

**ISOLATION AND CHARACTERIZATION OF STARCH, STARCH
BIOSYNTHETIC GENES AND PROTEASE INHIBITORS FROM
MARAMA BEAN (*TYLOSEMA ESCULENTUM*)**

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ABSTRACT

Marama bean (*Tylosema esculentum*) is a highly nutritious plant and is currently regarded as a prospective crop for the future in arid zone agri-ecologies of the world. Starch is a major storage component in higher plants and in marama bean it's mostly found stored in the tuber. Starch is used in both food and non-food industries. Starch biosynthesis involves groups of committed enzymes. Aims of the present study were to determine the physicochemical and pasting properties of native marama bean starch isolate and characterize marama starch biosynthesis genes and detect serine protease inhibitor activities in green and mature marama seeds. The total starch content of marama bean tubers was determined by amyloglucosidase/ α -amylase enzymatic digestion and amylose content by Concanavalin A precipitation. A complementary Deoxyribonucleic acid (cDNA) library was constructed from marama tuber for the screening and isolation of Soluble Starch Synthase I (SSSI) and a Polymerase Chain Reaction (PCR) based strategy was used to isolate Adenosine diphosphate-glucose pyrophosphorylase (AGPase) and Starch Branching Enzymes (SBEs) using degenerative primers designed at the conserved motif of corresponding cloned plant starch synthesizing genes. Detection of serine protease inhibitor activities in green and mature marama seeds was established using the reverse zymogram technique and fluorogenic substrate N-alpha-benzoyl-L-arginine 7-amido-4-methylcoumarin hydrochloride and cDNA clone encoding a serine protease gene from marama was isolated using newly developed degenerate PCR primers. Native marama starch content was 87.38 mg starch/gram fresh weight and the total amylose content was 35 %. Phosphate at the C-6 position determined as Glucose-6-Phosphate was 0.788 nmol G6P/mg. The starch granules were round to elliptical with smooth surfaces and their sizes ranged from 8 -20 μ m. The pasting properties of pasting temperature, host paste, peak, final viscosity, breakdown and set back showed higher values for marama starch in contrast to commercial potato starch. A cDNA clone encoding a SSSI from *T. esculentum* was isolated and identified by cDNA screening. The cDNA clone is 684 bp in length and encodes 228 amino acid residues. Sequencing of cloned cDNA showed 100% identity with potato SSSI. The phylogenetic tree indicated the divergence of SSI in higher plants proceed in line with evolutions of monocots from dicots. AGPase and SBEs genomic clones from *T. esculentum* were isolated and their sequence features revealed. AGPase small and large subunit clones both showed 96% identities with *Glycine max*, while SBEI and SBEII clones showed 91 and 93% identities to *Cicer arietinum* and *Medicago truncatula*. Phylogenetic trees for both AGPase and SBEs clones exhibited higher sequence similarity to the AGPase and SBEs of dicotyledons.

Reverse zymogram analysis revealed four putative serine inhibitor activities in mature seeds and not in green seeds. Fluorogenic substrate analysis showed significantly higher ($P < 0.05$) trypsin activity in mature marama bean (2326 ± 356 Fluorescence Units mg^{-1} protein) than green seeds (362 ± 73 FU mg^{-1} protein). The analyses showed lower ($P < 0.05$) serine protease activity in both cowpea (877 ± 138 FU mg^{-1} protein) and soybean (381 ± 36 FU mg^{-1} protein). A partial cDNA clone encoding a serine protease gene from marama bean showed 100% identity to trypsin inhibitor for cowpea. Physicochemical properties of marama starch revealed considerable high amylose content and pasting properties when compared to other tuberous starch producing crops. Molecular features of starch synthesizing genes in marama were similar to that of the same genes characterized in other plant species. Serine inhibitors activities in marama seed may as well be an indicative that marama seed may have potent serine protease inhibitors which could be utilized in pharmaceuticals and in human health. Physicochemical properties of marama starch would suggest that it could find applications in foods and non-food uses that require higher amylose content and high pasting properties. Isolation and characterization of marama starch biosynthetic genes provide theoretical foundation in understanding the structure and functions of SSSI, AGPase and SBEs. Serine protease inhibitor gene isolation and detection of serine protease inhibitor of marama seeds will broaden the pool for plant serine protease genes and might be an ideal choice for developing pharmaceutical products. Future studies need to establish the potential uses of marama starch, characterize marama starch biosynthetic gene structures, functions, regulation and expression. It would be interesting to study in the future any reason for the higher serine protease activity in mature seeds than in green seeds as established recently. This study has clearly contributed to starch biology by making known for the first time the physicochemical and functional properties of marama tuber starch. It also established for the first time the genetic signature of genes involved in starch biosynthesis of marama bean, which may confer unique properties to marama tuber starch. At the same time, the presence of serine protease inhibitors activities and serine protease inhibitor gene in marama seeds with possible application in pharmaceutical products were investigated for the first time making this investigation novel.

Keyword: *Tylosema esculentum*, marama, starch biosynthesis, SSSI, AGPase, SBEs, serine protease inhibitors

TABLE OF CONTENTS

| | |
|--|--------------|
| ABSTRACT | ii |
| TABLE OF CONTENTS | iv |
| LIST OF FIGURES | x |
| LIST OF TABLES | xiii |
| LIST OF ABBREVIATIONS | xv |
| ACKNOWLEDGEMENTS | xvii |
| DEDICATION | xviii |
| DECLARATION | xix |
| CHAPTER 1: GENERAL INTRODUCTION | 1 |
| CHAPTER 2: LITERATURE REVIEW | 7 |
| 2.1. <i>Tylosema esculentum</i> : A prospective green gold in southern Africa..... | 7 |
| 2.2. Overview of Starch metabolism..... | 10 |
| 2.3. Significance of starch and its major botanical sources | 12 |
| 2.4. Starch fine structure and granule organisation..... | 16 |
| 2.4.1. Amylopectin..... | 17 |
| 2.4.2. Amylose | 20 |
| 2.5. Some notable physicochemical and functional properties of starch | 22 |
| 2.5.1. Granular morphology and size distribution..... | 22 |
| 2.5.2. Amylose/amylopectin ratio of starches | 23 |
| 2.5.3. Pasting properties..... | 24 |
| 2.6. Pathway of starch biosynthesis | 26 |
| 2.7. Interaction between enzymes involved in starch biosynthesis..... | 28 |

| | |
|--|-----------|
| 2.7.1. ADP-glucose pyrophosphorylase..... | 33 |
| 2.7.2. Starch synthase..... | 36 |
| 2.7.2.1. Granule bound starch synthases (GBSS) | 38 |
| 2.7.2.2. Starch synthase I (SSI)..... | 39 |
| 2.7.2.3. Starch synthase II (SSII) | 40 |
| 2.7.2.4. Starch Synthase III (SSIII)..... | 42 |
| 2.7.2.5. Starch synthase IV (SSIV) | 43 |
| 2.7.3. Starch branching enzymes | 45 |
| 2.7.3.1. Starch branching enzyme I (SBEI) | 47 |
| 2.7.3.2. Starch branching enzyme II (SBEII)..... | 48 |
| 2.7.4. Starch de-branching enzyme..... | 49 |
| 2.8. Biotechnological approaches to increase starch biosynthesis | 51 |
| 2.9. Construction of cDNA library..... | 54 |
| 2.10. Protease inhibitors..... | 57 |
| 2.10.1. Characterization of Kunitz type serine protease inhibitor from marama | 58 |
| 2.10.2. Medical and pharmaceutical application of plant serine protease inhibitors . | 58 |
| 2.10.3. Reverse zymography for detecting protease activity | 60 |
| 2.11. Statement of the research problem..... | 60 |
| 2.12. Aims of the study | 62 |
| CHAPTER 3: MATERIALS AND METHODS | 64 |
| 3.1. Plant material | 64 |
| 3.2. Physicochemical properties of marama tuber starch..... | 64 |
| 3.2.1. Isolation of starch from marama tubers | 64 |
| 3.2.2. Total starch assay | 65 |
| 3.2.3. Amylose/Amylopectin assay..... | 67 |
| 3.2.4. Determination of phosphate at the C-6 -position of the glucose residues..... | 68 |
| 3.2.5. Pasting properties..... | 68 |
| 3.2.6. Starch granular characterization..... | 69 |

| | |
|--|----|
| 3.3. Construction of cDNA library and screening for the identification of tuber-specific starch synthase genes in <i>T. esculentum</i> | 70 |
| 3.3.1. <i>T. esculentum</i> seed germination and cultivation | 70 |
| 3.3.2. RNA extraction from tuber material | 70 |
| 3.3.3. Concentration of RNA | 71 |
| 3.3.4. Isolation of mRNA..... | 72 |
| 3.3.5. Construction of a cDNA library in <i>E. coli</i> expression vector | 72 |
| 3.3.6. Small scale isolation of plasmid DNA..... | 76 |
| 3.3.7. Large scale isolation of library plasmid DNA | 77 |
| 3.3.8. Restriction Endonuclease digestion analysis | 78 |
| 3.3.9. Bacterial culture and maintenance | 79 |
| 3.3.10. Preparation of chemically competent <i>E. coli</i> cells | 79 |
| 3.3.11. Heat shock transformation of chemically competent <i>E. coli</i> cells | 80 |
| 3.3.12. Preparation of electrocompetent <i>E. coli</i> G6MD2:: pACAG cells..... | 80 |
| 3.3.13. Electroporation of electrocompetent <i>E. coli</i> G6MD2::pACAG cells..... | 81 |
| 3.3.14. Screening of expression library and sequencing of inserts involved in starch synthesis..... | 82 |
| 3.4. Isolation and sequence analysis of genomic clones encoding AGPase large and small subunits and SBEs genes from <i>T. esculentum</i> | 82 |
| 3.4.1. Isolation of genomic DNA..... | 82 |
| 3.4.2. Oligonucleotide PCR primers design..... | 83 |
| 3.4.3. PCR amplification of marama AGPase and SBEs genes..... | 85 |
| 3.4.4. Gel electrophoresis of DNA..... | 85 |
| 3.4.5. Purification and extraction of PCR products | 86 |
| 3.4.5.1. PCR Products purification using QIAquick [®] PCR purification kit..... | 86 |
| 3.4.5.2. PCR Products purification using QIAquick [®] Gel Extraction kit | 87 |
| 3.4.6. Cloning of purified PCR products into the vector | 88 |
| 3.4.6.1. Ligation of PCR Products in the pCR [®] 2.1 cloning vector | 89 |

| | |
|---|------------|
| 3.4.6.2. Transformation of ligation reaction into <i>E.coli</i> competent cells..... | 90 |
| 3.4.6.3. Identification and selection of positive clones..... | 90 |
| 3.4.9. Determination of plasmid DNA concentration and insert size | 92 |
| 3.4.10. Sequencing of positive DNA clones..... | 93 |
| 3.4.11. Analysis of the isolated AGPase and SBEs gene sequences..... | 94 |
| 3.5. Detection of serine protease inhibitor activity and isolation of serine proteinase coding sequence from <i>T. esculentum</i> seeds | 95 |
| 3.5.1. Protein determination..... | 95 |
| 3.5.2. Serine protease inhibitor activity determination with gelatine SDS PAGE..... | 95 |
| 3.5.3. Fluorometric measurement of serine proteinase inhibitor activity..... | 96 |
| 3.5.3. Isolation of serine proteinase coding sequences | 97 |
| 3.5.3.1. RNA isolation | 97 |
| 3.5.3.2. Synthesis of cDNA from total RNA | 97 |
| 3.5.3.2. PCR reactions and purification | 98 |
| 3.5.3.3. Primer design | 98 |
| 3.5.3.4. DNA cloning..... | 99 |
| 3.5.3.5. DNA Sequencing and in silico analysis of serine proteinase coding sequence | 101 |
| 3.5.6. Statistical analysis..... | 102 |
| CHAPTER 4: RESULTS..... | 103 |
| 4.1 Physicochemical properties of <i>T. esculentum</i> tuber starch | 103 |
| 4.1.1. Starch, G6P and amylose content of the marama tuber | 103 |
| 4.1.2. Pasting properties..... | 104 |
| 4.1.3. Granular morphology..... | 106 |
| 4.2. Construction of cDNA library and screening for the identification of tuber-specific starch synthase genes in <i>T. esculentum</i> | 107 |
| 4.2.1. Characterization of marama bean tuber cDNA library | 107 |
| 4.2.2. Complementary DNA library transfer to an expression vector and qualitative analysis of the expression library..... | 108 |

| | |
|---|-----|
| 4.2.3. Screening of the <i>T. esculentum</i> tuber expression library for starch synthase..... | 109 |
| 4.2.4. Characterization of marama SSSI gene..... | 111 |
| 4.2.5. Multiple alignment of deduced amino acid sequences for marama SSSI gene.. | 112 |
| 4.2.6. Phylogenetic relationships of marama SSSI among plant SSSI proteins..... | 113 |
| 4.3. Isolation and sequence analysis of genomic clones encoding AGPase large and small subunits and SBEs genes for <i>T. esculentum</i> | 115 |
| 4.3.1. PCR amplification and molecular cloning of AGPase genes of marama bean .. | 115 |
| 4.3.2. Sequence analysis of marama AGPase small and large subunit | 116 |
| 4.3.3. Multiple alignment of deduced amino acid sequence for marama AGPase small subunit gene | 120 |
| 4.3.4. Multiple alignment of deduced amino acid sequence for marama AGPase large subunit gene | 122 |
| 4.3.5. Phylogenetic analysis of plant AGPase and marama AGPase gene clones | 124 |
| 4.4. Isolation and sequence analysis of genomic clones encoding SBEs genes for <i>T. esculentum</i> | 127 |
| 4.4.1. PCR amplification and molecular cloning of SBEs genes of marama bean | 127 |
| 4.4.2. Sequence analysis of marama Starch Branching Enzymes genes..... | 128 |
| 4.4.3. Multiple alignment of deduced amino acid sequences for marama SBEs genes | 132 |
| 4.4.4. Phylogenetic analysis of plant starch branching enzymes and marama SBEs gene clones | 134 |
| 4.5. Detection of serine protease inhibitor activity and isolation of serine proteinase coding sequence from <i>T. esculentum</i> seeds | 136 |
| 4.5.1. Detection of serine protease inhibitor activity by reverse zymogram technology | 136 |
| 4.5.2. Fluorometric measurement of serine proteinase activity | 137 |
| 4.5.3. Serine protease inhibitor coding sequence isolation | 139 |

| | |
|---|------------|
| CHAPTER 5: DISCUSSION | 141 |
| 5.1. Physicochemical properties of marama tuber starch..... | 141 |
| 5.2. Isolation of a gene encoding starch biosynthesizing enzyme SSSI from cDNA library constructed from <i>T. esculentum</i> tuber | 147 |
| 5.3. Isolation and sequence analysis of genomic clones encoding AGPase large and small subunits genes from <i>T. esculentum</i> | 152 |
| 5.4. Isolation and sequence analysis of genomic clones encoding SBEs genes from <i>T. esculentum</i> | 157 |
| 5.6. Detection of serine protease inhibitor activity and isolation of serine proteinase coding sequence from <i>T. esculentum</i> seeds. | 159 |
| 5.5. Unique contribution to the body of knowledge of Science | 162 |
| CHAPTER 6: CONCLUSION..... | 164 |
| CHAPTER 7: RECOMMENDATIONS | 166 |
| REFERENCES..... | 169 |

LIST OF FIGURES

| | |
|---|-----|
| Figure 1: Pathway for starch metabolism showing the major metabolites and enzymes involved in the conversion of sucrose to starch in storage organs. | 11 |
| Figure 2 : The composition and structure of starch granules. | 17 |
| Figure 3 : Chemical structure of amylopectin showing glucosyl residues linked by α -1,4-bonds to form chains which are connected by α -1, 6-bonds. | 18 |
| Figure 4 : Chemical structure of amylose showing glucosyl residues linked by α -1,4-glucosidic bonds in long linear chains. | 20 |
| Figure 5 : A typical RVA pasting profile showing the commonly measured parameters. | 26 |
| Figure 6 : A simplified diagram showing reactions and roles of enzymes involved in higher plant starch biosynthesis | 27 |
| Figure 7 : A summary of the role of major group of enzymes involve in starch biosynthetic pathway. | 28 |
| Figure 8 : A flow chart of cDNA library construction. | 55 |
| Figure 9 : The map above shows the elements of pENTR™ 222 insert vector. | 75 |
| Figure 10 : Circular map and polylinker sequence of the pBluescript SK (+/-) phagemid. | 76 |
| Figure 11 : Vector map of pCR 2.1 used for cloning of genomic DNA encoding AGPase large and small subunits and SBEs genes from marama. | 89 |
| Figure 12 : Vector map, promoter and multiple cloning sequences of PGEM® -T Easy vector used in this study showing the cloned inserts positions (red colour). | 101 |
| Figure 13 : Marama bean tubers (A) were isolated and used for the extraction of Starch (B). | 104 |
| Figure 14 : Pasting properties of native marama tuber and potato starch suspensions studied by Rapid visco-analyser. | 106 |

| | |
|--|-----|
| Figure 15 : Scanning electron micrographs of native marama tuber starch granules. | 107 |
| Figure 16 : Iodine vapour staining of <i>E. coli</i> G6MD2. | 110 |
| Figure 17 : Complementary DNA (17A) and deduced amino acid (17B) sequences of the SSSI gene in marama. | 111 |
| Figure 18 : Alignment of deduced amino acid sequence of marama SSSI with plant (monocots and dicots) SSSI and <i>E coli</i> glycogen synthase..... | 113 |
| Figure 19 : Phylogenetic relationships of SSSI protein of plant species and glycogen synthase protein of <i>E coli</i> | 114 |
| Figure 20 : DNA banding patterns of marama AGPase genes amplified by different primers using genomic DNA as a template from marama tuber..... | 116 |
| Figure 21 : Nucleotide sequence (21A) and deduced amino acid sequence (21B) of marama AGPase small subunit gene..... | 117 |
| Figure 22 : Nucleotide sequence (22A) and deduced amino acid sequence (22B) of marama AGPase large subunit gene. | 118 |
| Figure 23 : Comparison of the predicted amino acid sequences of plant ADP-glucose pyrophosphorylase small subunit and a marama genomic clone fragment predicted amino acid..... | 121 |
| Figure 24 : Comparison of the predicted/deduced amino acid sequences of plant ADP- glucose pyrophosphorylase large subunit and a predicted amino acid of marama genomic clone..... | 123 |
| Figure 25 : Phylogenetic tree based on the amino acid alignment of plant AGPase small subunits along with cloned marama AGPase small subunit gene..... | 125 |
| Figure 26 : Phylogenetic tree based on the amino acid alignment of plant AGPase large subunits along with cloned marama AGPase large subunit gene..... | 127 |
| Figure 27 : DNA banding patterns of marama SBEs genes amplified by different primers using genomic DNA as a template from marama tuber..... | 128 |

| | |
|--|-----|
| Figure 28 : Nucleotide sequence (28A) and deduced amino acid sequence (28B) for marama SBEI gene. | 129 |
| Figure 29 : Nucleotide sequence (29A) and deduced amino acid sequence (29B) for marama SBEII gene. | 130 |
| Figure 30 : Alignment of the deduced amino acid sequence of marama SBEI | 132 |
| Figure 31 : Alignment of the deduced amino acid sequence of marama SBEII | 133 |
| Figure 32 : A single phylogenetic tree of SBEs, and Glycogen Branching enzyme of <i>E coli</i> and Starch branching of alga..... | 135 |
| Figure 33 : Marama Reverse zymogram indicating location of putative serine protease inhibitor activity..... | 137 |
| Figure 34 : Trypsin activity in crude extracts of marama, cowpea and soybean. | 138 |
| Figure 35 : Isolation of a putative serine proteinase inhibitor gene from Marama bean cDNA by RT-PCR..... | 139 |
| Figure 36 : Confirmation of the presence of the cDNA insert released by <i>EcoRI</i> digestion of the pGEM-T Easy plasmid vector and analysed on a 1.5% agarose gel..... | 140 |
| Figure 37 : A 495 genomic sequence encoding a mature marama serine protease inhibitor gene..... | 140 |

LIST OF TABLES

| | |
|--|-----|
| Table 1 : Application of starch in various industries | 14 |
| Table 2 : Application of starch in the food industry | 15 |
| Table 3 : Average size and shape of starch granule from major botanical sources | 23 |
| Table 4 : List of starch biosynthesis enzymes, together with EC numbers, formal names and isoforms..... | 32 |
| Table 5 : Two families of SBEs from different plants..... | 46 |
| Table 6 : Genes from major plant starch sources involved in starch biosynthesis that have been cloned. | 52 |
| Table 7 : Mutations known to affect structural genes for enzymes involved in starch biosynthesis of major starch sources..... | 54 |
| Table 8 : Degenerate primer sequences and PCR annealing temperatures for marama AGPase and SBEs genes used in the study..... | 84 |
| Table 9 : Starch composition (starch content, G6P content and amylose content) of the <i>T.esculentum</i> tuber..... | 104 |
| Table 10 : Pasting properties of starches from <i>T. esculentum</i> and commercial potato..... | 105 |
| Table 11 : <i>T. esculentum</i> primary library insert sizes estimated following <i>Mlu</i> I digests of plasmid DNA and separation by agarose gel electrophoresis..... | 108 |
| Table 12 : <i>T. esculentum</i> expression library insert sizes were estimated following <i>Pvu</i> II digests of plasmid DNA and separation by agarose gel electrophoresis..... | 109 |
| Table 13 : Sequence comparisons of marama SSSI cDNA and protein with those of other plant species..... | 112 |

| | |
|---|-----|
| Table 14 : Result of search for similarity between marama deduced amino acid AGPase small and large subunit and GenBank accessions using Blastx | 119 |
| Table 15: Percentage similarity between the predicted marama AGPase large subunit protein and its homolog from selected plant species..... | 122 |
| Table 16: Percentage similarity of the predicted marama AGPase large subunit protein and its homolog from selected plant species..... | 124 |
| Table 17: Result of search for similarity between marama deduced amino acid SBEI and SBEII together with GenBank accessions using Blastx..... | 131 |

LIST OF ABBREVIATIONS

| | |
|--------|---|
| ADP | adenosine diphosphate |
| AGPase | ADP-glucose pyrophosphorylase |
| AGP-L | AGPase large subunit |
| AGP-S | AGPase small subunit |
| ATP | adenosine triphosphate |
| cDNA | complementary DNA |
| DBE | debranching enzyme |
| DMSO | dimethylsulphoxide |
| DP | degree of polymerization |
| DTT | dithiothreitol |
| EC | enzyme commission |
| EDTA | ethylenediaminetetraacetic acid |
| G-6-P | glucose-6-phosphate |
| 3-PGA | 3-phosphoglycerate |
| NCBI | National Center for Biotechnology Information |
| PCR | polymerase chain reaction |
| SBE | starch branching enzyme |
| SSS | soluble starch synthase |
| (v/v) | (volume/volume) |
| (w/v) | (weight/volume) |
| GBSS | granule-bound starch synthase |

| | |
|--------|---|
| kDa | kilodalton |
| LB | Luria Bertani |
| mRNA | messenger ribonucleic acid |
| cfu | colonies forming units |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcriptase - polymerase chain reaction |
| RVA | rapid visco analyzer |
| SEM | scanning electron microscope |
| SS | starch synthase |
| BLAST | Basic Local Alignment Search Tool |
| CTAB | Cetyl Trimethyl Ammonium Bromide |
| DAPA | Diaminopimelate |
| ISO | Isoamylase |
| PUL | Pullulanase |
| BBI | Bowman-Birk proteinase Inhibitor |
| GOPOD | Glucose oxidase/oxidase |
| FU | Fluorescence Unit |
| PAGE | Polyacrylamide gel electrophoresis |

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DEDICATION

This dissertation is dedicated to my family for being my beacon of inspiration, for planting in me a seed of diligence, and courageously telling me that the only limits I can set on my life are those that I set myself.

Sure I am that this day we are masters of our fate that the task which has been set before us is not above our strengths; that its pangs and toils are not beyond my endurance. As long as we have faith in our own cause and an unconquerable will to win, victory will not be denied us.

-Winston Churchill-

DECLARATION

I, Emmanuel Nepolo, declare hereby that this study is a true reflection of my own research, and that this work or part thereof has not been submitted for a degree in any other institution of higher education.

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Date.....

Emmanuel Nepolo

CHAPTER 1: GENERAL INTRODUCTION

Starch is an important carbohydrate, a primary energy source for plants and an important industrial material globally due to its abundance and readily availability (Jeon, Ryoo, Hahn, Walia and Nakamura, 2010). There is an ever increasing demand for starch, especially from non-food industries. Starch in storage organs (kernels, roots, tubers or stem) from different botanical sources is the most important dietary source of energy for human and animal consumption. Major cereals (wheat, rice, and maize) as well as major tuber crops (potato, cassava, and yam) provide four-fifths of the calories required by human beings (Mauro, 1996).

Two forms of starch, namely, transient starch and storage starch are known to be available in higher plants. During the day, transient starch is produced in photosynthetic tissues such as leaf chloroplasts, and degraded at night to provide carbon for non-photosynthetic metabolism. Transient starch is converted into sucrose in the dark and subsequently translocated within the plant to supply the energy and carbon demand required for growth and development (Tetlow, 2011). In contrast to transient starch, storage starch is a long-term carbon store existing in plants and is synthesized in non-photosynthetic plastids called amyloplasts (Jeon et al., 2010). Amyloplasts are found in tuberous tissues, or as carbon stores in seeds (Tetlow, 2011). Storage starch is vital to metabolism in higher plants as a supplier of long-term energy requirement.

For instance, storage starches in tuberous tissues may need to be accessed as environmental conditions dictate, while storage starches in developing seeds act as a long-term carbon store for the next generation (Gerard, Colonna, Buleon and Planchot, 2001). The parts that are harvested for most staple crops are regarded as starch-storing organs, providing an important polysaccharide for humans and this represents up to 80% of daily caloric intake, (Burrell, 2003). Seed storage (reserve) carbohydrates in cereal seeds are the most important group, followed by tubers, storage roots (e.g., cassava, taro), and seeds of legume (Zeeman, Kossmann and Smith, 2010).

Much agricultural land is dedicated to the growth of starch producing cereal, root and tuber crops. Of the estimated 2,500 million tonnes of starch crops harvested annually (Food and Agriculture Organization of the United Nations, values for 2007) most is consumed directly as food or used as animal feed, but there is an increasing demand from non-food industries for starch as a cheap, natural and renewable raw material (Zeeman et al., 2010). Aside from the agri-food sector, starch has also been sought as an environmentally-friendly industrial polymer. It can be fabricated into paper, paints, textiles, cosmetics, pharmaceuticals, biodegradable plastics and construction materials, making it a versatile and highly useful commodity (Shigechi et al., 2004). Starch is also used as a source of renewable energy in the form of ethanol where it is used as a major feedstock for first-generation biofuels due to the relative ease with which it can be converted to fermentable sugars (Smith, 2008).

The various uses of starch require different types with distinct physical and chemical properties. Starches from diverse botanical sources have different properties, making them suitable for different end uses. However, there are limitations in the physicochemical properties of natural starch which often make it unsuitable for the demands of industrial processing. Many of the desired physicochemical properties underpinning the different uses of starches can be produced and altered through chemical and enzymatic modifications and/or physical treatment (Frohberg, 2008). Modification of starch results in increased production costs. It would be economically valuable to produce plants with starch properties suitable for a specific use. Although, this can be accomplished using traditional plant breeding, this procedure is tedious and laborious, and does not ensure that plants with a desired trait will necessarily be obtained.

The expansion of the use of starch with desired physicochemical properties for both dietary and industrial uses has created an increased demand for it in the market place. Total world-wide utilization of starch in 2008 was 66 million tons; this reached approximately 75 million tons by 2012, signifying an annual growth rate of 2-3% (International Starch Market Index, 2012). This increase in demand is met with challenges of a shortage of available suitable raw material at a tolerable price and an ever increasing energy cost. Therefore, there is a need to find new sources of starch to supplement those already known to meet the global demand for both food and non-food uses. The advent of genetic engineering with emphasis on crop yield improvements, has given new insight into the development of plant lines with different sorts of starch properties.

This can be accomplished within a shorter period of time as compared to a traditional breeding program (Tetlow, 2011). Understanding the pathway by which this polymer is synthesized in plants could facilitate the improvement of starch crop for both food and non-food uses.

Recently enzyme inhibitors have received greater significance owing to their potential as useful tools for the study of enzyme structures and reaction mechanisms, and their utilization in pharmacology and agriculture (Ryan, 1990, Oliva et al., 2010). Specific and selective protease inhibitors are powerful tools for inactivating target proteases in the pathogenic processes of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular dystrophy, cancer, and AIDS. Protease inhibitors which specifically inhibit the proteases that are essential in the life cycle of organisms that cause mortal diseases such as malaria, AIDS, and cancer can be used in drug design towards prevention of propagation of these causative agents (Kaplan and Wanstrom, 1991). Plants are recognized as a potential source for most of the naturally occurring protease inhibitors which have been isolated and well characterized. Most of them were found to belong to the group of serine protease inhibitors which could inhibit trypsin and chymotrypsin (Michaud, 1998).

Based on this background information, the present study was designed to establish the physicochemical properties of starch and to identify starch biosynthetic genes of *Tylosema esculentum* (Burchell A. Schreiber) commonly known as marama. Marama is a prospective new crop in Southern Africa because of the outstandingly high nutritional value of its seeds (Hartley, Tshamekeng and Thomas, 2002) as well as the amount of starch (Coetzer, Robbertse and Grobbelaar, 1983) stored in the tuber. The potential of marama bean tuber starch has not been utilized due to a lack of knowledge about its physicochemical properties as well as the molecular features of the genes responsible for starch biosynthesis in this plant species. In an attempt to increase people's preference towards using underutilized food sources such as marama bean tubers, this study provides the basis for possible application of native marama starch for both food and non-food uses.

The commercial application of the starches from marama bean tubers will promote its demand. Small scale and rural farmers can produce these crops because marama bean grows well in very harsh conditions (poor soil, high temperature and low rainfall) requiring only small inputs by the farmers. This in turn would provide some food security for the local people and in sub-Saharan regions. The study also reported for the first time some possible starch biosynthetic enzyme genes for marama bean. Understanding of genes which synthesize starch in marama will facilitate the improvement of marama starch through genetic engineering to attain starch with desirable properties suitable for either food or non-food applications. These findings will be vital for domestication of marama bean as a starch crop.

Although a large number of protease inhibitors have been isolated and identified from several plants (Lorito et al., 1994), *T. esculentum* as source of protease inhibitor is not yet reported. In this context, the study reported for the first time serine protease inhibitor activities of *T. esculentum* towards recognizing this plant species as potential source of the naturally occurring protease inhibitors and their probable utilization in development of pharmaceutical products.

The next chapter (2) provides a Literature Review of topics relevant to this study. The research problem and objectives of the study are stated at the end of chapter 2. Chapter 3 presents the methodology that was applied in this study. Description and statistical analysis of the study data are presented in chapter 4. In Chapter 5, an overall discussion of results and practical applications as well as the implications of the study are presented. Recommendations for further research are presented in chapter 6. Recommendations chapter is then followed by a concluding chapter 7, summing up the findings of the study.

CHAPTER 2: LITERATURE REVIEW

2.1. *Tylosema esculentum*: A prospective green gold in southern Africa

According to taxonomy, Marama bean (*T. esculentum*) belongs to the genera Cercideae in the subfamily Caesalpinioideae within the family Fabaceae, and it is allied to *Cercis* and *Bauhinia* (Wunderlin, Larsen and Larsen, 1981). Species of the genus *Tylosema* were hitherto included in *Bauhinia*, but were later recognised as a separate genus (Castro, Silveira, Coutinho and Figueiredo, 2005). Five species within the genus *Tylosema* have been characterized and they are all indigenous to Africa. Coetzer and Ross (1977) distinguished four of these species, which were later reviewed by Castro et al. (2005). These four species are: *T. esculentum* (Burch.) Schreiber, *T. fassoglense* (Schweinf.) Torre & Hillc., *T. argenteum* (Chiov.) Brenan and *T. humifusum* (Pic.-Serm. & Roti. Mich.) Brenan. Castro et al. (2005) described the fifth species, *T. angolense* (Silveira & Castro) as a new species.

