 M. ha with a productivity of 13.35 t/ha producing 276 million tonnes (FAO, 2015). According to the FAO statistical data base, 66.21% of cassava was cultivated in Africa before but due to low productivity (8.82 t/ha), they are now producing only 53.37% of the total cassava production. Indonesia, Thailand and India cultivate cassava in an area of 240,000 ha with a productivity of 27.92T/ha producing a total of 6.7 MT. *Manihotae* is known to have about 200 species. Most important species is *M. esculenta* Crantz which is commonly known as cassava. Manihot is native to South America from where it extended to different parts of the World like, North America and finally sub-tropical humid regions like Asia and Africa. Production of cassava is mainly concentrated in over 13 states of India such as Andhra Pradesh, Assam, Karnataka, Kerala, Meghalaya, Nagaland, Tamil Nadu, Rajasthan, Arunachal Pradesh, Mizoram, Pondicherry, Tripura, Andaman and Nicobar Island (Alfredo *et. al.*, 2007).

2.2.2 Importance of cassava

In India, 60-70% of the total cassava is used for the commercially production of sago, starch, dried chips and flour. Increasingly cassava starch and starch derivatives such as dextrin, glucose and high fructose syrups has become the main product of cassava agro industry. Cassava starch is widely used for the production of paper, textile industry as well as the manufacture of bio-ethanol and adhesives. Presence of surplus fermentable sugars after saccharification is the main advantage of cassava over the other crops. Simple and low cost technologies were employed for the preparation of adhesives. Gelatinization of cassava starch by simple heating is necessary for the preparation of gums.

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Cassava has high commercial value ranging between 73.7 to 84.9% dry weight making it a potential source of industrial starch. Cassava starch is also used as a bland flavor in pharmaceuticals due to high clarity. Due to high resistance to stress and shear and freezing, it can be used in the film forming industry. Cassava starch has been widely used for the production of baby food and synthetic rice. Cassava is a good raw material for alcohol production (Sharkawy, 1993).

2.3. STARCH

The second largest biomass produced by the plants is starch. Starch biosynthesis is meant not only for the synthesis of starch but also for the ordered arrangements of monomer unit in a three dimensional manner. Starch is synthesized by the polymerization of glucose molecule and is stored in the form of osmotically inactive water insoluble granules in the amyloplast. In cassava, most of the starch is stored in tuber amyloplast (Rickard *et al.*, 1991). Cassava starch granules are one end flatted round structures of 5-40 μm (Moorthy, 1994). The size of granules increase up to six month after which size remains constant (Moorthy and Ramanujan, 1986).

2.3.1. Importance of starch

Starch is not only used for energy production, but also for many industrial purposes. In industry, starch is derived from dry matter accumulated in the storage organs like tubers, seeds and fruits (Kossmann and Lloyd, 2000). Starch is a biodegradable, bio-adhesive and is widely used in food and pharmaceuticals. Nearly 550,000 tons/year of starch is converted into thermoplastics of which majority are biodegradable (Beilen and Poirier, 2007). In weaving industry, starch is used to warp yarn. Native starch is used to produce facials and talcum powders. In food industry, they use resistant starch, which are beneficial for maintaining a proper hygiene. Pasta and rice have

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resistant starch. Resistant starch has the ability to regulate our blood sugar by increasing the insulin sensitivity thereby preventing diabetes and was also found to be effective in the treatment of colon cancer.

2.3.2. Structure of starch

Starch is an important form of carbohydrate. In semi-crystalline starch, glucose molecule is arranged in three dimensional structures in starch granules. Through photosynthesis, plants convert radiant energy into chemical energy that is stored in chemical bonds in molecule like glucose. Starch is synthesized during the day in leaves from glucose molecule and is translocated to plastids of storage organs including seeds, tubers, storage roots and fruits (Martin and Smith, 1995). Starch is composed of amylose and amylopectin.

Amylose molecules are lighter and extended structures. D-glucose molecules are linked with α-1, 4 linkages to form a linear glucan. (Martin and Smith, 1995). Around 500-20,000 α-1,4 -D glucose molecule are found in amylose and the number of glucose molecules depends on the species. However, there may be a few branches present in the amylose molecule (Hoover and Ratnayake, 2001). This may influence the molecular structure and behaviour of starch (Buléon et al., 1998). Due to the hydrogen bond amylose molecule gets gelatinized and form crystallites that are resistant to amylase. Extensive intra and inter molecular hydrogen bonding makes starch hydrophobic with a reduced solubility (Martin and Smith, 1995). Amylose is an important factor that determines the quality and imparts definite characteristics to starch.

Formation of non-random α-1, 6 branch points in amylose molecule forms amylopectin molecules. In amylose, the branching enzyme produces branching points after every 30 residues. An amylopectin molecule is a compactly arranged structure having a hydrodynamic radius of 21-75 nm (Parker and Ring, 2001). Each molecule has two million glucose residues.

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Amylopectin is arranged in alternative crystalline and amorphous structures. An amylopectin molecule can be divided into three chains namely A chain, B chain, C chain. A chain is short and unbranched outer chain. B chain is highly branched. C chain is single chain having a non-reducing end (Guilbot and Mercier, 1985). There are two types of amylopectin, the first form of amylopectin molecule is composed of 23-29 glucose molecules arranged to form denser crystallites whereas the other type is having hydrated open hexagonal type crystallites having 30-44 glucose units (Parker and Ring, 2001). Manners and Matheson in 1981 proposed many models of amylopectin structure. One of them is amylopectin molecule that are large flattened disks having α-1,4-glucan chain joined by frequent α-1,6 branch points. Starch contains trace amount of protein and minerals. There is 0.1% of protein present in the cassava starch (Blennow*et al.*, 2000).

Exposure of leaf into bright light causes synthesis of starch granules (Sachs, 1887). 24-48 hr exposure of leaf in the dark leads to disappearance of starch (Edwards and walker, 1983).Starch in potato tubers, sweet potato and in roots of yam, cassava, maize endosperm ranges between 65 and 90% of dry matter (Sivak and Preiss, 1998).

2.3.3. Biosynthesis of starch

Biochemical and molecular analysis of enzymes and genes encoding these enzymes, gives conclusion about basic mechanism of enzyme and relationship between each enzyme in a biosynthetic pathway. Studies proved that multiple enzymes are involved in the starch biosynthetic pathway. They are AGPase, starch synthase, branching enzyme, de-branching enzyme and disproportionating enzyme. Generally starch is formed from sucrose molecule.

2.3.3.a) ADP-Glucose Pyrophosphorylase

ADP-Glucose Pyrophosphorylase (AGPase) is the key enzyme in starch biosynthesis catalyzing the synthesis of ADP-glucose from ATP and Glc-1- phosphate. This is the committed step in starch biosynthesis.

2.3.3.b) Starch synthase

Starch synthase transfers ADP-glucose to non-reducing end of glucan chain. Based on the location of starch synthase, they are classified into two. One is Granule Bound Starch Synthase (GBSS) and the other is Soluble Starch Synthase (SSS). GBSS is bounded to starch granule where as Soluble Starch Synthase is bound to stroma of chloroplast or amyloplast as the soluble form (MacDonald and Preiss, 1985). In addition to this, in higher plants five isoforms of starch synthases have been identified on the bases of their sequence. They are *GBSS, SSI, SSII, SSIII,* and *SSIV/V* (Li *et al.*, 2001;Vrinten and Nakamura, 2000). Various studies showed that *SSI, SSII* and *SSIII* are involved in starch synthesis. *SSI* is responsible for the synthesis of short glucan chains in amylopectin. Activity of the enzyme varies according to the species (Commuri and Keeling, 2001). *SSII* catalyze the production of intermediate glucan chains. The degree of polymerization is in the range of 12-25, in cereals compared with the total SS activity. It is a minor contributor but the loss of its activity cause a drastic change in composition and amount of starch (Umemoto *et al.*, 2002). Activity of *SS III* changes according to species. In case of cereals, elimination of the enzyme causes minute, reproducible changes in the kernel and highly branched amylopectin is found in these plants (Gao *et al.*, 1998). In case of potato, a significant change in the amylopectin is due to chain length modification. For *SSIV/V*, no mutants were observed. *In vitro* analysis of *GBSS* in maize showed an extension in amylopectin (Donald and Preiss, 1985). Mutant analysis of plants lacking *GBSS1* gene showed an absence of amylose from which we can conclude that

in *in vivo* reaction, *GBSS* is mainly involved in the amylose synthesis (Shannon and Garwood, 1984). Reduction in *GBSS* activity by gene silencing by introducing antisense gene caused a drastic reduction in the amylose production (Salehuzzaman *et al.*, 1993).

2.3.3.c) Starch branching enzymes (BEs)

Branching enzyme is involved in the hydrolysis of α-1,4 bond and thereby transferring this cleaved chain to hydroxyl group on C6 of inner glucose in amylose forming an α-1,6 glucosidic bond. BE is involved in α-amylase domain (Jespersen *et al.*, 1993; Svenson, 1994). Based on the catalytic site and amino acid sequence, there are two types of starch branching enzyme viz., SBEI and SBEII. Differences in the specificity in two isoforms of enzyme, SBE1 result in a higher preference to amylose than SBEII and also possess high branching activity (Jobling*et al.*, 1999; Rahman *et al.*, 2001).

Transgenic plants whose genes are suppressed through mutation or gene silencing cause minimal effects on starch biosynthesis (Ball and Morell, 2003). However a significant change is noticed in the structure and physicochemical properties (Satoh *et al.*, 2003). In the case of potato, elimination of BE has lead to an increased level of amylose (Schwall*et al.*, 2000).

2.3.3.d) Starch de-branching enzymes (DBEs)

De-branching enzyme hydrolyses 1,6 glycoside linkage. DBE is classified in to two types viz., lyisoamylase type α-1,6 glycohydrolase and pullulanase type α-1,6 glycohydrolase based on the specificity. Glycogen, phytoglycogen and amylopectin are de-branched by isoamylase type DBE and amylopectin, glycogen and phytoglycogen are attacked by pullululase type DBE. During amylopectin synthesis, DBE selectively remove improperly

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positioned branches and this method is called 'glucan trimming' (Zeeman *et al.*,1998). From the Arabidopsis genome, ISA1, ISA2, and ISA3 are the three enzyme encoding isoamylase like protein.They are conserved in divergent plants. ISA1 and ISA2 proteins are the subunits of heteromultimeric isoamylase (Hussain *et al.*, 2003) are essential for starch synthesis in leaves, ISA3 is important for starch degradation.

2.3.3. e) Disproportionating enzyme (D-enzyme)

Enzyme catalyses the transfer of $\alpha(1,4)$ oligosaccharide from one end of a linear glycan chain to the another end of glycan chain. Accurate function of the enzyme is unclear. Analysis of mutant chlamydomonas lacking this enzyme indicates the involvement of enzyme in starch biosynthesis. But studies in *Arabidopsis* showed that this enzyme is involved in starch degradation.

Transitory starch is used for the growth of plants during night (Sulpice *et al.*, 2009). Leaf starch accumulates in spongy mesophyll cells and palisade cells. In storage organs, starch is stored in the amyloplast. Activities of key enzymes such as starch synthase and AGPase determine starch biosynthesis capacity and act as the rate-limiting step of the starch synthesis pathway (Crevillen *et al.,*2003). Leloir *et al.* (1950) discovered nucleoside di-phosphate sugar lead to the elucidation of starch biosynthetic pathway. ADP-glucose pyrophosphorylase, starch synthase and branching enzyme are the main enzymes involve in starch synthesis (Preiss, 1991). Carbon get incorporated in starch through ADP-glucose (Fig.1).

Glucose-1-P + ATP <-»■ ADP-Glucose + PP$_i$
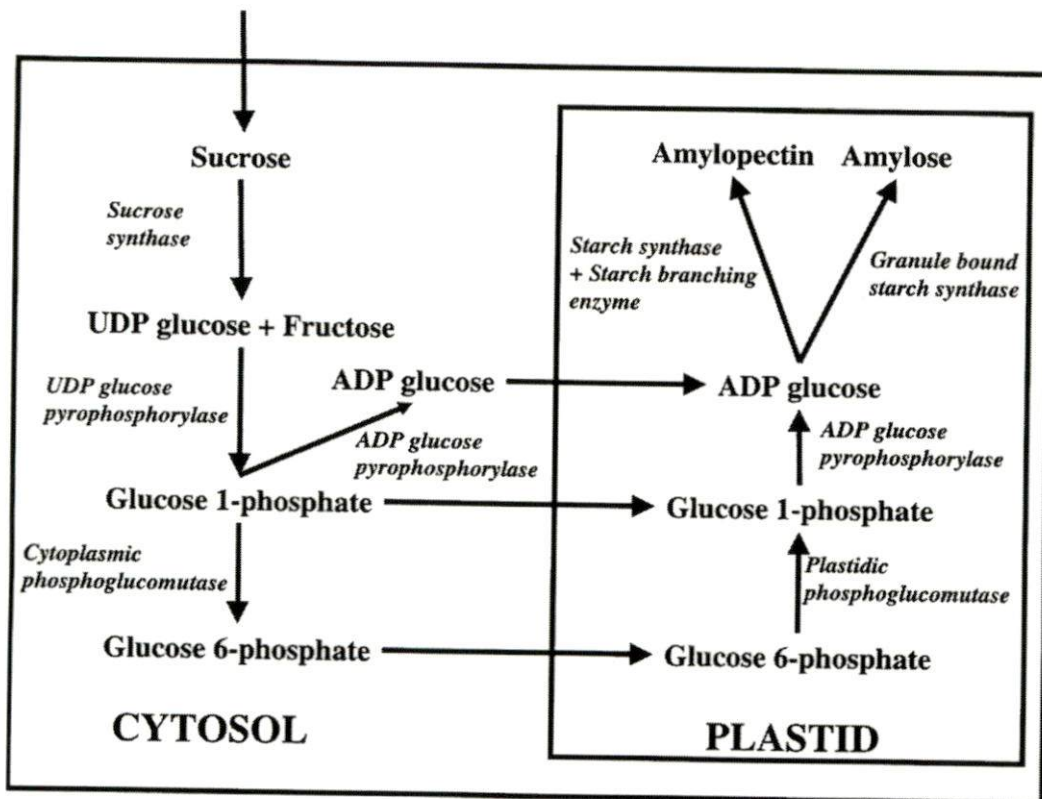
Fig:1 Proposed starch biosynthetic pathway adopted from Kossman and Lloyd (2000).