T. esculentum is the most well-known and documented within the genus *Tylosema*. Several common names have been applied to *T. esculentum*. This plant is also known as mangetti, braaiboontjie, ombanui/otjipiva/ozombanui (Herero), gami (!Kha-Khu), tsi/tsin (Kung Bushmen), marumama (Thonga), lai/muraki/litammani/rama/tammani (Tswana) and marama (Powell, 1987). The most widely used name in literature seems to be morama/marama.

Marama bean is a long lived perennial tuberous legume and a prospective new crop in southern Africa because of its outstandingly high nutritional value (Hartley et al., 2002). According to van der Maesen (2006), marama bean is a desiccation-tolerant species which thrives in extreme environments with high temperatures (typical daily maximum of 38°C in the growing season), low rainfall (100–900 mm) and long periods of drought. The plant is native to the Kalahari Desert and neighbouring semi-arid regions of Namibia, and the northern part of South Africa, but also occurs in Angola, Zambia and Mozambique (Holse, Søren and Ase, 2010). The marama plant is a creeper with stems up to 3 m long arising from a large tuber with forked tendrils which facilitate climbing opposite the leaves (Castro et al., 2005). Mature seeds of *T. esculentum* are encapsulated in woody hard pods which usually contain two, but sometimes as many as six, large (2–3 g) dark brown edible seeds (Wehmeyer et al., 1969) and the pods open at maturity.

Research studies (Holse et al., 2010; Mosele, Hansen, Schulz and Martens, 2011) have indicated that mature marama seeds are a rich source of proteins and lipids, both above 30%, making the nutritional content comparable to soya bean and groundnut. The seeds also have a considerable content of dietary fibre (19–27%), and mineral content is similar to that of groundnut and approaching that of soya bean (Holse et al., 2010). In spite of its significant potential, the marama bean has not yet been studied very extensively.

However, a number of studies have evaluated the domestication of the plant and its potential as a food crop (Francis and Campbell, 2003; Monaghan and Halloran, 1996; Powell, 1987) as well as the biology of the legume (Hartley et al., 2002; Mitchell, Keys, Madgwick, Parry and Lawlor, 2005; Travlos, Economou and Karamanos, 2007). Health potential of the bean has also been evaluated *in vitro* (Chingwaru et al., 2011).

Despite the potential of marama bean as a healthy nutritive crop for developing countries, none of its carbohydrates have been thoroughly studied. Mosele et al. (2010) reported an average starch content of 0.2% dry mass for mature marama seeds. However, there are no available reports on the physicochemical properties of marama starch to determine its potential uses. Potential use of the carbohydrate fractions in food applications could yield valuable income for rural communities gathering marama beans. Carbohydrates, especially polysaccharides are important in the food industry because they can be used as thickeners, stabilisers, texturisers and gelling agents (Viñarta, MMolina, Figueroa and Farina, 2006; Khurana and Kanawjia, 2007). To date, the researcher is not aware of scientific reports or literature on the genes (starch biosynthesis enzymes) by which marama produces its starch. These data are needed for the realization of this neglected legume's potential to improve nutrition, increase food security and diversify livelihoods of people living in these rural areas (van der Maesen, 2006).

2.2. Overview of Starch metabolism

Starch is the primary energy reserve in most plants and is the second most abundant carbohydrate in the biosphere after cellulose. Plants accumulate and mobilise starch in both non-photosynthetic storage tissues and photosynthetic tissue. In the plant, starch is deposited as granules in chloroplasts of photosynthetic tissues (leaves) as transient starch or in the amyloplasts of storage tissues (such as tuber, stem and seed endosperm) as storage starch. These granules vary in size, shape, composition and properties between species, organs and stage of development (Jane, Kasemsuwan, Leas, Zobel and Robyt, 1994).

During the day starch is deposited in the leaves by active carbon dioxide fixation through photosynthesis and degraded by respiration at night (Preiss, 1988). In sink tissues such as cereal grain endosperm, potato tubers, pea seed cotyledons and tuberous roots of cassava, sucrose is imported from the photosynthetic tissues and converted before it is taken up by the amyloplasts (plastid) and thereafter stored in the form of starch granules for a long period of time (Figure 1).

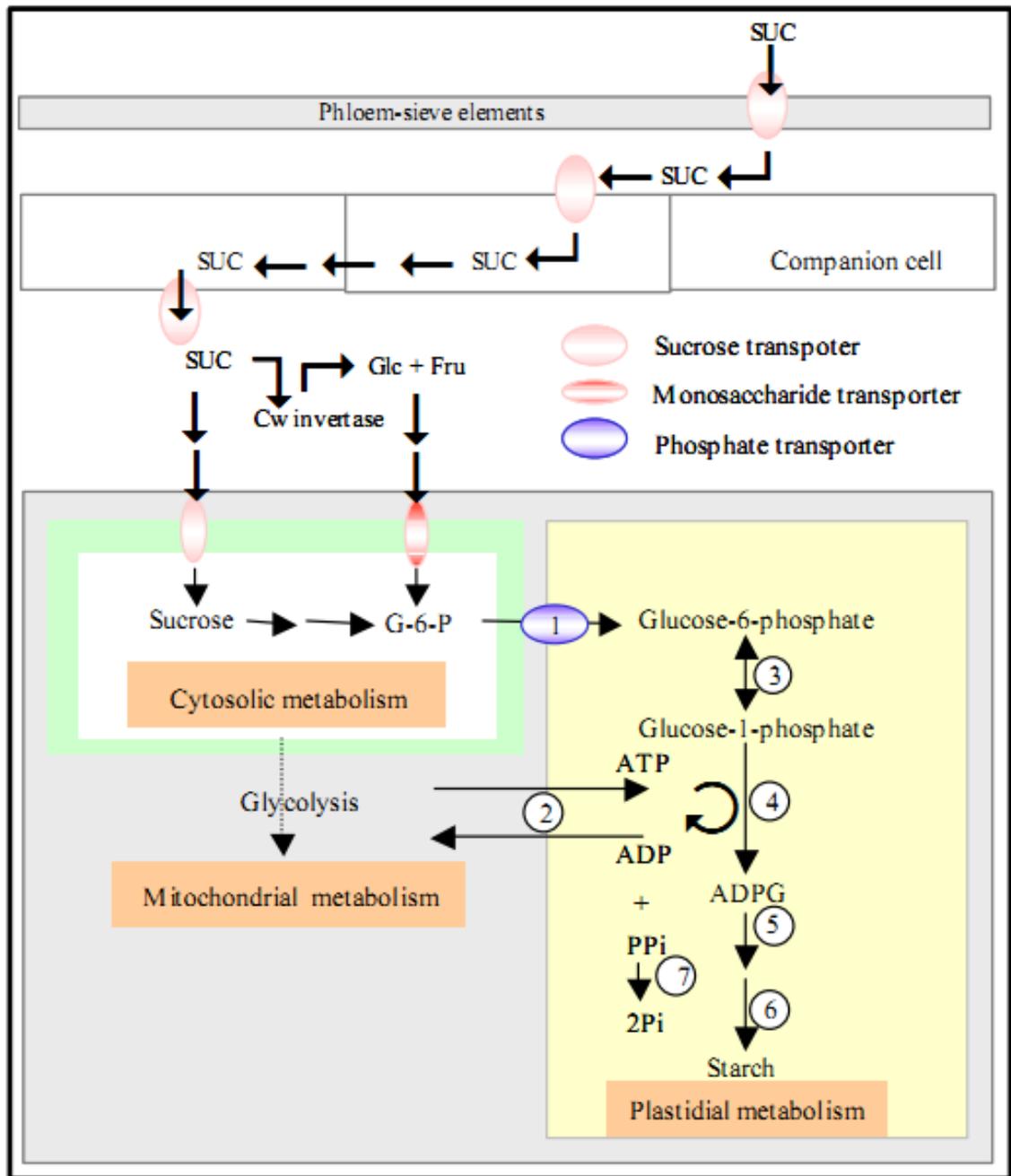


Figure 1: Pathway for starch metabolism showing the major metabolites and enzymes involved in the conversion of sucrose to starch in storage organs. 1, G-6-P transporter; 2, amyloplast adenylate transporter; 3, plastidial phosphoglucomutase; 4, ADP-glucose pyrophosphorylase; 5, starch synthases; 6, starch branching enzymes; 7, inorganic pyrophosphatase; (Source: Alisdair, Willmitzer and Trethewey, 2002).

2.3. Significance of starch and its major botanical sources

Starch is a substantial component of the human diet, (populations that rely on agricultural crops), in providing about 50% of daily energy uptake, mostly through unrefined cereal. Of the starch produced commercially in the world, approximately a third of it is used for non-food applications, while the remaining two thirds are used in the food industry (Zeeman et al., 2010). Table 1 provides an overview of the application of starch in various industries and the types of products made, while Table 2 illustrates the application of starch in the food industry. The use of starches in non-food applications is increasingly becoming important for consumers as they become more aware of 'green' issues such as sustainability of raw materials and biodegradation of materials (Jane et al., 1994). The need for starches in industrial applications such as in paper, textile, chemical, medical and pharmaceutical industries has resulted in research targeted towards producing starches with specific properties required for these specialized applications, as well as towards increasing the yields of starch producing plants.

Approximately 30 million tons of starch is isolated annually for a wide range of industrial applications. There are over 600 commercial products which have been processed from starch and the market for starch is still increasing especially as other resources (such as crude oil) are shrinking. Moreover, new markets for starch are arising, for example, the use of starch for the production of biofuels (Smith, 1998).

The majority of commercial starch is sourced from potatoes, maize and wheat (Martin and Smith, 1995) and as a result these are the systems used in most studies of starch biosynthesis and structure, with most emphasis being placed on potato and maize. However, other starch sources have also been exploited, notably rice, which as a simple monocotyledonous plant is a useful model for wheat and dicot, which like maize, exhibits useful visible starch mutant phenotypes. Barley has also been used as there is commercial interest in it as a source of starch in countries which do not have suitable growing conditions for crops such as potatoes and maize.

Intensive studies have been performed on crops such as cassava, which is widely grown in tropical climates with a view to genetic improvement of this crop (Munyikwa, Langeveld, Jacobsen and Visser, 1997) for starch production. Therefore, there is a need for new starch crops such marama bean in parts of the world where environmental and growing conditions are not suitable for crops such as potatoes or corn, to meet the ever increasing world market demand for starch (Martin and Smith, 2005).

Table 1: Application of starch in various industries

| Industry | Application |
|--------------------------|--|
| Adhesive | Binding |
| Agrochemical | Mulches, pesticide delivery, seed coatings |
| Building | Ceramics, coating (wood, metal), wallboard, fibreboard, ceiling tiles, wallpaper |
| Cosmetics | Face and talcum powder |
| Detergents | Surfactants, suspending agent, bleaching agents and bleaching activators. |
| Domestic products | Briquettes, diapers, typewriter ribbons, trash bags, twine, cords, string |
| Food | Viscosity modifier, edible film, glazing agent |
| Pharmaceuticals | Diluent, binder, drug delivery (encapsulation) |
| Medical | Plasma extender/replacer, transplant organ preservation (scaffold), absorbent sanitary products. |
| Paper and board | Binding, sizing, coating |
| Oil drill | Viscosity modifier |
| Plastics | Food packaging, biodegradable filler |

Adapted from Lawton (2004)

Table 2: Application of starch in the food industry

| Food product | Application and function |
|--|---|
| Mayonnaise and salad dressing | As thickener and stabiliser |
| Meat products | As water binder to increase yields, reduce cooking losses, improve texture, sliceability and succulence, and extend shelf life |
| Savoury snack | Increases dough viscosity (high amylopectin), strengthening dough to improve forming, cutting and to achieve a harder and more crunchy texture (high amylose), reduces oil pick up (high amylose) |
| Dairy products | To attain desired texture and viscosity |
| Gravy, soup and sauces | To increase opacity and change mouth feel, as bulking agent to aid dispersion |
| Confectionery | As structure builder in coatings, as moulding medium to support the shaping of confections as process aid |
| Beverage and encapsulated ingredients | Emulsion stabilizer |
| Baked food | As structure builder, texture and viscosity controller, shelf life enhancer and colouring |
| Butter and breading | To offer viscosity control which controls the quantity and thickeners of the butter layer, the adhesion efficiency, visual effects (smooth to blistered), texture, storage and reconstitution stability, to reduce oil pick up (high amylose starches). |

Adapted from Murphy (2000)

2.4. Starch fine structure and granule organisation

Starch is a complex carbohydrate polymer with high molecular weight occurring in plant storage organs such as seeds and tubers and in non-storage organs such as leaves and roots. It exists as water insoluble glucan polymers which form into a semi-crystalline granular structure in the plastid. A starch granule consists of two different glucosyl polymers amylose: which is essentially linear and amylopectin which is highly branched. Amylopectin and amylose together form semi-crystalline, insoluble granules with an internal lamellar structure (see Figure 2). The ratio of these polymers in a starch granule is largely genetically controlled, and normally amylopectin is the major component, typically making up 75% or more of the starch granule (Zeeman et al., 2010).

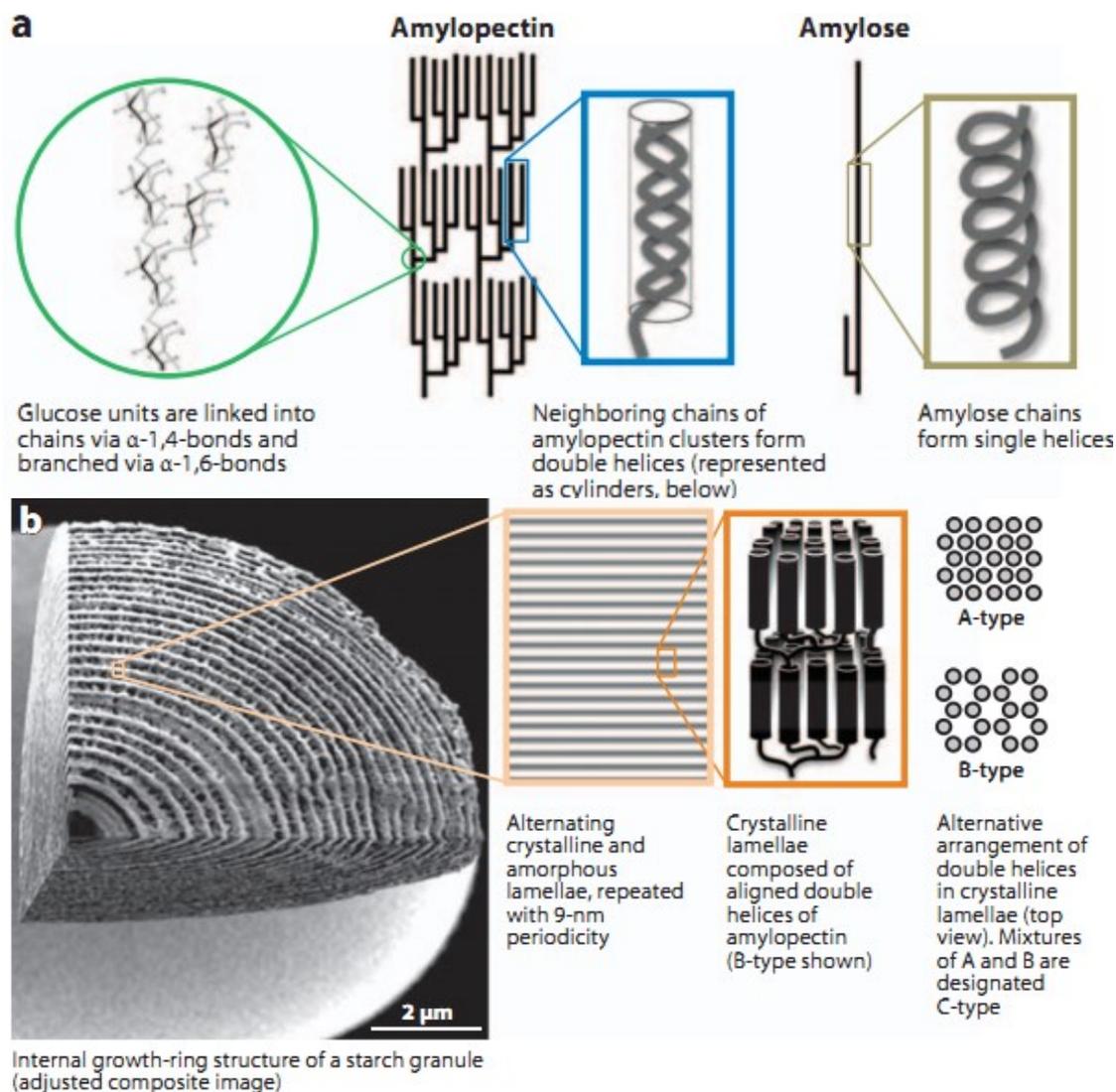


Figure 2 : The composition and structure of starch granules. (a) A schematic representation of amylose and amylopectin, and the structures. (b) The relationship between the starch granule (composite image of potato granules, left) and amylopectin structure. Crystalline and amorphous lamellae arrange to form blocklets that make up the growth rings, adapted from Zeeman et al. (2010).

2.4.1. Amylopectin

Amylopectin is a branched molecule with an estimated molecular weight of between 107 and 109 daltons (Buléon, Colonna, Planchot and Ball, 1998). The glucosyl residues of amylopectin are linked by α -1,4-bonds to form chains between 6 and >100 glucosyl residues in length (see Figure 3).

The α -1, 4-linked chains are connected by α -1, 6-bonds (branch points), similar to glycogen. Amylopectin has less branch points compared to glycogen and therefore contains a less dense pattern of glucan branches than glycogen (Buléon et al., 1998).

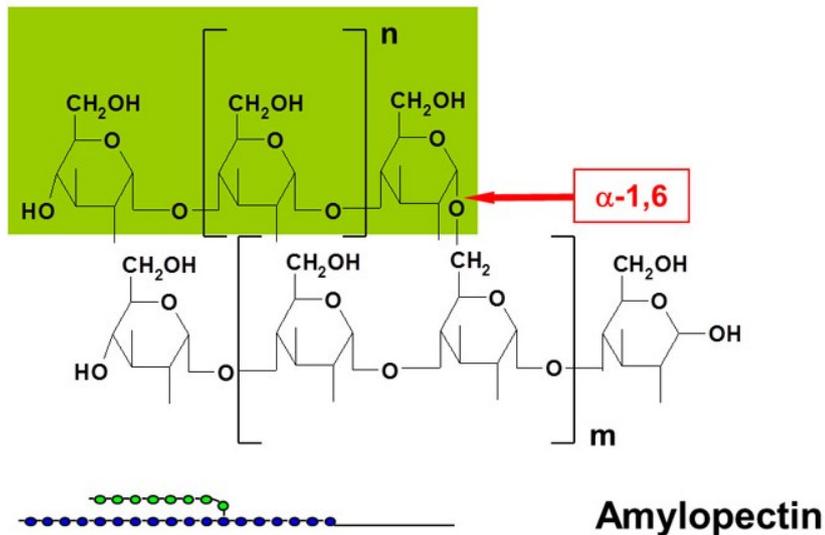


Figure 3: Chemical structure of amylopectin showing glucosyl residues linked by α -1,4-bonds to form chains which are connected by α -1, 6-bonds.

The branching pattern of amylopectin allows for the formation of secondary and higher-order glucan structures that makes up the nature of the starch granule. Although the exact molecular architecture of amylopectin is not known, the combination of chain lengths, branching frequency, and branching pattern gives rise to a treelike structure in which clusters of chains occur at regular intervals along the axis of the molecule (Zeeman et al., 2010). Typically, chains within these clusters average between 12 and 15 glucosyl residues.

The less abundant chains that span two clusters contain approximately 35–40 residues, while those that span three clusters contain approximately 70–80 residues (Buléon et al., 1998; Hizukuri, 1986). Within the starch granule, amylopectin molecules are radially organized such that the free (non-reducing) ends of the chains point toward the periphery. Pairs of adjacent chains within clusters form double helices that pack together in organized arrays, giving rise to concentric, crystalline layers (lamellae) within the granule. These lamellae alternate with amorphous lamellae formed by the regions of the amylopectin molecule that contain the branch points. The lamellar organization is repeated with a 9- to 10-nm periodicity. This semi-crystalline structure makes up the bulk of the matrix of the starch granule and is highly conserved in higher-plant starches (see Figure 2) (Zeeman et al., 2002).

Most granules contain concentric growth rings that are visible by light microscopy and by scanning electron microscopy after etching the granule matrix with acids or hydrolytic enzymes (Zeeman et al., 2002; Pilling and Smith, 2003). These rings have periodicities of several hundreds of nanometers. They are thought to reflect the organization of the alternating crystalline/amorphous lamellae into near-spherical blocklets, which vary periodically in diameter (between 20 and 500 nm) as the granule is laid down (Ridout, Parker, Hedley, Bogracheva and Morris, 2003).

2.4.2. Amylose

Amylose, the second glucan component of starch, is created by 100–10,000 glucosyl units and it is smaller than amylopectin its relative molecular weight estimates vary between 10^5 and 10^6 daltons (Buléon et al., 1998; Zeeman et al., 2002). Amylose is a lightly branched polymer (see Figure 4) and it is believed to exist primarily in an unorganized form within amorphous regions of the granule.

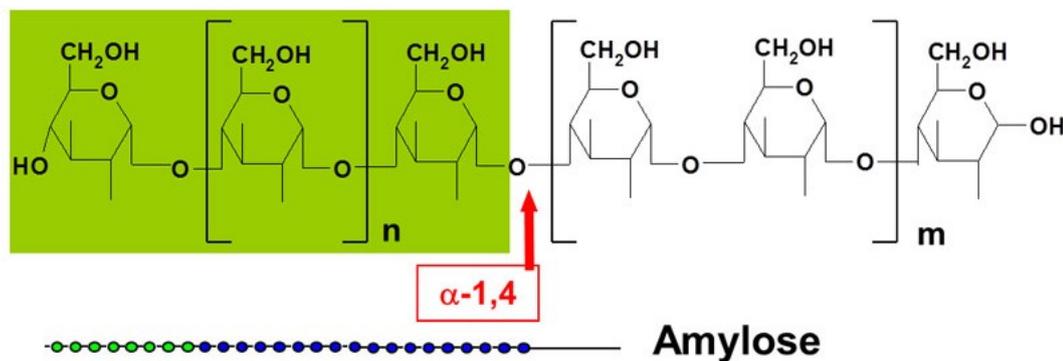


Figure 4 : Chemical structure of amylose showing glucosyl residues linked by α -1,4- glucosidic bonds in long linear chains.

Amylose content of starch is typically 20-30% (Zeeman et al., 2002). Some mutant plant genotypes, particularly of maize, contain starch of very high amylose content of up to 70%. Nevertheless, other genotypes in a wide range of plant species, called *waxy* genotypes (Nakamura et al., 1995), contain starch with less than 1% amylose (maize, barley, rice and amaranth). *Waxy* starches typically gelatinize easily, yielding clear pastes that will not gel. A *waxy* locus encoding a granule-bound starch synthase (GBSS) protein is found in most waxy mutants.

These mutants provide good materials to elucidate the physiological role for starch synthesis-related enzymes (Nakamura et al., 1995). Starches with high amylose content can form hydrogen-bonded insoluble aggregations, suitable for use in adhesives, plastics and as a source of dietary-fiber starch (Doane, 1994). The examination of amylose-free mutants of various species has shown that amylose is not crucial for the characteristic semi-crystalline structure of starch.

The waxy mutants of cereals (Nakamura et al., 1995), the *lam* mutant of pea (Smith, Denyer and Martin, 1995) and the *amf* mutant of potato (van der Leij, Visser, Ponsteln, Jacobsen and Feenstra, 1991) all lack or have severe reductions in amylose but still contain starch granules of normal morphology and higher level structure. The exact role of amylose in the organisation of the starch granule is not yet known (Nakamura et al., 1995). It is thought that the linear α -1,4 linked glucan amylose is embedded in the amylopectin matrix. However, amylose can influence the organisation of short chains of amylopectin and plasticity of the starch granule. Analysis of normal and low amylose lines of maize and pea indicated that an increase in amylose to amylopectin ratio increases the size of the crystalline lamella relative to the amorphous lamella while the 9 nm periodicity remains unchanged (Jenkins and Donald 1995). It has been proposed that amylose reduces the packing of amylopectin chains in the crystalline lamellae leading to a reduced density and a relative increase in size of the crystalline lamellae.

2.5. Some notable physicochemical and functional properties of starch

2.5.1. Granular morphology and size distribution

The shape, size, and other morphological characteristics of starch granules vary depending on many factors. In general, the morphology of starch granules primarily depends on the botanical source, but also on environmental conditions under which the crop was grown (see Table 3). Regular microscopy, scanning electronic microscopy (SEM), and polarized light microscopy are commonly used to observe the shape of starch granules and characterize them.

Legume starches appear to be oval, elliptical and smooth, (Gujska, Reinhard and Khan, 1994; Hoover and Ratnayake 2002). The size distribution and shape of granules have an impact on functional properties of a given starch. Various microscopic techniques are used as the main techniques for granule size determination. More advanced methods such as focused beam reflected analysis, and laser scattering/diffraction have been used to analyze starch granule size distribution more accurately (Ambigaipalan et al., 2011). Granule size is important in determining many functional properties of starch. Larger starch granules tend to be more crystalline than smaller ones. The smaller granules gelatinized at higher temperature with lower gelatinization enthalpy than larger granules (Chiotelli and Le Meste 2002). During food processing, large granules are more likely to be damaged during milling than small ones. This is important because the degree of starch damage will influence flour functionalities, such as water absorption and flour dough properties (Dexter, Preston, Martin and Gander, 1994; Oh, Seib, Ward and Deyoe, 1985).

Table 3: Average size and shape of starch granule from major botanical sources

| Botanical source | Diameter range (microns) | Shape |
|-------------------------|---------------------------------|----------------------------|
| Maize | 5-20 | spherical or polygonal |
| Potato | 15-75 | oval or spherical |
| Rice | 3-8 | Polygonal |
| Wheat A type | 22-36 | Disk |
| Wheat B type | 2-3 | Spherical |
| Barley A type | 10-48 | Oval |
| Barley B type | 2-10 | Spherical |
| Cassava | 5-40 | rounded with indentation |
| Banana | 15-45 | Irregular |
| Pea | 10-45 | irregular with indentation |
| <i>Phaseolus</i> | 10-45 | disc shaped |

Adapted from Frances and Bligh (1999)

2.5.2. Amylose/amylopectin ratio of starches

Starch granules are made up of primarily two polysaccharides: amylose and amylopectin. Amylose is an essentially linear polymer consisting of glucose units by α (1-4) linkage. Amylopectin is the larger branched molecule joined by both α (1-4) and α (1-6) linkages (Buléon et al., 1998). The ratio of amylose to amylopectin varies from one starch source to another. Generally, cereal starch granules contain approximately 20% amylose (Jenkins and Donald 1995). The total amylose contents of bean starches generally range from 23 to 30% (Hoover and Ratnayake 2002).

The differences in botanical sources, crop conditions, and analytical methods could influence the results. Amyloses to amylopectin ratio and polymer characteristics are considered as the factors in determining starch functional properties that are important for food applications. Amylopectin chain length is related to crystal structure (Hizukuri 1985). Amylopectin is ascribed to generate the ordered crystalline structure of starch granules; amylose is considered to disrupt this structural order. The ratio of amylose/amylopectin is also a crucial factor in determining pasting properties. High amylose content is associated with increased pasting temperature, low peak viscosity and shear thinning, and increased set-back in Rapid Visco Analysis (RVA) profiles (Jane et al., 1999).

2.5.3. Pasting properties

Pasting is an important property of starch, especially for food processing applications. Pasting occurs when starch is heated over the gelatinization temperature under mechanical stress, during which the granules swell and disintegrate in excess water (Jane et al., 1999). Brabender visco-amylography and Rapid Visco Analysis (RVA) are used to analyze the pasting properties of starch. Normally, in standard pasting profile analyses, viscosity of the tested sample is recorded at three different temperature stages (see Figure 5). The RVA curve describes pasting, a phenomenon following gelatinization, involving granular swelling, exudation of amylose and amylopectin, and total disruption of the starch granule. Pasting temperature is the point when the temperature rises above the gelatinization temperature, inducing starch granule swelling and resulting in increased viscosity.

The peak viscosity indicates the maximum viscosity reached during the heating and holding cycle and is indicative of the water holding capacity of starch (Batey, 2007), and peak temperature occurs at peak viscosity. The breakdown viscosity is normally regarded as a measure of the disintegration of the starch granules as they are heated (Singh et al., 2008) due to the rupture of granules and the release of soluble amylose. The degree of RVA breakdown is related to the solubility of the starch, and the more soluble the starch is, the more it will thin on shearing (Charles, 2004). As the mixture is cooled, re-association between starch molecules, especially amylose, results in the formation of a gel and the subsequent increase in viscosity. Total setback involves retrogradation, or re-ordering, of the starch molecule.

Various factors, such as botanical sources, granule size, degree of crystallinity, presence of fat and protein, and branch chain-length distribution of starch polymers have been shown to have an impact on starch pasting properties (Jane et al., 1999; Karim et al., 2007). Amylose and lipids have been revealed to inhibit granule swelling, while high levels of amylopectin are known to increase granule swelling in cereal starches (Tester and Morrison 1990). Differences in these starch physicochemical properties have a major impact on pasting properties.

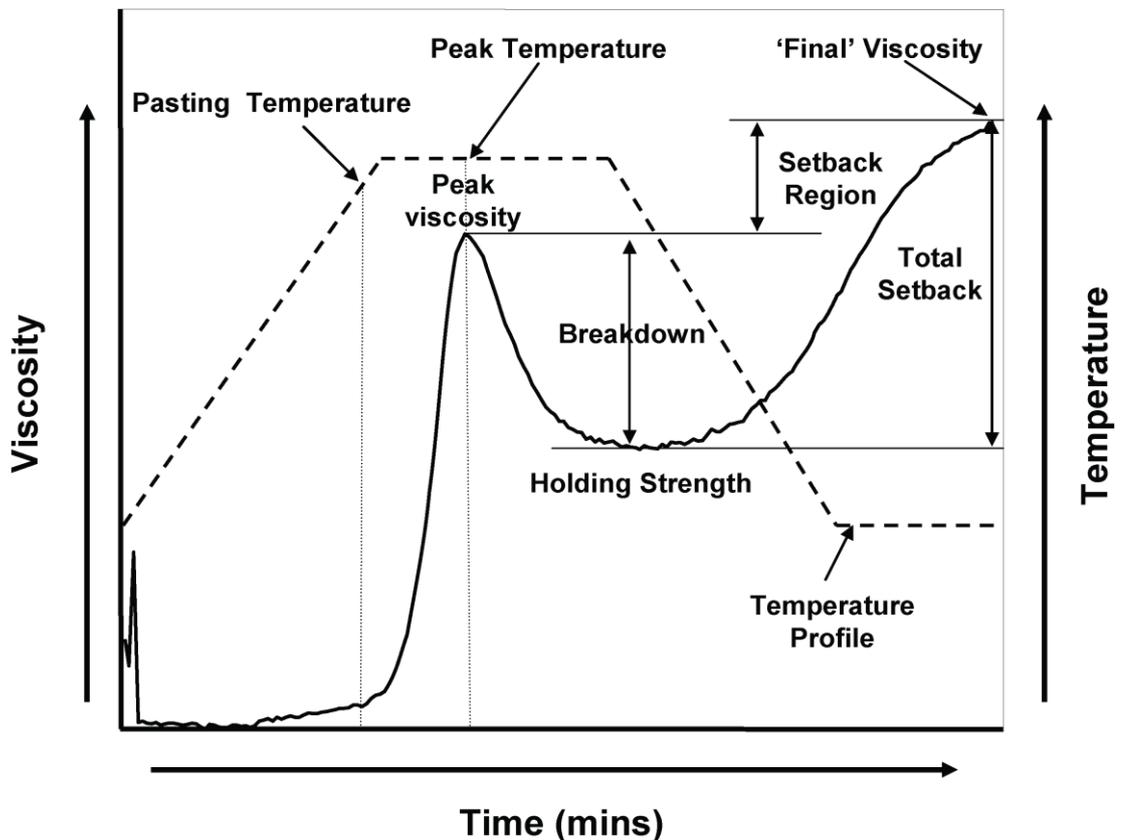


Figure 5: A typical RVA pasting profile showing the commonly measured parameters. Source: Batey (2007).

2.6. Pathway of starch biosynthesis

The biochemistry of starch synthesis is relatively well understood although it is a complex process (Buléon et al., 1998). A highly complex and organized coordination of a suite of starch biosynthetic enzymes is required to synthesize starch in the amyloplast. Four major groups of enzymes are currently known to be involved in starch biosynthesis, adenosine 5' disphosphate glucose pyrophosphorylase (AGPase), starch synthase (SS), starch branching enzyme (SBE) and starch debranching enzyme (DBE) (Vrinten and Nakamura, 2000) (See Figure 6). Major groups of enzymes involved in amylose and amylopectin biosynthesis process are shown in Figure 7.

These major enzymes involved in the biosynthetic process catalyze specific reactions and several isoforms of these enzymes exist in many plants, leading to a highly complex biosynthetic process. The initial point of starch biosynthesis is glucose which is derived from photosynthesis in the green parts of plants. This glucose is transported to and deposited in storage tissue including grain endosperm and tuberous roots. In the amyloplast, glucose is activated by the addition of ADP by ADP-glucose pyrophosphorylase (AGPase) (James, Denyer and Myers, 2003). The ADP-glucose is then used by starch synthases which add glucose units to the growing polymer chain to build the starch molecules (Buléon et al., 1998).

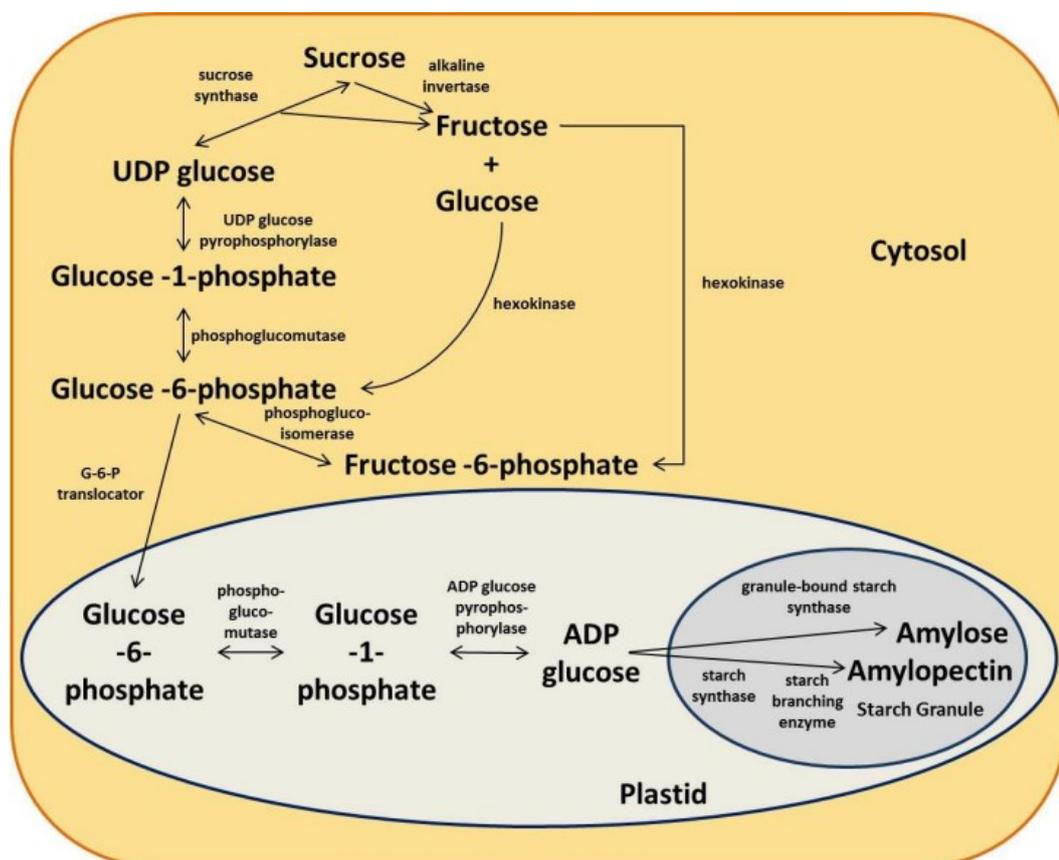


Figure 6: A simplified diagram showing reactions and roles of enzymes involved in higher plant starch biosynthesis. (Modified from <http://www.jic.ac.uk/STAFF/trevor-wang/images/full/starchpath2.jpg>)

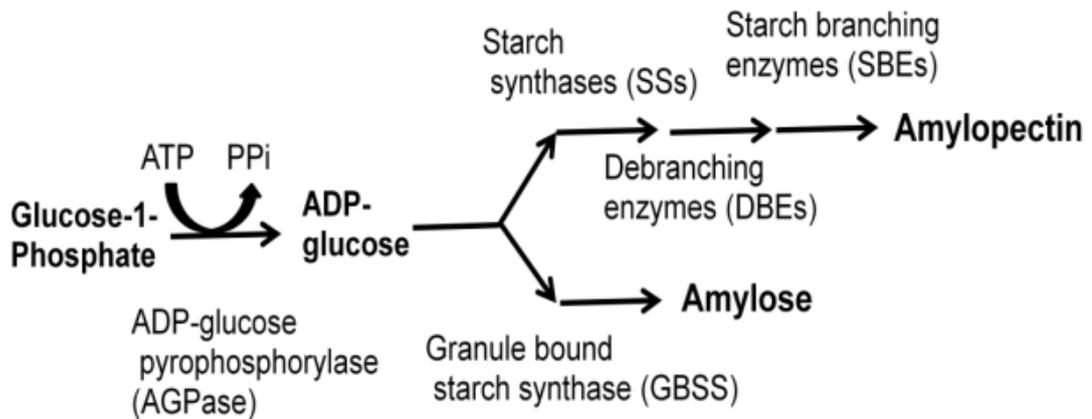


Figure 7: A summary of the role of major group of enzymes involve in starch biosynthetic pathway.

2.7. Interaction between enzymes involved in starch biosynthesis

The complexity of starch granule semi-crystalline structure is startling because only two polymers are involved in starch biosynthesis and only two types of linkages can be found. Thus, starch biosynthesis enzymes (AGPase, SS, SBE and DBE) may have distinct involvement in starch biosynthesis (see Table 4). Commonly, the first step of starch biosynthesis, which is considered to be a rate-limiting step, is the synthesis of ADP-glucose catalyzed by ADP-glucose pyrophosphorylase (AGPase). The substrate for starch biosynthesis in higher plants is ADP-glucose. The glucosyl moiety is transferred onto existing glucan chains by starch synthases (ADP-glucose:[1→4]- α -d-glucan 4- α -d-glucosyltransferase (Fu, Ballicora, Leykam and Preiss, 1998).

Higher-plant starch synthases are encoded by five gene classes, designated GBSS (for granule-bound starch synthase), SSI, SSII, SSIII, and SSIV. Phylogenetic analyses separate the GBSS, SSI, and SSII classes from the SSIII and SSIV classes (Ball and Morell, 2003; Patron and Keeling, 2005; Letterier, Holappa, Broglie and Beckles, 2008). GBSS binds tightly to the starch granule and is considered being the only enzyme that is responsible for amylose synthesis (see Figure 6). The amylose component of starch is synthesized by GBSS. Mutants and transgenic plants lacking this enzyme are essentially amylose free (Denyer, Johnson, Zeeman and Smith, 2001) Amylose-free starch granules are normal in appearance, illustrating that only amylopectin is necessary for granule formation.

GBSS differs from the other SS isoforms in its exclusive localization to the granule and in its mode of action. Unlike other starch synthase isoforms, GBSS transfers glucosyl residues from ADP-glucose to its glucan substrate processively, generating long chains (Denyer, Waite, Edwards, Martin and Smith, 1999). This occurs within the semi-crystalline matrix formed by amylopectin (Tatge, Marshall, Martin, Edwards and Smith, 1999), explaining why the newly formed amylose chains are not acted upon by stromal BEs. GBSS can use soluble malto-oligosaccharides as substrates for amylose production (Denyer, Dunlap, Thorbjornsen, Keeling and Smith, 1996; Zeeman, Smith, S.M and Smith, A.M, 2002). It can also act on the existing side chains of amylopectin (van der Wal, D'Hulst, Vincken, Buléon, Visser and Ball, 1998) and contribute to the formation of long chains in amylopectin (Fulton et al., 2002; Hanashiro et al., 2008).

It has been proposed that, in evolutionary terms, long chain formation in amylopectin may have been the original function of GBSS (Ral et al., 2006). Amylose synthesis may render starch denser and improve the efficiency of carbon storage, explaining the conservation of GBSS in higher plants. The other SS isoforms (often termed soluble SS) are thought to be responsible for generating and extending glucose chains in amylopectin and are either soluble in the plastid stroma, or part soluble and part associated with the granule. Genetic and biochemical data indicate that each SS isoform has different properties and a distinct role in amylopectin synthesis and may have non-essential role in amylose biosynthesis (Tomlinson and Denyer, 2003).

Analysis of the distribution of chain lengths of amylopectin in mutant and transgenic plants lacking specific isoforms has led to the idea that the SSI, SSII, and SSIII classes preferentially elongate short, medium, and long chains, respectively (Tomlinson and Denyer, 2003). The branching of amylopectin proceeds concurrently with chain elongation (Nielsen, Baunsgaard and Blennow, 2002). Branching is catalysed by branching enzymes (BE; α -1,4-glucan: α -1,4-glucan-6-glycosyltransferase), which cleave existing linear α -1,4-glucan chains and transfer the cleaved segment of six or more glucose units to the C6 position of a glucosyl residue of another (or the same) glucan chain to form a α -1,6-branch.

Two classes of starch branching enzymes have been found in higher-plants, designated SBEI and SBEII (SBEIIa and SBEIIb in monocots) sometimes defined as family B and A, respectively. Class I enzymes preferentially transfer longer chains than class II enzymes (Tomlinson and Denyer, 2003). Genetic analysis has shown that the two classes of SBE make distinct contributions to the synthesis of amylopectin (Tomlinson and Denyer, 2003). The evolution of multiple, specialized isoforms of SS and SBE can be seen as a crucial factor in determining the architecture of amylopectin and therefore the capacity to synthesize starch rather than glycogen. Most SBEIs (e.g., SBEIs from kidney bean, rice, wheat, maize, barley, and potato) belong to family B but two SBEIIs from pea and sweet-potato are also grouped into this family based on their amino acid sequences. Family A includes most SBEIIs (SBEIIa and SBEIIb) along with pea SBEI, rice SBE3, SBE4, and others (Nielsen et al., 2002).

Besides SS and BEs, other glucan-modifying enzymes participate in the starch biosynthetic process. Debranching enzymes (DBEs; α -1,6- glucanohydrolase), which cleave branch points, are important determinants of the structure of amylopectin. DBEs isoamylase and limit- dextrinase (LDA, also called pullulanase) are two types of DBEs in plants (Hussain et al., 2003). They are distinguishable by their amino acid sequences and substrate specificities. The ISA type has three classes, designated as ISA1, ISA2, and ISA3 (Hussain et al., 2003). ISA1 and ISA2 are strongly implicated in amylopectin synthesis. In potato tubers and *Arabidopsis* leaves, ISA1 forms a complex with ISA2 resulting in a heteromultimeric enzyme (Delatte, Trevisan, Parker and Zeeman, 2005; Hussain et al., 2003; Watterbled et al., 2005).

In the endosperm of rice, and probably other cereals, ISA1 exists both as a homomultimer and heteromultimer with ISA2 (Utsumi and Nakamura, 2006). The primary role of LDA and ISA3 appears to be in starch degradation. ISA1 is most active on glucan substrates with relatively long external chains, such as solubilized amylopectin, whereas LDA and ISA3 have high activities on glucans with short external chains (such as β -limit dextrins). ISA2 appears to be catalytically inactive (Hussain et al., 2003). It probably modulates the action or stability of ISA1, rather than contributing directly to debranching (Delatte et al., 2005; Watterbled et al., 2005). However, both LDA and ISA are considered to be involved in determining amylopectin fine structure, loss and reduction of these DBEs result in the production of phytyglycogen at the expense of amylopectin synthesis.

Table 4: List of starch biosynthesis enzymes, together with EC numbers, formal names and isoforms

| Enzyme | EC number | Formal name | Isoform |
|--------------------------------------|-----------------------------|---|------------------------------|
| ADP-glucose pyrophosphorylase | EC:2.7.7.27 | ATP: α -D-glucose-1-phosphate adenylyltransferase | Bt2, Sh2, AGP1, AGP2 |
| Starch synthase | EC:2.4.1.21 | ADP-glucose:(1 \rightarrow 4)- α -D-glucan 4- α -D- glucosyltransferase | GBSSI, SSI, SSII,SSIII, SSIV |
| Branching enzyme | EC:2.4.1.18 | (1 \rightarrow 4)- α -D-glucan:(1 \rightarrow 4)- α -D-glucan-6- α -D-[(1 \rightarrow 4)- α -D glucano]-transferase | SBEI, SBEIIa, SBEIII |
| Debranching enzyme | EC:3.2.1.68 and EC:3.2.1.41 | Glycogen α -1,6-glucanohydrolase and α -dextrin endo-1,6- α -glucosidase | ISA1,ISA2, ISA3, PU1 |

Adapted from Keeling and Myers (2010)

2.7.1. ADP-glucose pyrophosphorylase

ADP-glucose pyrophosphorylase plays a very important role in starch biosynthesis because it is the key enzyme that limits the speed of starch synthesis through controlling the production of ADP-glucose. ADP-glucose is a soluble precursor and the basic sugar nucleotide for different classes of starch synthases, a group of enzymes which are involved in elongation of the α -glucan chains in both transient and storage starch biosynthesis in higher plants (Preiss, 1988). ADP-glucose is produced from glucose-1-phosphate (G-1-P) and adenosine triphosphate (ATP) by the catalytic activity of AGPase. Therefore, AGPase catalyzes the key metabolic step in the synthesis of starch in higher plants and glycogen in bacteria by providing ADP-glucose, the substrate for all SSs (Preiss, 1988). The reversible reaction of ADP- glucose and inorganic pyrophosphate (PPi) synthesis from ATP and G-1-P by the catalytic activity of AGPase is shown in the reaction below (Fu et al., 1998).



AGPase is present in all starch synthesizing tissues in higher plants: in spinach leaves (Morell, Bloom, Knowles and Preiss, 1987; Copeland and Preiss, 1981), *Arabidopsis thaliana* leaves (Lin, Casper, Somaville and Preiss, 1988) and also in potato tubers (Okita et al., 1990). AGPase is a hetero-tetrameric enzyme that contains two large regulating subunits (AGP-L or LSU) and two small (AGP-S or SSU) catalytic subunits.

The sizes of large and small subunits in spinach leaves and potato tubers are 54-55 kDa and 50-51 kDa respectively (Okita et al., 1990), while in wheat developing endosperm is 58 kDa and 55 kDa, (Tetlow et al., 2003). AGPase subunits in photosynthetic and non-photosynthetic plant sources are both regulated positively by 3-Phosphoglycerate (3-PGA) and negatively regulated by inorganic phosphate (Pi) in leaf chloroplasts (Cross et al., 2004). The level of 3-PGA in chloroplast stroma increases during the light period in photosynthetic tissues and the Pi level decreases providing a sensitive signal which activates AGPase and stimulate the synthesis of starch (Cross et al., 2000). In non-photosynthetic tissues such as the amyloplast in cereal endosperm (Tetlow et al., 2003) and potato tubers (Tiessen et al., 2011) similar regulation by 3-PGA and Pi has been reported.

Hence, the ratio of these two allosteric effectors is believed to play an important role in controlling AGPase activity and starch synthesis in photosynthetic tissues (Preiss, 1991). Nevertheless, evidence from wheat and barley endosperms suggests that measurable activity, the majority of which is cytosolic, is much less sensitive to 3-PGA activation and Pi-inhibition than other forms of AGPase (Tetlow et al., 2003; Kleczkowski, Villand, Luth, Olsena and Preiss 1993). However, the plastidial AGPase from the storage tissues of dicots (e.g., potato tuber) appears to be as sensitive to the allosteric effectors as their counterparts in the chloroplast (Ballicora et al., 1995). The sensitivity of plastidial AGPase to allosteric regulation in other plastid types, such as leucoplasts and chromoplasts is unknown.

Since AGPase catalyses the rate-limiting step in starch biosynthesis, mutants that lack AGPase activity produced less starch than normal plants (Ball et al., 1991; van den Koornhuyse et al., 1996). On the contrary, a transgenic potato with unusually high AGPase activity produced increased starch content (Stark, Timmermann, Barry, Preiss and Kishore, 1992). Biochemical and genetic evidence indicates that AGPases are usually located in the plastids and in the cytosol (Tiessen et al., 2011). In dicots, AGPase is exclusively located in the plastid and represents 98% of the total AGPase activity in the cell (Thorbjørnsen, Volland, Denyer, Olsen and Smith, 1996; Tiessen et al., 2011). In contrast, the localization of AGPase is predominantly in the cytosol in the endosperms of many cereals; in wheat endosperm, 60-70% of the AGPase activity is cytosolic (Geigenberger, 2011) while it represents 80-95% in maize and developing barley endosperm (Denyer et al., 1996; Beckles, Smith and Rees, 2001; Tiessen et al., 2011).

However, the large and small subunit sizes are slightly smaller in plastidic AGPase than in cytosolic form in the amyloplast (Beckles et al., 2001; Tetlow et al., 2003). The difference in ADP-glucose location between non-cereals and cereals indicates that there exist two pathways for the synthesis of ADP-glucose in the cereal endosperm. This difference is probably caused by the optimal usage of sucrose in the cereal endosperm to accelerate starch accumulation when there is plenty of sucrose.

2.7.2. Starch synthase

Starch synthases catalyse the synthesis of an α -(1-4) linkage between the non-reducing end of a pre-existing glucan chain and the glucosyl moiety of ADP-glucose, causing the release of ADP in higher plants. Among the entire starch biosynthesis enzymes, SS has the highest number of isoforms (Fujita et al., 2011). Two forms of starch synthase have been found in most organs; first, the granule-bound starch synthases (GBSS) which binds tightly to starch granules and encoded by the *Waxy* (*Wx*) gene are involved in amylose biosynthesis (Nakamura, Yamori, Hirano and Hidaka, 1993).

The second class of starch synthases are soluble starch synthase (SSS) consisting of four major isoforms SSSI, SSSII, SSSIII, and SSSIV which are involved in amylopectin synthesis. Isoforms of the major classes of SSSs are highly conserved in higher plants (Ball and Morell, 2003). In wheat endosperm, five starch synthase activities have been identified. A 60 kDa GBSS encoded by *waxy* gene is exclusively found in the starch granule (Clark, Robertson and Ainsworth, 1991; Yan et al., 2000). A 75kDa SSI and a 180kDa SSIII could be detected in soluble fraction at the mid-endosperm development stage (Li et al., 2000). SSI is also found in the starch granule (Rahman et al., 1995). SSII is a 100-105kDa protein found in the starch granule (Denyer et al., 1996; Rahman et al., 1995) but it could also be detected in the soluble fraction of the endosperm extract during the early endosperm development stage (Li et al., 1999).

The sequence of the fifth starch synthase, SSIV, has been deposited in GenBank but this enzyme seems to be predominantly expressed in the leaves and has low sequence identity with other starch synthases expressed in the endosperm. In maize, five classes of starch synthases have also been identified. In addition to GBSS, cDNAs encoding SSI, SSIIa, SSIIb and dull1 SS (SSIII) has been isolated (Gao, Wanat, Stinard, James and Myers, 1998; Harn et al., 1998; Knight et al., 1998). All maize soluble starch synthases possess a divergent N-terminal extension that is absent in GBSS and *Escherichia coli* glycogen synthase (GSs).

In potato tuber, the primary amino acid sequences of GBSSI and SSII isoforms have been compared and the data revealed that the core region (≈ 60 kDa) was similar to all other known starch synthases and bacterial glycogen synthases (Edwards et al., 1999). Their amino acid sequences include an N-terminal motif (KTGGL) thought to be required for binding of ADP/ADP-glucose (Edwards et al., 1999). A region of approximately 60kDa is highly conserved in C-terminus of all these enzymes in higher plants and green algae, whereas this region is distributed across the protein sequence in bacterial glycogen synthases (Tetlow, 2011). The K-X-G-G-L motif is thought to be required for binding of ADP/ADP-glucose in bacterial glycogen synthase and in higher plant SSs (Furukawa, Tagaya, Tanizawa and Fukui, 1993; Busi et al., 2008).

The phylogenetic and sequence analysis of plants SS (*A. thaliana*, wheat and rice) and algal SS and prokaryotic GS isoforms on the basis of predicted amino acid sequence suggests that SSIs, SSIIIs and GBSSIs have distinct evolutionary origins as compared to SSIIIs and SSIVs (Leterrier et al., 2008). Particularly, valine residue within the highly conserved K-X-G-G-L motif appears to have faced strong evolutionary selection in SSIIIs and SSIVs and may affect primer/substrate binding of these SSs compared to SSIs, SSIIIs and GBSSIs (Leterrier et al., 2008).

2.7.2.1. Granule bound starch synthases (GBSS)

There are two isoforms of GBSS: GBSSI and GBSSII of which are only found in the granule matrix of starch biosynthesizing tissues. These isoforms are homologous and have approximately 66-69% amino acid sequence identity but their encoding genes are situated at different loci. The gene encoding GBSSI is predominantly expressed in endosperm whereas GBSSII is expressed in leaves and other non-storage tissues (Vrinten and Nakamura, 2000). GBSSI coded by the *Waxy* gene is the most well characterised starch biosynthesis enzyme in plants and has significant effect on starch composition and quality. GBSSI is responsible for elongating amylose in storage tissues and GBSSII in tissues such as pericarp, leaf, stem, and root (Yandeau-Nelson et al., 2010; Vrinten and Nakamura, 2000). Therefore, the above mentioned information makes GBSSI the most important enzyme responsible for endosperm amylose content. GBSSI has been widely studied in different plant species (Nakamura, 2002; Saito, Konda, Vrinten, K. Nakamura and T. Nakamura, 2004; Shapter et al., 2009).

The *waxy* mutant results in a lack of amylose production (Vrinten and Nakamura, 2000; Vrinten, Nakamura and Yamamori, 1999). However, the *Waxy* or low amylose starches are still able to form a granule and maintain its semi-crystalline property, suggesting that amylose is not required for insoluble granule synthesis (Denyer et al., 1999).

2.7.2.2. Starch synthase I (SSI)

The role of starch synthase isoforms in the synthesis of the starch granule is not clarified. There has been considerable effort to isolate mutant plants specifically lacking one of the isoforms of the starch synthase in order to determine their individual functions. These have provided some insights into the possible functions of the starch synthases. SSI is primarily responsible for the synthesis of the shortest glucan chains those with the degree of polymerization (DP) of up to ten or less than ten glucosyl units (Commuri and Keeling, 2001) in transient starch synthesis in leaves (Dauvillée et al., 2005).

The soluble SSI in maize is 76 kDa in size (Mu et al., 1994) and the degree of association of SSI in the starch granule is significant, representing 85% of total SSI content in maize endosperm (Mu-Forster et al., 1996). The amino acid sequence of SSI in maize is 75.7% identical to rice SSI (Knight et al., 1998). In japonica rice lacking SSII (Nakamura et al., 2005), SSI accounts 70% of the total SSs activity (Fujita et al., 2006). However, the relative contribution of SS isoforms is different in different species, for example SSIII contributes more than 70% of total SS activity in potato (Abel, Springer, Willmitzer and Kossman, 1996).

SSI from japonica rice produces chains with a degree of polymerization (DP) 8-12 from short and DP 6-7 chains emerging from the branch point in the A and B1 chains of the amylopectin (Fujita et al., 2006). Thus, starch synthase I may be mainly involved in the synthesis of the exterior A- and B-chains of amylopectin. Further, SSI mutant showed a decreased number of DP 8-12 glucan chains and an increase of both DP 6-7 and DP 16-19 chains in endosperm amylopectin in japonica rice (Fujita et al., 2006). SSI is also involved in incorporating glucosyl units into small chains filling up the cluster structure, but is not involved in making longer interior chains. However, SSI mutants in transgenic potato plants displayed no visible phenotypic changes in starch structure (Kossmann, Abel, Springer, Lloyd and Willmitzer, 1999), while playing a more important role in the leaves. The overlapping function of SSI and SSIII were revealed by creating double-recessive homozygous mutants from SSI null mutants with SSIII null mutants in japonica rice (Fujita et al., 2011). The seeds from these mutants remained sterile and the heterozygous mutants produced fertile opaque seeds further confirming that SSI or SSIII is required for starch biosynthesis in rice (Fujita et al., 2011).

2.7.2.3. Starch synthase II (SSII)

Starch synthases II have been characterized in several plants, including *Chlamydomonas*. Two SSII isoforms are present (SSIIa and SSIIb) in higher plants. SSIIa predominates in cereal endosperm while SSIIb is mostly confined to vegetative and photosynthetic tissues (Morell et al., 2003). SSII is also partitioned in both the starch granule bound protein fraction and in the soluble protein fraction located in the plastid (Li et al., 1999).

SSIIa plays a role in medium chain length extension and appears to be involved in elongating glucan chains produced by SSI leading to the production of medium length chains of DP=12-25 (Zhang et al., 2004). A starch synthase IIa activity has been shown to be lacking in barley *sex6* mutants endosperm. The endosperm has a shrunken phenotype and reduced starch content due to substantial decrease in amylopectin content.

However, the amylose content was increased up to 62.5% and 71% in comparison with 25% in the wild-type (Morell et al., 2003). Moreover, these mutants have altered chain-length distribution, whereas the amount of shorter glucan chains (DP= 6-11) increased from 24.15% (in wild-type) to 38.18% and 38.96%. The medium length glucan chains (DP= 12-30) decreased from 69.12% (in wild-type) to 54.14% and 53.42% in M292 and M342 mutants respectively (Morell et al., 2003). Interestingly, the lack of SSII causes an alteration in the distribution of the branching enzymes SBEIIa, SBEIIb, and SSI in the starch granule in the soluble fraction in barley amyloplasts (Morell et al., 2003). This suggests that either SSII mutation prevents binding of these proteins to the starch granules or prevents the formation of protein complexes in the amyloplast stroma and get trapped in the granule (Morell et al., 2003). It is believed that all these effects are mainly due to the complete loss of starch synthase II activity, but the alteration of the distribution of the other starch synthases and the branching enzymes may also play a role in the synthesis of the altered amylopectin.

The effects on chain length distribution of *ss2* mutants observed in barley are similar to *sugary2* (*su⁻²*) mutants of maize endosperm lacking SSIIa (Zhang et al., 2004) indicating a common function for SSII in starch granule assembly. The *su⁻²* mutants exhibit a significant increase in DP= 6-11 shorter chains and a decrease in DP=13-20 medium length chains (Zhang et al., 2004). In the *A. thaliana* mutant *ss2* (*Atss2*), the growth rate and the starch quantity were not affected, but increased the amylose/amylopectin ratio, total amylose (43% of total amylose) and significantly decreased DP=12-28 medium length glucan chains were in the endosperms of many cereals (Zhang et al., 2008). These results suggested that the loss of SSII activity can be restored by any other conserved SS, specifically SSI, GBSSI, or SSIII or SSIV in transient starch biosynthesis (Zhang et al., 2008). Hence, it can be concluded that this starch synthase II is responsible for the synthesis of intermediate-size chains.

2.7.2.4. Starch Synthase III (SSIII)

The major isoform of soluble starch synthase in potatoes, labelled as starch synthase III, has a molecular size of 139 – 140 kDa (Abel et al., 1996) and more than 200 kDa in maize endosperm (Cao et al., 1999). SSIII is expressed throughout in all developmental stages of these plants, and in contrast to GBSS1 and SSII, SSIII it is highly expressed in sink and source leaves. SSIII is coded by the *DUI* gene in maize endosperm (Cao et al., 1999). The *du1* mutations alter starch structure indicating that *DUI* provides a definite function(s) that cannot be compensated for by the remaining soluble SS activity (Abel et al., 1996).

Abel et al. (1996) reported a significant decrease in total SS activity by 13-29% in an *ss3* mutant in comparison to 100% of SS activity in the wild-type, without any significant effect on the potato phenotype, amylose content or the tuber yield in potato. The granule morphology was changed in *ss3* single mutants producing small granule structures (Abel et al., 1996). SSIII appears to be a vital enzyme in transient starch biosynthesis; starch granule initiation requires the presence of either SSIII or SSIV (Szydlowski et al., 2011). The conclusions made based on these observations were that SSIII is a major factor for the synthesis of starch in granules with normal morphology but the exact role it plays in the synthesis of starch remains obscure.

2.7.2.5. Starch synthase IV (SSIV)

A class of starch synthase amino acid sequences designated as SSIV have been reported in expressed sequence tag (EST) databases from several species including *Arabidopsis*, *Chlamydomonas*, wheat and cowpea. SSIV is exclusively present in the stroma of the plastids (Roldan et al., 2007; Leterrier et al., 2008). The role of SSIV in chain length distribution is not clear, but it may play a selective role in priming starch granule formation (Roldan et al., 2007). The cDNA sequence of wheat SSIV preferentially expressed in leaves is most similar to rice SSIV2, which share a similar exon-intron arrangement (Leterrier et al., 2008) suggesting that the SSIV present in leaves and endosperms may have slight variation in amino acid sequences (e.g. as similarly observed in SBEIIa and SBEIIb). There is limited information on the SSIV gene products as few have been isolated or characterized. Starch synthases show high similarity to each other.

For instance, the SSIV protein in *A. thaliana* is a 112.99 kDa in size showed 71%, 58.2%, 56.8% and 58.3% sequence identity to *Vigna unguiculata* (accession number AJ006752), wheat (accession number AY044844), rice (SSIVa, accession number AY373257) and rice (SSIVb, accession number AY373258) respectively (Roldan et al., 2007). A mutant of *A. thaliana* deficient in SSIV has been isolated. Two independent mutant alleles of SSIV in *A. thaliana*, *Atss4-1* and *Atss4-2* showed no decrease in total soluble SS activity but instead lower growth rates were observed in the mutant plants grown under a 16-h day/8-h night photo regime when compared to the wild type, (Roldan et al., 2007).

The starch contents in the leaves were significantly reduced in both mutants by 35% for the *Atss4-1* and 40% for the *Atss4-2* line with respect to their wild types at the end of the illuminated period. There was also a decrease in leaf starch of 35 – 40%. When the SSIV protein was restored by transformation to the mutant, both growth rate and starch levels were restored, indicating that the mutant alterations were due to SSIV deficiency. Analysis of the mutant starch in leaves, revealed that amylose/amylopectin ratio was not changed in *Atss4* mutants (Roldan et al., 2007), and there were only minor effects on the amylopectin structure (a slight decrease in the number of DP 7 – 10 chains). However, there were significant alterations in the morphology of the starch granules with respect to size and in the number of granules in the chloroplast. Whereas normal wild type chloroplasts would have 4–5 starch granules per chloroplast, the SSIV mutants contained a single starch granule per chloroplast concluding that the mutation at the *AtSS4* locus affects both the number and size of starch granules synthesized in the chloroplast.

These observations suggested that the SSIV may be involved in the priming of the starch granule (Roldan et al., 2007). SSIV is a newly identified SS isoform existing in the plastids. Therefore, the exact function of SSIV in storage starch biosynthesis has yet to be identified. The expression of SSIV during the endosperm development is high at the later stage of the grain filling (Dian, Jiang and Wu, 2005). Thus, it is proposed that the function of SSIV is to establish an initial structure for starch synthesis.