ADP-glucose pyrophosphorylase is the key enzyme responsible for the conversion of glucose-1-phosphate and ATP into ADP glucose. This reaction is the first dedicated step in the starch biosynthesis pathway. Once nucleotide sugar is activated, it will attach to the non-reducing end of α-1,4 glycan residue and generate linear α-1,4 linkages called amylose. Starch branching enzyme introduces α-1, 6 inter linear chains resulting in the formation of amylopectin. Branching occurs according to the chain length of glucan molecule. Another enzyme is the debranching enzyme which cleaves α-1, 6 linkage. There is a balance between starch branching enzyme and starch debranching enzyme which is essential for producing starch granules.

2.3.4. Modification in starch biosynthesis

Major developments in the production of high starch varieties occurred during last 5 years. There are successful and unsuccessful biotechnological approaches for high starch accumulation in heterotrophic organs of plant. A strategy for increasing the tuber starch content is by increasing the transport of ATP to plastid. For this, a transgenic potato was created which up regulate the genes responsible for the ATP/ADP transporter protein. A chimeric gene was produced through the fusion of ATP/ADP transporter gene from *Arabidopsis* and promoter from cauliflower mosaic virus. This lead to the accumulation of 2 fold more ADP glucose and 16-36% more starch production (Geigenberger *et al.*, 2001). In 2010, transgenic wheat was produced with a high sucrose up-take capacity. Transgenic plants were constructed which ectopically expressed the barley sucrose transporter HvSUT1 under the control of an endosperm specific promoter. Transgenic plants produced wheat with high sucrose and protein concentration (Weichert*et al.*, 2010). Most recent method for production of high starch varieties is by the over-expression of *SuSy* activity.

2.4. Adenosine Di-phosphate glucose pyrophosphorylase (AGPase)

Plant AGPase and its regulation ensure the co-ordination between carbon assimilation, starch synthesis and sucrose synthesis. The enzyme maintains the biosynthesis in equilibrium wherein the forward and reverse reaction rates are equal. The flux control coefficient of AGPase in *Arabidopsis thaliana* leaf starch synthesis was found to be 0.6 confirming the regulatory role of AGPase enzyme.

2.4.1. Enzymology

AGPase is the first commited step in the starch biosynthesis pathway. It is an important regulatory step in the starch biosynthesis pathway (Ghosh and Preiss 1966). Bacterial AGPase enzyme is homotetrameric that is encoded by *glgC* gene in *Escherichia coli* having mol. wt of 200 k Da of which each

subunits have a weight of 48 kDa (Preiss, 1991). But plants have heterotetrameric enzyme of molecular weight about 210-240 kDa (Copeland and Preiss, 1991), Isothermal titration calorimetry shows that wild type heterotetrameric enzyme shows two type of substrate binding sites where as homotetramer of either large subunit or small subunit shows either one of the two type of binding sites (Cakir, 2015). Two subunits are encoded by two genes (Bhave *et al*., 1990). Larger subunit is constantly expressed in all tissues and smaller subunit is highly expressed in specific tissues like tuber and leaves. AGPase activity is high in young leaves than mature leaves and in stem petioles. A study in molecular cloning and characterization of AGPase in pea cotyledons found that *psagps1* and *psagps2* genes encode the small subunit and that *psagps1* expressed in all tissues while *psagps2* expressed in seeds. Large subunit encoded by *psagpl1* and it is highly expressed in leaves (Burgers, 1997; Singh, 2002). Kavakli *et al.* (2001) investigated the function of AGPase and discovered that large subunit interact with small subunit and play a role in binding of glucose-1-phosphate.

Non-tuberous roots do not have AGPase activity (Munyikwa *et al*., 2001). For the enzyme to be highly potential, it requires the presence of both subunits. Lack of one subunit not only reduce the activity of AGPase enzyme, but also reduce starch synthesis. This result was revealed from the *in vivo* experiments in *Arabidopsis thaliana* (Lin *et al*.1988; Li and Press, 1992.) similar event has been noticed when either of the subunit was absent in AGPase in maize endosperm (Press *et al*. 1990). In the case of mutants, only one of the enzyme is absent. There are multiple tissues where specific forms of AGPase are present in plants. Endosperm, embryo, leaf small unit are encoded by three separate genes. (Hannah *et al*., 2001).There is also evidence that AGPase present in different tissues have the same transcript (Doan *et al*., 1999). The mutants like *brittle 1*, *brittle2* and *shrunken 2* shows that active AGPase is synthesized in cytosol in the case of grasses and transported into plastids. Shrunken 2 have mutations that eliminate the synthesis of cytosolic large

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subunit and brittle 2 have mutations that eliminate the synthesis of cytosolic small subunit. This leads to the inactivation of cytosolic AGPase and reduced starch content (Tsai and Nelson, 1966). Hannah (2001) reported that AGPase from maize encoded by two genes. One gene encoding cytosolic AGPase and other encoding plastidal. Except exon1 all other sequences are similar which is due to the insertion and duplication due to a transposable element. Johnson *et al.* (2003) discovered that in barley the normal rate of starch synthesis is achieved only in presence of functional cytosolic AGPase.

2.4.2. Structure of AGPase

AGPase enzyme is a heterotetramer having two large and two small subunits (Ballicora *et al.*, 2004). To Large subunit is responsible for the allosteric regulation and small subunit has both catalytic and regulatory property in AGPase enzyme. Homotetramer of small subunit of AGPase alone show high enzyme activity, lower affinity towards activator 3-phosphoglycerate and higher sensitivity towards inorganic phosphate (Ballicora *et al.*, 1995). 85-95% sequence similarity has been shown between small subunits from different species and 50-60% sequence similarity has been show between large and small AGPase subunits (Ballicora*et al.*, 2004). In the case of cassava, large and small units of cassava showed 54% of homology. AGPase is subjected to post-transcriptional redox regulation. Through this modification, an intra molecular cysteine bridge formed between cys-82 of two small subunits lead to the dimerisation of the small subunit monomer (Fu *et al.*, 1998).

2.4.3. Regulationof AGPase

Activity of AGPase is regulated by key metabolites from carbon assimilatory pathways (Sivak and Preiss, 1998). 3-PGA and fructose-6-phosphate are the best activators. 3-PGA increases the enzyme action and

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increase the affinity of enzyme towards substrate (Dickinson and Preiss,1969). Two small subunits were expressed constitutively, but the four large subunit genes were differentially expressed in various tissues. Different developmental stages show different combination of these six proteins each with unique enzyme activity (Zhou, 2016). A 40 fold increase in the catalytic activity is insensitive to modulation by metabolites thereby increasing the starch production and mutation reduces starch production up to 50% (Smith *et al.,* 1989). For the production of mutant varieties of potato having reduced starch content, antisense technology was employed. AGPase S-gene was introduced into the plants which inhibit AGPase that lead to the reduction of ADP-glucose available for the production of amylase and amylopectin. Transgenic plant has AGPase activity only less than 2% of wild type and 40% reduction in dry weight. When compared with wild type of potato, transgenic tuber store glucose and sucrose instead of starch (Visser*et al.,* 1990). AGPase activity in *Shrunken-2* and *Brittle-2* contains only 10% of wild type and it produces only 25% of starch from the wild type. (Cameron and Teas, 1954).

Higher concentration of 3-PGA overcomes the inhibition by inorganic Pi (Preiss,1988). In the regulatory level, lysine region near to the C- terminus region is highly conserved between species (Smith white and Preiss,1992). Site-directed mutagenesis in the conserved region of AGPase causes catalysis and regulation. So it is revealed that this conserved region is important for the enzymatic activity of AGPase enzyme. AGPase show common folding pattern with other species showing that they are evolved from a common ancestor (Ballicora *et al.*, 2004). Introduction of a few point mutations alone with low level recombination's resulted in a change in the quaternary structure, effector sensitivity and allosteric effector sensitivity. These changes finally lead to evolution (Sulamone *et al.*, 2001). Small subunit dimers have higher affinity towards substrate, high sensitivity to 3-PGA activators and reduced sensitivity to Pi inhibition than monomers (Tiessan *et al.*, 2002). Sucrose and glucose level modulate rate of starch biosynthesis is post-translational redox

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modulation (Tiessen *et al.* 2003). Mutation in the plastidic large subunit cause male sterility in rice pollen grains (Lee *et al.*2016)

2.4.4. Localization of AGPase

Localization of AGPase is subdivided into leaf, root and endosperm. In the endosperm of many grasses, there is a second form of AGPase which is present in the cytosol contributing to about 85-95% of the total AGPase activity (Burton *et al.*, 2002). AGPase is localized in the photosynthetic and non-photosynthetic tissues. In the photosynthetic tissues like leaves, AGPase is present in the chloroplast and this enzyme is highly sensitive to regulators. Jardin (1997) found that *Agpb1* promoter appear to be specific for sink tissue. (Neuhaus*et al.*, 1990). Mature chloroplast containing photosynthetic tissues have the ability to supply ATP and precursors for starch synthesis. Synthesis occurs during illumination. Starch is synthesized in the chloroplast and is remobilized to the sink organs where it is utilized for the growth and non-photosynthetic metabolism. This type of starch is known as transitory starch. In non-photosynthetic organs like tubers and the endosperms of the cells, AGPase enzyme is sited at amyloplast. The enzyme present in the sink tissue is less controlled by regulators (Denyer *et al.*, 1995). Precursor and energy for starch biosynthesis are transported to amyloplast from cytosol. The starch is used for long term storage.

2.5. Modifications in AGPase activity

In the process of developing of high starch varieties one of the best method is enhancing AGPase activity because AGPase enzymatic reaction is the rate limiting step in the overall starch biosynthesis pathway.

Transgenic gene was produced by introducing *glgC16* gene under the control of promoter. *glgC16* gene is modified form of *E.Coli glg C* gene which is created through site directed mutagenesis. Modified form of enzyme lack allosteric regulatory control and the enzyme is unaffected by negative regulatory elements such as fructose bisphosphate. Recent studies show that ectopic expression of *glgC* gene did not increased the starch content in *aps1* *Arabidopsis* mutant (Li *et al*., 2012). Development of transgenic potato with high AGPase activity is the first attempt. Chimeric gene was developed through the fusion of *glg C* gene with 35S promoter gene (Stark *et al*., 1992). Plants expressing mutant AGPase had higher AGPase activity between 0-70% than the control plant. AGPase activity leads to 260% increase in the total root starch biomass. Under green house condition, increasing biomass production leads to increase in root number and root size whereas no significant increase in root starch density was observed. Increasing the root biomass is correlated with a relative increase in the root AGPase activity. Next attempt was to express *glgC16* under the control of tuber specific patatin promoter. However, there was no increase in the tuber starch content and AGPase activity (Sweetlove *et al*., 1996).The method of using *glgC16* for high starch production is now widely used in different varieties like rice (Nagai *et al*. 2009), maize (Wang *et al*., 2007) and cassava (Ihemere *et al*., 2006).

Enhanced AGPase activity in wheat endosperm to increased seed yield trough transferring wheat with modified Maize *Shrunken 2*gene which alter AGPase large subunit with decreased sensitivity towards negative allosteric effectors and increased large subunit and small subunit interaction (Smidansky

et al., 2001). Over expression of either one of *Sh2* or *Bt2* enhance AGPase activity, seed weight and starch content (Li, 2010). Potato *upreg1* regulate mutated enzyme and introduce in rice and increase sink driven yield productivity in crop (Lee, 2010).

Another attempt to increase starch synthesis is by the production of mutant AGPase enzyme lacking negative regulatory control. The enzyme is modified by inserting three bases to the allosteric regulation site of the AGPase gene that lead to the modification in negative inhibition site of AGPase by the presence of additional serine or tyrosine residues. Expression of this mutated gene leads to increase in the seed weight. But percentage of starch is unaffected. This gives an idea that single mutation can produce higher yield (Giroux *et al.*, 1996).

Park *et al.* (2006) modulate soluble sugar in strawberry fruit through antisense suppression of AGPase and starch content of the fruit decreases to 27-47% and total sugar content increased to 16-37% in transgenic plant.

In maize, endosperm specific large subunit of AGPase is encoded by *shrunken 2* (*Sh 2*) and the gene is mutated to *Sh2r6hs*. Mutated gene encodes an enzyme which is allosterically insensitive and this leads to an increase in the seed weight by 11-18% without changing the percentage of starch. Ectopic expression of mutant gene has been conducted in rice (Smidansky *et al.*, 2002) and wheat (Meyer *et al.*, 2004).

These are the main findings based on AGPase and my objective is to compare the gene from high and low starch varieties of cassava.

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MATERIALS AND METHODS

3. MATERIALS AND METHOD

The study entitled characterization of AGPase in high and low starch varieties of cassava was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crop Research Institute, Sreekariyam, Trivandrum (annual rainfall of 1250 mm; altitude 50 MSL; mean annual temperature of 22-31°C; coordinates: latitude of 8°32'N and longitude of 76°55'E) during 2016-17 Details of experimental material and methods used in the study are elaborated in this.

3.1. Planting

High starch line 9S-127 and low starch variety MNga-1 of cassava (Plate 1) were selected for the present study with the objective of assessing the variations in AGPase genes from low and high starch cultivars of cassava. The selected samples were planted in the field of ICAR-CTCRI, Trivandrum. The laboratory experiments were performed at the Division of Crop Improvement, ICAR-Central Tuber Crop Research Institute

3.2 Sample collection

9S-127 and MNga-1 were collected .9S-127 is a high starch containing, CMD resistant, high yielding, constantly flowering improved cassava line of starch content ranging from 28-30%. MNga-1is a CMD resistant cassava, which is an exotic variety received from IITA Nigeria. Released in the name Sree Padmanabha from ICAR-CTCRI in 2007, starch content ranged from 21-23%.