2.7.3. Starch branching enzymes

The branching structural arrangement of amylopectin is generated by starch branching enzymes (SBEs). These enzymes generate α -(1 \rightarrow 6)-linkages by cleaving internal α -(1 \rightarrow 4) bonds and transferring the released reducing ends to C6 hydroxyls to form the branched structure of the amylopectin molecule. Analysis of the primary amino acid sequences of higher plant SBEs reveals two major classes; SBEI (also known as SBE B) and SBEII (also known as SBE A) with multiple isoforms (e.g. SBEI, SBEIIa, and SBEIIb) (see Table 5) of which some are plant, tissue and/or developmental specific in their expression patterns (Regina et al., 2005; Gao et al., 1997). Starch-storage tissues of cereals, pea and potato contain at least two isoforms of starch branching enzyme, SBEI and SBEII (Martin and Smith, 1995; Larsson et al., 1996). In *Arabidopsis* (Fisher, Boyer and Hannah, 1996), maize (Gao et al., 1997) and barley (Sun, Sathinsh, Ahlandsberg and Jansson, 1998), SBEII can be divided into two types (SBEIIa and SBEIIb) that differ slightly in catalytic properties.

Most isoforms of SBE such as SBEI in wheat, barley, and rice endosperm as well as SBE2 in kidney bean were observed in the soluble fraction of amyloplast extracts (Rahman et al., 1995). Nevertheless, in kidney bean SBE1 is found to be located in the starch granule fraction (Hamada et al., 2001). Moreover, some SBEs i.e. (SBEI and SBEII in pea embryos, SBEII in wheat, barley, maize, and rice endosperm, and SBEIIb in maize endosperm are found in both the soluble and granule fractions (Denyer et al., 1993; Mu-Forster et al., 1996). The SBEs bound to granules are not found to have any activity in starch biosynthesis but are simply trapped during synthesis of the starch granule.

Table 5: Two families of SBEs from different plants

| Family A | | | Family B | |
|---|--------|-----------------------|----------|-----------------------|
| Organs | SBEs | Protein ID in GenBank | SBEs | Protein ID in GenBank |
| Potato (<i>Solanum tuberosum</i>) | SBEII | CAB40746.1 | SBEI | CAA70038.1 |
| Pea (<i>Pisium sativum</i>) | SBEI | CAA56319.1 | SBEII | CAA56320.1 |
| Kidney bean (<i>Phaseolus vulgaris</i>) | SBE2 | BAA82348.2 | SBEI | BAA82349.1 |
| <i>Arabidopsis thaliana</i> | SBE2-1 | NP181180.1 | | |
| | SBE2-2 | NP195985.2 | | |
| Rice (<i>Orzya sativa</i>) | SBE3 | BAA03738.1 | SBEI | AAP68993.1 |
| Wheat (<i>Triticum aestivum</i>) | SBE2 | AAG27623.1 | SBEI | CAB40980.1 |
| Barley (<i>Hordeum vulgare</i>) | SBEIIa | AAC69753.1 | | |
| | SBEIIb | AAC69754.1 | | |
| Maize (<i>Zea mays</i>) | SBEa | AAB67316 | SBEI | AAO20100.1 |
| | SBEIIb | AAC33764.1 | | |

All SBEs in the plant kingdom belong to the α -amylase family whose members have the $(\beta/\alpha)_8$ barrel structure containing the active center (Jespersen et al., 1993).

Four sequences (HSHAS/GFRFDGVT/GEDVS/AESHDQ) in both family A and B SBEs were found as the most conserved domains. The high sequence diversity displayed by the SBEs is in N and C-terminal domains. Therefore, differences in the enzymatic properties between individual SBEs would be determined by these domains (Kuriki, Stewart and Preiss, 1997).

2.7.3.1. Starch branching enzyme I (SBEI)

The two classes of SBE differ in terms of the length of the glucan chain transferred *in vitro* and also show different substrate specificities. SBEII proteins transfer shorter chains and exhibit a higher affinity towards amylopectin, while SBEI exhibits higher rates of branching with amylose and transfers longer chains (Guan and Preiss 1993; Takeda, Guan and Preiss, 1993). SBEI has a lower affinity for amylose and tends to produce shorter constituent chains compared to SBEIIa or SBEIIb when reacted with amylose *in vitro* (Gao et al., 1997). In maize, SBEI is expressed moderately during middle stages of kernel development (12–20 DAA), strongly during the later stages of kernel development (22–43 DAA) and is moderately expressed in vegetative tissues (Kim, Fischer, Gao and Guiltinan, 1998). When compared to the total SBE activity in mutants of SBEI, SBEIIa and SBEIIb in maize, a loss of enzyme activity has been identified for only SBEIIa and SBEIIb (Blauth et al., 2002) showing that the lack of SBEI was compensated by other two SBE isoforms.

Alternatively, SBEI does not have a significant role in determining starch quantity or quality in leaves or endosperm (Blauth et al., 2002). SBEI is highly conserved in plants and has been shown to interact with other starch biosynthetic enzymes (Liu et al., 2009; Tetlow et al., 2004) indicating that SBE plays some function in regulating the starch biosynthetic process. Therefore, it has been suggested that the participation of SBEI in amylopectin synthesis is not central to the process (Liu et al., 2009). It may be that the functions of this enzyme are adequately compensated for by the properties of SBEIIa or SBEIIb, or it may be that the enzyme does not interact with substrates until SBEIIa or SBEIIb have acted. In any event, the role of SBEI remains obscure. These data suggested that SBEI does not play a central role in this *in vitro* system, leaving the role of SBEI in the starch biosynthetic pathway still an open question.

2.7.3.2. Starch branching enzyme II (SBEII)

In monocots the SBEII class is made up of two closely related gene products SBEIIa and SBEIIb (Rahman et al., 2001). However, recent studies in developing wheat endosperm demonstrated that their expression patterns are considerably different where SBEIIa is expressed at a higher level than SBEIIb (Regina et al., 2005). Mutations in SBEII isoforms show a more pronounced phenotypic change. In maize, mutation of the gene encoding SBEIIb (also known as *amylose extender (ae)* or *amo* in barley) produces a high-amylose starch phenotype. In wheat, such starches can only be produced by suppression of both genes encoding the SBEIIa and SBEIIb forms (Regina et al., 2006). In potato, down-regulation of the equivalent SBE form produces a high-amylose starch (Schwall et al., 2000).

Phenotypic changes in SBEIIa mutations are dependent on the source of starch. In maize there was a visible change in leaf starch in SBEIIa mutants. However, no significant changes occurred in storage starches of maize kernels (Blauth et al., 2001). This observation suggests a primary role for SBEII in leaf (transient) starch synthesis with either no critical role of SBEII in amylopectin biosynthesis in the endosperm or a role that can easily be compensated for by other SBEs in its absence.

2.7.4. Starch de-branching enzyme

In addition to the three starch biosynthesizing enzymes (AGPase, SS and SBE), there is some evidence indicating that starch de-branching enzyme (DBE) may have a function in starch biosynthesis, especially in amylopectin synthesis. Starch debranching enzymes play an important role in the development of crystalline amylopectin. There are two types of DBEs known to exist in plants, the isoamylase-type (ISO) hydrolyzes α -(1 \rightarrow 6) linkages in amylopectin and pullulanase-type (PUL) hydrolyzes α -(1 \rightarrow 6) linkages in amylopectin and pullulan, a fungal polymer of malto-triose residues. There are at least three isoamylase-type DBE isoforms (ISO1, ISO2, and ISO3) (Wong et al., 2003). In a pullulanase-type starch de-branching enzyme absent mutant, *sugary1*, amylopectin is not synthesized but replaced with a water-soluble α -1,4, α -1,6 linked glucan known as phytoglycogen which contains more frequent and randomly located branches throughout the molecule compared with amylopectin that has repeated unit structure referred to as amylopectin cluster (Wong et al., 2003).

Rice and maize mutants lacking ISO1 (*sugary1*) demonstrate an increase in the disordered water soluble, highly and randomly branched phytyglycogen (Nakamura, 2002). This suggests that starch de-branching enzyme directly participates in starch biosynthesis. Zeeman et al. (1998) reported the role of starch de-branching enzymes in the determination of the fine structure of amylopectin by screening mutant populations of *Arabidopsis* and obtained two mutants lacking an isoamylase-type de-branching enzyme. The loss of this isoamylase resulted in a 90% reduction in the accumulation of starch and an increase in phytyglycogen. Both normal starch and phytyglycogen accumulated simultaneously in the same chloroplasts in the mutant lines, suggesting that isoamylase has an indirect rather than a direct role in determining amylopectin structure. The precise roles for the isoamylase-type and pullulanase-type DBEs in starch biosynthesis are not yet clear. Based on these de-branching enzyme deficiency mutants, the function of de-branching enzymes in amylopectin synthesis has been proposed in two very different models.

The first model, the glucan trimming model proposes that DBEs remove any branches that would prevent crystallization of the developing granule (Ball et al., 1996; Myers, Morell, James and Ball, 2000). An alternative to the glucan-trimming model proposes that DBEs clear away any soluble glucan not attached to the granule (Zeeman et al., 1998). The theory is based on the concept that SSs and SBEs will continue to synthesize glucan polymers if sufficient substrate is present therefore causing phytyglycogen accumulation. It was thought for a long time that starch synthase and starch branching enzyme are the only two enzymes required to synthesize the starch polymers.

This has been proved by several mutants in higher plants. However, the *dbe* mutants such as *sugary1* mutant of maize indicated that the reduced or eliminated isoamylase activity in the mutants displayed abnormal starch which contains highly branched phytyglycogen and small amounts starch granules (Burton et al., 2002).

2.8. Biotechnological approaches to increase starch biosynthesis

Starches from different botanical sources have different polymer compositions and structures hence, different physicochemical properties (gelatinization temperature, viscosity of cooked pastes and gels, etc.). These properties referred to as the functionality of the starch, determines the range of applications for which a given starch is used (Zeeman et al., 2010) Manipulation of either yield or quality of starches within the plant requires substantial understanding of fundamental processes involved in the biosynthesis of starch in storage organs (seeds, stems, rhizomes and tubers). The first step in understanding these fundamental processes is through isolation and characterization of major genes which are involved in starch biosynthesis (Frances and Bligh, 1999). At present, genes which are known to be involved in starch biosynthesis within the amyloplast have now been cloned from various plant sources such maize, potato, rice, chickpea, wheat, barley, cassava and rice (see Table 6).

Table 6: Genes from major plant starch sources involved in starch biosynthesis that have been cloned.

| Enzyme | Source |
|----------------------------------|--|
| AGPase large subunit | rice, potato, maize, wheat, barley, cassava, chicken pea |
| AGPase small subunit | rice, potato, lentil, chicken pea, wheat barley, cassava |
| GBSS | rice, lotus, potato, maize, wheat, pea |
| SSS | rice, potato, amaranth, |
| Branching enzyme type A | rice, pea, maize |
| Branching enzyme type B | rice, maize, potato, pea |
| Debranching enzyme (pullulanase) | Rice |
| Debranching enzyme (isoamylase) | Maize |

Adapted from Frances and Bligh (1999)

Information on starch biosynthetic genes present in various plant sources will eventually lead to the understanding the interactions and mode of action of enzymes involved in starch biosynthesis. This understanding has been the driving force behind a lot of experiments performed to date involving the manipulation of starch biosynthesis enzymes by either the use of antisense inhibition of gene expression or the use of naturally occurring or chemically induced mutants (Frances and Bligh, 1999), (see Table 7). Most attempts to enhance starch accumulation have focused on engineering AGPase activity in plants, an enzyme which provides the substrate for SSs and is subject to tight allosteric regulation.

There is evidence that increasing the supply of ATP to the plastid can stimulate the production of ADP-glucose and hence the rate of starch biosynthesis. Firstly, overexpression of a plastidial envelope adenylate translocator from *Arabidopsis* in potato increased ADP-glucose levels by two-fold and increased starch content by 16 - 36% compared with control tubers (Geigenberger et al., 2001). Secondly, the down regulation of a plastidial adenylate kinase, an enzyme that interconverts two molecules of ADP into ATP and AMP, resulted in a tenfold increase in ADP-glucose levels and a doubling of the starch content in potato tubers, in both greenhouse and field trials (Geigenberger et al., 2001).

It is possible that the down regulation of adenylate kinase leads to enhanced transport of ATP into the plastids in exchange for ADP which is released from ADP-glucose upon glucan polymerization. Increased starch content in plants could have a very dramatic effect on the financial side of plant production. It would enable the cost of tuber harvesting and transport to be offset by the profit from an increase in starch contents. In marama tuber, starch content could potentially be increased by increasing ADP-glucose, provided that the interactions and mode of action of enzymes involved in starch biosynthesis are understood.

Table 7: Mutations known to affect structural genes for enzymes involved in starch biosynthesis of major starch sources.

| Mutation | Enzyme affected | Plant | Phenotype |
|------------------------------|---------------------------------|--------------|------------------------------|
| <i>waxy (wx)</i> | GBSS | maize | no amylose |
| <i>waxy (wx)</i> | GBSS | Rice | no amylose |
| <i>amylose free (amf)</i> | GBSS | potato | no amylose |
| <i>low amylose (lam)</i> | GBSSI | Pea | low amylose |
| <i>amylose extender (ae)</i> | SBE IIb | maize | high amylose |
| <i>amylose extender (ae)</i> | SBE III | Rice | high amylose |
| <i>brittle-2 (bt-2)</i> | AGPase small subunit | maize | low starch |
| <i>shrunk-2 (sh-2)</i> | AGPase large subunit | maize | low starch |
| <i>rugosus (r)</i> | SBE I | Pea | low starch |
| <i>rugosus (rb)</i> | AGPase small subunit | Pea | low starch |
| <i>rugosus-5 (rug-5)</i> | GBSS II | Pea | altered starch granule shape |
| <i>sugary-1 (su-1)</i> | Debranching enzyme (isoamylase) | maize | high phytoglycogen |

Adapted from Frances and Bligh (1999)

2.9. Construction of cDNA library

Complementary DNA (cDNA) libraries are expressed libraries compared with genomic DNA libraries. A cDNA library represents the information encoded in the mRNAs and the information is obtained in a particular time from a particular tissue or organism in which the expected genes are expressed.

To analyze the expression information contained in mRNA, firstly, mRNA molecules are converted into cDNA molecules and are inserted into a self-replicating lambda or plasmid vector. Consequently, each lambda or plasmid DNA contains a cDNA molecule and the recombinant DNA molecules compose a cDNA library. The cDNA library can be screened by hybridization using different probes. Alternatively, the inserts in a cDNA library can be fully sequenced to obtain the information of gene transcription in a particular time and tissue or organism (see Figure 8).

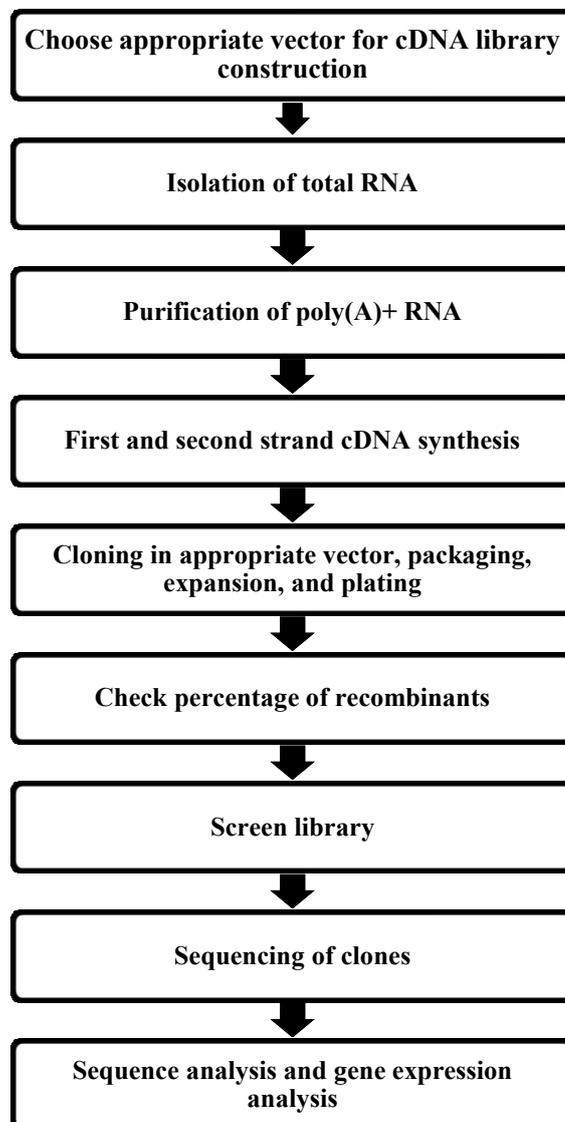


Figure 8 : A flow chart of cDNA library construction.

Gateway Cloning Technology is a universal system for cloning and sub-cloning DNA/cDNA fragments in many expression vectors (Ohara and Temple, 2000). This technology uses the λ -recombination system transfer cDNA fragments between vectors which contain recombination site for the λ -recombinase machinery while reading frame and orientation. This approach is characterized by several advantages over conventional restriction-assisted cloning methods which employ restriction enzyme. Some of these advantages are that it (i) eliminates restriction digestion for directional cloning, (ii) generates lower level of chimeric clones, (iii) produces higher amount of full-length cDNA clones and (iv), gives higher cloning efficiency (Hartley, Temple and Brasch, 2000; Ohara and Temple, 2001).

Moreover, this method facilitates the construction of cDNA input into different expression vectors, especially when it is not clear which system or host background will provide sufficient expression levels. This approach does reduce enormous amount of work and prevents significant barriers to progress. Therefore, in this study a Gateway cloning technology was used to construct *T. esculentum* tuber specific cDNA expression libraries to screen for starch synthase genes and activity.

2.10. Protease inhibitors

Protease inhibitors in plants play an important role in the regulation of growth and development, response to stress, and defense against insects and pathogens. These inhibitors are thought to be involved primarily in defense against herbivores, which rely on proteases to digest the proteins they consume, and for protection against wounds inflicted by insects. In seeds, where protease inhibitors are often found in high concentrations, they keep the plants own proteases in check to prevent degradation of storage proteins. Plants contain a variety of serine proteinase inhibitors which are divided into 16 classes (Ryan, 1990), with soybean trypsin inhibitors (Kunitz), Bowman–Birk inhibitors, potato inhibitors I and II being four of the better studied classes. Bowman–Birk proteinase inhibitors (BBI) were first isolated and characterized in soy-bean seeds (Birk, Gertler and Khalef, 1963; Bowman, 1946) and later found in other leguminous plants (Norioka and Ikenaka, 1983).

Since the discovery of soybean trypsin inhibitor by Kunitz in 1946 (Kunitz, 1946), many protease inhibitors have been isolated from different leguminous species and although several studies focused on the endogenous and nutritional importance of these proteins (Breiteneder and Radauer, 2004), their exact physiological role has not been well understood (Birk, 2003). Structural properties of inhibitors that control the activity of the proteinases are targeted as potential pharmaceutical products (Oliva and Sampaio, 2008; Turk, 2006).

2.10.1. Characterization of Kunitz type serine protease inhibitor from marama

Nadaraja, Weintraub, Hakala, Sherman and Starcher (2009), isolated a Kunitz-type serine protease inhibitor from marama bean that is specific for elastase and distinct from the marama bean trypsin inhibitor. This inhibitor has a typical amino acid composition when compared to other serine protease inhibitors with a cysteine content of only one or possibly two residues per molecule and a blocked amino acid terminus. Inhibition studies have further indicated virtually no inhibition of chymotrypsin by the inhibitor but strong inhibition of elastase was observed (Nadaraja et al., 2010). This inhibitor further appears to be heterogeneous due to carbohydrate differences, demonstrating two bands on an SDS gel with molecular weight of 17.8 kDa and 20 kDa, respectively. Partial sequence, derived from mass spectrometry, has indicated that the protein is a Kunitz-type inhibitor distinct from other known plant serine protease inhibitors.

2.10.2. Medical and pharmaceutical application of plant serine protease inhibitors

Serpins (serine protease inhibitors) are a class of proteins involved in the regulation of serine and other types of proteases (Breiteneder and Radauer, 2004). In humans, the majority of serpins regulate the functions of proteases involved in the body's response to injury. This includes roles in coagulation, fibrinolysis, inflammation, wound healing, and tissue repair (Oliva and Sampaio, 2008.)

Elastase is part of the chymotrypsin-like clan which includes chymotrypsin, trypsin and elastase. Among them is the human neutrophil elastase/leukocyte elastase (HNE; E.C. 3.4.21.37) which is involved in several diseases (Mosolov and Valueva, 2005).

Elastase has generally a broad range of extra cellular matrix substrates including elastin, proteoglycans, collagen and fibronectin. Leukocyte elastase a major serine protease in human is predominantly present in the azurophilic granules of neutrophils and monocytes. So far, a specific marama bean inhibitor has been recently isolated and characterized with a low $K(i)$ when tested against the serine protease elastase and for both pancreatic and neutrophil elastase (Nadaraja et al., 2010). An important role has been suggested for human elastase in various inflammatory disorders. This includes pulmonary emphysema, sepsis, arthritis and certain skin diseases (Oliva et al., 2010). In humans, elevated elastase activity results in skin sagging and reduced skin elasticity by degrading elastin which is an elastic substance in the lungs and some other organs that requires supports of their structural frameworks (Oliva et al., 2010).

In general, elastase inhibitors (serine protease inhibitors) are efficient tools for studying the physiological function of elastase. However, these inhibitors can also be potential therapeutical candidates for pulmonary emphysema, adult respiratory stress, rheumatoid arthritis and other diseases and further as an additive in creams for skin treatment. The use of protease inhibitors as therapeutic agents, in particular their use in inhibition of cellular transformation, blood clotting disorders, osteoporosis, retroviral disease and cancer is undergoing thorough investigation (Hocman, 1992). Due to these applications of protease inhibitors in medicinal and agricultural fields, the search for novel protease inhibitors has received widespread interest.

2.10.3. Reverse zymography for detecting protease activity

The Reverse zymography procedure uses synthetic substrates to detect protease activity. This procedure involves resolving the proteins by denaturing or non-denaturing Polyacrylamide gel electrophoresis (PAGE) followed by incubating the gel in solution with a target protease (Michaud, 1998).

After allowing the entrapped enzyme to react with a specific chromogenic or fluorogenic substrate, either in solution (Wilkesman and Kurz, 2009) or by contact with a substrate embedded membrane, protease activity is revealed by destaining the gel and the inhibitors are visualized as clear (or dark), proteolysis-free areas against a dark-or clear-background, depending on the specific substrate used. Fluorogenic substrates have proven useful for this kind of detection system in allowing the status of inhibitors found in crude extracts at very low concentrations to be monitored (Weder and Kaiser, 1995). Reverse zymography is generally reproducible and allows semi-quantitative analysis of protease inhibitor activities, making this analytical approach useful in the study of several protease inhibitor-related processes.

2.11. Statement of the research problem

Although starch and starch biosynthesis have been researched for several decades, the packing of amylopectin and formation of starch granule still remain unclear to date. Especially in marama bean, the research of starch physico-functional properties and starch biosynthetic genes is scanty to the researcher's knowledge.

Therefore, the problem that gives rise to this study is that, firstly, there is no information available on the amount of starch amylose/amylopectin present in marama tubers at the same time there is no literature available on the physicochemical properties of native marama tuber starch. Secondly, there is no scientific report about the molecular features of marama starch biosynthesis genes available in public database. The rational use of starch requires a prior knowledge of its properties, and starch physicochemical and pasting properties are most important properties to consider when determining starch uses in food systems and other industrial application. Information on the concentration of starch and amylose present in marama tuber together with the physicochemical properties of the native marama tuber starch would form a basis for possible applications and for further investigation of physicochemical modification to improve the functional properties of starch. The analysis of starch biosynthesis indicates that some starch properties are controlled by the enzymes involved in starch biosynthesis pathway. Therefore, to analyse marama bean starch at the molecular level, the study focussed on the analysis of type of starch synthesizing enzymes in marama bean. Thirdly, to the researcher's knowledge no report on serine protease inhibitor activities and activity profile of marama bean seed has been carried out so far. Protease inhibitors from natural sources such seeds of legume plants are known to be source of bioactive constituents with pharmaceutical and industrial applications. In the study, marama seeds have been used as a source for the detection of a useful natural product (serine protease inhibitor) to determine if the serine protease inhibitor is the most potent in marama or if even more potent serine protease inhibitors are expressed in the marama bean.

This will provide a basis for possible consideration of marama as a source of protease inhibitors with potential application in cosmetic or pharmaceutical industry. To the researcher's knowledge neither identification nor characterization of any serine protease inhibitor gene family has been carried out in marama to date. Information on serine protease inhibitor genes in marama may be vital and needed in developing marama cultivars with expected proportions of inhibitors based on their different demands for pharmaceutical and industrial applications.

2.12. Aims of the study

This dissertation, aimed to investigate starch biosynthesis at the molecular level for marama bean by isolating starch synthesis genes in marama bean and examining their sequence structures. It also focused on determining and comparing serine protease inhibitor activity of marama bean seeds with those of other legumes and establishes an activity profile of marama bean serine protease inhibitors. Furthermore, it aimed to isolate and characterize serine protease inhibitor gene from marama seed cDNA. Therefore, specific objectives of this study were to:

1. Determine starch and amylose concentration of marama tuber and evaluate the physicochemical properties of native marama tuber starch.
2. Isolate and characterise of a gene encoding a major starch biosynthesizing enzyme SSSI from cDNA library constructed from marama tuber.

3. Isolate AGPase genes from marama using a PCR based strategy, analyse their sequences and establish their evolutionary relationship to those of other plant species.
4. Isolate SBEs genes from marama using a PCR based strategy, analyse their sequences and establish their evolutionary relationship to those of other plant species.
5. Test serine protease inhibitor activity in marama seeds and seeds of different plant species using a fluorogenic synthetic protease substrate to determine and compare total inhibitor activity of seeds and establish activity profile of serine protease inhibitors of marama bean mature and developing seeds by activity gel electrophoresis (reverse zymogram technology).
6. Isolate and characterize serine protease inhibitor cDNA gene clone from marama seed.

CHAPTER 3: MATERIALS AND METHODS

3.1. Plant material

Marama bean (*T. esculentum*) was grown in marama experimental field of Omipanda and Otjovanatje in Omaheke region, Namibia (Nepolo, Takundwa, Chimwamurombe, Cullis and Kunert, 2009). Tuber samples were collected from the field after three months after planting (March, April, and May) and used directly in experimental analysis or stored at -80°C until analysis. Mature dry and green marama seeds were also collected from the experimental field and stored at -80°C until used for analyses.

3.2. Physicochemical properties of marama tuber starch

3.2.1. Isolation of starch from marama tubers

Marama tuber starch was isolated by the method as described by Edwards, Marshall and Sidebottom, (1995) with some modifications. Two hundred grams of peeled and sliced tuber was homogenized with 500 mL of extraction buffer (50mM Tris/HCl, pH 7.5, containing 1mM diaminoethanetetraacetic (EDTA), 1mM dithiothreitol (DTT) and 0.1% (w/v) sodium metabisulfite) using a Waring commercial blender.

The homogenate was filtered through four layers of cheese cloth and the filtrate was centrifuged for 5 minutes at 1000 g. The supernatant was discarded and the starch was re-suspended in buffer (50mM Tris/HCl, pH 7.5, containing 1mM EDTA and 1mM DTT), then centrifuged for 5 min at 1000 g. This was repeated a further three times. After the last wash with buffer, the starch was re-suspended in cold acetone and left to settle at -20°C and the supernatant was discarded. This was repeated twice, until the starch started to appear white. The starch was allowed to air dry and stored in air-tight containers until analysis.

3.2.2. Total starch assay

Concentration of starch in marama tuber was determined using amyloglucosidase/ α -amylase method as described by McCleary, Solah and Gibson, (1994) (Total Starch, Megazyme International Ireland Inc.). Fresh marama tubers were finely milled using a mortar and a pestle. A 100-mg milled sample was wet with 0.2 mL of 80% (v/v) ethanol and treated by boiling for 6 minutes in 300 units thermostable α -amylase to partially hydrolyse the starch. The pH of the samples was adjusted by adding 4 mL of 200 mM sodium acetate buffer pH 4.5. Dextrins were quantitatively hydrolysed to glucose by incubation at 50°C for 30 min with 20 units amyloglucosidase at pH 4.5. The samples were adjusted to 100 mL total volume and centrifuged at 2,000 x g for 10 minutes. The amount of glucose in the supernatant was determined using glucose oxidase/peroxidase (GOPOD) reagent (Megazyme International Ireland Inc.).

One litre of GOPOD reagent contains $\geq 12,000$ units glucose oxidase, ≥ 650 units peroxidase, and 0.4 mmol 4-aminoantipyrine in glucose reagent buffer (1 M potassium dihydrogen orthophosphate, 200 mM para-hydroxybenzoic acid). A 0.1-ml aliquot of the supernatant as described above was incubated with 3 mL GOPOD reagent at 50°C for 20 minutes. The absorbance at 510 nm of each sample was read against the reagent blank. The concentration of starch in a sample was calculated by comparing the amounts of glucose produced from the sample and from glucose standard (1 mg/mL). Concentration of starch was expressed as a percentage of fresh flour weight.

$$\text{Starch \%} = \Delta A \times F \times 1000 \times (1/1000) \times (100/W) \times (162/180)$$

$$= \Delta A \times (FIW) \times 0.9$$

where ΔA = absorbance read against the reagent blank

F = conversion from absorbance of glucose standard to μg

= 100 μg of glucose/ absorbance for 100 μg of glucose

1000 = volume correction (0.1 mL taken from 100 mL)

1/1000 = conversion from μg to ml

100/W = factor to express starch as a percentage of flour weight

W = the weight in mg of the flour analysed

162/180 = adjustment from free glucose to anhydro-glucose which occurs in starch

3.2.3. Amylose/Amylopectin assay

Amylose was determined using the Concanavalin A precipitation method as described by Gibson, Solah and McCleary, (1996) (Amylose/Amylopectin Assay Kit, Megazyme International Ireland Inc.). A 25-mg sample was solubilized by boiling for 15 minutes in 1 mL dimethyl sulfoxide (DMSO). Lipids were removed by precipitating the starch in 6 mL of 95% (v/v) ethanol. The starch pellet was recovered by centrifugation at 2,000 x g for 5 minutes, then, redissolved by boiling for 15 minutes in 2 mL DMSO. The starch solution was diluted to 25 mL total volume with Concanavalin A solvent (180 mM sodium acetate, 900 mM NaCl₂, 0.9 mM CaCb, 0.9 mM MgCl₂ and 0.9 mM MnCl₂ pH 6.4). Starch from a 0.5-mL aliquot was hydrolysed with 16.5 units amyloglucosidase and 2.5 units α -amylase. The sample was incubated with 4 mL GOPOD reagent at 40°C for 20 minutes, and the absorbance of the sample was read at 510 nm against the reagent blank. Amylopectin in another aliquot (1.0 mL) was precipitated at room temperature for 1 h in the presence of 1.3 mg/mL Concanavalin A. After centrifugation at 14,000 x g for 10 minutes, amylose content in the supernatant was determined by treating the sample with amyloglucosidase/ α -amylase solution. The percentage of amylose was expressed as a proportion of total starch. The amount of amylopectin was indirectly determined by subtracting the amounts of amylose from total starch concentration.

$$\text{Amylose, \% (w/w)} = \frac{\text{Absorbance (Con A Supernatant)}}{\text{Absorbance (Total Starch Aliquot)}} \times \frac{6.15}{9.2} \times \frac{100}{1}$$

where, 6.15 = dilution factor for Con A-treated sample, and 9.2 = dilution factor for total starch extracts respectively.

3.2.4. Determination of phosphate at the C-6 -position of the glucose residues

Phosphate at the C-6 position was determined as Glc-6-P after acid hydrolysis of the starch as described by Nielsen, Wischmann, Enevoldsen and Møller, (1994) with modifications. Starch (125 mg) was suspended in 0.5 mL of 0.7 M hydrochloric acid and kept at 100°C for 4 hours. An aliquot (30 µL) was mixed with 230 µL of assay buffer containing 100 mM MOPS-KOH (pH 7.5), 10 mM MgCl₂, 2 mM EDTA in a microtitre well and neutralized with 100 µL of 0.7 N KOH. NAD (final concentration 0.4 mM) and 2 units of Glc-6-P dehydrogenase from *Leuconostoc mesenteroides* (Sigma) were then successively added. Glc-6-P was determined by the absorption change at 340 nm caused by the Glc-6-P dehydrogenase-mediated reduction of NAD⁺. Glucose residue was determined as the *Glc-6-P* formed by acid hydrolysis of starch. Potato starch was concurrently analysed for phosphate as a control. Standard curves demonstrated that the Glc-6-P dehydrogenase contained no interfering enzyme activities.

3.2.5. Pasting properties

The pasting properties of marama starch were determined using a rapid visco-analyzer (Perten RVA 4500, Australia). Test profile STD1 was used for determination of pasting characteristics. An amount of 4.0 g of starch was directly weighed into aluminum RVA canisters and 25 mg of distilled water was added to form a starch suspension.

The starch suspension was stirred in the RVA container initially at 11000 x g for 10 seconds and finally at 3000 x g for the remainder of the test. The temperature profile was started from 50°C for 1 minutes followed by raising the temperature linearly to 95°C in 3 minutes and 42 seconds, holding for 2 minutes and 30 seconds, cooling the system to 50°C in 3 minutes and 48 seconds and holding at 50°C for 2 minutes.

Peak viscosity (PV), temperature at PV ($P_{temp.}$), hot paste viscosity (HPV) or holding strength, cool paste viscosity (CPV) or final viscosity, breakdown (BD) or (PV-HPV), Set back (SB) or (CPV-HPV) and stability ratio HPV/PV were determined from the viscosity profile curve and expressed in centipoise (cP). The ThermoLine windows software was used to process the data (Perten RVA 4500, Australia).

3.2.6. Starch granular characterization

The starch granules were observed using a Scanning Electron Microscope (Leo-Zeiss). Starch granules were sprinkled onto a double-sided tape attached to a stub and coated with gold using a sputter coater (s150A, Edwards) and placed in the SEM chamber. Photomicrographs were taken using a SEM apparatus at an accelerating voltage of 25 kV.

3.3. Construction of cDNA library and screening for the identification of tuber-specific starch synthase genes in *T. esculentum*

3.3.1. *T. esculentum* seed germination and cultivation

Seeds were placed in a mixture of 50% sand and 50% potting soil and seedlings allowed to grow for approximately three months in a glasshouse. *T. esculentum* tubers were removed from the soil, sliced into smaller discs, and immediately frozen in liquid nitrogen and stored at -80 °C or used immediately for RNA extraction.

3.3.2. RNA extraction from tuber material

Total RNA was extracted using a modified CTAB RNA extraction procedure (Gasic et al., 2004). CTAB buffer (2% w/v cetyl trimethylammonium bromide; 2% (w/v) Polyvinylpyrrolidone; 100 mM TRIS-HCl, pH 8.0; 25 mM EDTA; 2 M NaCl) was prepared and autoclaved. β -mercaptoethanol was added to a final concentration of 2% (v/v) directly before use. Total RNA was extracted by grinding frozen tuber slices in liquid nitrogen with pestle and a mortar. An amount of 1.5 mL CTAB buffer was added to approximately 200 mg of ground material. CTAB buffer and tuber ground material mixture was mixed vigorously by vortexing for 30 seconds.

Samples were incubated for 30 minutes, at 65°C and vortexed for 30 seconds at 5 minute intervals. Centrifugation was performed at 16000 x g for 10 minutes at room temperature before the supernatant was transferred to a fresh tube and one volume of chloroform/isoamylalcohol (24:1) was added.

The sample was vortexed for 30 seconds and then centrifuged at 16 000 x g for ten minutes at 4°C before the supernatant was collected and the chloroform/isoamylalcohol extraction repeated. RNA was precipitation by the addition of LiCl to the collected supernatant to a final concentration of 2 M and incubated at 4°C overnight. The sample was centrifuged at 16 000 x g for 1 hour at 4°C and the supernatant was discarded. The pellet was washed with 70% (v/v) ethanol by centrifuging at 16 000 x g for 10 minutes at 4°C, and the ethanol was discarded. The resulting RNA pellet was allowed to dry for 30 minutes and re-suspended in 30 µL of DEPC-treated dH₂O.

3.3.3. Concentration of RNA

The RNA concentration in each sample was determined spectrophotometrically. RNA samples were combined together into separate RNase free 1.5 mL microcentrifuge tube and 1/10 volume of DEPC-treated 3 M Sodium acetate (pH 4.8) was added along with RNA grade glycogen (Thermo Scientific) at a final concentration of 0.1µg/µL. The solution was mixed gently with 2.5 volumes of ethanol and incubated at -80°C overnight. The mixture was centrifuged at 16000 x g at 4°C for one hour, the supernatant was discarded and the pellet washed with 70% (v/v) ethanol. RNA was recovered by centrifugation for 30 minutes at 16000 x g and 4°C respectively. After the supernatant was discarded, the pellet was centrifuged for 20 minutes at 16000 x g at 4°C and the remaining ethanol was also removed.

RNA was re-suspended in DEPC-treated dH₂O and the concentration was determined spectrophotometrically. Approximately 2 µg of the re-suspended precipitated RNA was separated on a 1% (w/v) agarose gel to analyse the quality of the extracted RNA.

3.3.4. Isolation of mRNA

The messenger RNA (mRNA) was isolated from the total RNA using a commercially available kit (GenElute™ mRNA miniprep kit, Sigma Aldrich). All procedures were performed according to manufacturer's protocol. Briefly, total RNA (5- 500 µg) isolated from tuber was used for isolation of polyadenylated mRNA using oligo (dT) covalently linked to 1 µm polystyrene beads to capture polyadenylated mRNA by hybridization. mRNA-bead complexes were washed three times, on a microcentrifuge spin filter, and eluted into 10 mM Tris-HCl, pH 7.5, to yield pure mRNA which was subsequently used for cDNA synthesis and library construction.

3.3.5. Construction of a cDNA library in *E. coli* expression vector

The *T. esculentum* cDNA library was constructed with approximately 1 µg of starting mRNA using CloneMiner™ cDNA Library Construction Kit (Invitrogen). All procedures were performed according to the manufacturer's protocol. Briefly, purified mRNA from marama tuber was reverse transcribed using SuperScriptIII and poly-thymine oligo primers containing the *attB2* recombination site (first strand cDNA synthesis).

After a second strand cDNA synthesis, the other recombination site *attB1* was ligated to the 5' end of the cDNA overnight. T4 DNA polymerase was used to blunt end the cDNA ends followed by a phenol/chloroform extraction and ethanol precipitation to isolate the cDNA. The cDNA pool was size-fractionated by column chromatography using the provided Sephacryl S-500 HR resin column to by to remove excess primers, adapters, and small cDNAs. Different fractions were pooled together and precipitated using ethanol. The cDNA was analysed to determine the yield of the cDNA using a Nano-Drop. This pooled cDNA flanked by *attB* recombination site was then used in Gateway's BP recombination reaction to put it into a donor plasmid. About 75-100 ng of cDNA obtained from different pooled fractions was used in the BP recombination reaction. The BP reactions facilitated recombination between *attB*-flanked cDNAs and *attP* sites in the donor plasmid (pENTRTM222) to generate a Gateway entry library (see Figure 9). A total of 100 ng of pooled cDNA and 150 ng of pENTRTM222 plasmid were added to the BP Clonase enzyme mix and incubated at 25 °C for 16 -20 hours. Following proteinase K inactivation, DNA was ethanol precipitated and transformed into Electro-MAX DH10B *E. coli* T1 phage resistant cells that were plated on LB plates containing kanamycin (50 µg/mL). Electroporation parameters were as follow: 2.0 kV, 200Ω and 25 µ. Thereafter, the cDNA library titer for each plate was calculated using the equation below:

$$\text{cfu/mL} = \frac{\text{colonies on plates} \times \text{dilution factor}}{\text{volume plated (mL)}}$$

The titer for each plate was used to calculate the average titer for the entire cDNA library.

Plasmids DNA was isolated from 25 positive clones formed on the overnight plates and used for verification of cDNA inserts and resulting in the entry library (pENTR™222-cDNAs). The remaining library cells were washed off from the plates with 0.9% NaCl and frozen down with 40% glycerol in 1 mL aliquots and stored at -80 °C. To transfer cDNA library into the expression vector, a Gateway® compatible vector was constructed into the *EcoRV* site of pBluescript SK(-) (see Figure 10). Briefly, 100 ng of entry library was incubated with 100 ng of *attR* containing destination vector pBluescript SK(-)::DEST, 4 µL of LR reaction buffer and 4 µL of LR Clonase enzyme mix at 25 °C for 60 minutes. Thereafter, 2 µL of proteinase K solution was added and incubated at 37 °C for 10 minutes to stop the reaction. LR reaction mix (1 µL) was then transformed into 50 µL of stain ElectroMAX DH10B competent cells and plated out on Ampicillin (100 µg/ml) containing LB plates and resulted in the destination expression library harbouring plasmid pBluescript SK(-)::DEST for functional screening. The Gateway® cassette was ligated in sense orientation with regard to the *Lac* promoter and the orientation of cDNA entry clones was confirmed by sequencing.

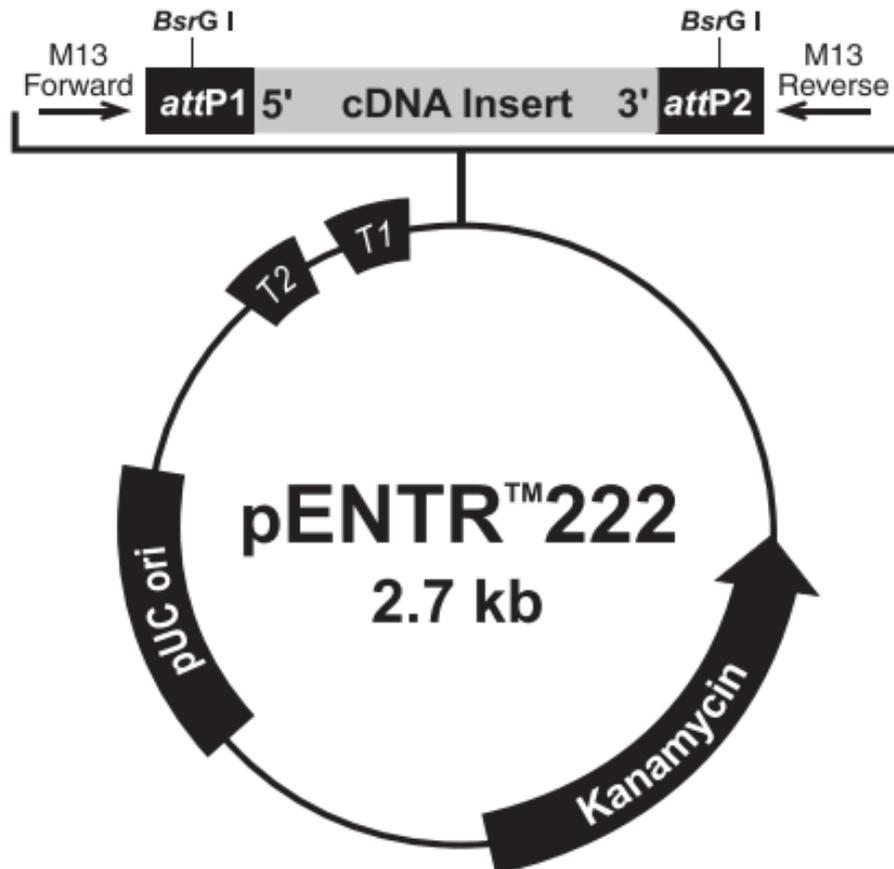
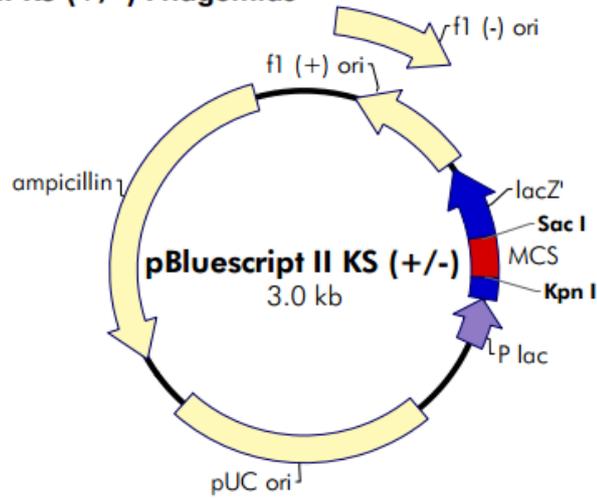


Figure 9: The map above shows the elements of pENTR™ 222 insert vector. The map above shows the elements of pENTR™ 222 insert vector. Source: Gateway™ pENTR™ Vectors instruction manual-Invitrogen, 2002.

pBluescript II KS (+/-) Phagemids



pBluescript II KS (+/? Multiple Cloning Site Region (sequence shown 598? 26)

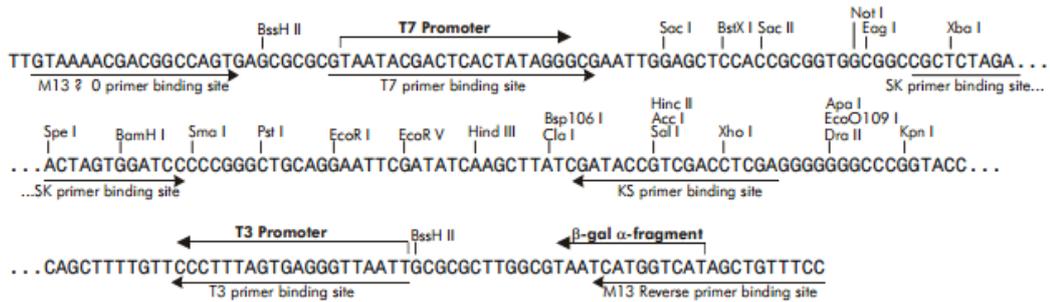


Figure 10: Circular map and polylinker sequence of the pBluescript SK (+/-) phagemid. Circular map and polylinker sequence of the pBluescript SK (+/-) phagemid. The polylinker of the pBluescript phagemid has 21 unique cloning sites flanked by T3 and T7 promoters and a choice of 6 different primer sites for DNA sequencing. Source: pBluescript II phagemid vectors instruction manual-Agilent Technologies, 2008.

3.3.6. Small scale isolation of plasmid DNA

Bacterial colonies were inoculated into 2 ml LB broth containing 50 µg/mL kanamycin and incubated overnight at 37 °C with shaking at 200 rpm. The culture was decanted into a 2 mL microcentrifuge tube which was centrifuged at 16 000 x g for two minutes. The pellet was re-suspended in 200 µL of 50 mM Tris-Cl pH 8.0; 10 mM EDTA; 0.1 g/L RNase A by vortexing.

This was followed by an addition of 200 μ L of 200 mM NaOH; 1% (w/v) SDS and the tube mixed gently by inversion followed by the addition of 200 μ L of 3 M KAc, pH 5.5. The tube was mixed gently by inversion and placed on ice for five minutes prior to centrifugation at 16000 x g for 10 minutes. The supernatant was transferred to a tube containing 0.7 volumes of isopropanol and mixed by inversion. This was left at room temperature for 5 minutes and then centrifuged at 16000 x g for 10 minutes. After removal of the supernatant, the DNA pellet was washed with 70% (v/v) ethanol. Following removal of the supernatant the pellet was left to air-dry on the bench after which it was re-suspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The concentration of the plasmid in solution was determined by spectrophotometry.

3.3.7. Large scale isolation of library plasmid DNA

The library was transformed into *E. coli* and were plated onto solid LB media containing appropriate antibiotics and incubated overnight at 37 °C. Colonies were re-suspended by washing with LB broth and then pelleted by centrifugation at 4000 x g for 20 minutes at room temperature. The cells were re-suspended in 8 ml of 50 mM glucose; 25 mM TRIS-Cl, pH 8, 0; 10 mM EDTA and incubated on ice for 5 minutes. Following incubation, 16 ml of 0.2 M NaOH; 1% (w/v) SDS was added and incubated on ice for 5 minutes. After that 24 mL of 3M KAc pH 4.8, was added and the samples incubated on ice for a further 5 minutes. The reaction mixture was filtered through four layers of Miracloth (Fisher Scientific) and diluted with 0.7 volumes of isopropanol.

Precipitation of plasmid DNA took place for 30 minutes at room temperature, following which the DNA was isolated by centrifugation at 4000 x g for 30 minutes at room temperature. The DNA was re-suspended in 1 mL of dH₂O and mixed with 1 ml of 5 M LiCl. RNA was precipitated at room temperature for 10 minutes and the tubes centrifuged at room temperature for 10 minutes and 14 000 x g. One volume of chloroform: isoamylalcohol: phenol (24:1:25) was added and the sample vortexed after which it was centrifuged for three minutes at 14 000 x g. The upper phase was removed and 0.1 volumes of 8 M LiCl and 2.5 volume of ethanol were added before the sample was mixed by inversion. Samples were incubated at room temperature for 10 minutes before being centrifuged for 10 minutes at 14 000 x g. The supernatant was removed and each pellet washed with 70% ethanol. After removal of ethanol, DNA was air dried for 5 minutes and re-suspended in TE buffer (10 mM Tris-Cl, pH 8.0; 1 mM EDTA).

3.3.8. Restriction Endonuclease digestion analysis

For the analysis of cDNA inserts in each library, 25 clones were randomly selected and cultured in 2 mL of LB medium containing 50 µL/mL of ampicillin (*E .coli* expression libraries and Gateway primary library) or 25 µL/mL of kanamycin (Gateway Entry libraries) individually. The harbouring plasmids were isolated and used for digestion analysis. For each enzyme digestion reaction, 1.5 µg of plasmid was incubated with 10 U of the indicated enzymes (*PvuII* and *MluI*), 2 µL 10X corresponding enzyme buffer (Fermentas) and nuclease-free water to a total volume of 20 µL according to the manufacturer's instructions.

The final reaction mix was incubated at 37 °C in a heat block for 30-60 minutes to digest the plasmid DNA. Restriction digestion reaction was then analysed on a 1% agarose gel at 80 volts for 2 hours to determine insert sizes.

3.3.9. Bacterial culture and maintenance

Bacterial colonies were cultivated on solid LB media (10 g/L Tryptone; 5 g/L Yeast Extract powder; 10 g/L NaCl; 15 g/L bacto agar) and subsequently inoculated into liquid LB broth (10 g/L Tryptone; 5 g/L Yeast Extract powder; 10 g/L NaCl). *E. coli* strain *G6MD2* (CGSC# 5080) cells were maintained on solid LB media supplemented with Diaminopimelate (DAPA) to a final concentration of 0.01% (w/v).

3.3.10. Preparation of chemically competent *E. coli* cells

E. coli was inoculated in 2 mL liquid LB broth and incubated at 37°C overnight. 1 mL of this was inoculated into 125 mL of liquid SOB media (5 g/L Yeast Extract Powder; 20 g/L Tryptone; 10 mM NaCl; 2,5mM KCl; 20 mM MgSO₄; pH 7.5) and grown to an OD₆₀₀ of 0.3. The cells were placed on ice and centrifuged for 10 minutes at 1500 x g and 4°C. The supernatant was removed and the cells gently re-suspended in 40 ml of ice-cold CCMB80 buffer (10 mM KAc, pH 7.0; 80 mM CaCl₂.2H₂O; 20 mM MnCl₂.4H₂O; 10 mM MgCl₂.6H₂O; 10% (v/v) glycerol; pH 6.4) and incubated on ice for 20 minutes.

The re-suspended cells were centrifuged for 10 minutes at 1500 x g and 4°C and re-suspended in 10 mL of CCMB80 buffer (10 mM KAc, pH 7.0; 80 mM CaCl₂.2H₂O; 20 mM MnCl₂.4H₂O; 10 mM MgCl₂.6H₂O; 10% (v/v) glycerol; pH 6.4). The re-suspended cells were diluted with CCMB80 buffer until a mixture of 50 µL of the re-suspended cells and 200 µL LB broth yielded an OD₆₀₀ of between 1.0-1.5. Aliquots of 50 µL of re-suspended cells were frozen in liquid nitrogen and stored at -80°C.

3.3.11. Heat shock transformation of chemically competent *E. coli* cells

Chemically competent *E. coli* cells were thawed on ice for 5 minutes. Plasmid DNA or ligation products were added to the cells and incubated on ice for a further 20 minutes. The reaction was then incubated at 42°C for a minute before incubation on ice for 2 minutes. LB media (350 µL) was added to the cells and incubated for one hour at 37°C. The cells were plated onto solid LB media containing either 50 µL/ml of ampicillin or 25 µL/mL of kanamycin.

3.3.12. Preparation of electrocompetent *E. coli G6MD2*:: pACAG cells

The plasmid pACAG (Kossmann et al., 1999), a pACYC-184 derivative containing *E. coli glgC16*, was introduced to chemically competent *E. coli G6MD2* cells by heat shock. A single colony was inoculated into liquid LB containing 0.01% (w/v) DAPA; 35 µg/mL chloramphenicol and grown overnight. Two millilitres of this was added to 250 mL LB and grown to an OD₆₀₀ of 0.6. Cells were chilled on ice and collected by centrifugation at 4°C and 4000 x g for 15 minutes.

The supernatant was discarded and the cells re-suspended in an equal volume of 250 mL cold 10% glycerol. Centrifugation was repeated and after the supernatant was discarded, the pellet was re-suspended in half a volume of cold 10% (v/v) glycerol. A third centrifugation step was performed and the cell pellet was taken up in 2 mL of ice-cold 10% (v/v) glycerol. Aliquots of 50 μ L were frozen in liquid nitrogen and stored at -80°C.

3.3.13. Electroporation of electrocompetent *E. coli* G6MD2::pACAG cells

Electrocompetent cells were thawed on ice and 20 μ L mixed with approximately 1 μ L (100 ng) of expression library plasmid DNA. The cell mixture was transferred to a 0.2 cm pre-chilled electroporation cuvette and electroporated (Capacitance, 25 μ F; Resistance, 200 Ω ; V, 2.5 kV). Bacterial cells were re-suspended in SOC media (5 g/L Yeast Extract Powder; 20 g/L Tryptone; 10 mM NaCl; 2,5mM KCl; 20 mM MgSO₄; 20 mM glucose; pH 7.5) containing 0.01% (w/v) DAPA immediately following electroporation, and incubated at 37 °C with shaking at 225 rpm for 1 hour. Cells were diluted in LB media and plated onto solid LB media (35 μ g/mL chloramphenicol; 50 μ g/mL ampicillin; 0.01% w/v DAPA; 20 mM glucose) and grown overnight at 37°C.

3.3.14. Screening of expression library and sequencing of inserts involved in starch synthesis

E. coli G6MD2::pACAG cells transformed with the expression library and grown overnight on solid LB media (35 µg/mL chloramphenicol; 50 µg/mL ampicillin; 0.01% w/v DAPA; 20 mM glucose) were stained with vapours from iodine crystals. Blue colonies were selected and the plasmids extracted on small scale. The isolated plasmids were diluted 10^{-1} then transformed into *E. coli* G6MD2::pACAG cells by electroporation and stained with iodine vapours to confirm the presence of blue colonies. This procedure was repeated until re-transformation yielded only blue colonies. Plasmids responsible for producing blue colonies were transformed into chemically competent *E. coli* DH5α cells. Plasmid DNA was sequenced by the Central Analytical Facility of the University of Stellenbosch to identify the form of starch synthase present in marama specific tuber expression library.

3.4. Isolation and sequence analysis of genomic clones encoding AGPase large and small subunits and SBEs genes from *T. esculentum*

3.4.1. Isolation of genomic DNA

Total genomic DNA was isolated from tuber samples using DNA-easy mini kit (Qiagen). The integrity of isolated DNA was qualitatively checked by separation on 0.8% (w/v) agarose gel which was visualized under ultraviolet light after staining with ethidium bromide.

The isolated DNA was also quantified spectrophotometrically by measuring the A_{260}/A_{280} ratio and the absorbance at 260 nm with a Nanodrop ND-1000. Samples were stored at -20°C until use.

3.4.2. Oligonucleotide PCR primers design

Sets of degenerate PCR primers for amplification of AGPase and SBE genes were designed using primer 3.0 (<http://frodo.wi.mit.edu/primer3/>) and CODEHOP (Staheli, Boyce, Kovarik and Rose, 2011) web based softwares. The primers were selected from highly conserved regions using a simultaneous alignment tool through computer analysis with Clustal W tool (Thompson, Higgins and Gibson, 1994). The primers for AGPase small subunit were designed from the alignment of the predicted cDNA sequences of *Cicer arietinum* (accession number: AF356004.1), *Phaseolus vulgaris* (accession number: AB103473.1) and *Pisium sativum* (accession number: X96764.1), which identified several conserved domains. Degenerate primers for amplification of marama AGPase large subunit were designed from conserved domains of predicted cDNA sequences of *C. arietinum* (accession number: AF356003.1), *G. max* (accession number: NM001254462.2), *Lens culinaris* (accession number: GQ861440.1), *P. vulgaris* (accession number: AB103472.1) and *P. sativum* (accession number: X96766.1).

Degenerate primers for amplification of marama SBEI genes were designed from conserved domains of predicted cDNA sequences of *Vigna radiata* (accession number: AY667492.1), *Medicago truncatula* (accession number: XM_003625723.1), *P. sativum* (accession number: X80009.1) and *P. vulgaris* (accession number: AB029549.1), while those of SBEII were designed from conserved domains of *V. radiata* (accession number: AY662199.1), *M. truncatula* (accession number: XM_003625722.1), *P. sativum* (accession number: X80010.1) and *P. vulgaris* (accession number: AB029548.1). The cDNA sequences from which degenerate primers were designed were obtained from the NCBI Gen Bank database. All oligonucleotide primers were synthesized by Life Technologies (Pleasanton CA, USA). Primers sequences and annealing PCR conditions are listed in Table 8.

Table 8: Degenerate primer sequences and PCR annealing temperatures for marama AGPase and SBEs genes used in the study

| Name | Orientation | Primer sequence (5' – 3') | Annealing temperature (°C) |
|--------|-------------|---------------------------|----------------------------|
| ADPG-S | Sense | CTTGGCATTATACTTGGAGGTG | 57 |
| ADPG-S | Antisense | GTTTCCCTTGCAGCTTCTTCTTG | 59 |
| ADPG-L | Sense | ATGAGTAACTGCATAAATAGTGGC | 57 |
| ADPG-L | Antisense | ATNGTTCCAATTTCTTCCCA | 57 |
| SBEI | Sense | TATGAAGGTGGTCTTGATGGTC | 59 |
| SBEI | Antisense | CAGAGCTACTCATTCCCACATG | 60 |
| SBEII | Sense | TTATGCTGAGAGTCATGAC | 49 |
| SBEII | Antisense | TGAAAATTGAAGACAAAGAC | 50 |

3.4.3. PCR amplification of marama AGPase and SBEs genes

The Polymerase Chain Reactions were carried out to amplify coding genes of AGPase and SBEs from isolated genomic DNA of marama using designed degenerate oligonucleotide primers. PCR reactions were performed in a final volume of 50 μ L containing 1 x PCR reaction buffer, $MgCl_2$ concentration of 2.5 mM, 0.2 μ M of each primer, 200 μ M of each dNTP, 2 U *Taq* polymerase (Life Technologies, Burlington, Ontario, Canada) and 100 ng genomic DNA. PCR reactions were performed on a G-storm thermal cycler (VWR International, Mississauga, Ontario, Canada), which was programmed for touchdown and standard PCR (Sambrook, Fritsch and Maniatis, 1989). PCR conditions were: initial denaturation step at 95 °C for 3 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, an annealing step at 54 – 47 °C and an extension step at 72 °C for 1 minute 30 sec. The initial annealing temperature of 54 °C was reduced by 2 °C after every 5 cycles to reach a temperature of 47 °C for the final 5 cycles. PCR reactions were completed with a final extension step at 72 °C for 5 minutes to facilitate cloning in the pCR[®]2.1 vector (Life Technologies, Burlington, Ontario, Canada) by completing the *Taq* polymerase catalysed addition of single dATPs to the 3' ends of PCR products.

3.4.4. Gel electrophoresis of DNA

Sizes of PCR products were determined by electrophoresis using 1.2% (w/v) agarose gel containing ethidium bromide staining (Sambrook and Russell, 2001). Typically, 9 μ L of the PCR amplification reactions was mixed with 1 μ L of 10 x loading dye.

All gels were prepared and electrophoresed in 1 x TAE buffer at 75 -100 V for 40 - 90 minutes. Appropriate PCR DNA markers were used for estimating the size of PCR products. Gels were visualized using ultraviolet light and photographed using Gel Doc XRS gel imaging system (Biorad, USA).

3.4.5. Purification and extraction of PCR products

PCR products of expected sizes were purified using two methods. Single band PCR products were purified using QIAquick PCR purification kit (Qiagen), while multiple PCR products were extracted from 0.8% (w/v) agarose gels using QIAquick gel extraction kit (Qiagen). Procedures for both kits were followed according to the manufacturer's instructions.

3.4.5.1. PCR Products purification using QIAquick[®] PCR purification kit

PCR reactions were purified based on the supplier's protocol (Qiagen). Briefly, 5 volumes of buffer PB (provided with the kit) were added to 1 volume of the PCR reaction (e.g. 50 μ L PCR reaction adds 250 μ L PB buffer) and mixed well by vortex. The DNA was bound by applying the sample to the QIAquick column (provided with the kit) placed in a 2 mL tube. The column was centrifuged at 13 000 x g for 60 seconds until the sample had passed through the column. The flow through was discarded and the column was placed back in the same tube. The column was washed by adding 750 μ L of buffer PE and further centrifuged at 13 000 rpm for 60 seconds.

Residual wash buffer was removed from the column by centrifuging for 60 seconds after discarding the flow through. The column was then placed in a clean 1.5 mL Eppendorf tube and DNA was eluted by adding 50 μ L buffer EB (10 mM Tris Cl, pH 8.5) to the center of the column and further centrifuged for 1 min. The quality and concentration of the purified DNA was analyzed on a gel and subsequently used for cloning AGPase and SBEs genes.

3.4.5.2. PCR Products purification using QIAquick[®] Gel Extraction kit

The desired DNA fragment was excised from the agarose gel using a sterile sharp blade and placed into a pre-weighed 1.5 mL Eppendorf tube. The Eppendorf tube weight was determined before and after the placement of a gel slice containing DNA fragment to determine the weight of the gel slice. To a 1.5 mL Eppendorf tube containing the gel slice, 3 volumes of QG buffer (provided with the kit) was added to 1 volume of a gel (100 mg gel~100 μ L). Gel slices were incubated at 50°C for 10 minutes and vortexed every 3 minutes until they had dissolved. To completely dissolve gels, 1 volume of isopropanol was added to samples and mixed well. The DNA was bound by applying the sample to the QIAquick column (provided with the kit) and centrifuged at 13 000 rpm for 60 seconds until the sample has passed through the column. The flow through was discarded and the column was placed back in the same tube to repeat the process for sample volumes more than 800 μ L. Washing of the column involved adding 750 μ L of PE buffer and centrifuging at 13 000 rpm for 60 seconds. Residual wash buffer was removed from the column by further centrifuging for 60 seconds after discarding the flow through.

The column was placed in a clean 1.5 mL Eppendorf tube and DNA was eluted by adding 50 μ L buffer EB (10 mM Tris Cl, pH 8.5) to the center of the column and centrifuge for 1 minute. The quality and concentration of the purified DNA was analyzed on a gel and subsequently used for cloning AGPase and SBEs genes.

3.4.6. Cloning of purified PCR products into the vector

Purified PCR products were cloned into the pCR[®]2.1 vector (Life Technologies, Burlington, Ontario, Canada) for sequencing purposes. The pCR[®]2.1 cloning vectors were used to clone AGPase and SBEs genes from marama bean. Fragments of AGPase and SBEs genes were cloned into *Eco* RI site in this work (see Figure 11).