Plate 1. Low starch variety MNga-1

Plate 2: High starch variety 9S-127

3.3. Estimation of starch in dried cassava samples

The dry matter and starch content of high starch line 9S-127 and low starch variety MNga-1 (Sree Padmanabha) cassava line were determine to analyze the variation in dry matter and starch content.

3.3.1. Dry matter analysis

To estimate dry matter content two cassava lines 9S-127 and MNga-1 cassava varieties were used. Immediately after tuber harvesting samples were chopped into small pieces. 50 g were kept at 65°C for 72 h., in hot air oven and the dry weight was measured and percentage was calculated using the following formula.

$$\text{Dry matter percentage} = \frac{\text{Dry weight (DW)}}{\text{Fresh weight (FW)}} \times 100$$

The samples were crushed and stored in plastic bottles. The flour is further used for starch estimation.

3.3.2. Starch estimation

3.3.2. a) Preparation of samples

Powdered samples were used for starch estimation. One gram of well dried sample was taken into a 100 ml conical flask, to which 20 ml of 80% alcohol was added and kept overnight, to extract sugar content. Similarly leaf samples are used for the estimation of starch, in which 80% of acetone is used to remove pigments. Dried leaf powder can be use for starch estimation.

After incubation filter the samples using whatman No. 1 filter paper and the filtrate is used for the sugar estimation. Residue on the filter paper was washed with 2 lots (10 ml each) of distilled water to remove adhering sugar molecule and filtrate is added to original solution. To this flask 20 ml of 2N HCl was added and boiled for

half an hour till the colour changes to brown and the flask is cooled and made up to 100 ml using distilled water. This supernatant was directly used for starch estimation.

3.3.2. b) Titrimetric Assay

In another flask 5ml NaOH and 10 ml potassium ferriccyanide were taken and mixed contents were mixed thoroughly and kept over the flame for boiling. When the reagent began to boil, flame was lowered and 2 drops of methylene blue was added to the flask. The solution immediately turned to blue green. Starch hydrolate was taken on the pipette and titrated by adding drop by drop in to the flask contain NaOH and potassium ferriccyanide till light yellow colour was appeared. The pipette reading was noted and calculated.

3.3.2. c) Calculations

The starch content of the sample is calculated using the formula,

$$\text{Starch (g/100g FW)} = \frac{\text{Volume of ferriccyanide x made up volume x 0.9x100}}{\text{Titre value x weight of sample x1000}}$$

For expressing starch on dry matter basis,

$$\text{Starch (g/100g DM)} \quad \frac{\text{Starch content (g/100 g fresh weight) x100}}{\text{Dry matter (\%)}}$$

3.4. Standardization of total RNA isolation.

RNA is less stable and it is easily degradable during isolation. Two methods were used for extracting RNA from cassava leaves quantity and quality of extracted RNA were estimated. The manual method LiCl method and GeneJET Plant RNA Purification Mini Kit (Thermo fisher) were used

3.4.1. LiCl method for RNA precipitation

- The materials used for RNA extraction such as pestle and mortar, tips and eppendoff tubes were soaked overnight in 0.1% DEPC treated water to inactivate the RNase. The water was drained out next day and air dried. Then the materials were double autoclaved to inactivate the DEPC.

- The working table and micropipettes were cleaned with 70% alcohol and latex gloves were used during the RNA extraction in order to avoid RNase contamination from floor and hand.

- 100 mg of fresh leaf tissue was ground in DEPC treated mortar and pestle with liquid N_2.Transferred it into fresh tube and then 1 ml of pre-warmed CTAB RNA extraction buffer added to the ground sample.

- The samples were vortexed and incubated for 10 min at 65°C and centrifuged for 15 min at 15000 rpm at room temperature.

- The supernatant was transferred into a fresh 2 ml tube and equal volume of chloroform isoamyl alcohol was added into the tube The sample was mixed gently and centrifuged at 20,000 x g for 10 min at 4 °C

- The supernatant was transferred into fresh tube and to this 0.25 volume of 10 M ice cold LiCl was added.

- The sample was mixed well and incubated overnight at -20 °C

- After the incubation samples were centrifuged at 30,000 x g for 30 min at 4 °C then pellet was washed with 75% ethanol and centrifuged at 10,000 x g at 4 °C for 10 min and repeated twice.

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- Air dried the pellet at 37 °C for 30 min and dissolved in DEPC treated water by incubating the sample for 1 hr at 37 °C.
- The RNA sample was then stored at -20 °C

3.4.2. RNA extraction using GeneJET Plant RNA Purification Mini Kit (Thermo Scientific)

- Fresh plant tissues about 100 mg were put into liquid nitrogen and ground thoroughly with DEPC treated Mortar and Pestle.
- Immediately transferred the tissue powder into a 1.5 ml micro centrifuge tube containing 500 µl of Plant RNA Lysis Solution and mixed thoroughly by overtaxing for 10-20 sec.
- Samples were incubated for 3 min at 56°C and centrifuged for 5 min at 14,000 rpm.
- Supernatant was collected and transferred to a clean micro centrifuge tube. 250 µl of 96% ethanol was added to the sample and mixed thoroughly by pipetting.
- Transferred the prepared mixture to a purification column inserted in a collection tube and centrifuged the column for 1 min at 11000 rpm. discarded the flow through solution and reassembled the column and collection tube.
- To the purification column 700 µl of Wash Buffer (WB 1) was added and then centrifuged for 1 min at 11000 rpm. Flow-through and the collection tube were discarded. Purification column was placed into a clean 2 ml collection tube.
- About 500µl of Wash Buffer 2 was added into the purification column and centrifuged for 1 min at 11,000 rpm. Flow-through was discarded, column and collection tube were reassembled. Repeated the step and re-spin the column for 1 min at 14,000 rpm, flow through and collection tube were discarded and purification column transferred into RNase-free 1.5 ml collection tube.

- 50 µl of nuclease-free water was added to the centre of the purification column membrane to elute the RNA and centrifuged for 1 min at 11000 rpm
- Repeated the elution step to get more yields
- Purification column was discarded and purified RNA was stored at -20°C until use.

3.5. Analysis of extracted RNA

3.5.1. Agarose gel electrophoresis

The integrity and purity of isolated RNA was checked in 1.2 percentage agarose gel. The gel was prepared by weighing out 1.2% of agarose in a conical flask and dissolved it using TAE buffer up to the desired level so that 1.2% agarose solution was obtained. Agarose was dissolved by boiling. The flask was allowed to cool and 1µl of Ethidium Bromide (EtBr) was added into the gel and mixed to make sure the even distribution of EtBr. The gel was poured to the casting tray with combs and was allowed to solidify. About 3µl of isolated RNA sample was mixed with 2µl of 6X loading dye and loaded in the gel.

The gel was run for 30 minutes at 80V. The run was stopped after the dye front reached 3/4th of the gel. Then it was visualized under ultraviolet light and image was documented using alpha imager gel documentation system.

3.5.2. Quantification of RNA

The Thermo Scientific NanoDrop™ 1000 Spectrophotometer was used to measure the quality and quantity of samples with high accuracy and reproducibility. RNA absorbs UV in the range of 260 nm. The concentration of our RNA was calculated by measuring the absorbance at 260 nm and is multiplied with dilution factor, based on the relationship that an A_{260} of 1.0 equals 40µg/ml pure RNA. RNA purity was evaluated by taking ratio of absorbance at A_{260} to A_{280}

3.6. cDNA synthesis

The RNA isolated from leaf samples were subjected to cDNA conversion using RevertAid M-MuL V reverse transcriptase enzyme (Thermo Scientific). After thawing components of the kit, it was mixed and briefly centrifuged and stored on ice. Added the following reagents into a sterile, nuclease- free tube on ice in the indicated order:

Template RNA   : 5 µl

Primer Oligo (dT) 18 primer   : 1 µL

Waternuclease-free   : 6 µL

Mixed gently, centrifuged briefly and incubated at 65°C for 5 min. Chilled on ice, spin down and placed the vial back on ice.

Then add the following components in the following order:

5X Reaction Buffer   :   4 µL

RiboLock RNase Inhibitor (20 U/µL)   :   1 µL

10 mM dNTP Mix   :   2 µL

RevertAid M-MuLV RT (200 U/µL)   :   1 µL
_____

Total volume   :   20 µL
_____

All the reagent were added, mixed and spin down using Rivotek spinner and incubated for 60 min at 42°C and terminated the reaction by heating at 70°C for 5 min with the help of Bio-Rad

3.7. PCR Amplification using gene specific primer

3.7.1. Primer designing

Gene specific primer were designed (Table 1) for the gene encoding the large subunit and small subunit. The homologues sequences of cDNA from AGPase were retrieved from NCBI (National Centre for Biotechnology Information). The consensus sequences were choosing to design specific primer targeting the corresponding nucleotide sequence of cassava AGPase. Consensus sequence were developed using CAP 3 software. The primer was designed by an online software primer three plus from four contig sequence. Annealing temperature of designed primer (Table:1) ranged from 47- 50°C.

3.7.2. PCR standardization

For standardization of PCR conditions the genomic DNA of MNga-1 sample was used with 16 primers. Based on the data from Tm calculator (Thermofisher) annealing temperature at 51°C was found necessary to perform gradient PCR to standardize annealing temperature. The gradient was carried out in temperature range of 45-55°C to choose the most appropriate annealing temperature that give specific band with minimum ambiguity.

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3.7.3. PCR reaction

Gene specific primer designed using the contigs (Table 3) were used for PCR amplification. The cDNA is used for amplifying AGPase specific regions. PCR was carried out in a thermal cycler C1000^{TM}BioRad).

The composition of the reaction mixture was as follows:

Components/ Concentration		volume
Template DNA (10 ng/ µl)	:	2.0 µl
Primer(10µM)	:	0.5 µl
10X Taq buffer	:	2.0 µl
dNTP mix	:	0.4 µl
Taq polymerase (1u)	:	0.2 µl
Distilled Water	:	14.5 µl
Total Volume	:	20 µl

Table 1: Gene specific primer designed for the study

Sl. no	Primer name	Forward primer	Reverse primer	Tm (°C)	Size
1	AGPS1a	AGGTGGTGCAGGGACTAGAT	CGTCAAGGCCAAGTATGGTC	56	572
2	AGPS1b	CTAGCACTGGCATGAGGGTA	CAAGTAGCTCTGTCCCTTGG	54	423
3	AGPS1c	GGATGAGGAACGTGCAACTG	AGGACCGGAGTCCAACTACT	54.3	542
4	AGPS1d	GTGGTGCAGGGACTAGATTG	AAGGGTGGAGAAGTGCCTTG	52.4	1400
5	AGPS2a	GAAGCGTGCTACTGCCTTTG	CCAGGAAACTTGTCCCGAAG	54.7	581
6	AGPS2b	GGCTACTGGGAAGACATTGG	GACAGCACCCTCCGAAATAC	55.4	225
7	AGPS2c	TCTTGGGTCTTGACGATCAG	GTGACATCAGCATCGAGCAT	53.6	349
8	AGPS2d	GACATCACTGTGGCTGCTCT	GACAGCACCCTCCGAAATAC	52.6	522
9	AGPS3a	AGTCCAGAGAACCCCGATTG	CGAGGTTGGGTGTAGATTGG	54.23	617
10	AGPS3b	CTTGGGATGAGAGTGCAAGC	GACGGTGACAATCCCACTCT	57.8	525
11	AGPS3c	CCTTACGTTCCCAAGGAGTC	CTCTCCTTGCGGTTTCTCTG	53.1	662
12	AGPS3d	CACGTGCAGTATCCAGCAGA	GACGGTGACAATCCCACTCT	52.8	1451
13	AGPS4a	GGGGAAGAGGAAGCTATGTC	GCTCTTGCAACACCTTCCAG	54.31	1472
14	AGPS4b	GAGGAAGCTATGTCGGCAAC	AGATGGTCACCAGCCAGAAC	55.8	1478
15	AGPL1	GGATCAGAGGGAGTCTCAAC	GGCCTATCTGCTTCTTGCAC	54.12	512
16	AGPL2	CTTACCAGAAGGGCAGCAAC	CTGGCCTATCTGCTTCTTGC	56.27	1175

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The PCR amplification was carried out in 6 steps as follows.

Step	Temperature	Time	Specification
1	94	5	Initial denaturation
2	94	1	Denaturation
3	47	2	Annealing
4	72	2	Extension
5	72	10	Final extension
6	4	α	Hold

Steps 2, 3, 4 — Run for 30 cycle

Schematic representation of PCR amplification

94°C 5min | 94°C 1 min | 47°C 2 min | 72°C 2min | 72°C 10 min | 4°C α
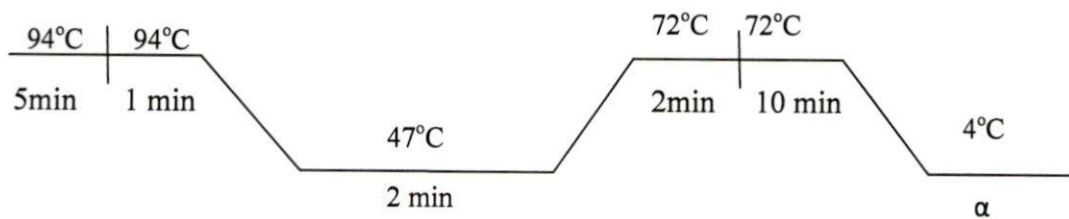
3.8. Gel elution of PCR amplified fragment

The PCR products were extracted with QIAquick gel extraction kit for sequencing of desired fragment.

- DNA fragments in the 1.5% gel were excised with clean sharp scalpel.
- The gel slices were put into a pre-weighed 2ml tubes and weight recorded. Three volumes of QG added to the gel and incubated at 50°C and vortexes the tubes every 2 minute.
- After completing dissolution of gel, 1 volume of isopropanol was added to the samples and mixed well and transferred the samples to QIAquick column and centrifuged for 1 min.
- After centrifugation 500 µl of QG buffer was added to QIAquick column and centrifuged for 1 min. QIAquick column was washed with 750 µl of PE buffer.
- Repeated this step.
- After washing procedure, eluted the DNA with 50 µl of EB buffer repeated the step for increasing DNA quantity.
- Elute was subjected to PCR using the same reaction mix under required conditions and PCR product analyzed using 1.5% agarose.

3.9. Sequencing

a) Cycle sequencing

Cycle sequencing is the modification of traditional Sanger sequencing method. This method uses di-deoxynucleotides in a polymerization reaction to create a nested set of DNA fragments with di- deoxynucleotides at the 3' terminus of each fragment. It employs a thermo-stable DNA polymerase, which can be heated to 95°C and retain the enzyme activity after repeated us

The PCR reaction cocktail for cycle PCR is given below.

Reagents	Concentration	Volume
Ready reaction mix (BigDye Terminator)	2.5X	0.5 μl
Dilution mix	5X	1 μl
Template	100-200 ng/μl	2 μl
Primer (either forward/Reverse)	2 μM-10 μM	2 μl
MilliQ water		4 μl
Total		10 μl

The following thermo-cycling PCR profile was set for cycle sequencing.

Step	Temperature(°C)	Time	Specification
1	96	1 min	Initial denaturation
2	96	10 sec	Denaturation
3	47	5 sec	Annealing
4	72	4 min	Extension
5	4	for infinity	Hold
Run for 25 cycle			

b) Clean-up

The cycle sequencing products were subjected for cleanup prior to sequencing to remove unincorporated dye terminators and salt that may compete for the capillary electrophoretic injection. The following protocol for clean up (Appendix IV) was followed.

- A master-mix I (10 µl sterile water and 2 µl 12M EDTA; per reaction) was prepared. 12 µl of master mix I was added to each PCR tube containing 10 µl product.

- A second master-mix II (2 µl 3M sodium acetate; PH 4.6 and 50 µl ethanol) per reaction was prepared. 52 µl of master-mixII was added to each reaction.

- The contents were mixed well and incubated for 15 min at room temperature.

- Then centrifuged at 12000g for 20 min at room temperature.

- Supernatant was removed and 250 µl 70% ethanol was added and centrifuged at 12000 rpm for 10 min at room temperature.

- Supernatant was removed and the pellet was dissolved in 10- 20 µl HiDi^{TM} formamide and mixed by vortexing.

- 10 µl of each sample was loaded onto respective wells. The 96 well-plate was sealed with septa strip and placed in a plate holder and assembled plate was covered from above using a plate retainer. The assembled plate unit was then placed in auto-sampler of Genetic Analyzer (Applied Biosystems-3500).

- The sequencing analysis for the SSR primer was set in the machine and the samples were run. The data file ("filename.abi") was collected at the end of the program.

3.10. Sequence analysis

3.10.1. Nucleotide sequence similarity

To analyse Sequence similarity sequence generated using three primers from high and low starch varieties of cassava were compared using BLAST N. It finds regions of similarity between our sequences with sequences in the NCBI. The program compares nucleotide to nucleotide sequence databases and calculates the statistical significance.

3.10.2. Identification of sequence variation

To identify Single Nucleotide Polymorphism (SNP) using bioinformatics tool Clustal Omega for comparison. It is a multiple sequence alignment program. It produces biologically meaningful multiple sequence alignments of divergent sequences. Sequences of amplicon developed using AGPSIb primer in MNga-1 and 9S-127 was aligned and differences in the nucleotides were identified. Similarly sequences of amplicon developed from AGPS2d and AGPL1 from low starch variety MNga-1 and high starch variety 9S-127 were compared.

3.10.3. Translation of sequence

To identify the difference in amino acid sequence between MNga-1 and 9S-127, translate the nucleotide sequences from the two amplicon developed using AGPS1b, AGPS2d and AGPL1 primers in MNga-1 and 9S-127. ExPASy translational tool translate sequence into corresponding amino acid.

3.10.4. BLAST-p

Amino acid sequences developed from single nucleotide sequences using ExPASy translation tool were blasted for finding active amino acid. These amino acid sequences compared using BLAST-P for finding the similarity with the AGPase amino acids in the NCBI. BLAST-P finds regions of similarity between study amino

acid sequences in former study with the amino acid sequences in the NCBI. The program compares amino acid sequences to amino acid sequence databases and calculates the statistical significance.

3.10.5. Clustal Omega

Clustal Omega is a multiple sequence alignment program. It produces biologically meaningful multiple sequence alignments of divergent sequences. Functional amino acid sequences developed from single primer in two different varieties high starch 9S-127 and low starch MNga-1 were aligned and differences in the amino acid were identified.

RESULTS

4. RESULTS

The present study was carried out to identify the nucleotide polymorphism in high starch variety 9S-127 and low starch variety MNga-1. The results of experiment conducted are described below.

4.1 Selection of plant material

High starch variety 9S-127 and low starch variety MNga-1 were planted in ICAR-CTCRI for the comparison of AGPase sequence in high and low starch variety.

4.2 Estimation of starch content

In this study, 9S-127 and MNga-1 were used. Starch content of both samples was estimated. Dry weight percentage of 9S-127 is 41% and MNga-1 is 31% after 72h incubation. Fresh weight percentage of starch in 9S-127 is 29.5 and for MNga-1, 23.6. From the result, 9S-127 has high starch when compared to MNga-1.

4.3 Standardization of RNA isolation

GeneJET plant RNA purification mini Kit (Thermo Scientific) and manual methods (LiCl) used for the isolation of RNA from MNga-1 and 9S-127 varieties. Plates 3 shows the RNA profile on agarose gel using LiCl and Plate2 shows the RNA profile on agarose gel using kit method. RNA isolation using kit method gave better spectrometric reading.

4.3.1Quantification of RNA

The concentration on RNA present in each sample was determined by taking the absorbance reading at 260nm and 280nm in Nano-Drop spectrometer. The concentration of RNA isolated using manual method ranged from 231-391 ng/µl (Table 2) and RNA isolated using GeneJET plant RNA

purification mini kit method, MNga-1 and 9S-127, was 3661.4, 4574.1 ng/μl respectively (Table 3). It concludes that GeneJET plant RNA purification mini kit gives good concentration of RNA.

4.3.2 Quality of isolated RNA

The quality of RNA was tested in 1% agarose gel using ethidium bromide dye. The gels were documented using gel documentation. RNA isolated using manual method (LiCl) gave poor quality of RNA and RNA isolated using GeneJET plant RNA purification mini kit method gave good quality RNA.

Table 2. Spectrophotometric readings of RNA isolated using LiCl method.

Sample name	Concentration (ng/μl)	Path L (mm)	A_{260}/A_{280}	A_{260}/A_{230}
9S-127	269.46	10	2.49	0.19
9S-127	391.00	10	2.29	0.32
MNga-1	231.26	10	2.41	0.27
MNga-1	329.27	10	2.31	0.29

Table 3. Spectrophotometric readings of RNA isolated using kit method

Sample name	Concentration (ng/μl)	Path L (mm)	A_{260}/A_{280}	A_{260}/A_{230}
9S-127	4574.13	10	2.383	2.1
MNga-1	3661.35	10	2.401	2.3

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GEL PICS

Gel profile of RNA
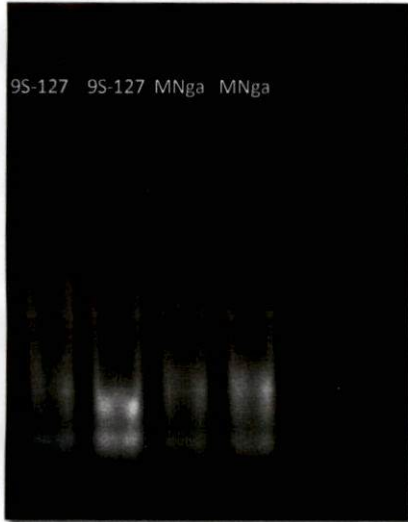
Plate: 3

Plate 3: 1.2% gel image of RNA isolation using manual method
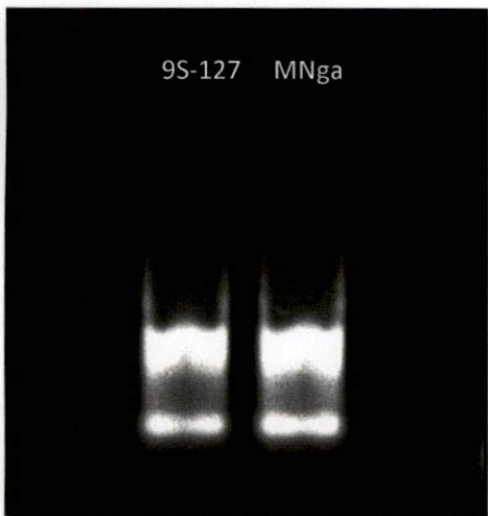
Plate:4

Plate 4: 1.2% gel image of RNA isolation using kit method

4.4 cDNA synthesis

mRNA isolated from high starch variety 9S-127 and low starch variety MNga-1 were reverse transcribed to cDNA. The quality of cDNA was checked in 1% agarose gel and the quantity determined using Nano-Drop spectrometer

4.4.1 Quantification of cDNA

The concentration of cDNA present in the sample was determined by taking absorbance reading at 260nm and 280nm in Nano-Dropspectrometer (Table.4). After quantification all the samples were diluted to uniform concentration of 100 ng/μl.

4.4.2 Checking the quality of cDNA

The quality of each DNA sample was tested in 1% agarose gel with ethidium bromide dye. The gel was documented in gel documentation system (Plate.5).

Table 4. Spectrophotometric readings of cDNA

Sample name	Concentration (ng/μl)	Path L (mm)	A_{260}/A_{280}	A_{260}/A_{230}
9S-127	1591.116	10	1.945	2.097
MNga-1	1630.18	10	1.929	2.077
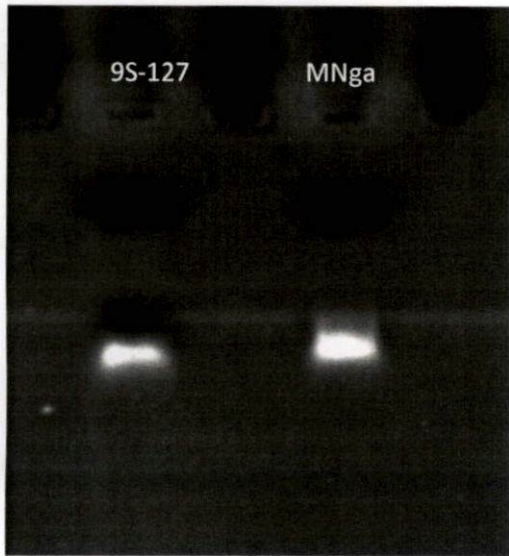
Plate:5 Gel profile of cDNA

Plate 5: Fig c: 1.2% gel image of c DNA

4.5. PCR

Amplification of AGPase gene was carried out using PCR, from 9S-127 and MNga-1 using sixteen degenerative primers (Table 1).

4.5.1. Primer designing

A total 16 primers were designed from four contig sequence of AGPase using primer 3 plus software. Contig sequences (Fig.1) were developed from all available cDNA sequences of AGPase using CAP 3 software.

4.5.2. PCR optimization

Amplification of gene specific primer was performed to optimize PCR conditions and the PCR products were run on 1.2% agar to check amplification. Among 16 three primers AGPS1b, AGPS2d and AGPL gave good result after optimizing annealing temperature (Ta) and produce amplification in 47°C.

The high starch variety 9S-127 and low starch variety MNga-1 were amplified with primers AGPS1b, AGPS2d, and AGPL shows specific bands of bands size 423,581,512 at 47°C using DNA template concentration 100ng/μl. These conditions were used for the amplification of AGPase gene in 20μl reaction volume for purification and sequencing (Plate 6).

4.6. Sequencing

Gene specific regions were obtained after amplification with specific primers. Amplification with AGPS1b showed a band size of 423bp (Fig.2) and AGPS2d gave a band size of 581bp (Fig.3) and AGPL gives a band of 512bp (Fig.4) These regions were sequenced in ABI3500 genetic analyzer. The sequence data of these varieties obtained in 'abi file format' were used for

Contig I

GCTTATCCCTCGGCAATGGATGTGCCTTTGGCATCTAAAACATTCCCCTCGCCCTCACCTTCCAAGCGTGAAC
AATGCAACGTTGATGGCCATAAGAGCTCATCGAAGCATGCAGATCTCAATCCACATGCTAATGATAGTGTTC
TCGGAATTATTCTTGGAGGTGGTGCAGGGACTAGATTGTATCCCCTGACGAAGAAGCGTGCGAAGCCTGCAG
TGCCACTGGGTGCCAACTACAGGCTTATTGATATTCCTGTCAGTAATTGTCTGAACAGCAACATATCAAAGAT
CTATGTGCTCACGCAGTTCAACTCAGCTTCTCTTAATCGTCATCTCTCACGAGCCTATGGGAGCAACATTGGA
GGTTACAAGAATGAAGGATTTGTTGAAGTCCTTGCTGCACAGCAGAGCCCAGATAACCCTGACTGGTTTCAG
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CTGTTGCTGCCTTGCCCATGGATGAGGAACGTGCAACTGCATTTGGCCTTATGAAAATCGATGAAGAAGGGA
GGATAATTGAATTCGCAGAGAAACCAAAAGGAGAACAGTTGAAAGCTATGATGGTTGATACGACCATACTT
GGCCTTGACGATGCGAGGGCAAAGGAAATGCCTTATATTGCTAGCATGGGTATCTATGTTATTAGCAAACAT
GTGATGCTTCAGCTTCTCCGCGAGCAATTTCCTGGAGCTAATGACTTTGGAAGTGAGGTTATTCCTGGTGCAA
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TCTATAATGCAAATTTGGGAATTACCAAAAAGCCAATACCCGATTTCAGTTTCTATGACCGTTCTGCTCCCAT
TTACACACAACCTCGACACTTGCCTCCTTCAAAGGTTCTTGATGCTGATGTGACAGACAGTGTTATTGGTGAA
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ATAATAGAGGACACATTGCTAATGGGTGCGGACTACTATGAGACTGAAGCTGATAAGAAACTCCTTGCTGAA
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ATTGGAGATAACGTGATGATAATCAATGTTGACAATGTTCAAGAAGCGGCAAGGGAGACAGATGGATATTT
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TAAGAGCAATAAAAAAGGAGTGCCATGCAAGGCACTTCTCCACCCTTTTTCTCCCTTGATGTATTAGGAACT
GTGATGTACAAGCAACTGTGATGCACTTAGGCGAAGTGCCCCTGGATTCAGCTTTCTCTTTGCTTGTAGTTGA
TTTCCAGCAGAC