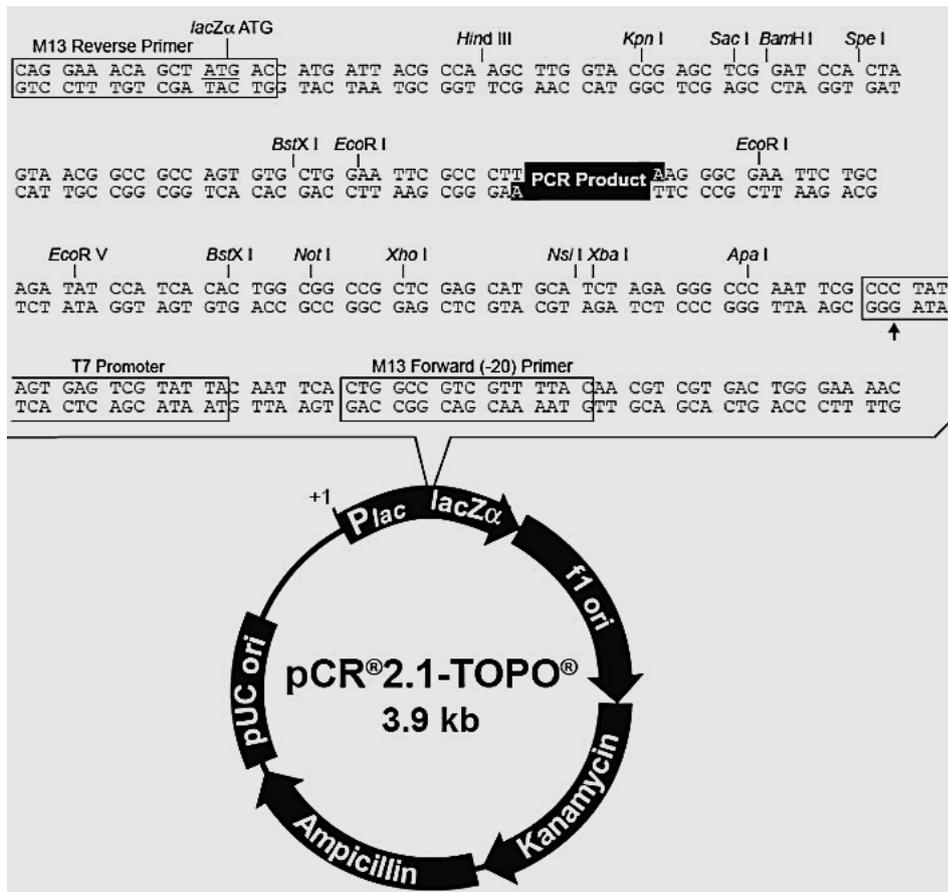


Figure 11: Vector map of pCR 2.1 used for cloning of genomic DNA encoding AGPase large and small subunits and SBEs genes from marama. Source: TOPO[®] TA Cloning[®] Kit manual-Invitrogen, 2012.

3.4.6.1. Ligation of PCR Products in the pCR[®]2.1 cloning vector

Fresh PCR products of AGPase and SBEs genes (less than 1 day old) were used for ligation into the vector. A 10 μ L ligation reaction mixture containing 2 μ L 5X T4 DNA Ligase Reaction Buffer, 2 μ L pCR[®]2.1 vector (25 ng/ μ L), 1 μ L Express Link[™] T4 DNA Ligase (5 U/ μ L), 3 μ L fresh PCR product (10–100 ng/ μ L) and 2 μ L deionised water was prepared. The ligation was incubated at room temperature for 1 hour and placed on ice before transformation.

3.4.6.2. Transformation of ligation reaction into *E.coli* competent cells

E.coli competent cells provided with the kit (One Shot[®] Competent Cells) were used for the transformation of the ligation reaction following manufacturer's procedures. Eppendorf tubes containing the ligation reactions were centrifuged briefly and placed on ice. Vials of 50 μ L vial of frozen One Shot[®] Competent Cells stored at -80 °C were thawed on ice and used for transformation. Ligation reactions (2 μ L each) were pipetted directly into the vial of competent cells and mixed by stirring gently with the pipette tip. The vials were incubated on ice for 30 minutes. After incubation, the cells were heat shocked for 30 seconds at 42°C without shaking, then allowed to recover for 5 minutes by placing them immediately on ice. An amount of 250 μ L of S.O.C. medium (at room temperature) was added to each vial. The vials were incubated horizontally in a shaking incubator for 1 hour at 37°C and 225 rpm. After the 1 hour incubation of cells at 37°C, 20–100 μ L from each transformation was plated on LB agar plates. These plates contained 20 μ L each of 5-bromo-4-chloro-3-indolyl-P-D-galactoside (X-Gal) and 100 μ g/mL ampicillin. Plates were incubated for 16 hours overnight at 37°C.

3.4.6.3. Identification and selection of positive clones

The pCR[®]2.1 TOPO vector contains the *LacZ* gene which provides α -complementation of the β -galactosidase subunit by the plasmid, with that encoded by the appropriate host cell.

This allows for recombinant bacteriophage to be distinguished from non-recombinant by colour selection, whereas colourless (white) colonies indicate recombinant phage while blue colonies are indicative of non-recombinant phage (Close, Christmann, and Rodriguez, 1983), on plates containing X-gal. White colonies were identified as positive transformants and were selected for plasmid isolation and restriction analysis.

3.4.7. Isolation of plasmid DNA

At least 10 white single recombinant *E. coli* colonies were selected for plasmid DNA preparation and isolation. Single colonies were inoculated into 3 mL LB broth containing 100 µg/mL of ampicillin and grown at 37 °C overnight in a shaking incubator with shaking set at 225 rpm. Single recombinant *E. coli* cells grown overnight were harvested and centrifuged at 8000 rpm for 3 minutes to pellet the cells. Pelleted bacterial cells were suspended in 250 µL of resuspension buffer P1 to lyse the cells. Cells were transferred to a microcentrifuge tube, followed by the addition of 250 µL of lysis buffer P2 and mixed immediately by inverting the tube 6 times. Subsequently, the cells were centrifuged at 13 000 rpm for 10 minutes and supernatants were collected. Supernatants were applied to the QIAprep spin column by pipetting and centrifuged for 60 seconds and the flow through was discarded. The QIAprep spin column was then washed sequentially with 500 µL of PB buffer, 750 µL of PE buffer and centrifuged for 60 seconds, with flow through being discarded in-between washing steps.

The column was further centrifuged at full speed (13 000 rpm) for an additional 1 minute to remove residual washing buffer and placed in a clean 1.5 mL centrifuge tube. Plasmid DNA was then eluted by adding 50 μ L of EB buffer (10 mM Tris-Cl, PH 8.5) to the center of each column left to stand for a minute and centrifuged for 1 minute.

3.4.9. Determination of plasmid DNA concentration and insert size

The concentration of the plasmid DNA isolated was determined by agarose electrophoresis method, by running 5 μ L of plasmid DNA samples on agarose gel stained with ethidium bromide and visualized under UV doc system. The insert sizes were determined by *Eco*RI digestion and tested by the method of colony PCR. For restriction analysis with *Eco*RI, a 10 μ L reaction mix contained 0.2 μ L of *Eco*RI enzyme, 1 μ L of 10 x reaction buffer, 3 μ L of plasmid DNA and 5.8 μ L of deionised water was prepared. The reaction mixture was incubated in a water bath with a temperature set at 37 °C for 1 hour. Insert sizes were also tested by the method of colony PCR, using 2 μ L of colonies grown overnight in 3 mL LB broth containing 100 μ g/ml of ampicillin as a template. Degenerate oligonucleotide primers designed for AGPase and SBEs genes in this study and M13 oligonucleotide primers supplied with the cloning kit were used for testing insert sizes by colony PCR. A touchdown PCR was performed for the degenerate oligonucleotide primers as described above and a standard PCR for M13 universal oligonucleotide primers.

A standard PCR reaction was performed in a total volume of 25 μ L, containing 1 x PCR buffer, 2.5 mM MgCl₂, and 2.5 mM dNTP, 1.25 units of *Taq* DNA polymerase and 1 μ M of each M13 oligonucleotide primer. Amplification was carried out with initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, an annealing at 56°C for 30 seconds, an extension step at 72°C for 1 minute and 30 seconds. The final extension step was run at 72°C for 5 minutes. Insert sizes were analysed by colony PCR and determined by running PCR products on a 0.8% (w/v) agarose gel for at 75 -100 V for 35 -60 minutes. Gels were visualized using ultraviolet light and photographed using a ChemiDoc XRS system (Bio-Rad, USA). Appropriate PCR DNA markers were used for estimating the size of inserts prior to sequencing.

3.4.10. Sequencing of positive DNA clones

Positive isolated DNA clones were sent for sequencing using M13 primers which are based on the sequence of the pCR 2.1 TOPO vector provided in the kit. The cloned DNA was sequenced from the M13 forward and reverse primers by the dideoxynucleotide chain termination method (Sanger, Nicklen and Chase, 1977) which was carried out at Genome Quebec (Montreal, QC). The obtained DNA sequences were subjected to *in-silico* analysis for genes identification.

3.4.11. Analysis of the isolated AGPase and SBEs gene sequences

Internet-based bioinformatic resources were used to analyse the obtained DNA sequences. Vector sequences in obtained sequences were recognised and removed by VecScreen program of NCBI (<http://www.ncbi.nlm.nih.gov/VecScreen.html>). Homology searches for nucleotide sequences from genomic clone and their deduced amino acid sequences in the GenBank were conducted using the NCBI Blastx and Blastp search programs program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Isolated AGPase and SBEs gene sequences were predicted using FGENESH program of Softberry web server (Solovyev, Kosarev, Seledsov and Vorobyev, 2006). This tool was also used for DNA sequences translation to predicted protein sequences. Deduced amino acid sequence with various degrees of identity of AGPase and SBEs genes from various plants were used for phylogenetic analysis. These sequences were retrieved from the GenBank and deduced from the cDNA and protein sequences.

Multiple sequence alignment for deduced amino acid sequences were generated by Clustal W algorithm of sequences analysis software CLUSTAL W (Thompson et al., 1994) available at www.genome.ad.jp and adjusted manually. The resulting data was analysed using equal weighted neighbour joining (NJ). NJ trees were constructed using the MEGA 5.2 software (Tamura et al., 2011) and bootstrap values were calculated from 1000 replicate analyses.

3.5. Detection of serine protease inhibitor activity and isolation of serine proteinase coding sequence from *T. esculentum* seeds

3.5.1. Protein determination

De-shelled marama bean samples weighing approximately 3 g were grounded into a fine powder in liquid nitrogen using a mortar and pestle. Grounded material was collected in Eppendorf tubes and 500 μ L of the extraction buffer (50 mM Tris-HCl, pH 7.8) was added. The tubes were centrifuged for 10 minutes at 4°C at a maximum speed of 10 000 x g. After centrifugation, the supernatant (protein) was removed and stored at -20°C. Protein concentration was determined using the Bradford assay (Bradford, 1976). Protein standard calibration was prepared using BSA (1 mg/mL) as a standard. For the assay in a plate reader, 5 μ L of sample or standard and 250 μ L of Bradford reagent were added to wells in triplicate. Samples were incubated at room temperature for 5 minutes and the absorbance was measured at 595 nm. Final concentration of protein was expressed in mg/mL

3.5.2. Serine protease inhibitor activity determination with gelatine SDS PAGE

Protease inhibitor profiles were established by screening protein extracts from developing and mature beans for inhibitory activity against a commercially available serine protease, trypsin. Serine protease inhibitor activity in mature and green marama seeds was detected by using a reverse zymogram (Michaud, 1998). For that, a gelatine-containing SDS gel was placed into proteolytic buffer containing 2 mg/100 mL of the commercially available serine proteases (trypsin, chymotrypsin and elastase) for hydrolysing the gelatine.

Seed protein extracts were mixed with 5X sample buffer and loaded onto a 12% SDS-PAGE containing 1% gelatine for protein separation. Electrophoresis was run on ice at 100 V for 3 hours. The gel was incubated overnight in a proteolytic buffer containing trypsin and then stained with 0.5 % Coomassie Blue. Regions where serine protease inhibitors have reacted with trypsin remain blue on a clear background after gel de-staining. For comparison, mature soybean and cowpea seeds were screened for protease inhibitor activity.

3.5.3. Fluorometric measurement of serine proteinase inhibitor activity

Serine protease activities were investigated using substrate *N*_α-Benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride (Bz-Arg-MCA) (specific for trypsin) (Michaud, 1998). This is a highly sensitive fluorometric substrate. When hydrolyzed by its specific proteases, bound α -amino 4-methylcoumarin (MCA) is released, which is highly fluorescent and MCA released is determined using fluorescence spectrophotometry. Hydrolysis of the substrate was used to assay crude marama bean protein extracts derived from marama bean seeds and other legume seeds. Stock solutions were prepared at 100 μ M in 1% DMSO. The hydrolysis of Bz-Arg-AMC at the final concentration of 100 mM Tris-HCl pH 7.8 was measured at 25 °C by the increase of emitted fluorescence at 450 nm after excitation at 360 nm. The total reaction and sample volume used was 100 μ L. The control reaction was made up of 10 μ L of trypsin (0.2 μ g/ μ L), 8 μ L of the substrate (100 μ M) dissolved in DMSO and 82 μ L of buffer Tris-HCl, pH 7.8.

The experimental reaction contained 10 μL protein extracts (0.2 $\mu\text{g}/\mu\text{L}$), 8 μL of the substrate and 72 μL of buffer Tris-HCl, pH 7.8. Fluorometric assay was performed with a spectro-fluorometer (BMG FluoStar Galaxy) for 10 minutes, with readings every 20 seconds. Reaction rates represented by the slope of the curve were recorded as Fluorescence Units (FU) per unit time and all reactions were performed in triplicate.

3.5.3. Isolation of serine proteinase coding sequences

3.5.3.1. RNA isolation

The plant material was frozen and ground using liquid nitrogen in a mortar with a pestle. Total RNA was isolated from 50 mg of the powder using the RNeasy[®] Plant Mini Kit (Qiagen) following the recommended protocol of the supplier (Qiagen, Germany). The quality and concentration of the RNA was assessed on a 1% agarose gel and a spectrophotometer (Nanodrop[®], ND 1000), respectively, thereafter the RNA was stored at -80°C .

3.5.3.2. Synthesis of cDNA from total RNA

The first-strand cDNA was synthesized from 0.2 μg of the total RNA using the Improm-II[™] Reverse Transcription system and by applying the recommended protocol of the supplier (Promega, UK). Random hexamers were annealed to the RNA at 70°C for 5 minutes followed by chilling on ice for 5 minutes.

Reverse transcription was performed in a 20 μ L reaction mixture containing 7.5 mM $MgCl_2$, 0.215 mM dNTPs, 2.5 units of reverse transcriptase and 10 units of RNase inhibitor (RNasin). A PCR reaction was run for 5 minutes at 25°C, 1 h at 42°C, 15 minutes at 70 °C and the reaction was cooled at 4°C. The quality of the cDNA was checked by using 1 μ L of cDNA in a PCR reaction with *G. max* 40s primers. PCR amplification was carried out for 5 min at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 1 minute at 72°C and a final extension of DNA strands for 10 minutes at 72°C.

3.5.3.2. PCR reactions and purification

All PCR products were fractioned on either on a 1% agarose gel using 1 X TAE buffer. To enable viewing the amplification products, ethidium bromide was incorporated into the gel at 0.1 μ L/mL prior to casting. The stained gels were visualized under U.V.-light to identify amplification products and to isolate separated PCR products from the gel. Gel pictures were acquired using a photo documentation system.

3.5.3.3. Primer design

A pair of primers was designed using the CLC Main Workbench software program version 6.71, based on the nucleotide sequence within the conserved region of serine proteinase inhibitors of common legumes.

The amino acid sequence of the conserved region was obtained from a multiple alignment of legume serine proteinase inhibitors, amino acid sequences of *P. vulgaris* (accession number CAD32697.1), *Vigna unguiculata* (ABD85194.1) and *Lablab purpureus* (AAK97770.1). Primer pairs designed were: MBBI Forward (5'-GATGAACCTTCTGAATCTTCT-3') and MBBI Reverse (5'-AGAATGACAAGAATTAAGTCT-3'). PCR was conducted with 1 μ L cDNA in a 20 μ L reaction mixture containing 2.0 mM MgCl₂, 0.2 mM dNTPs, 0.3 μ M each of primer and one unit of Bio-*Taq* DNA polymerase enzyme. PCR amplification was carried out for 5 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, 1 minute at 72°C and a final extension of DNA strands for 10 minutes at 72°C.

3.3.5.4. DNA cloning

For cloning, PCR products were run on a 2% agarose gel and bands of interest were incised from the gel, purified and cloned into the pGEM[®]-T Easy plasmid vector, (Figure 12) following recommendation from the supplier (Promega). This involved ligating 30 ng DNA extracted from the gel with 10 ng of plasmid DNA in the presence of 1 μ L NaCl in a 10 μ L reaction mixture for 30 minutes at 25°C. Ligated plasmid DNA was subsequently transformed into competent *E. coli* cells using the heat shock method. This involved placing a 1.5 mL Eppendorf tube containing 50 μ L of *E. coli* cells on ice for 30 minutes after which the pGEM[®]-T Easy vector was added mixed gently and the mixture was maintained on ice for another 30 minutes.

The Eppendorf tube containing the bacteria plasmid mixture was immersed for 45 seconds in a 42°C water bath and immediately cooled on ice for 2 minutes. S.O.C medium (900 µL) was added to the cells and the mixture was incubated for 1 hour at 37°C on a shaker (1500 x g. To select for transformed *E. coli* cells, 50 µL of the cell suspension was spread on a plate containing 25 mL Luria Bertani (LB) medium (g/liter:10 tryptone, 5 yeast extract, 10 NaCl, 15 agar), supplemented with 50 µg/mL kanamycin/ampicillin. The plates were incubated overnight at 37°C. Five bacterial colonies were selected and each inoculated in 5 mL liquid LB in the presence of kanamycin/ampicillin and grown for 17 hours under shaking at 2000 x g. pGEM –T Easy plasmid containing the cloned DNA insert was isolated from *E. coli* using the GenElute plasmid mini prep kit (Sigma-Aldrich). The presence of the insert was confirmed by restriction enzyme analysis digesting 1 µL of the plasmid with 1 Unit of *Eco*R1 in a 20 µL reaction mixture for 1 hour at 37°C.

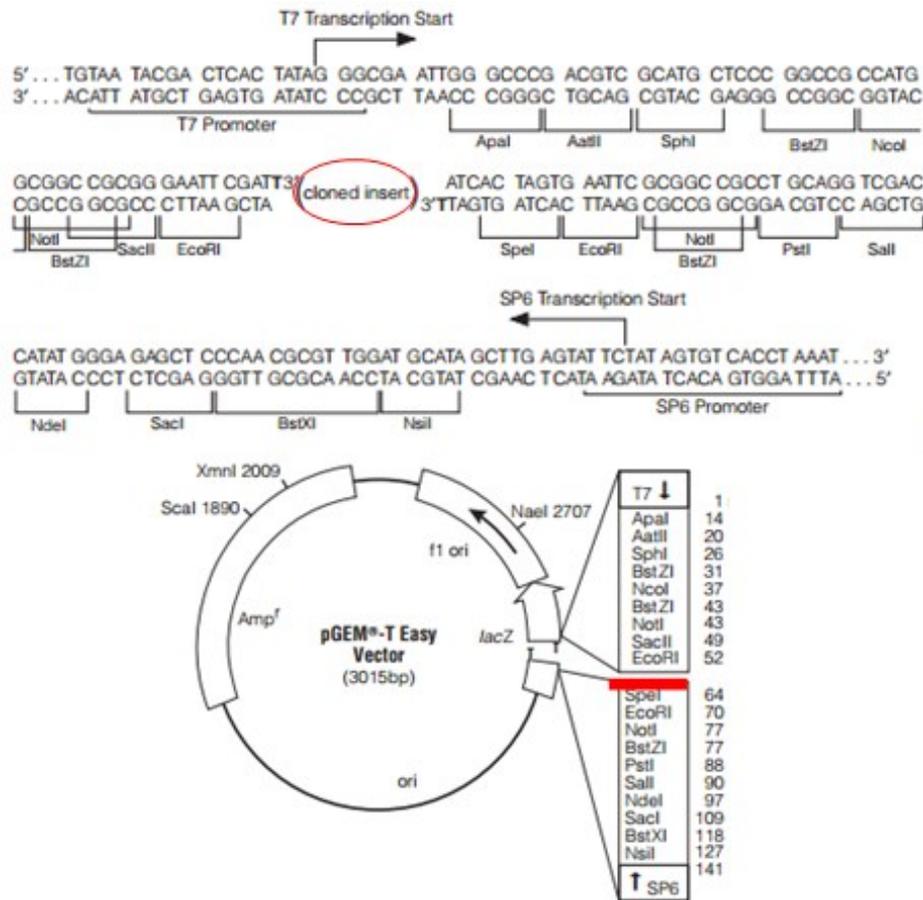


Figure 12: Vector map, promoter and multiple cloning sequences of PGEM®-T Easy vector used in this study showing the cloned inserts positions (red colour). Source: Improm-II reverse transcription system instruction manual-Promega, 2011.

3.3.5.5. DNA Sequencing and *in silico* analysis of serine proteinase coding sequence

Positive bacterial colonies (white colonies) were sequenced in both the reverse and forward orientation with M13 universal primers using a 454 GS FLX sequencing platform. Obtained sequences were used to search the nucleotide database of the National Center for Biotechnology (www.ncbi.nlm.nih.gov) using Blastn program. The sequences that gave positive serine proteinase inhibitor hits were identified and used in the CLC program for translation into amino acid sequences.

3.3.6. Statistical analysis

All estimates of sample variability are given in terms of the SD of the mean. The significance of differences in proteinase activity of mature and green marama seeds was determined by the student's two-tailed *t* test. P value < 0.05 was considered significant.

CHAPTER 4: RESULTS

4.1 Physicochemical properties of *T. esculentum* tuber starch

4.1.1. Starch, G6P and amylose content of the marama tuber

Starch was successfully isolated from marama tuber using a modified method from Edwards et al. (1995). The starch isolated was white in colour to naked eyes with a smooth texture (see Figure 13, plate A and B). The total starch content, determined by enzymatic digestion was 87.38 mg starch/gFW as amyloglucosidase/ α -amylase method. Phosphate at the C-6 position determined as Glc-6-P after acid hydrolysis in marama tuber starch was 0.788 nmol G6P/mg starch (see Table 9). Determination of the amylose/amylopectin content by enzymatic digestion showed that in marama tuber amylopectin is the major constituent comprising about 65% (w/w) with amylose constituting the remaining 35% (w/w) respectively.

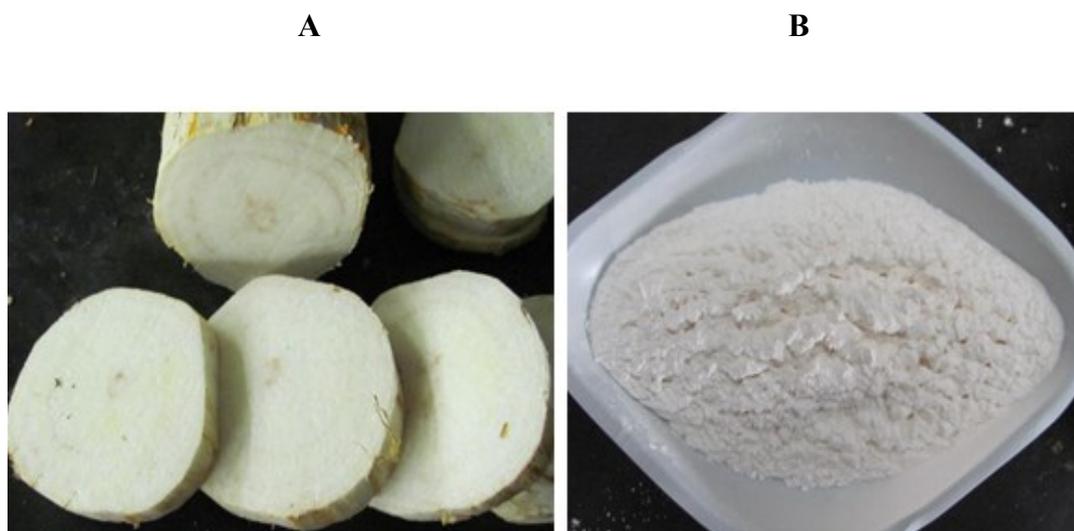


Figure 13: Marama bean tubers (A) were isolated and used for the extraction of Starch (B).

Table 9: Starch composition (starch content, G6P content and amylose content) of the *T.esculentum* tuber

| Starch content (mg starch/gFW) | G6P Content (nmol G6P/mg starch) | Amylose (% w/w) |
|-----------------------------------|-------------------------------------|--------------------|
| 87.38 ±18.24 | 0.788 ± 0.159 | 35.74 ± 5.18 |

The data presented are the mean of samples taken from 4 independent tubers. From each tuber five samples were taken and analysed individually. Final data represents the mean and computed standard deviation.

4.1.2. Pasting properties

The pasting properties of the native marama starch dispersion and commercial potato at the same concentrations are presented in Table 10. To compare the pasting properties of the different starches, gelatinization temperature (GT), peak viscosity (PV), hot paste viscosity (HPV), cold paste viscosity (CPV), break-down (PV-HPV) and setback (CPV-HPV) were calculated from the viscosity-temperature versus time curves obtained (see Figure 14).

Marama tuber starch showed higher pasting temperature (91.15°C) than potato starch. The peak viscosities (PV) observed for marama and potato starches were 2087 and 1075 cP respectively. The hot paste viscosity of two starch samples was comparable with marama starch showing a higher value of 829.3 cP. The breakdown viscosity (BD) of potato was 1025.5 cP, lower than 1257.7 cP of marama starch. The marama starch showed higher value of cold paste viscosity/final viscosity 1276.3 cP than potato starch. The setback viscosity (SB) was much higher for marama starch (447 cP), than that observed for potato starch (69 cP).

Table 10: Pasting properties of starches from *T. esculentum* and commercial potato

| Starch Sample | P_{Temp} (°C) | PV(cP) | HPV(cP) | CPV(cP) | BD(cP) | SB(cP) |
|----------------------|------------------------------|---------------|----------------|----------------|---------------|---------------|
| Marama | 91.15 | 2087 | 829.3 | 1276.3 | 1257.7 | 447 |
| Potato | 72.5 | 1075 | 49.5 | 69 | 1025.5 | 19.5 |

P_{Temp} = Pasting temperature; PV= Peak viscosity; HPV =Hot paste viscosity; CPV= Cool paste viscosity; BD=Breakdown;SB= Setback.

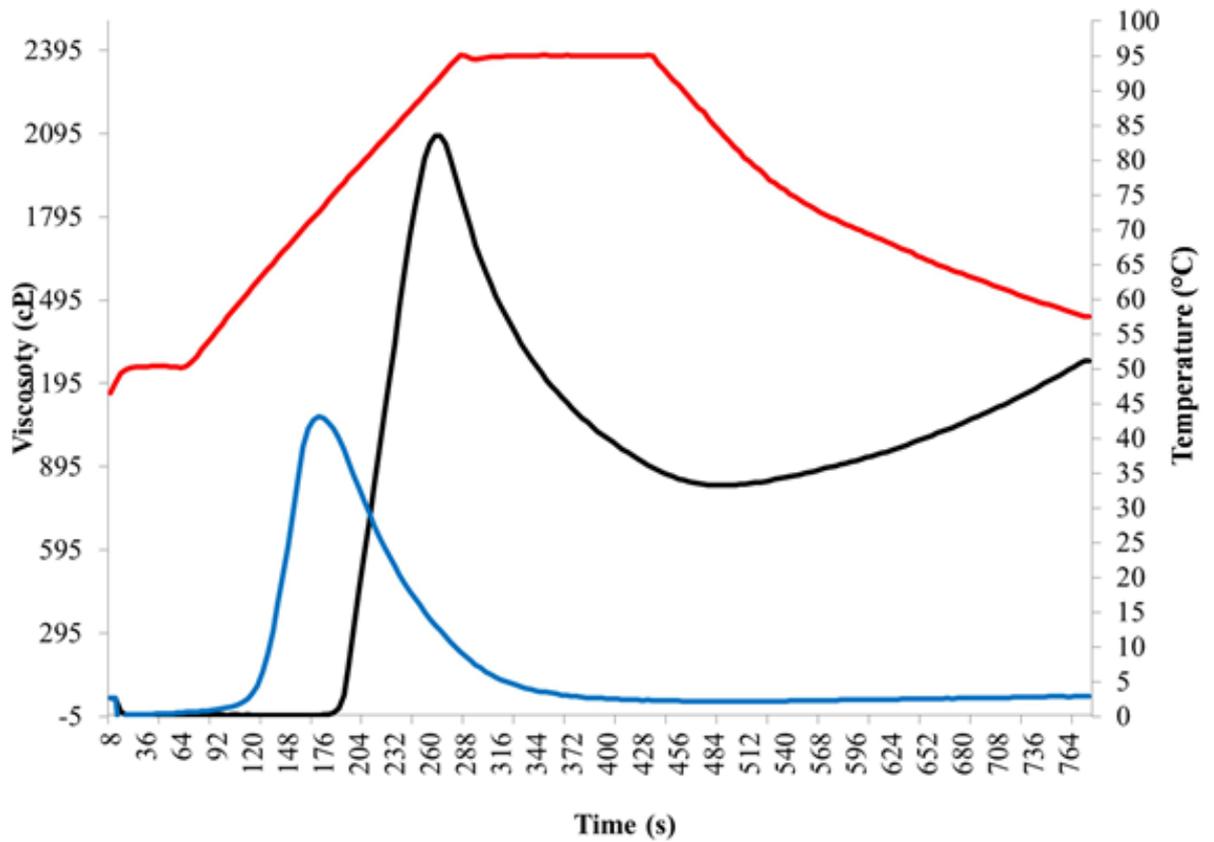


Figure 14: Pasting properties of native marama tuber and potato starch suspensions studied by Rapid visco-analyser. Red = temperature; Black = marama tuber starch; Blue= potato starch.

4.1.3. Granular morphology

The micrographs of the starch granules of marama tuber exhibited a variety of shapes, varying from small round (8 μm) to the larger elliptical and oval (20 μm). The surface of starch granules appeared to be smooth and showed no evidence of fissures or ruptures. Some of the starch granules had deep indentations, (see Figure 15).

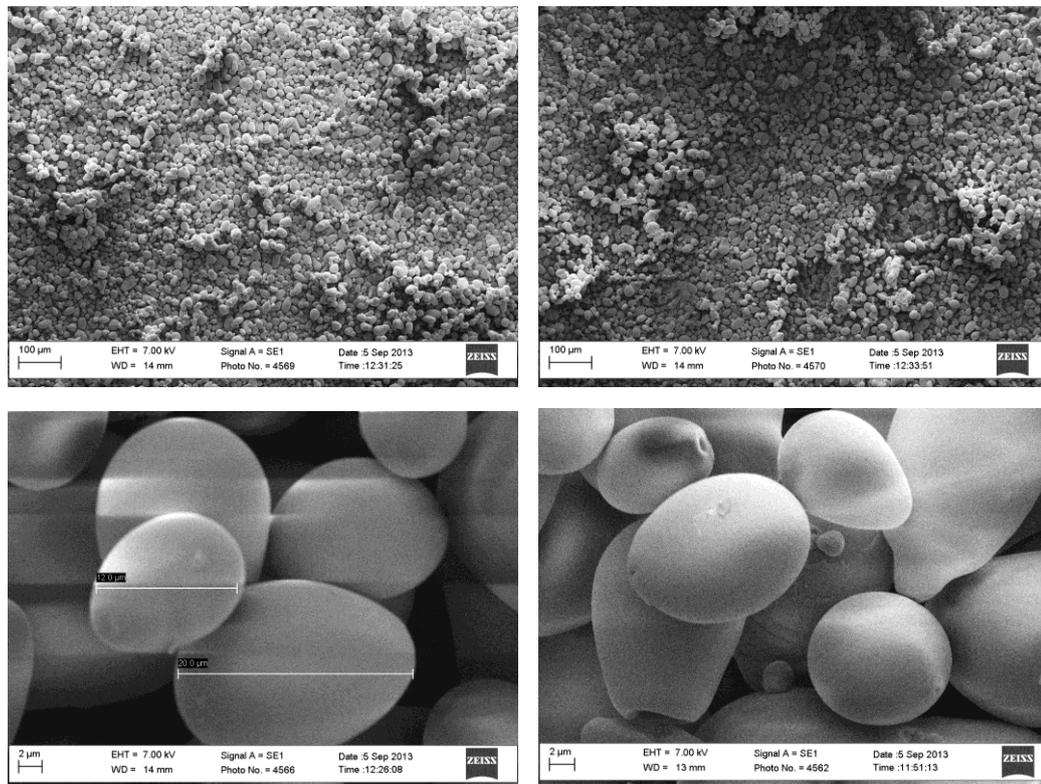


Figure 15: Scanning electron micrographs of native marama tuber starch granules.