Contig II

CACGAGGGTTTATTCAGGCACATAGAGAAACTGATGCAGACATCACTGTGGCTGCTCTGCCCATGGATGAGA
AGCGTGCTACTGCCTTTGGTTTGATGAAGATTGACGACGAGGGACGCATTATTGAGTTTGCTGAGAAACCTA
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TCAAAGATGCTCGATGCTGATGTCACAGATAGTGTTATTGGCGAGGGATGTGTGATAAAGAACTGTAAAATT
CACCATTCAGTCGTTGGGCTTCGATCTTGTATTTCGGAGGGTGCTGTCATTGAAGACACATTACTGATGGGAG
CAGACTACTATGAGACGGATGCTGACA

Contig III

AGCTGTAATTAAGTGCAATCACACTCTACCACACACTCTCTATAGTATCTATAGTTGAGAGCAAGCTTTGTTA
ACAATGGCGGCTTCCATTGGAGCCTTAAAATCTTCACCTTCTTCTAACAATTGCATCAATGAGAGAAGAAAT
GATTCTACACGTGCAGTATCCAGCAGAAATCTCTCATTTTCGTCTTCTCATCTCGCCGGAGACAAGTTGATGC
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AATTCAACTCTGCCTCTCTGAATCGCCACCTTTCACGAGCATATGCTAGCAACATGGGAGGATACAAAAACG
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AAGAACTGTAAGATTCACCATTCCGTGGTTGGGCTCAGATCATGCATATCAGAGGGAGCAATTATAGAAGAC
TCACTTTTGATGGGGGCAGATTACTATGAGACTGATGCTGACAGGAAGTTGCTGGCTGCAAAGGGCAGTGTC
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GATTGTCACCGTCATCAAGGATGCTTTGATTCCAAGTGGAATCATCATCTGAAGGAATGCGTTTTAACTTGGT
TGTCCTCCAAGATTTTGGCTAAACAGCCATGAGGTAGAAACGTGCTGAACTTTTATTTTCCTGAGCTGTAGAA
ATCTAGTGTACATCTTTCTGTTATGATACTTCTCATTACCCCCACAAAAGAAGACTGGATGCTGTAAAAATTA
TTCGTCTAGAATAAAAAATAAATTACTCAAAAAAAAAAAAAAAAAAAAAAA

Contig IV

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CATCTGTATCGAATGGATTACGAGAGATTTATACAAGCACACAGGGAAAGTGATGCTGATATTACTGTTGCT
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TGCTTT

Fig 1: contings developed using CAP3 software.
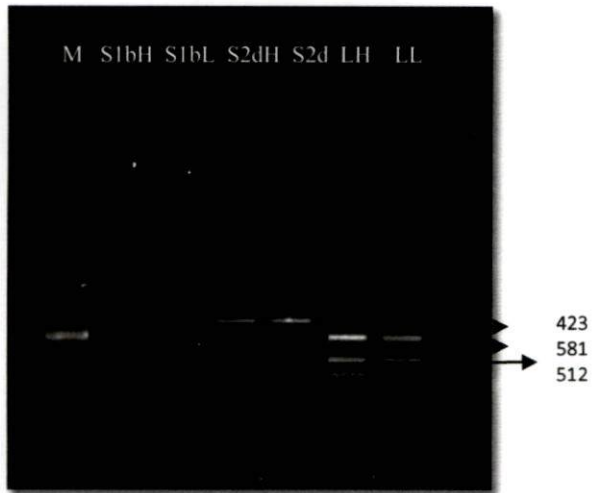
Plate: 6 Gel profile of amplified product.

Plate 6 : M: DNA 50 bp ladder, S1bH: amplicon of 9S-127 amplified with AGPS1b,
S1bL: amplicon of MNga-1 amplified with AGPS1b, S2dH: amplicon of 9S-127
amplified with AGPS2d, S2bL: amplicon of MNga-1 amplified with AGPS2d. LH:
amplicon of 9S-127 amplified with AGPL, LL: amplicon of MNga-1 amplified with
AGPL

Sequences

MNga-1

GGGATGAGAGTGCAAGCTTATTTATATGATGGCTACTGGGAAGATATTGGAACAATTGGGGCATTTTACAAT
GCCAATCTGGGTATAACTAAAAAGCCAGTGCCAGATTTCAGCTTCTATGATCGTTCATCTCCAATTTATACTC
AGCCTCGATATTTGCCTCCATCCAAGATGCTTGATGCTGATGTTACTGACAGTGTTATTGGTGAGGGGTGTGT
TATTAAGAACTGTAAGATTCACCACTCTGTGGTTGGGCTTCGATCCTGCATATCAGAAGGTGCAATCATAGA
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9S-127

TCCATTGGGATGAGAGTGCAAGCTTACTTATATGATGGCTACTGGGAAGATATTGGAACAATTGGGGCATTT
TACAATGCCAATCTGGGTATAACTAAAAAGCCAGTGCCAGATTTCAGCTTCTATGATCGTTCATCTCCAATTT
ATACTCAGCCTCGATATTTGCCTCCATCCAAGATGCTTGATGCTGATGTTACTGACAGTGTTATTGGTGAGGG
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ATAGAGGATACATTACTAATGGAAGCAGATTACTATGAGACTGATGCTGACAGGAGGTTTCTGGCAGCCAAG
GGTAGT

Fig 2: sequencing of amplicon developed from Primer AGPS1b

MNga-1

AAATTGGTTCCAGGGCACAGCTGATGCTGTCAGACAGTACTTGTGGTTGTTTGAAGAGCACAATGTTCTGGA
ATTCTTGATTCTTGCTGGGGATCATTTATACCGCATGGATTATGAAAGGTTTATTCAAGCACACAGAGAAACT
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9S-127

GGGCACAGCTGATGCTGTCAGACAGTACTTGTGGTTGTTTGAAGAGCACAATGTTCTGGAATTCTTGATTCTT
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GCGCATAATTGAATTTGCTGAGAAGCCAAAAGGGGAGCAATTGAAAGCTATGAAGGTTGATACTACAATTCT
AGGTCTTGATGATGAGAGAGCTAAAGAGATGCCTTACATTGCTAGTATGGGGATATATGTCGTCAGCAAAAA
TGTGATGTTAGATCTTCTAAGAGATAAGTTTCCTGGAGCCAATGACTTTGGAAGTGAAGTTATTCCTGGTGCT
ACTTCCATTGGGATGAGAGTGCAAGCTTAC

Fig 3: sequencing of amplicon developed from Primer AGPS2d

MNga-1

GGATGCTATAAACTAATAGACATTCCAATGAGCAACTGTTTCAACAGTGGCATAAACAAAATATTCGTACTG
ACTCAATTCAACTCAGCTTCCCTTAATCGTCACCTTGCACGCACATACTTTGGAAATGGTATTAAATTTGCTG
ACGGTTTTGTGGAGGTCCTGGCAGCAAATCAAACGCCTGGAGAAGCAGGAATGCAGTGGTTCCAGGGAACT
GCAGATGCTGTGAGGCAATTTATTTGGGTATTTGAGGATGCCAAGAACAGAAATGTTGAGAATATATTGATC
TTGTCCGGAGATCATCTTTACCGAATGGATTATCTGGATTTTTTGCAGCATCATGTTGACAGTAATGCTGATA
TCACGATTTCATG

9S-127

TGGGAGGATGCTATAAACTAATAGACATTCCAATGAGCAACTGCATCAACAGTGGCATAAACAAGATATTCG
TACTGACCCAATTCAACTCTGCTTCCCTTAACCGGCACATTGCACGCACATACTTTGGAAATGGTATTAAATT
CAGAGACGGTTTTGTGGAGGTCCTAGCTGCAACTCAAACGCCTGGAGAAGCAGGAATGCAGTGGTTCCAAG
GAACTGCAGATGCTGTGAGGCAATTTATTTGGGTTTTTGAGGATGCCAAGAACAGAAATGTTGAGAATATAT
TGATCTTGTCCGGAGATCATCTTTATCGAATGGATTATCTGGATT

Fig 4: sequencing of amplicon developed from Primer AGPL1

identification of sequence based polymorphism through bioinformatic analysis.

4.6.1. Sequence analysis

The resulting sequence data were trimmed to remove contaminations and low complexity regions. The sequence results were visualized in Bio-Edit sequence alignment editor software. The obtained sequences were run through BLAST program of NCBI.

4.6.2. Similarity Analysis

BLAST map query sequence of MNga-1 amplified with AGPS1b shows 100 hit similar to glucose-1-phosphate adenyl transferase small subunit 2, chloroplastic (*Manihot esculenta*) (XP021629456.1), 9S-127 amplified with AGPS1b shows 100 hit similar to ADP-glucose pyrophosphorylase small subunit (AAK11298.1)(Fig.5) and MNga-1 and 9S-127 fragment amplified using AGPS2d gives higher hits related to *Manihot esculenta* isolate km94ADP glucose pyrophosphorylase small subunit m-RNA complete cds (KU2431231) sequence showed 100% similarity (Fig. 6).

MNga-1 and 9S-127 amplified with AGPL showed similarity to *Manihot esculenta* isolate KM94 AGPase large subunit protein mRNA complete cds (KU243121.1) (Fig. 7).

Nucleotide BLAST

Fig: 5 AGPS1b primer fragment
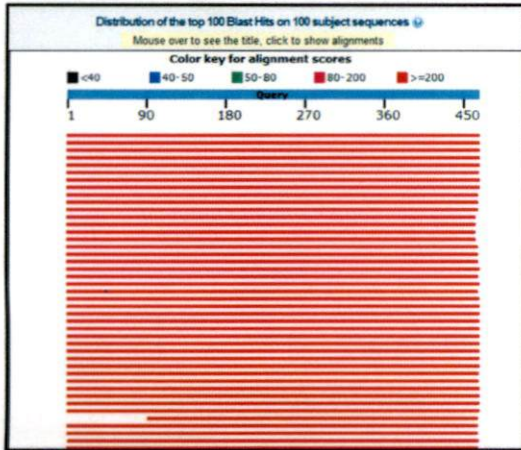
9S-127   MNga-1
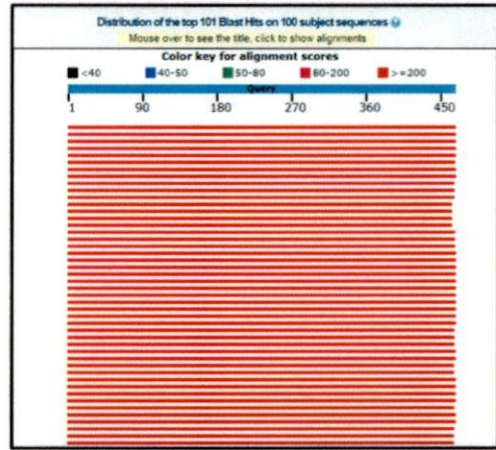
Fig: 6 AGPS2d primer fragment

9S-127   MNga-1

69
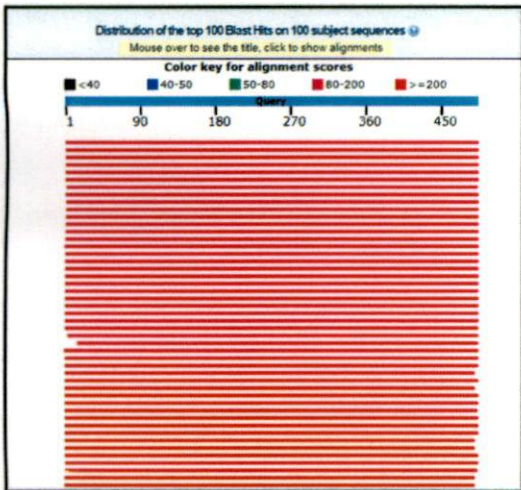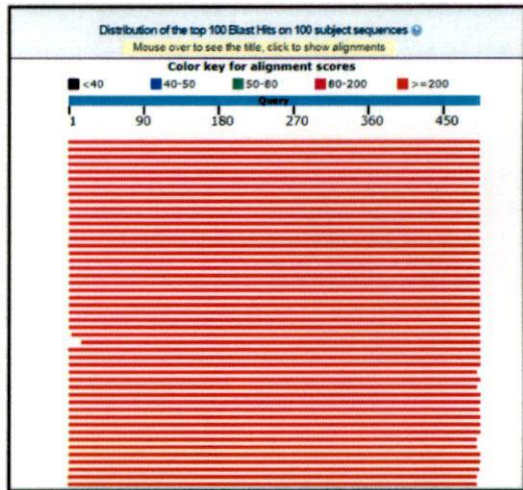
Fig: 7 AGPL1 primer fragment

MNga-1
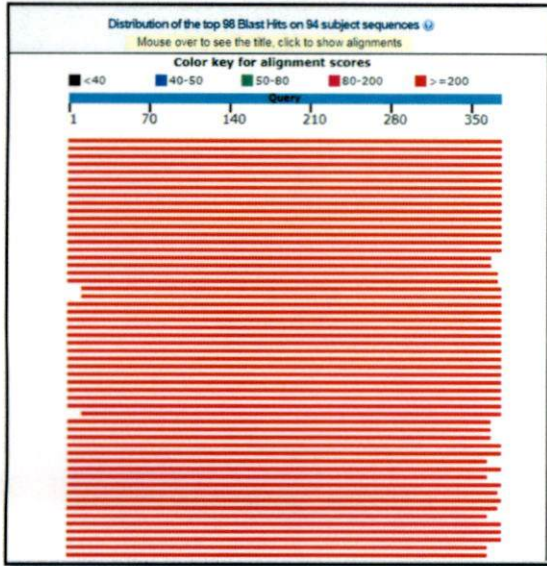
9S-127
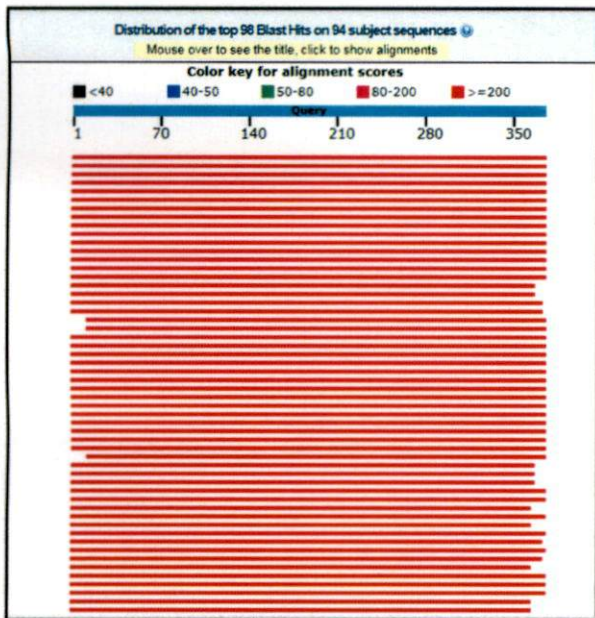
Table 5. Similarity of sequence with NCBI Database

Primer	Variety	Similarity with in Database	Accession number
AGPS1b	MNga-1	Glucose-1-phosphate adenyl transferase (*Manihot esculenta*)	XP021629456.1
	9S-127	ADPglucose pyrophosphorylase small subunit	AAK11298.1
AGPS2d	MNga-1	km94 AGPase small subunit (*Manihot esculenta*)	KU2431231
	9S-127	km94 AGPase small subunit (*Manihot esculenta*)	KU2431231
AGPL	MNga-1	KM94 AGPase large subunit (*Manihot esculenta*)	KU243121.1
	9S-127	KM94 AGPase large subunit (*Manihot esculenta*)	KU243121.1

4.6.2. Nucleotide variation identification

To investigate the nucleotide variation within the gene encoding AGPase in high and low starch varieties of cassava, the sequence variations were analyzed using the sequences developed from each primer in high and low starch cassava varieties. Clustal Omega is a bioinformatics tool used for comparison of nucleotide sequences. Sequence developed from 9S-127 and MNga-1 by using AGPS1b (SS) were compared and found that two SNP region (Fig. 8), from AGPS2d (SS) gives nine SNP regions (Fig. 9) and sequence developed using AGPL (LS) identified 16 SNP region in high and low starch variety, it shows high sequence variation in large subunit of nucleotide (Fig. 10) than small subunit (Table 6.).

Comparison of nucleic acid sequence

T/C
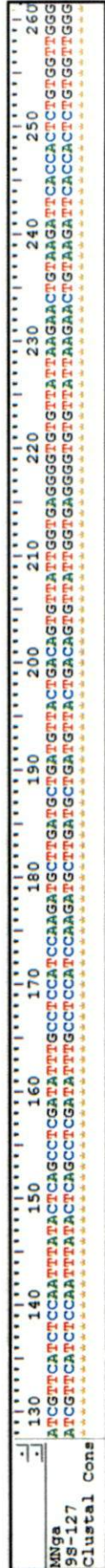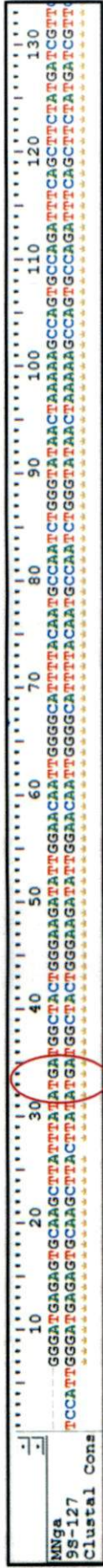
G/A

Fig: 8 Comparison of sequence developed from AGPS1b.

Fig: 9 Comparison of sequence developed from AGPS2d
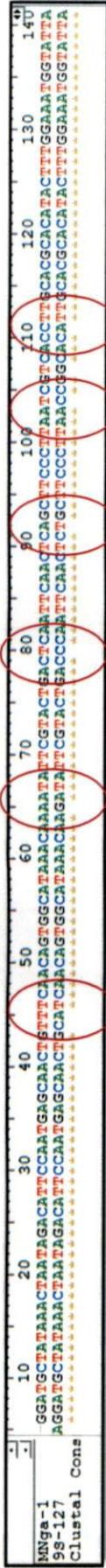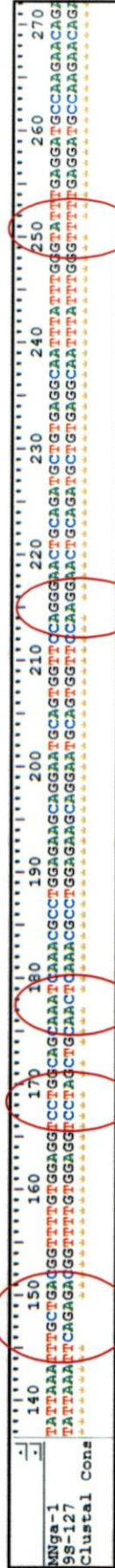
Fig: 10 Comparison of sequence developed from AGPL1.

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4.6. Translation of sequence

The sequences were translated to corresponding amino acid sequence through ExPASy software (Fig.11,12,13). Codon in the nucleic acid determines the amino acids. Nucleotide sequences can be read in six reading fame three in forward and three in reverse. Among the six reading frame one is used in functional gene and mostly preferred longest amino acid sequence is mostly referred functional amino acid sequences were identified through BLAST P (Fig.14,15,16) The six sequenced nucleotides were converted to amino acids in all reading frames. From which functional amino acid were identified through BLASTP (Fig.17).

4.6.1. Protein comparison

To investigate the change in aminoacid between high starch 9S-127 and low starch MNga-1 translated sequence were compared using CLUSTAL OMEGA multiple sequence alignment tool and variation in the protein sequence was identified. Sequence variation in small subunit of MNga-1 at 296th position methionine is replaced by leucine, at 298th position tyrosine is replaced by phenylalanine, at 263rd position Arginine is replaced by serine and at 157th glutamic acid is replaced by glycine (Fig. 18, 19). Methionine and leucine are highly similar and shows same property. Similarly, tyrosine and phenyl alanine are highly similar and have same property.

In case of large subunit MNga-1 at 137th position isoleucine is replaced by phenylalanine, at 159th Iso-leucine is replaced by leucine, 169th arginine is replaced by alanine and at 181th threonine is replaced by asparagine (Fig. 20). Isoleucine and phenylalanine are highly similar. Isoleucine and leucine have similar properties.

Invitro translation of sequences

9S-127

MNga-1

Fig : 11 Amino acid sequences developed from AGPS1b primer

9S-127

MNga-1

Fig : 12 Amino acid sequences developed from the primer AGPS2D

MNga-1

Translate   Home | Contact

Translate Tool - Results of translation

Open reading frames are highlighted in red. Please select one of the following frames - in the next page, you will be able to select your initiator and retrieve your amino acid sequence:

5'3' Frame 1
G C Y K L I D I P Met S N C F N S G I N K I F V L T Q F N S A S L N R H L A R T Y F G N G I K F A D G F V E V L A A N Q T P G E A G Met Q W F Q G T A D A V R Q F I W V F E D A K N R N V E N I L I L S G D H L Y R Met D Y L D F L Q H H V D S N A D I T I S

5'3' Frame 2
D A I N Stop Stop T F Q Stop A T V S T V A Stop T K Y S Y Stop L N S T Q L P L I V T L H A H T L E Met V L N L L T V L W R S W Q Q I K R L E K Q E C S G S R E L Q Met L Stop G N L F G Y L R Met P R T E Met L R I Y Stop S C P E I I F T E W I I W I F C S I Met L T V Met L I S R F H

5'3' Frame 3
Met L Stop T N R H S N E Q L F Q Q W H K Q N I R T D S I Q L S F P Stop S S P C T H I L W K W Y Stop I C Stop R F C G G P G S K S N A W R S R N A V V P G N C R C C E A I Y L G I Stop G C Q E Q K C Stop E Y I D L V R R S S L P N G L S G F F A A S C Stop Q Stop C Stop Y H D F Met

3'5' Frame 1
H E I V I S A L L S T Stop C C K K S R Stop S I R Stop R Stop S P D K I N I F S T F L F L A S S N T Q I N C L T A S A V P W N H C I P A S P G V Stop F A A R T S T K P S A N L I P F P K Y V R A R Stop R L R E A E L N Stop V S T N I L F Met P L L K Q L L I G Met S I S L Stop H

3'5' Frame 2
Met K S Stop Y Q H Y C Q H D A A K N P D N P F G K D D L R T R S I Y S Q H F C S W H P Q I P K Stop I A S Q H L Q F P G T T A F L L L Q A F D L L P G P P Q N R Q Q I Stop Y H F Q S Met C V Q G D D Stop G K L S Stop I E S V R I F C L C H C Stop N S C S L E C L L V Y S I

3'5' Frame 3
Stop N R D I S I T V N Met Met L Q K I Q I I H S V K Met I S G Q D Q Y I L N I S V L G I L K Y P N K L P H S I C S S L E P L H S C F S R R L I C C Q D L H K T V S K F N T I S K V C A C K V T I K G S Stop V E L S Q Y E Y F V Y A T V E T V A H W N V Y Stop F I A S

9S-127

Translate   Home | Contact

Translate Tool - Results of translation

Open reading frames are highlighted in red. Please select one of the following frames - in the next page, you will be able to select your initiator and retrieve your amino acid sequence:

5'3' Frame 1
W E D A I N Stop Stop T F Q Stop A T A S T V A Stop T R Y S Y Stop P N S T L L P L T G T L H A H T L E Met V L N S E T V L W R S Stop L Q L K R L E K Q E C S G S K E L Q Met L Stop G N L F G F L R Met P R T E Met L R I Y Stop S C P E I I F I E W I I W I

5'3' Frame 2
G R Met L Stop T N R H S N E Q L H Q Q W H K Q D I R T D P I Q L C F P Stop P A H C T H I L W K W Y Stop I Q R R F C G G P S C N S N A W R S R N A V V P R N C R C C E A I Y L G F Stop G C Q E Q K C Stop E Y I D L V R R S S L S N G L S G

5'3' Frame 3
G G C Y K L I D I P Met S N C I N S G I N K I F V L T Q F N S A S L N R H I A R T Y F G N G I K F R D G F V E V L A A T Q T P G E A G Met Q W F Q G T A D A V R Q F I W V F E D A K N R N V E N I L I L S G D H L Y R Met D Y L D

3'5' Frame 1
N P D N P F D K D D L R T R S I Y S Q H F C S W H P Q K P K Stop I A S Q H L Q F L G T T A F L L L Q A F E L Q L G P P Q N R L Stop I Stop Y H F Q S Met C V Q C A G Stop G K Q S Stop I G S V R I S C L C H C Stop C S C S L E C L L V Y S I L P

3'5' Frame 2
I Q I I H S I K Met I S G Q D Q Y I L N I S V L G I L K N P N K L P H S I C S S L E P L H S C F S R R L S C S Stop D L H K T V S E F N T I S K V C A C N V P V K G S R V E L G Q Y E Y L V Y A T V D A V A H W N V Y Stop F I A S S

3'5' Frame 3
S R Stop S I R Stop R Stop S P D K I N I F S T F L F L A S S K T Q I N C L T A S A V P W N H C I P A S P G V Stop V A A R T S T K P S L N L I P F P K Y V R A Met C R L R E A E L N W V S T N I L F Met P L L Met Q L L I G Met S I S L Stop H P P

Fig : 13 Amino acid sequences developed from the primer AGPL

Identification of functional amino acid sequence

9S-127
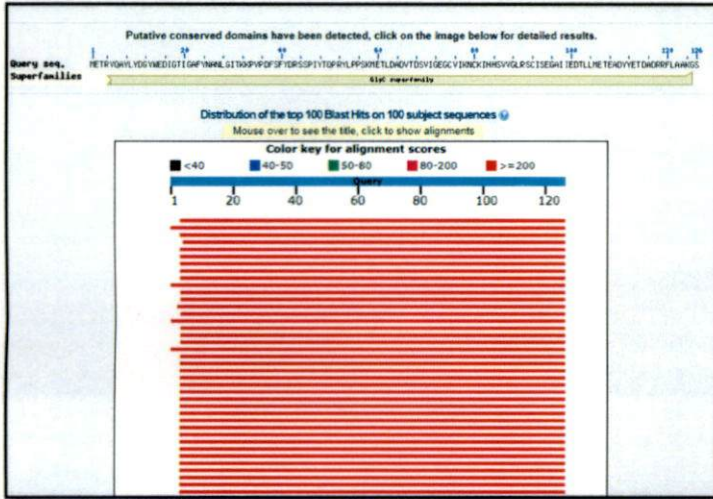
MNga-1
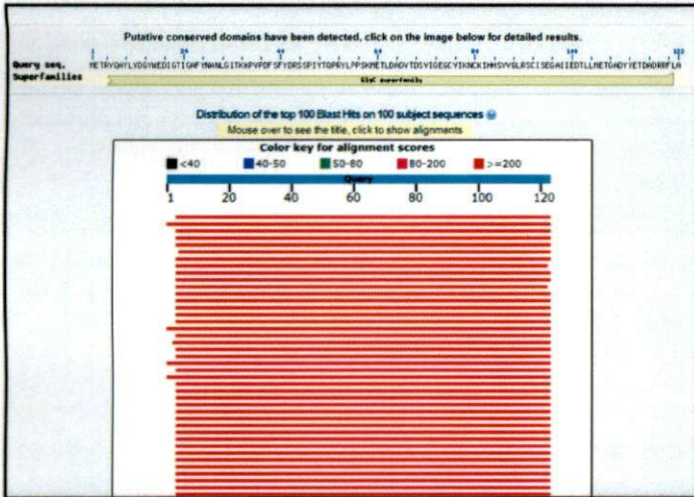
Fig: 14 Identification of functional amino acid developed from AGPS1b primer