4.2. Construction of cDNA library and screening for the identification of tuber-specific starch synthase genes in *T. esculentum*

4.2.1. Characterization of marama bean tuber cDNA library

A tuber-specific cDNA library from *T. esculentum* mRNA was successfully constructed employing the CloneMiner™ II cDNA library kit (Life technologies). The titer of primary library, constructed was approximately 1.5×10^6 cfu. Insert fragment sizes of the library were determined by isolating plasmid DNA from 20 randomly selected colonies and consequent digestion with *Mlu*I.

The average insert size was approximately 1.5 kb and sizes ranged from 0.4 kb to 3.4 kb, with 90% of the sequences exceeding 1 kb (see Table 11). Each of the isolated plasmids contained an insert which was an indication that the recombination rate was close to 100%.

Table 11: *T. esculentum* primary library insert sizes estimated following *Mlu*I digests of plasmid DNA and separation by agarose gel electrophoresis. The fragment sizes were determined by comparison to a known DNA ladder (λ DNA/*Pst*I digest, Sigma Aldrich)

| Clone | Insert Size (kb) | Clone | Insert Size (kb) |
|-------|------------------|-------|------------------|
| 1 | 1.20 | 11 | 1.40 |
| 2 | 0.40 | 12 | 1.20 |
| 3 | 1.10 | 13 | 1.30 |
| 4 | 1.30 | 14 | 1.50 |
| 5 | 1.50 | 15 | 1.70 |
| 6 | 1.20 | 16 | 3.00 |
| 7 | 1.40 | 17 | 3.30 |
| 8 | 3.40 | 18 | 2.00 |
| 9 | 1.55 | 19 | 1.40 |
| 10 | 1.20 | 20 | 0.50 |

4.2.2. Complementary DNA library transfer to an expression vector and qualitative analysis of the expression library

The final expression library was produced from the plasmids contained within 1×10^6 colonies from the primary expression library. Based on the *Pvu*II digestion analysis of 20 randomly selected clones, the average insert size was maintained at approximately 1.7 kb.

Of these fragments, ranging between 0.4 kb and 5.7 kb, 60% appeared to exceed the size of 1 kb (see Table 12). Recombination rate was again shown to be closer to 100%.

Table 12: *T. esculentum* expression library insert sizes were estimated following *PvuII* digests of plasmid DNA and separation by agarose gel electrophoresis. The average insert size calculated by comparison to a known DNA ladder (λ DNA/*PstI* digest, Sigma Aldrich)

| Clone | Insert Size (kb) | Clone | Insert Size (kb) |
|-------|------------------|-------|------------------|
| 1 | 2.40 | 11 | 0.80 |
| 2 | 1.80 | 12 | 0.85 |
| 3 | 1.30 | 13 | 1.80 |
| 4 | 0.70 | 14 | 0.45 |
| 5 | 1.90 | 15 | 0.45 |
| 6 | 0.95 | 16 | 4.75 |
| 7 | 0.40 | 17 | 2.2 |
| 8 | 0.95 | 18 | 1.65 |
| 9 | 3.10 | 19 | 1.50 |
| 10 | 1.85 | 20 | 5.70 |

4.2.3. Screening of the *T. esculentum* tuber expression library for starch synthase

Screening of the expression library was performed in the mutant *E. coli* G6MD2, a strain in which the *glg* operon has been deleted and is unable to manufacture glycogen (Schwarz, 1966). The bacteria were first transformed with the pACAG plasmid (Kossmann et al., 1999), which confers the expression of *glgC16*.

This encodes a mutant, unregulated, form of *E. coli* ADP-glucose pyrophosphorylase which produces large amounts of ADP-glucose (Creuzat Sigal et al., 1972). Bacteria grown on a glucose rich media accumulate linear glucans when an active form of starch synthase is expressed and stain dark blue (see Figure 16) when exposed to iodine (Kossman et al., 1999). Over 250 000 clones from the *T. esculentum* library were screened in *E. coli* G6MD2::pACAG cells and yielded 3 heavy blue stained colonies. These colonies were grown overnight in LB broth, plasmid extracted and sequenced thereafter.

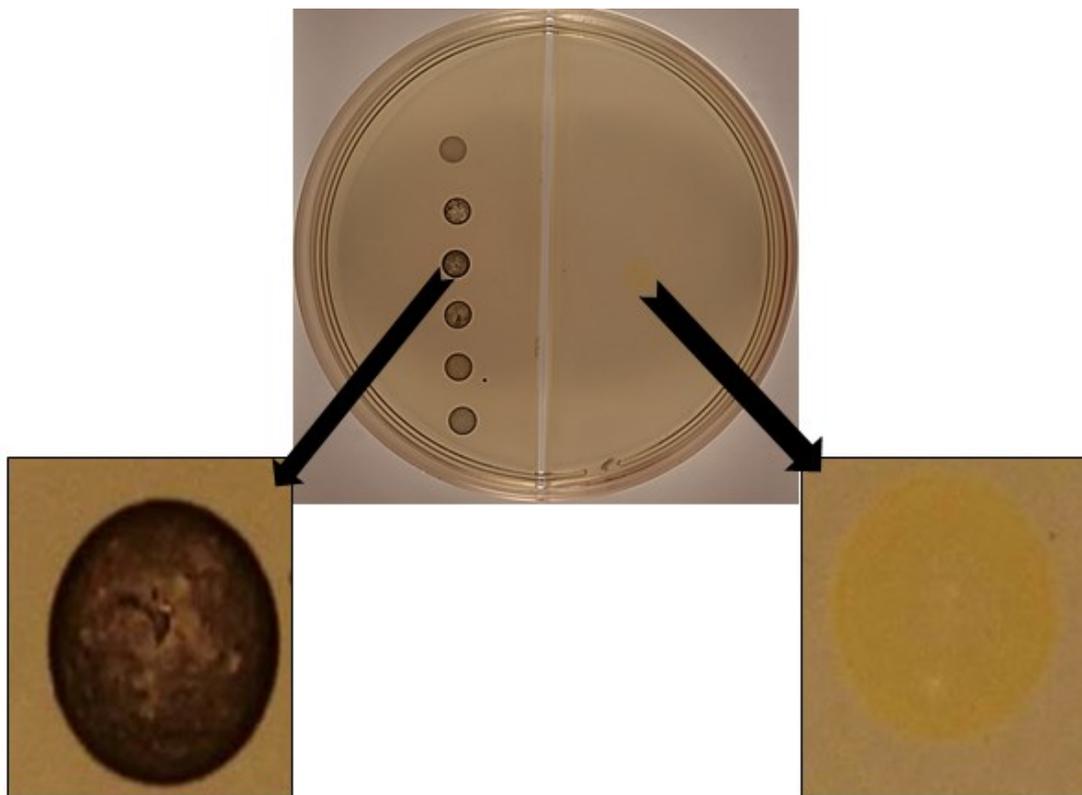


Figure 16: Iodine vapour staining of *E. coli* G6MD2. *E. coli* G6MD2::pACAG cells stain a pale golden yellow upon iodine staining, whereas *E. coli* G6MD2::pACAG expressing *T. esculentum* soluble starch synthase I stain dark blue.

4.2.4. Characterization of marama SSSI gene

Sequencing of marama tuber specific cDNA clones which expressed soluble starch synthase I resulted in one positive nucleotide coding sequence of 684 bp with 228 amino acid residues indicated by one letter code, (see Figure 17) as predicted by FGENESH program of Softberry web server.

17A: A 687 bp cDNA sequence encoding SSSI for marama,

```
5'-  
GAGCATATTGCTTCGCATTACTCCATCAATGACCTCTCCGGAAAGGTTTCAGTGCA  
AGACTGATCTGCAAAAGGAACTGGGCCTTCCAATTCGACCTGATTGTCTCTGAT  
TGGATTTATTGGAAGGCTGGACTACCAGAAAGGTGTTGACATAATCCTGTCAGC  
AATTCCAGAACTTATGCAGAATGATGTCCAAGTTGTAATGCTTGGATCTGGTGA  
GAAACAATATGAAGACTGGATGAGACATACAGAAAATCTTTTTAAAGACAAATT  
TCGTGCTTGGGTTGGATTTAATGTTCGTTTCTCATAGGATAACAGCAGGATGCGA  
CATACTATTGATGCCCTCAAGATTTCGAACCGTGTGGCTTAAACCAATTGTATGCA  
ATGAGATATGGCACCATACCTATTGTTTCATAGCACGGGGGGCCTAAGAGACACA  
GTGAAGGATTTTAATCCATATGCTCAAGAAGGAATAGGTGAAGGTACCGGGTGG  
ACATTTTCTCCTCTAACGAGTGAAAAGTTGCTTGATACTGAAGCTGGCAATCG  
GGACTTATACAGAACATAAGTCATCTTGGGAGGGATTGATGAGGAGAGGTATGG  
GAAGGGACTATTCCTGGGAAAATGCAGCCATTCAATATGAACAAGTTTTACCT  
GGGCCTTTATAGATCCTCCATATGTCAGATGA
```

17B: Predicted 228 amino acid sequence for marama SSSI gene

```
EHIA SHYSINDLSGKVQCKTDLQKELGLPIRPDCPLIGFIGRLDYQKGVDIILSAIPELM  
QNDVQVVMLGSGEKQYEDWMRHTENLFKDKFRAWVGFNVPVSHRITAGCDILLM  
PSRFEP CGLNQLYAMRYGTIPIVHSTGGLRDTVKDFNPYAQEGIGEGTGWTFSP L TSE  
KLLDTL KLAIGTYTEHKSSWEGLMRRGMGRDYSWENAAIQYEQVFTWAFIDPPYV  
R
```

Figure 17: Complementary DNA (17A) and deduced amino acid (17B) sequences of the SSSI gene in marama.

The deduced *T. esculentum* SSSI amino acid sequence used as a query sequence in BlastP showed the top scoring results with *S. tuberosum*, (100%), *M truncatula*, (77%) *C. arietinum* (76%) and *G. max* (76%) (see Table 13).

Table 13: Sequence comparisons of marama SSSI cDNA and protein with those of other plant species

| NCBI/GenBank accession with highest similarity (GenBank accession number) | Protein sequence identity (%) | <i>E</i> -value ^a |
|--|----------------------------------|------------------------------|
| <i>Marama SSSI</i> | - | - |
| <i>S. tuberosum</i> (P935681) | 100 | 6e-149 |
| <i>M. truncatula</i> (XP_003602259.1) | 77 | 3e-113 |
| <i>C. arietinum</i> (XP_004502657.1) | 76 | 3e-110 |
| <i>G. max</i> (XP_003523360.1) | 76 | 2e-111 |
| <i>M. esculenta</i> (ABV25893.1) | 75 | 5e-110 |
| <i>P. vulgaris</i> (BAD18845.1) | 74 | 9e-109 |

^a The expected *E*-value refers to the number of matches by chance alone. The lower the *E*-value the more significant the match is.

4.2.5. Multiple alignment of deduced amino acid sequences for marama SSSI gene

The cDNA clone encoding marama SSSI gene was aligned by ClustalW analysis with other SSSI gene sequences available on NCBI gene bank to identify sequence similarities and differences. Two of the most highly conserved amino acid region present in all other plant species and in glycogen synthase of bacteria were also present in the marama SSSI sequence.

The first conserved region contains a conserved sequence, PSRFEP CGLNQLY and the second contains a conserved sequence STGGLRDTVKDFN (see Figure 18).

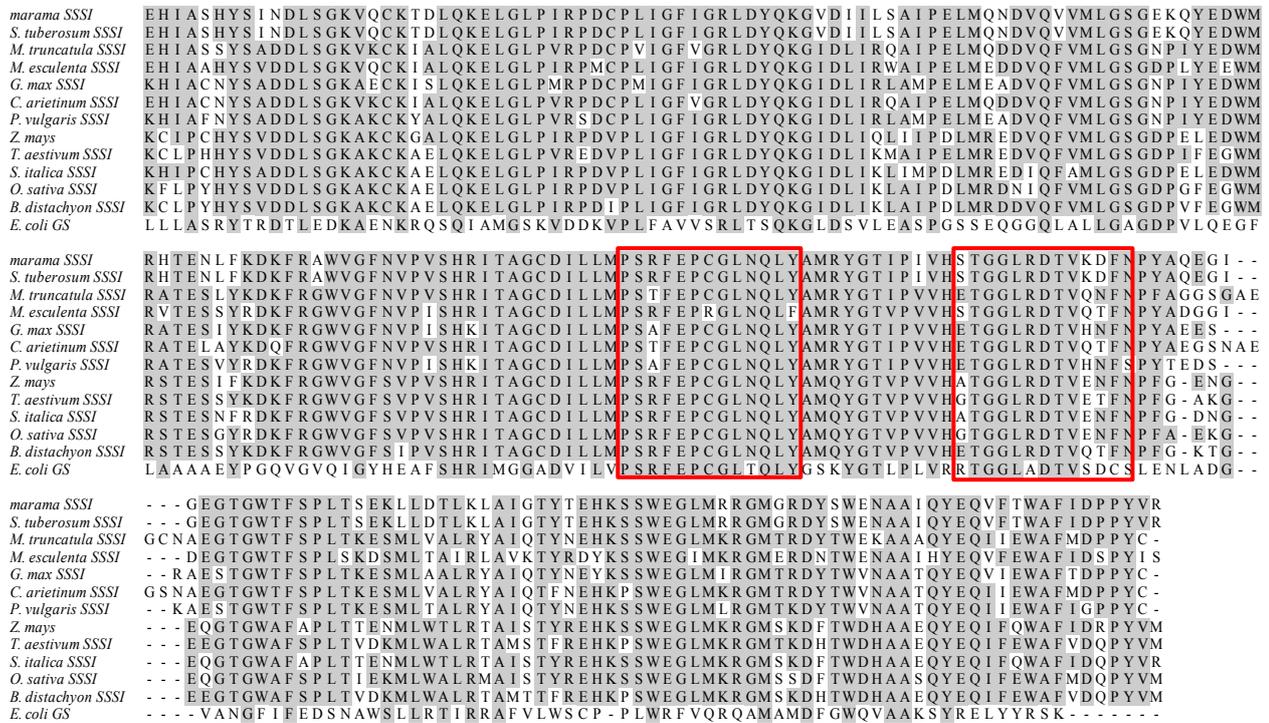


Figure 18: Alignment of deduced amino acid sequence of marama SSSI with plant (monocots and dicots) SSSI and *E. coli* glycogen synthase. The two conserved regions for starch synthases and glycogen are highlighted in red. The GenBank accession number of retrieved sequences are: *M. truncatula* (XP_003602259.1), *C. arietinum* (XP_004502657.1), *G. max* (XP_003523360.1), *P. vulgaris* (BAD18845.1), *M. esculenta* (ABV25893.1), *S. tuberosum* (P935681), *Z. mays* (AFW85727.1), *S. italica* (XP_00496777.1), *O. sativa* (AEB33739.1), *B. distachyon* (XP_003561063.1), *T. aestivum* (ADD54661.1) and *E. coli* (AAA23870).

4.2.6. Phylogenetic relationships of marama SSSI among plant SSSI proteins

Phylogenetic relationships between the amino acid sequences of plant SSSI proteins are shown in Figure 19. Plant SSSIs were separated into two clusters, dicots (cluster I) and monocots (cluster II).

Marama SSSI was member to a sub-cluster within the reminder of the dicot species. There was 100% sequence homology between potato SSSI and marama SSSI as indicated earlier by Blastp search results.

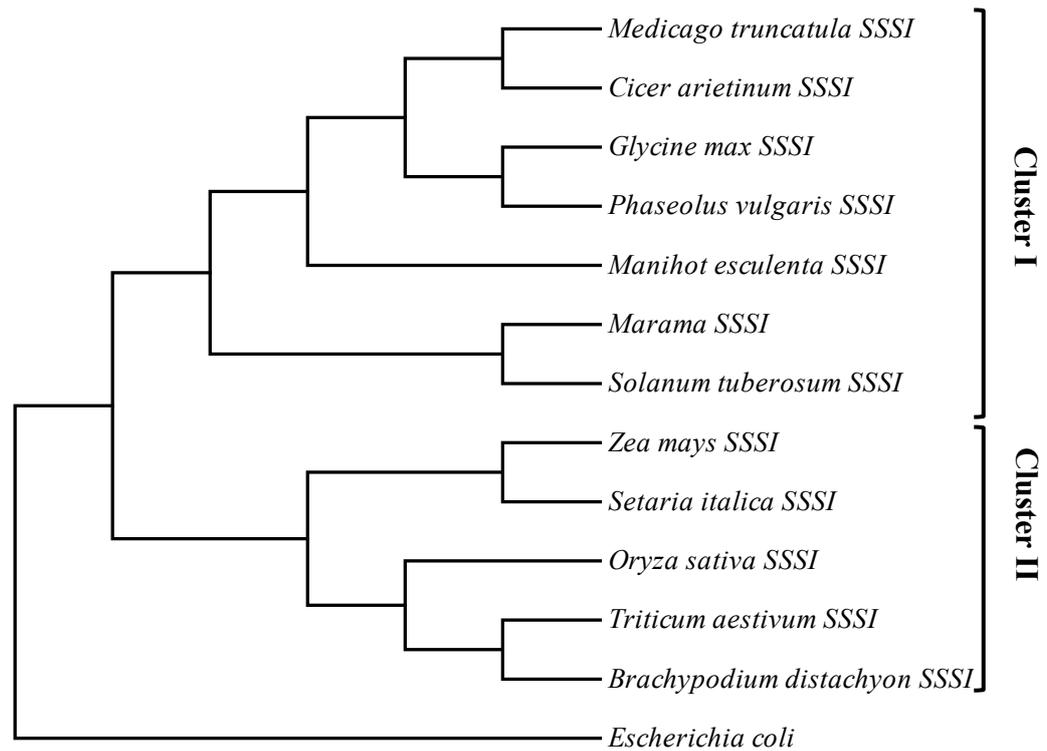


Figure 19: Phylogenetic relationships of SSSI protein of plant species and glycogen synthase protein of *E. coli*. The amino acid sequence of marama SSSI protein was determined in this study and other plant sequences were obtained from NCBI GenBank databases. The accession numbers for retrieved sequences are: *M. truncatula*, XP_003602259.1, *C. arietinum*, XP_004502657.1, *G. max*, XP_003523360.1, *P. vulgaris*, BAD18845.1, *M. esculenta*, ABV25893.1, *S. tuberosum*, P935681, *Z. mays*, AFW85727.1, *S. italica*, XP_00496777.1, *O. sativa*, AEB33739.1, *B. distachyon* XP_003561063.1, *T. aestivum* ADD54661.1, and *E. coli*, AAA23870.

4.3. Isolation and sequence analysis of genomic clones encoding AGPase large and small subunits and SBEs genes for *T. esculentum*.

4.3.1. PCR amplification and molecular cloning of AGPase genes of marama bean

PCR amplification for AGPases small subunit resulted in major DNA bands of approximately 0.6 kb, 0.7 kb; 0.9 kb and 1.1 kb amplified by ADPG-SU-For and ADPG-SU-Rev respectively (see Figure 20). Subsequently, two major DNA bands of approximately 1.4 kb and 2.0 kb were amplified by ADPG-LU-For and ADPG-LU-Rev (Figure 20). Identical results were produced in three replicate experiments. PCR products were purified, ligated to pCR2.1 TOPO vector and transformed effectively. To identify the identity of marama DNA inserts, 20 clones for AGPase with distinctive colony PCR and restriction profiles for all primer combinations were subjected to nucleotide sequence analysis. The resulting sequences were subsequently analysed using bioinformatics tools.

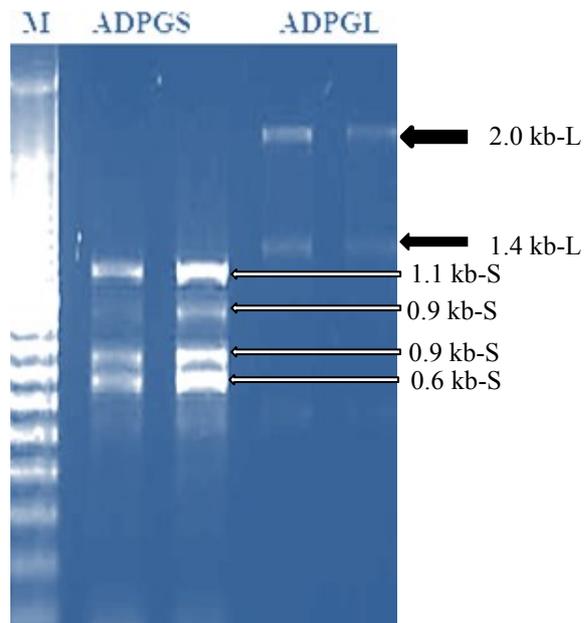


Figure 20: DNA banding patterns of marama AGPase genes amplified by different primers using genomic DNA as a template from marama tuber. M represent a DNA ladder, ADPGS and ADPGL represents small and large subunits.

4.3.2. Sequence analysis of marama AGPase small and large subunit

Internet-based bioinformatic resources and computer simulation analysis were used to study DNA sequences obtained after sequencing. The PCR fragment of marama AGPase small subunit encompassed a 366 bp encoding AGPase small subunit precursor with 121 amino acid residues as predicted by FGENESH program of Softberry web server (see Figure 21). The FGENESH program also predicted a 360 bp for marama large AGPase with 120 amino acid residues (see Figure 22).

21A: A 366 bp Nucleotide sequence encoding AGPase small subunit

```
5'-
ATGGGTGGCTACAAAAATGAAGGTTTTGTCGAGGTTCTAGCTGCTCAGC
AGAGTCCTGAGAATCCTAATTGGTTCCAGGGCACAGCAGATGCTGTGAG
GCAATATTTATGGCTTTTTGAGGAGCACAATGTTTTAGAGTTCCTAGTTC
TGGCTGGTGATCATTGTATAGAATGGATTATGAGAAATTCATTCAAGCA
CACAGGGAAACAGATGCTGACATCACTGTGGCTGCATTGCCAATGGATG
AAAAGCGTGCCACTGCATTCCGGTCTGATGAAGATTGATGAAGAAGGACG
CATAATTGAATTTGCCAAGAAGCTGCAAGGGAAACAAGCCGAATTCCAG
CACACTGGCGGCCGTTACTAG
```

21B: A deduced 121 amino acids sequence encoding AGPase small subunit

```
MGGYKNEGFVEVLAAQQSPENPNWFQGTADAVRQYLWLFEEHNVLEFLVL
AGDHLYRMDYEKFIQAHRETDADITVAALPMDEKRATAFGLMKIDEEGRIIE
FAKKLQGKQAEFQHTGGRY
```

Figure 21: Nucleotide sequence (21A) and deduced amino acid sequence (21B) of marama AGPase small subunit gene.

22A: A 360 bp Nucleotide sequence encoding AGPase large subunit

```
5'-
ATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGC
TTATGAGTAACTGCATAAATAGTGGCATTACAAGATATTTGTACTGACT
CAGTTCAACTCTGCGTCTCTGAACCGTCATATTGCCCGCACCTATTTGG
AAATGGCATCAGCTTTGGAGATGGAATTGTGGAGGTCCTTGCAGCAACT
CAAACACCAGGAGAAGCGGGGAGAAATTGGTTCCAAGGGACTGCCGAT
GCTGTCAGACAATTTACATGGGTATTTGAGGATGCCAAGAACACCAACG
TTGAGAATGTATTGATCTTGGCAGGGGATCATCTATAACCGAATGGATTAC
ATGGACCTTATTCAG
```

22B: A deduced 120 amino acids sequence encoding AGPase large subunit

```
MHARAAASVMDICRIRLMSNCINSGINKIFVLTQFNSASLNRHIARTYFGNGI  
SFGDGIVEVLAATQTPGEAGRNFQGTADAVRQFTWVFEDAKNTNVENVL  
ILAGDHLRMDYMDLIQ
```

Figure 22: Nucleotide sequence (22A) and deduced amino acid sequence (22B) of marama AGPase large subunit gene.

These sequences have no homology with sequences of other genes encoding AGPase large subunit in NCBI using BLASTN. However, an amino acid sequence search using BLASTX showed high homology with other gene encoding AGPase small and large subunit. BLASTX search analysis of the marama AGPase small subunit sequence on URL <http://blast.ncbi.nlm.nih.gov/Blast.cgi> showed 96% homology with *G. max* (Accession No.XP003519654.1) AGPase small subunit isoform 1 amino acid sequence. It also showed 95% homology with *C. arietinum* amino acid (Accession No XP004491558.1) small subunit sequence and 86-89% homology with other plant species (see Table 14).

BLASTX search analysis of the marama AGPase large subunit sequence showed 96% identity with *G. max* (Accession No.XP003527197.1) AGPase large subunit amino acid sequence. It also showed significant homologies with *M. truncatula* (Accession No XP003606943.1) large subunit, *C. arietinum* (Accession No XP004507462.1) large subunit and with other sequences (see Table 14). The result thus indicates that DNA fragments amplified from marama are partial sequences encompassing the conserved sequence of the AGPase small and large subunit gene in marama.

Table 14: Result of search for similarity between marama deduced amino acid AGPase small and large subunit and GenBank accessions using Blastx

| GenBank accession with highest similarity (GenBank accession number) | Percentage of amino acid identity (%) | <i>E</i>-value^a |
|--|--|-----------------------------------|
| Marama AGPase small subunit | - | - |
| <i>Glycine max</i> ADP-glucose pyrophosphorylase small subunit isoform 1 (XP003519654.1) | 96 | 1e-72 |
| <i>Cicer arietinum</i> ADP-glucose pyrophosphorylase small subunit isoform 1 (XP003606943.1) | 95 | 1e-71 |
| <i>Medicago truncatula</i> ADP-glucose pyrophosphorylase small subunit (XP003617925.1) | 91 | 9e-71 |
| <i>Vitis vinifera</i> ADP-glucose pyrophosphorylase small subunit (XP002263255.1) | 95 | 3e-72 |
| <i>Frugaria vesca</i> ADP-glucose pyrophosphorylase small subunit (NP195632.1) | 95 | 5e-72 |
| Marama AGPase large subunit | - | - |
| <i>Glycine max</i> ADP-glucose pyrophosphorylase large subunit (XP003527197.1) | 96 | 5e-65 |
| <i>Medicago truncatula</i> ADP-glucose pyrophosphorylase large subunit (XP003606943.1) | 94 | 9e-64 |
| <i>Cicer arietinum</i> ADP-glucose pyrophosphorylase large subunit 1 (XP004507462.1) | 91 | 9e-62 |
| <i>Solanum lycopersicum</i> ADP-glucose pyrophosphorylase large subunit (NP001233918.1) | 85 | 4e-60 |
| <i>Arabidopsis thaliana</i> ADP-glucose pyrophosphorylase large subunit 3 (NP195632.1) | 84 | 4e-59 |
| <i>Ricinus communis</i> ADP-glucose pyrophosphorylase large subunit (XP002512925.1) | 83 | 7e-55 |

^a The expected *E*-value refers to the number of matches by chance alone. The lower the *E*-value the more significant the match is.

4.3.3. Multiple alignment of deduced amino acid sequence for marama AGPase small subunit gene

The genomic fragment encoding marama AGPase small subunit gene sequence was aligned by ClustalW analysis with other sequences encoding AGPase small subunit available on NCBI gene bank to identify sequence similarities and differences. The analysis and comparison of sequences indicated that the deduced amino acid of marama AGPase small subunit had similar structure to other AGPase small subunit from other plants (see Figure 23). The predicted genomic fragment encoding marama AGPase small subunit revealed putative conserved domains within the predicted marama AGPase small subunit protein, which includes Nucleotidyl transferase (Accession No. pfam00483) and ADP-glucose pyrophosphorylase (Accession No. cd02508) under Glycosyltransferase family A (Accession No. cl11394). This could probably be an indication that the isolated genomic fragment is a gene fragment of ADP-glucose pyrophosphorylase small subunit in marama.

Alignment and visual examination of predicted deduced amino acids and literature comparison further revealed that marama AGPase small subunit gene fragment has three highly conserved Nucleotidyl transferase domain sites. The amino acid sequence of marama AGPase small subunit contains the domain WFQGTADAV which is believed to be the ATP binding site, Catalytic site domain LAGDHLRMDY and IIEFAKKLQGKQ and the IIEFAKLQGEQ Glc-1-P site look alike domain respectively (see Figure 23).

| | |
|-----------------------------|---|
| <i>Marama AGPaseS</i> | MGGYKNEGFVEVLAAQQSPENPNWFQGTADAVRQYLWLF E |
| <i>Glycine max</i> | MGGYKNEGFVEVLAAQQSPENPNWFQGTADAVRQYLWLF E |
| <i>Cicer arietinum</i> | LGGYKNEGFVEVLAAQQSPENPNWFQGTADAVRQYLWLF E |
| <i>Medicago truncatula</i> | MGGYKNEGFVEVLAAQQSPENPNWFQGTADAVRQYLWLF E |
| <i>Cucumis sativus</i> | MGGYKNEGFVEVLAAQQSPENPNWFQGTADAVRQYLWLF E |
| <i>Solanum lycopersicum</i> | MGEYKNEGFVEVLAAQQSPENPNWFQGTADAVRQYLWLF E |
| <i>Zea mays</i> | IAGYKNEGFVEVLAAQQSPENPNWFQGTADAVRQYMWLF E |
| <i>Marama AGPaseS</i> | EHNVLEFLVLAGDHLRMDYEKFIQAHRETDADITVAALP |
| <i>Glycine max</i> | EHNVLEFLVLAGDHLRMDYEKFIQAHRETDADITVAALP |
| <i>Cicer arietinum</i> | EHNVLEFLVLAGDHLRMDYEKFIQAHRETDADITVAALP |
| <i>Medicago truncatula</i> | EHNVLEFLVLAGDHLRMDYERFIQAHRES DADITVAALP |
| <i>Cucumis sativus</i> | EQNVLEFLVLAGDHLRMDYERFIQAHRETDADITVAALP |
| <i>Solanum lycopersicum</i> | EHNVLEYLIVLAGDHLRMDYEKFIQAHRETDADITVAALP |
| <i>Zea mays</i> | EHNIMEFLVLAGDHLRMDYQKFIQAHRETDADITVAALP |
| <i>Marama AGPaseS</i> | MDEKRATAFGLMKIDEEGR IIEFAKKLQ GKQ |
| <i>Glycine max</i> | MDEKRATAFGLMKIDEEGR IIEFAEKPKGEQ |
| <i>Cicer arietinum</i> | MDEKRATAFGLMKIDEEGR IIEFAEKPKGDQ |
| <i>Medicago truncatula</i> | MDEARATAFGLMKIDEEGR IIEFAEKPKGEQ |
| <i>Cucumis sativus</i> | MDEKRATAFGLMKIDEEGR IIEFAEKPKGEQ |
| <i>Solanum lycopersicum</i> | MDEKRATAFGLMKIDEEGR IIEFAEKPKGEQ |
| <i>Zea mays</i> | MDEQRATAFGLMKIDEEGR IVEFAEKPKGEK |

Figure 23: Comparison of the predicted amino acid sequences of plant ADP-glucose pyrophosphorylase small subunit and a marama genomic clone fragment predicted amino acid. Three Nucleoside Triphosphate domains are shown in red boxes. The GenBank accession numbers of the retrieved sequences are: *G. max*, XP_003519654.1, *C. arietinum*, XP_003606943.1, *M. truncatula*, XP_003617925.1, *C. sativus*, XP_004144558.1, *S. lycopersicum*, ABC26921.1, *Z. mays*, AAK69628.1.

The degree of sequence identity between the predicted AGPase small subunit protein from marama and its homolog from various plant species ranged from 86.49 to 96.40% (see Table 15). These results were consistent with those obtained in BLASTX analysis.