AGPS2d

MNga-1
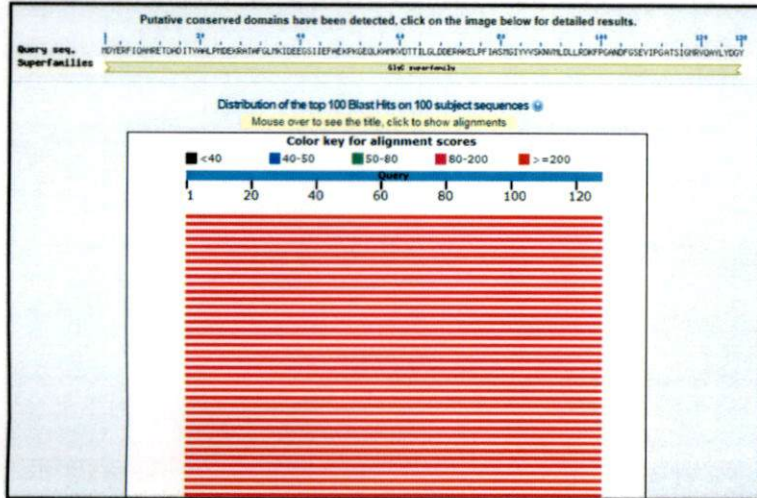
9S-127
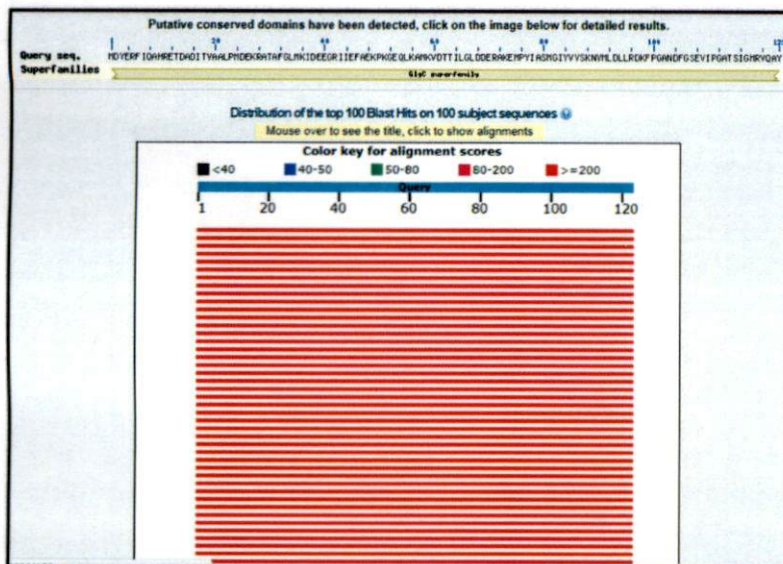
Fig: 15 Identification of functional amino acid developed from AGPS2d primer

9S-127
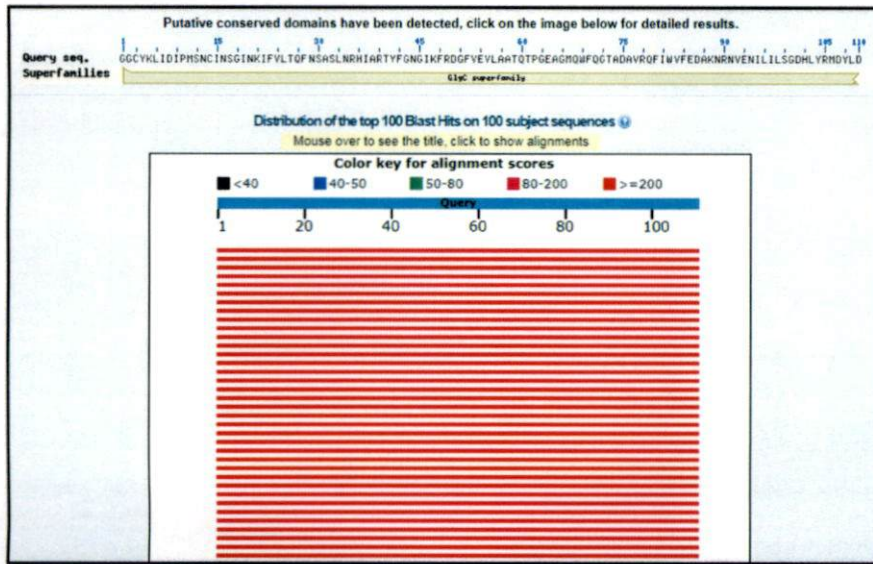
MNga-1
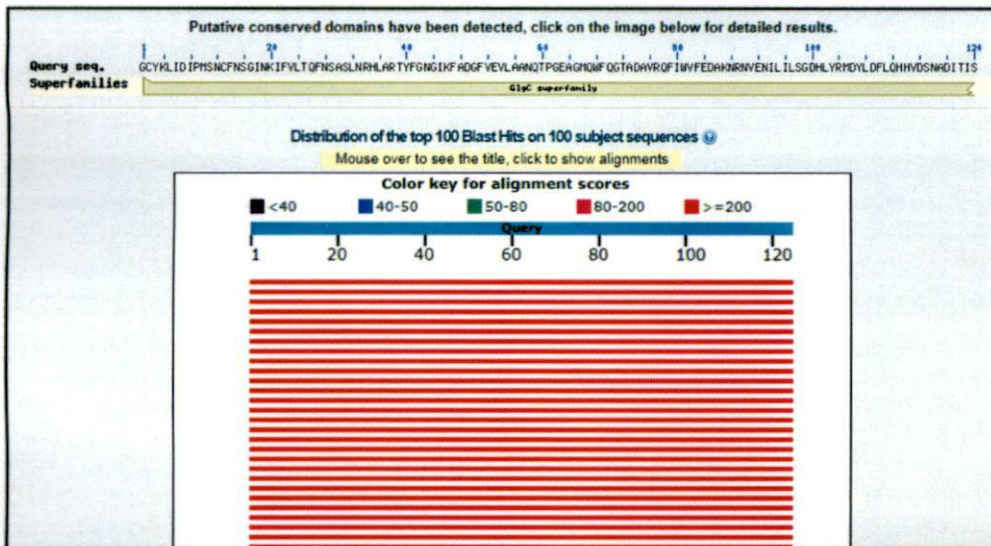
Fig: 16 Identification of functional amino acid developed from AGPL1 primer

Protein sequences corresponding fragment

MNga-1

MRVQAYLYDGYWEDIGTIGAFYNANLGITKKPVPDFSFYDRSSPIYTQP
RYLPPSKMLDADVTDSVIGEGCVIKNCKIHHSVVGLRSCISEGAIIEDTL
LMGADYYETDADRRFLA

9s-127

MRVQAYLYDGYWEDIGTIGAFYNANLGITKKPVPDFSFYDRSSPIYTQ
PRYLPPSKMLDADVTDSVIGEGCVIKNCKIHHSVVGLRSCISEGAIIEDT
LLMEADYYETDADRRFLAAKGS

Fig 17 a. Translation of amplicon developed from primer AGPS1b

MNga-1

MDYERFIQAHRETDADITVAALPMDEKRATAFGLMKIDEEGSIIEFAEK
PKGEQLKAMKVDTTILGLDDERAKELPFIASMGIYVVSKNVMLDLLRD
KFPGANDFGSEVIPGATSIGMRVQAYLYDGY

9S-127

MDYERFIQAHRETDADITVAALPMDEKRATAFGLMKIDEEGRIIEFAEK
PKGEQLKAMKVDTTILGLDDERAKEMPYIASMGIYVVSKNVMLDLLRD
KFPGANDFGSEVIPGATSIGMRVQAY

Fig 17 b. Translation of amplicon developed from primer AGPs2d

MNga-1

GCYKLIDIPMSNCFNSGINKIFVLTQFNSASLNRHLARTYFGNGIKFAD
GFVEVLAANQTPGEAGMQWFQGTADAVRQFIWVFEDAKNRNVENILIL
SGDHLYRMDYLDFLQHHVDSNADITIS

9S-127

GGCYKLIDIPMSNCINSGINKIFVLTQFNSASLNRHIARTYFGNGIKFRD
GFVEVLAATQTPGEAGMQWFQGTADAVRQFIWVFEDAKNRNVENILIL
SGDHLYRMDYLD

Fig 17 c. Translation of amplicon developed from primer AGPL1

Fig 17: Functional amino acid were identified through BLAST-P

Comparison of protein sequences

```
             |....|....|....|....|....|....|....|....|....|....|....|....|
                  10        20        30        40        50        60
98-127    MRVCAYLYDGYWEDIGTIGAFYNANLGITKKPVPDFSFYDRSSPIYTCPRYLFPSKMLDADVTDSVIGEGCVIKNCKIHHSVVGLRSCISEGAIIEDTLIMEADYYETDADRRFLAAKGS
MNga      MRVCAYLYDGYWEDIGTIGAFYNANLGITKKPVPDFSFYDRSSPIYTCPRYLFPSKMLDADVTDSVIGEGCVIKNCKIHHSVVGLRSCISEGAIIEDTLIMGADYYETDADRRFLA----
Clustal Cons ********************************************************************************************************  ***********
                  70        80        90       100       110       120
```
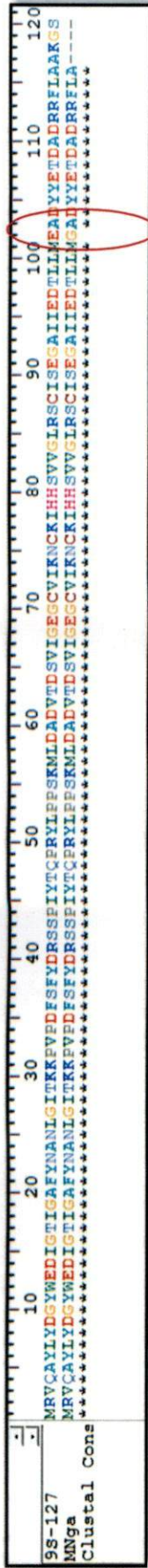
Fig: 18 Comparison of protein sequences developed fromAGPS1b Fragment

Amino acid change

M/L Y/F

R/S

```
             |....|....|....|....|....|....|....|....|....|....|....|....|
                  10        20        30        40        50        60
98-127    MDYERFIQAHRETDADITVAALPMDEKRATAFGLMKIDEEGRIIEAEKPKGECLKAMKVDTTILGLDDERAKEMPYIAEMGIYVSKNVMLDLLRDKFGANDFGSEVIPGATSIGMRVCAY----
MNga      MDYERFIQAHRETDADITVAALPMDEKRATAFGLMKIDEEGSIIEFAEKPKGECLKAMKVDTTILGLDDERAKELPFIAEMGIYVSKNVMLDLLRDKFPGANDFGSEVIPGATSIGMRVCAYLYDGY
Clustal Cons *****************************************  *** **************************   :; ********************* ********************
                  70        80        90       100       110       120
```
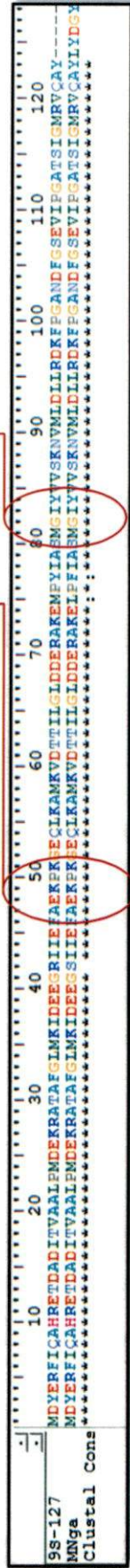
Fig: 19 Comparison of protein sequences developed from AGPS2d Fragment

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Fig: 20 Comparison of protein sequences developed from AGPL Fragment

Table 6. change in nucleotide and changes in aminoacid in both lines

Subunit	SNP (MNga-1/9S-127)	Position	Amino acid (MNga-1/9S-127)	Position
Small Subunit 11 mutations	T/C	387	Leucine/methionine	296
	G/A	622	Phenylalanine/ tyrosine	298
	A/G	1147		
	G/A	1177		
	T/C	1222	Serine/Arginine	263
	A/C	1247		
	G/A	1285		
	A/T	1339	Glutamicacid/glycine	
	T/A	1346		157
	T/A	1353		
	T/C	1354		
Large Subunit 16 mutations	T/C	406	Phenylalanine/ Isoleucine	137
	T/A	407		
	A/C	427		
	T/C	442		
	A/T	457	Leucine/Isoleucine	159
	T/C	466		
	C/A	473	Alanine/Arginine	169
	G/A	477		
	T/C	505	Asparagine/ Isoleucine	181
	G/A	506		
	C/G	507		
	T/A	508		
	G/A	532		
	A/C	540		
	G/A	577		
	T/C	676		

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DISCUSSION

5. DISCUSSION

Tuber crops are the second most important source of carbohydrate in the world's food. According to FAO (1981) showed that nearly 561 million tonnes of root crops were developed world in tropical regions root crops are more important than cereals. Nearly 82 million tones of root crops are produced per annum so that improvement of root crop in nutritional quality is more necessary for human welfare. Among this cassava is the most important source of energy and 6[th]most important source of calorie in human diet. Cassava is highly tolerant to poor soil condition and harsh climatic conditions. Cassava can harvested from 6 to 24 month after planting and gives high food security (Sharkawy, 1993).

Starch is used as staple food and food ingredient. 57% of starch is used in food industry and 43% in non-food sector (Wanous, 2004). Due to several industrial applications demand of starch is very high. To modify the quality and quantity of starch a strong knowledge in molecular basis of starch synthesis pathway is necessary to support breeding program focusing to develop new starch varieties with specific function.

ADP glucose pyrophosphorylase is the key enzyme involving the rate limiting step in starch biosynthesis pathway and starch accumulation. Starch content and quality significantly related to AGPase activity. A number of authors reported the importance of enzyme in starch accumulation in cassava and other root crops. According to Vaibhav et al. (2010) found that in AGPase activity on starch accumulation and growth are correlated from the study on AGPase activity related to starch accumulation and grain growth in wheat cultivar. The importance of this enzyme in starch biosynthesis pathway is demonstrated through up-regulation of ADP glucose in potato tuber which leads to 80% increase in starch content. SNP at the coding region determines the activity of enzyme. Mutation in E. coli AGPase at 412th position decreases specific activity of enzyme up to four order of magnitude (Frueauf et al., 2001) several studies have shown that genetic

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manipulation in AGPase could improve starch production and plant yield through enhanced utilization of photo assimilates (Gibson *et al.*, 2011).

Cassava AGPase enzyme is a heterotetramer that has two types of subunits one is small subunit and another is large subunit. Cassava AGPase large subunit gene is located in small derivative linkage group E and small subunit is located in the female derivative linkage group P. Cassava AGPase small subunit is 1958 bp long and an Open Reading Frame (ORF) of 1572 bp is translated to polypeptide of 524 amino acid. Molecular weight of AGPase small subunit is 57.3 kDa. cDNA of AGPase large subunit is 2385 bp long and has an ORF of 1539 bp translated to protein of 531 amino acid. The molecular weight of large subunit is 58.7kb.

The successful PCR amplification of gene shows that the candidate gene primer used were specific to three partial genomic sequences of AGPase. Gene amplification of AGPase in high starch variety 9S-127 and low starch variety MNga-1, three specific primers generated corresponding amplicons in both the lines. All three sequences of AGPase specific amplified region showed 100% similarity with AGPase sequence in cassava, A similar work was carried out by Zhou *et al.* (2016), and Cheng *et al.* (2015) by elucidating genomic organization of corresponding AGPase through isolating cDNA encoding regions of two small and four large subunits of AGPase from sweet potato. Cheng *et al.* cloned AGPase small subunit and large subunit through Rapid amplification of cDNA end technique.

Considerable sequence identity was found between the large and small subunit of plant and both sequences are equally sensitive to altering amino acid (Georgelis*et al.,* 2007). Small subunit of enzyme is fully catalytic. Active site in the enzyme is maintained by the amino acid sequence and its conformation. A study on direct molecular evolution of ADP glucose pyrophosphorylase reveals that gene shuffling in *TG-15* gene leads to the change in AGPase activity. One amino acid change in the coding region correlated well with observed changes like increasing sensitivity towards 3-PGA activation as well as decreased Pi

inhibition this allow increased carbon storage in starch reserves (Salamone *et al.*, 2002). Change in the amino acid sequence may result in change in conformation this leads to the change in activity of the enzyme. Chemical modification and mutation studies shows amino acid in the enzyme play an important role in the allosteric regulation and binding of substrate. Hydrophobicity of the amino acid depends on its activity.

In MNga-1 four amino acid change in the small subunit, sequence variation in small subunit of MNga-1 at 296th position where methionine is replaced by leucine, at 298th position tyrosine is replaced by phenylalanine, at 263rd position arginine is replaced by serine and at 157th glutamic acid is replaced by glycine. These change in the amino acid leads to the changing activity of enzyme by either increasing or decreasing the activity of enzyme. Here methionine and leucine have similar properties so this amino acid change may not cause much change in the enzyme property. Likely, tyrosine and phenyl alanine are highly similar and share similar property and may not lead drastic change. But the change in amino acid with dissimilar properties leads to changing catalytic activity and change in starch production and this may be the reason for low starch content in MNga-1. Lysine at the 195th position on small subunit of AGPase amino acid present in the binding site of substrate glu-1- phosphate (Parson and Press, 1978). This residue is conserved in large and small subunits. Affinity of glucose-1- phosphate decreases 135 fold when lysine is changed to arginine (Fu *et. al.*, 1998). Aspartic acid in the 142nd position of small subunit of AGPase is mainly involved in the catalysis and is close to substrate binding site. Site directed mutagenesis of aspartic acid decrease the specific activity 10,000 fold but other parameters do not change (Frueaf *et al.*, 2003). Mutation of lysine at 404 position and lysine at 441 of small subunit cause lesser affinity towards 3-PGA (Ballicora *et al.*, 1998). In *E. coli* arginine at 294th position by alanine or glutamine produce a mutanenzyme which changes inhibitor activity specific to NADPH (Frueauf *et al.*, 2001)

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In potato Glc 1-P binding capacity increases 10 fold with respect to wild type by mutation of aspartic acid of the small subunit (Laughlin *et al.*, 1998) and replacement of aspartic acid in the 121th position of enzyme to asparagine cause the enzyme to be less responsive to both glucose-1-phosphate and ATP, while alanine at 106 position to threonine altered sensitivity to ATP. Proline in the 43rd position of AGPase substituted by serine leads to 8 fold decreased sensitivity of enzyme to 3-PGA. From these results we can conclude that change in one amino acid can lead a great impact on enzyme activity.

From sequence comparison it is found that large subunit of the enzyme is more divergent. Sequence conservation of both AGPase subunit are different and show a divergence between large and small subunits. Mutations in the small subunit have greater effect on activity compared with large subunit (Fu *et al.*, 1998; Frueauf *et al.*, 2003). Mutation in large subunit affect allosteric regulation of enzyme. In case of large subunit MNga-1at 137th position isoleucine is replaced by phenylalanine, at 159th iso-leucine is replaced by leucine, 169th arginine is replaced by alanine and at 181th threonine is replaced by asparagine. Isoleucine and phenylalanine are highly similar. Isoleucine and leucine have similar properties so it do not cause much effect on enzyme activity.

In potato tuber large subunit become inactive due to presence of Threonine at 106th position and it become active when threonine is replaced by Lysine (Ballicora*et al.*, 2005). Site directed mutagenesis in 114th position of position of amino acid leads to decreasing affinity of enzyme towards Glc-1-p and activator fru-1,6 bis phosphate (Lee *et al.* 1986). Amino acid aspartic acid is mainly involved in the catalysis.

Six proline residue are found in the N- terminal region of large subunit of the potato tuber AGPase. Mutation in the proline region cause change in catalytic activity P (17)L, P(26)L, P(55)L mutation moderately affect kinetic properties. P(55)L and P(66)L mutation cause significant changes in the allosteric properties,

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P(66)L mutation up-regulate the properties towards the 3-PGA. While P(52)L mutation down regulate the property (Mary *et al.* 1998).

Single change leads to drastic changes in the enzyme activity. By change in single amino acid through site direct mutagenesis in either one of the two subunit of AGPase. Large subunit has only allosteric activity. Through site directed mutagenesis the large subunit can change into catalytic form (Hwang *et al*, 2008).

Fresh weight percentage of high starch in 9S-127 is 29.5 and for MNga-1 23.6. it is change in the amino acid in the enzyme causes the changes in the starch accumulation and change in the starch content.

To study gene activity of AGPase and other starch related enzyme in the biosynthetic pathway MNga-1 and 9S-127 may be hybridized. Based on its parent and hybrid /progenies, variation in the gene may be utilized as marker for identifying high starch lines.

SUMMARY

6. SUMMARY

Cassava is an important root crop apart from the dietary aspect cassava demanded in diverse industrial application. It is an abundant source of starch. A key regulatory enzyme in the starch biosynthesis enzyme is AGPase. Starch accumulation is directly proportional to AGPase enzyme activity. Characterisation of AGPase gene opens to the world of crop improvement and production of high starch variety.

The study entitled "Isolation and characterization of ADP-glucose pyrophosphorylase (AGPase) enzyme in high and low starch variety of cassava" was carried out at the Division of Crop Improvement, ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram during 2016-2017. The objective of the study was to analyse AGPase gene sequence variations in high starch cassava line 9S-127 and low starch cassava variety MNga-1. The gene amplification of AGPase is carried out with candidate primer.

From the Cassava improvement programme of ICAR-CTCRI, two varieties were selected for the study, high starch variety 9S-127 and low starch variety MNga-1. For the Amplification of AGPase, gene specific primers were designed using Primer 3 plus Bioinformatics software from the conserved regions of AGPase collected from NCBI. For the amplification of gene RNA isolated from high starch 9S-127 and low starch MNga-1 with manual and kit method were used. Manual method gives very poor quality and quantity of RNA. Kit method gave good yield of RNA. RNA was then converted into cDNA using reverse transcriptase enzyme. The gene specific primers were used for gene amplification. Sixteen primers were screened and three of them gave clear and reproducible bands in 1.2% agarose gel. Three primers AGPS1b, AGPS2d, AGPL showed bands in the size of 423bp, 581bp, 512bp. These specific bands were excised from the gel and purified with QIAquick Column. Purified gene fragments were used for sequencing in genetic analyser.

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The sequences were compared with nucleotide data base in NCBI. This revealed that our amplified sequence showed similarity with AGPase sequence of *Manihot esculenta*. The sequences of high starch and low starch variety were compared using Clustal omega. After the analysis it was found that between MNga-1 and 9S-127 there is a difference in six base pairs. These sequences were *in-vitro* translated using ExPASy translate tool in bioinformatics. Among the translated amino acid functional amino acid were identified through BLAST-P bioinformatics tool. Each functional amino acid sequence developed from amplified sequences of MNga-1 and 9S-127 using three corresponding primer were compared using Clustal omega bioinformatics software. From the analysed data four amino acid alteration in large subunit and small subunit of MNga-1 and 9S-127 were identified.

. Sequence variation in small subunit of MNga-1 were methionine at 296th position is replaced by leucine, at 298th position tyrosine is replaced by phenylalanine, at 263rd position Arginine is replaced by serine and at 157th glutamic acid is replaced by glycine were identified. Methionine and leucine are highly similar and shows same property. Similarly, tyrosine and phenylalanine are highly similar and have same property.

In case of large subunit MNga-1at 137th position iso-leucine is replaced by phenylalanine, at 159th Iso-leucine is replaced by leucine, 169th arginine is replaced by alanine and at 181th threonine is replaced by asparagine. Iso-leucine and phenylalanine are highly similar. Iso-leucine and leucine have similar properties. Change in amino acid sequence leads to the change in conformation of the enzyme that will affect the enzyme activity.

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APPENDICES

8 APPENDIXES

APPENDIX I

Reagents for RNA isolation

a.	Tris HCl	:	100 mM
b.	EDTA	:	25mM
c.	NaCl	:	2M
d.	CTAB	:	2%
e.	B Mercapto ethanol	:	2% ⎤
f.	PVP	:	2% ⎦ Freshly add

Prepared in DEPC treated water

100ml Extraction buffer

Solution A

a.	NaCl	:	40 ml
b.	Tris HCl	:	10ml
c.	EDTA	:	5ml

Solution B

a.	CTAB	:	2g
b.	PVP	:	2g
c.	Distilled water	:	40 ml

Solution A and Solution B are mix together and make up to 100 ml with distilled water. 2% B Mercapto-ethanol was added to freshly prepared buffer

TE Buffer (10X)

a. Tris – HCl(pH 8.0)   :   10 mM
b. EDTA   :   1 mM

Final volume made up to 100ml with distilled water

APPENDIX II

Solutions for Agarose gel electrophoresis

1.TAE Buffer (50 X)

a. Tris base   :   242g
b. Glacial acetic acid   :   57.1ml
c. 0.5 M EDTA (pH 8.0)   :   100ml

Final volume made up to 1000ml with distilled water and autoclave before use.

2.TBE Buffer (10 X)

a. Tris base   :   107g
b. Boric acid   :   55g
c. 0.5 M EDTA (pH 8.0)   :   40ml

Final volume made up to 1000ml with distilled water and autoclave before use.

3. Ethidium bromide

Weigh 10 mg of ethidium bromide powder (Sigma-aldrich) and dissolve in 1ml of distilled water.

4. Loading dye

a.	Formamide	:	50 ml
b.	Xyline cynol	:	50 mg
c.	Bromophenol blue	:	50 mg
d.	0.5 M EDTA (pH 8.0)	:	1 ml

APPENDIX III

Reagents used in clean-up for sequencing

a.	EDTA	:	125M
b.	Sodium acetate	:	3M (PH 4.6)
c.	Absolute ethanol	:	95%
d.	Ice cold ethanol	:	70%

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF ADP-GLUCOSE PYROPHOSPHORYLASE ENZYME IN HIGH AND LOW STARCH VARIETY OF CASSAVA

by

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9. ABSTRACT

Cassava (Manihot esculenta) is very important tropical tuber crop with high carbohydrate level and major industrial raw material for the starch and ethanol .In starch biosynthetic pathway ADP-glucose Pyrophosphorylase (AGPase) is the key enzyme in starch biosynthesis. AGPase enzyme catalyzes the conversion of ATP and glucose-1-phosphate into ADP glucose. The starch accumulation depends on activity of enzyme. Present study on analyzing AGPase gene sequence variation in high starch line 9S-127 and low starch variety MNga-1. Sixteen candidate primer based on the conserved sequence of AGPase cDNA sequence were screened. Among these primer sequences three primers were were showed the amplification of AGPase gene fragments in both parents with a size of 423bp, 581bp, 512bp. Sequencing of these fragment shows 100% similarity with *Manihot esculenta AGPase* gene. Comparison of each primer amplicon from high and low starch lines shows difference in 27 base pair and Amino acid comparison showed not all changes in the nucleotide makes amino acid change only changes in eight amino acid sequence four in large subunit and four in small subunit. Mutations in the gene change the amino acid sequence and thus change Sequence variation in small subunit. Replacement of amino acid with similar property may not affect the activity of enzyme. The changes in amino acid property cause changes in the intra-molecular bonding and hydrophobicity of the amino acid changes the conformation of the enzyme this may either increase or decrease the activity of enzyme.