Table 15: Percentage similarity between the predicted marama AGPase large subunit protein and its homolog from selected plant species

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---------------------|---|-------|--------|-------|-------|-------|-------|-------|
| Marama AGPL | 1 | 94.17 | 94.17 | 95.15 | 91.26 | 91.26 | 83.50 | 74.04 |
| Glycine max | 2 | 94.17 | 100.00 | 94.17 | 92.23 | 92.23 | 84.47 | 75.96 |
| Medicago truncatula | 3 | 94.17 | 100.00 | 94.17 | 92.23 | 92.23 | 84.47 | 75.96 |
| Phaseolus vulgaris | 4 | 95.15 | 94.17 | 94.17 | 93.20 | 93.20 | 83.50 | 75.00 |
| Lens culinaris | 5 | 91.26 | 92.23 | 92.23 | 93.20 | 98.06 | 84.47 | 73.08 |
| Cicer arietinum | 6 | 91.26 | 92.23 | 92.23 | 93.20 | 98.06 | 86.41 | 73.08 |
| Ipomea batatas | 7 | 83.50 | 84.47 | 84.47 | 83.50 | 84.47 | 86.41 | 74.04 |
| Zea mays | 8 | 74.04 | 75.96 | 75.96 | 75.00 | 73.08 | 73.08 | 74.04 |

The similarity was obtained by comparison of deduced amino acid sequences using joint alignments and pairwise comparison of CLC Main Workbench 6.71.

4.3.4. Multiple alignment of deduced amino acid sequence for marama AGPase large subunit gene

ClustalW analysis for the deduced mature marama amino sequence AGPase large subunit calculated the best match with other sequences encoding AGPase large subunit available on NCBI GenBank and aligned them to identify their similarities and differences. The analysis of sequences indicated that the deduced amino acid of marama AGPase large subunit had similar but not identical structure to those of known AGPase large subunit genes such as that of *G. max* and other legume species such as *P. sativum*, *M. truncatula*, *L. culinaris* and *C. arietinum* (see Figure 24). It was indicated that there are high levels of homology in amino acid sequences of all AGPase large subunit proteins from marama and others. All AGPase large subunit amino acid sequences have three conserved domains belonging to ADP-glucose pyrophosphorylase, a subfamily of a glycosyl transferase family (Accession No. cd02508), Nucleotidyl transferase family (Accession No. pfam00483) and Glycosyltransferase superfamily A (Accession No. cl11394).

The amino acid sequence of marama AGPase large subunit also revealed two Nucleotidyl transferase domain sites, namely the ATP binding site domain WFQGTADAV and Catalytic site domain LAGDHLRMDY (see Figure 24).

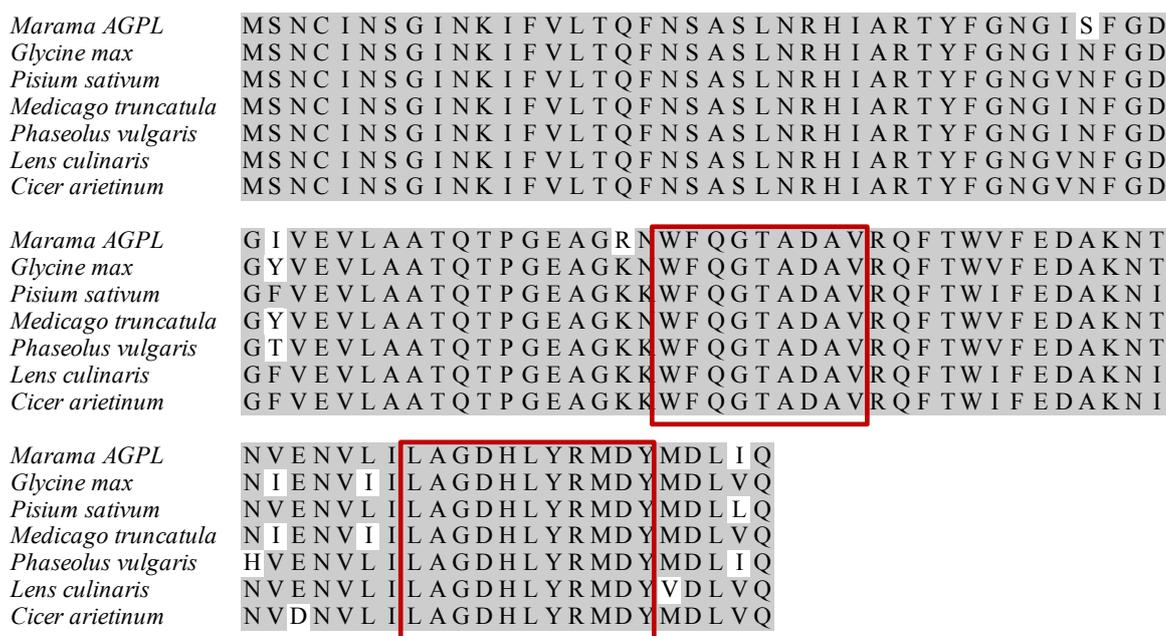


Figure 24: Comparison of the predicted/deduced amino acid sequences of plant ADP-glucose pyrophosphorylase large subunit and a predicted amino acid of marama genomic clone. Nucleotidyl transferase domain sites are shown in red boxes. The GenBank accession numbers of the retrieved sequences are: *G. max*, AFK46347.1, *C. arietinum*, XP_004500804.1, *M. truncatula*, XP_003606943.1, *P. vulgaris*, BAC66692.1, *L. culinaris*, AWC82825.1, *P. sativum*, CAA65541.1.

The degree of sequence identity between the predicted AGPase small subunit protein from marama and its homolog from various plant species ranged from 74.04 to 94.17% (see Table 16). These results were in agreement with those obtained in Blastx analysis.

Table 16: Percentage similarity of the predicted marama AGPase large subunit protein and its homolog from selected plant species

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|----------------------|---|-------|-------|-------|-------|-------|-------|-------|
| Marama AGPS | 1 | 96.40 | 94.59 | 93.69 | 94.59 | 92.79 | 92.04 | 86.49 |
| Glycine max | 2 | 96.40 | 97.30 | 97.30 | 98.20 | 96.40 | 93.81 | 90.09 |
| Cicer arietinum | 3 | 94.59 | 97.30 | | 94.59 | 95.50 | 92.92 | 90.09 |
| Medicago truncatula | 4 | 93.69 | 97.30 | 94.59 | | 97.30 | 94.59 | 91.15 |
| Cucumis sativus | 5 | 94.59 | 98.20 | 95.50 | 97.30 | | 94.59 | 92.04 |
| Arabidopsis thaliana | 6 | 92.79 | 96.40 | 95.50 | 94.59 | 94.59 | | 93.81 |
| Solanum lycopersicum | 7 | 92.04 | 93.81 | 92.92 | 91.15 | 92.04 | 93.81 | |
| Zea mays | 8 | 86.49 | 90.09 | 90.09 | 88.29 | 88.29 | 90.99 | 85.84 |

The similarity was obtained by comparison of deduced amino acid sequences using joint alignments and pairwise comparison of CLC Main Workbench 6.71.

4.3.5. Phylogenetic analysis of plant AGPase and marama AGPase gene clones

In order to investigate the phylogenetic relationships among plant AGPase small and large subunits genes, phylogenetic analyses were performed based on AGPase small and large subunits amino acid sequences and their isoforms. A phylogenetic tree of 13 AGPase small subunit amino acid sequences with the cloned marama AGPase small subunit gene fragment was constructed based on collected AGPase small subunit sequences as well as isoforms identified or predicted from monocots and dicots. A Neighbour Joining tree rooted to AGPase small subunit like protein from *Chlamydomonas reinhardtii* (Accession No. XP_001691854.1) divided plant AGPase small subunit proteins into two distinct clusters which are composed of dicotyledonous (cluster I) and monocotyledonous (cluster II) AGPase small subunit proteins (Figure 25). The monocot AGPase small subunits were clustered into a different cluster from dicot AGPase small subunits.

The dicot cluster was further divided into two sub-clusters, sub-cluster I, which contained legume species and sub-cluster II with non-legume species. The marama AGPase small subunit falls under the dicot sub-cluster (sub-cluster I) consisting of AGPase small subunit proteins from legumes such as soybean, common bean and lentil.

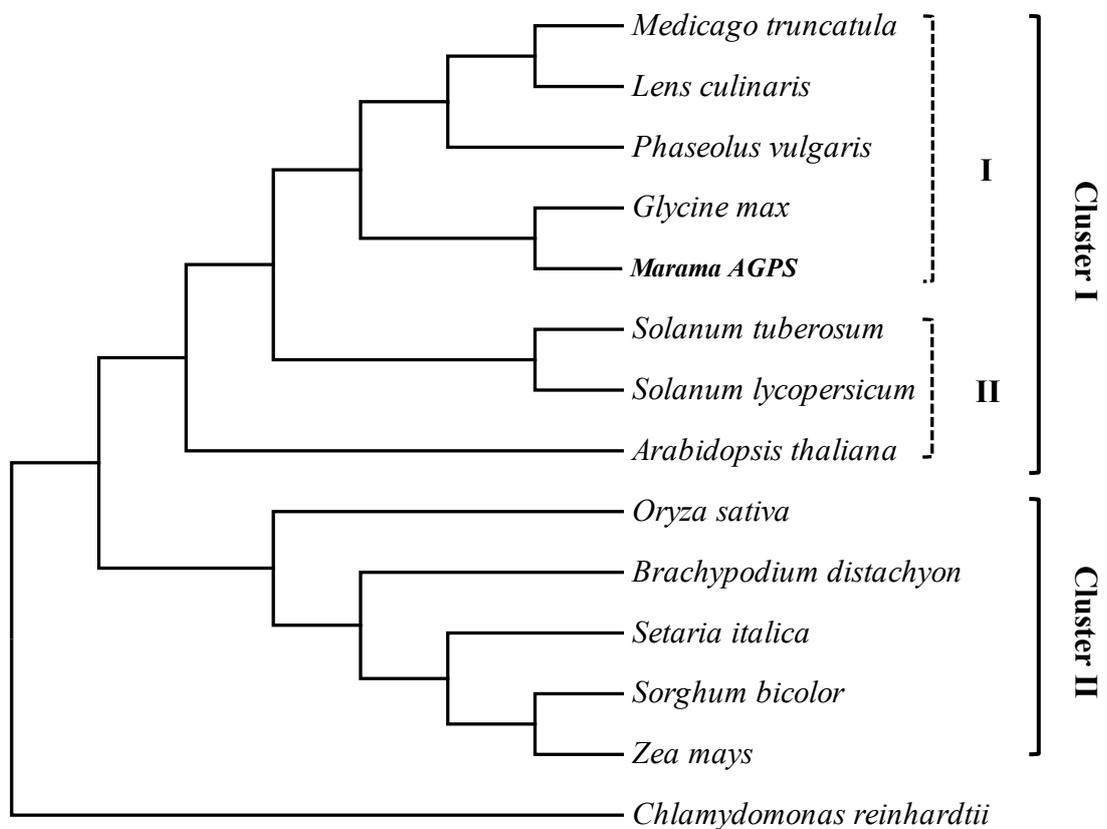


Figure 25: Phylogenetic tree based on the amino acid alignment of plant AGPase small subunits along with cloned marama AGPase small subunit gene. The rooted tree was constructed using MEGA 5.2 software with a neighbour-joining method. The parameter pairwise deletion and JTT (Jones, Taylor and Thornton) amino acid substitution model were used. Sequences aligned included AGPase small subunit proteins which have been identified or predicted from dicotyledons *M. truncatula* (XP_004144558.1), *L. cullinaris* (ACX48912.1), *P. vulgaris* (BAC66693.1), *Glycine max* (XP_003519654.1), *S. tuberosum* (AAA66057), *S.lycopersicum* (NP_001234696.1), *A. thaliana* (NP_199641.1), monocotyledons, *B. distachyon* (XP_003577955), *S. italica* (004973256.1), *S. bicolor* (XP_002462140.1) and *Z. mays* (NP_00405178.1).

A phylogenetic tree was also constructed on the basis of sequence similarity of AGPase large subunit proteins and marama AGPase large subunit gene fragment. The tree was constructed using the neighbour-joining method and *E. coli* (Accession No. EDV67779.1) was used as an out-group. The AGPase large subunit proteins were separated into two clusters, one cluster consisted entirely of AGPase large subunit from dicots (cluster I) and the second cluster consists entirely of AGPase large subunit from monocots (cluster II) (see Figure 26). The marama AGPase large subunit was found in the group of dicots. In this group, marama AGPase large subunit was closely related to the common bean *PvAGPSI*.

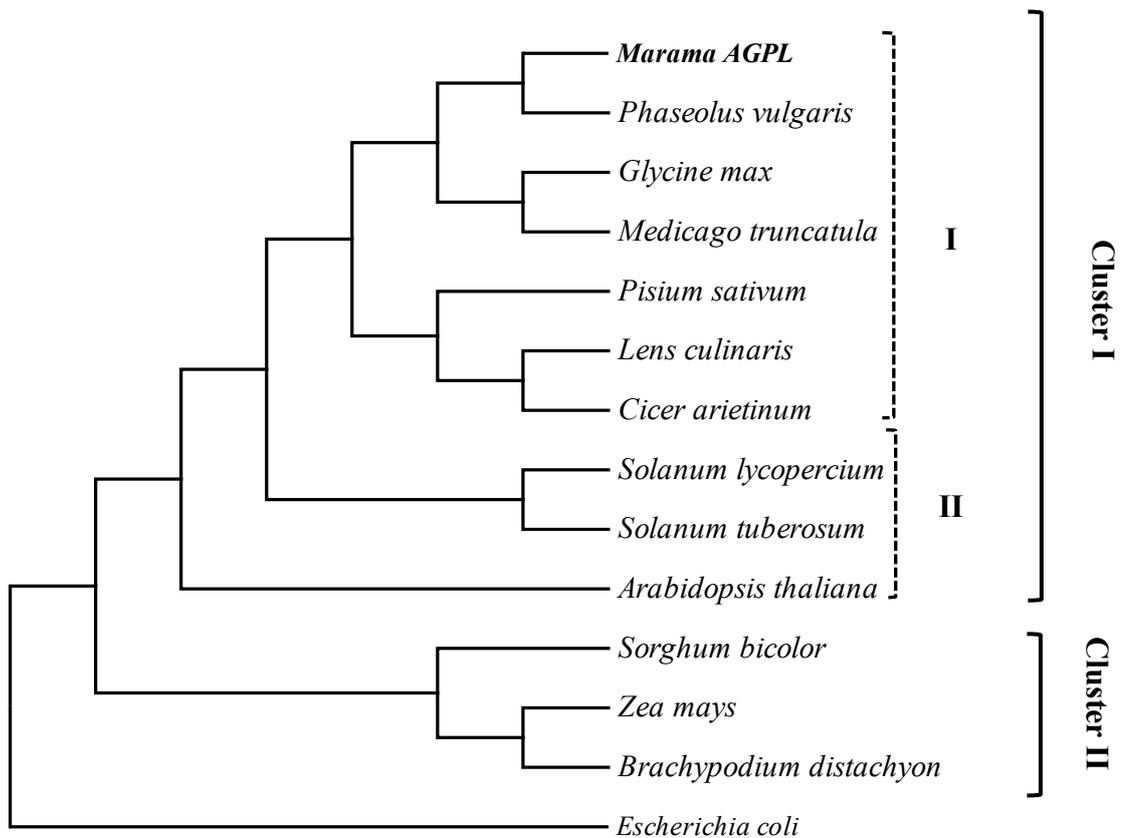


Figure 26: Phylogenetic tree based on the amino acid alignment of plant AGPase large subunits along with cloned marama AGPase large subunit gene. The rooted tree was constructed using MEGA 5.2 software with a neighbour-joining method. The parameters pairwise deletion and JTT (Jones, Taylor and Thornton) amino acid substitution model were used. Sequences aligned included AGPase large subunit proteins which were identified or predicted from dicotyledons *M. truncatula* (XP_003606943.1), *L. culinaris* (ACW82825.1), *P. vulgaris* (BAC66692.1), *S. lycopersicum* (ABC26921.1), *S. tuberosum* (CAA43490.1), *C. arietinum* (NP_004500804.1), *A. thaliana* (NP_199641.1), monocotyledons, *B. distachyon* (XP_004981865.1), *S. italica* (001051184.1) and *Z. mays* (ABD66656.1).

4.4. Isolation and sequence analysis of genomic clones encoding SBEs genes for *T. esculentum*

4.4.1. PCR amplification and molecular cloning of SBEs genes of marama bean

PCR amplification for SBEs resulted in single DNA bands for both SBEI and SBEII with approximate size of 0.5 and 0.9 kb, respectively amplified by primer pairs SBEI-For and SBEI-Rev and SBEII-For and SBEII-Rev (see Figure 27).

Identical results were produced in three replicate experiments. PCR products were purified, ligated to pCR2.1 (TOPO) vector and transformed successfully. To identify the identity of marama DNA inserts, 5 clones for SBEs with unique colony PCR and restriction profiles for all primer combinations were subjected to nucleotide sequence analysis. The resulting sequences were then analysed using bioinformatics tools.

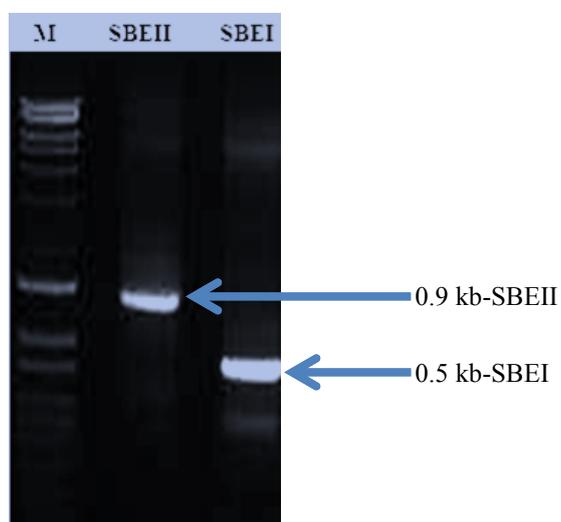


Figure 27: DNA banding patterns of marama SBEs genes amplified by different primers using genomic DNA as a template from marama tuber. M represent a DNA ladder, while SBEI and SBEII represents starch branching enzyme I and II.

4.4.2. Sequence analysis of marama Starch Branching Enzymes genes

DNA sequences of isolated SBEs gene fragments were studied using internet bioinformatic resources. The SBEI gene fragment encompassed a 291 bp encoding 96 amino acid residues as predicted by FGGENESH (Figure 28).

BLASTX search analysis of a genomic clone sequence encoding SBEI had high similarity to other SBEs genes, specifically to SBEI genes. For example, the sequence had 91% percentage of identity to *C. arietinum* SBE I, 90% to *P. vulgaris* SBE I and 88% to *G. max* SBEI (see Table 17). On the other hand, the SBEII gene fragment encompassed a 414 bp encoding 137 amino acid residues as predicted by FGENESH (Figure 29). BLASTX search analysis of a genomic clone sequence encoding SBEII had high similarity to other SBEII genes. For example, compared to protein sequences of *M. truncatula* and *G. max* SBEII, the percentage identities was 93 and 91% respectively (see Table 17).

28A: A 291 bp Nucleotide sequence encoding SBEI for marama

```
5'-  
ATGGCGGCCGCGGGAATTCGATTTTATGCTGAGAGTCATGACCAG  
GCCATTGTTGGGGACAAGACTATAGCATTTCCTAATGGATAAG  
GAAATGTATTCTGGTATGTCTTGCCCTGATTGATGCTTCTCCTTCCAT  
TGAACGAGGAATTGCTCTTCACAAGATGATACACTTCATAACTAT  
GGCATTAGGTGGGGAGGGCTATCTTAATTCATGGGTAATGAGGT  
AAGTACACTGTTCCATGCTTCCGCCAATAAACTGCTGCTGTTGGT  
CCTGCATATTTGCAGTAA
```

28B: A deduced 96 amino acids sequence encoding SBEI for marama

```
MAAAGIRFYAESHDAQIVGDKTIAFFLMDKEMYSGMSCLIDASPSIER  
GIALHKMIHFITMALGGEGYLNFMGNEVSTLFHASANKTAAVGPAYL  
Q
```

Figure 28: Nucleotide sequence (28A) and deduced amino acid sequence (28B) for marama SBEI gene.

29A: A 414 bp Nucleotide sequence encoding SBEII for marama

```
5'-  
GCCGCGATTCGCCCTTATGCTGAGAGTCATGACCAAGCTTTGGTTG  
GTGACAAGACCATAGCATTTTGGTTGATGGACAAGGACATGTACG  
ACTTCATGGCTTTAGACAGACCAACTACTCCTCTTGTAGATCGTGG  
TATAGCATTACACAAAATGATTAGGCTTATCACCATGGGACTCGG  
TGGTGAAGGGTATTTGAACTTTATGGGAAATGAATTTGGCCATCCT  
GAGTGGATTGATTTTCCAAGGGGTGATCAATATCTTCCTAATGGCA  
CAGTAATACCTGGGAACAATTATAGTTTTGATAAATGCAGGCGTA  
GATTTGACTTAGTAAGTTTCTTAGTTAGTTCAGTTGCAATTTGTTGC  
AATATTCCAACCTTTATCAGAGTTCAACTCGTTGGACATTATTTGT  
GA
```

29B: A deduced 137 amino acids sequence encoding SBEII for marama

```
AAIRPYAESHQALVGDKTIAFWLMDKDMYDFMALDRPTTPLVDRG  
IALHKMIRLITMGLGGEGYLNFMGNEFGHPEWIDFPRGDQYLPNGTVI  
PGNNYSFDKCRRRFDLVSFLVSSVAICCNIPFIRVQLVGHYL
```

Figure 29: Nucleotide sequence (29A) and deduced amino acid sequence (29B) for marama SBEII gene.

Table 17: Result of search for similarity between marama deduced amino acid SBEI and SBEII together with GenBank accessions using Blastx.

| GenBank accession with highest similarity (GenBank accession number) | Percentage of amino acid identity (%) | <i>E</i> -value ^a |
|---|--|------------------------------|
| Marama SBEI | - | - |
| <i>C. arietinum</i> SBEI (XP004500878.1) | 91 | 9e-35 |
| <i>P. vulgaris</i> SBEI (BAA82349) | 90 | 3e-34 |
| <i>V. radiata</i> SBEI (AAT76445.1) | 88 | 1e-33 |
| <i>G. max</i> SBEI (XP_003523080.1) | 88 | 6e-33 |
| <i>I. batatas</i> SBEI (BAE96956.1) | 81 | 2e-29 |
| <i>M. esculenta</i> SBEI (CAA54308.1) | 79 | 9e-31 |
| Marama SBEII | - | - |
| <i>M. truncatula</i> SBEII (XP_003625770.1) | 93 | 1e-65 |
| <i>V. radiata</i> SBEII (AAT76444.1) | 93 | 9e-63 |
| <i>P. vulgaris</i> SBEII (BAA82348.2) | 92 | 3e-62 |
| <i>P. sativum</i> SBEII (CAA56319.1) | 91 | 2e-62 |
| <i>G. max</i> SBEII (XP_003521449.1) | 91 | 6e-62 |
| <i>S. tuberosum</i> (CAB40748.1) | 88 | 3e-59 |

^a The expected *E*-value refers to the number of matches by chance alone. The lower the *E*-value the more significant the match is.

4.4.3. Multiple alignment of deduced amino acid sequences for marama SBEs genes

Using BLASTP for alignment, a conserved domain, the alpha amylase catalytic domain found in bacterial and eukaryotic starch branching enzymes was present in a protein sequence encoding marama SBEI. The highly conserved domain of marama SBEI fragment contained a conserved domain sequence “AESHDQ” found in all starch branching enzymes (see Figure 30). Therefore, the result of sequence alignment indicated that the predicted could be a fragment of SBEI gene for marama. Therefore, this result of sequence alignment indicated that the predicted peptide encoded by the DNA fragment from marama is a starch branching enzyme II.

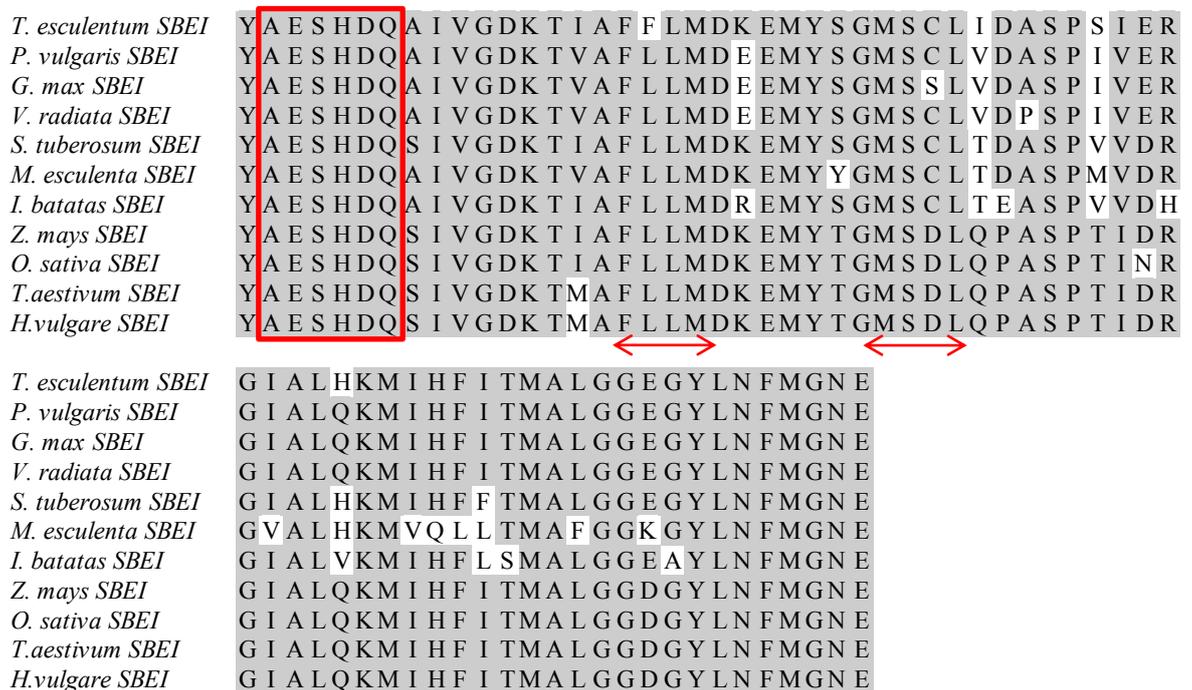


Figure 30: Alignment of the deduced amino acid sequence of marama SBEI with *V. radiata* SBEI (AAT6445.), *G. max* SBEI (XP_003523080.1), *P. vulgaris* SBEI, (BAA82349.1), *I. batatas* SBEI (BAE96954.1), *S. tuberosum* SBEI (CAA70038.1), *M. esculenta* SBEI (CAA543084), *Z. mays* SBEI (AAO20100.1), *T. aestivum* SBEI (CAB40980.1), *H. vulgare* SBEI, (AAP72268.1) and *O. sativa* SBEI (ABL74558.1). The red box indicates a conserved region of α -amylase family, while the arrows indicate α -helix and β -sheet look-alike found among SBEI isoforms.

Using BLASTP for alignment, marama SBEII fragment sequence contained one of the common conserved catalytic domain sequences “AESHDAQ” of the alpha amylase family found in all starch branching enzymes. The deduced amino acid sequence encoding SBEII for marama also showed region GDQYLPNGTVI corresponding to the SBEII-specific sequence P/EQXLPS/NGKF/II/VP (see Figure 31).

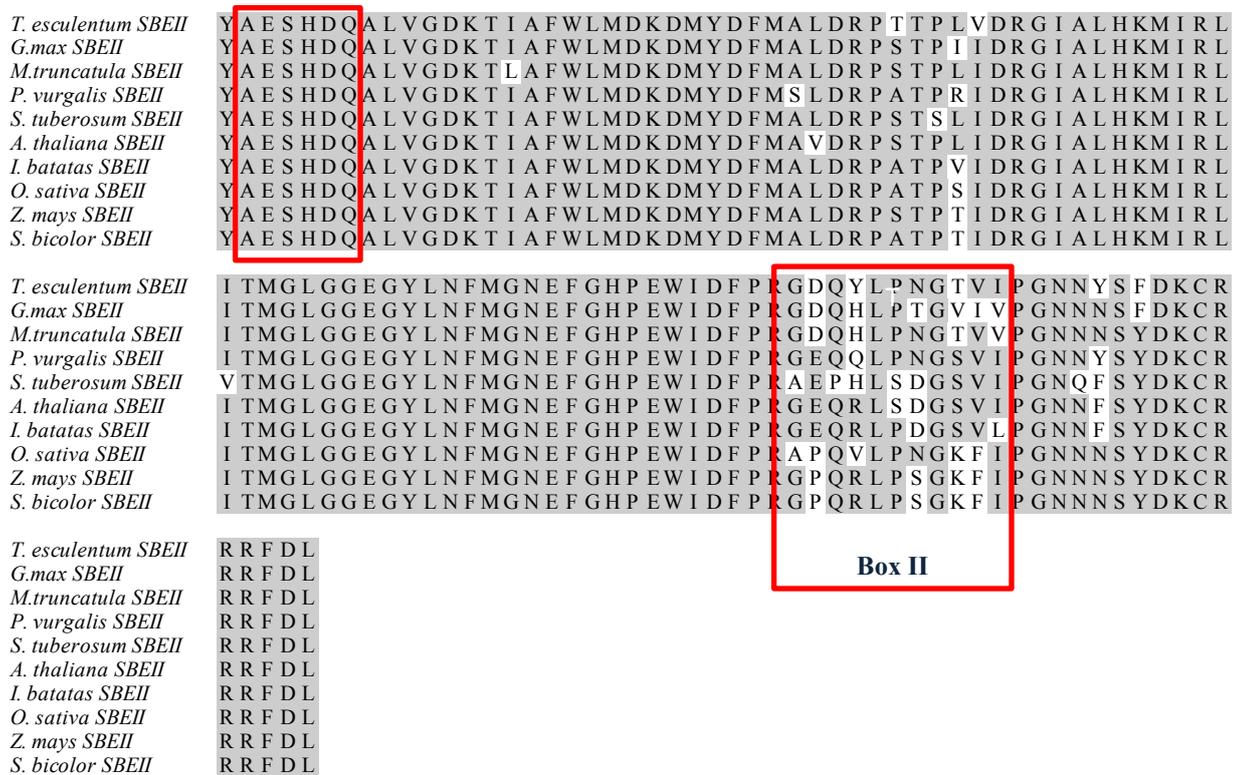


Figure 31: Alignment of the deduced amino acid sequence of marama with *A. thaliana* SBEII, (AAB03100.1), *I. batatas* SBEII (BAB40334.1), *G. max* SBEII (XP_003521449.1), *P. vulgarensis* SBEII (BAA82348.2), *M. truncatula* SBEII (XP_003625770.1), *Z. mays* SBEIIb, (AAP72200.1), *S. bicolor* SBEIIb (AAP72267.1), *O. sativa* SBEII (ACE88265.1) and *S. tuberosum* (CAB40748.1). The small red box indicates a conserved region (AESHDAQ) of α -amylase family, while the box II indicate GDQYLPNGTVI sequence corresponding to the SBEII-specific sequence P/EQXLPS/NGKF/II/VP.

4.4.4. Phylogenetic analysis of plant starch branching enzymes and marama SBEs gene clones

Twenty-eight amino acid sequences of starch branching enzymes from various plants and one sequence of glycogen branching enzyme from *E. coli* were analysed using the maximum parsimony method in this study. The GBEs of *E. coli* and red alga *Gracilaria gracilis* were used as out-group in the analysis. All starch branching enzymes from plants are separated into two families. The phylogenetic tree demonstrated that angiosperms SBE sequences are grouped into two super clusters designated; Family A which is composed of SBEII and family B which composed of SBEI (see Figure 32). Family A, contained starch branching enzyme II sequences from both monocots and dicots. Similarly, Family B is composed of starch branching enzyme I sequences from both monocots and dicots.

Moreover, SBE sequences from monocots are separated from those of dicots in both SBEI and SBEII clusters, resulting in two monophyletic sub clusters within each cluster. SBEI cluster is composed of two sub-clusters, dicot SBEI and monocot SBEI cluster. Likewise, SBEII cluster comprises of two sub-clusters, denoted dicot SBEII and monocot SBEII sub-cluster. SBEI from marama was a sister to the sub-cluster consisting of the remainder of SBEI from dicot species. SBEII from marama is placed in the family A super-cluster and has the highest evolutionary relatedness to *M. truncatula* and *G. max* SBEII. These results suggest that both SBEI and SBEII genes have separated following the divergence of monocots from dicots.

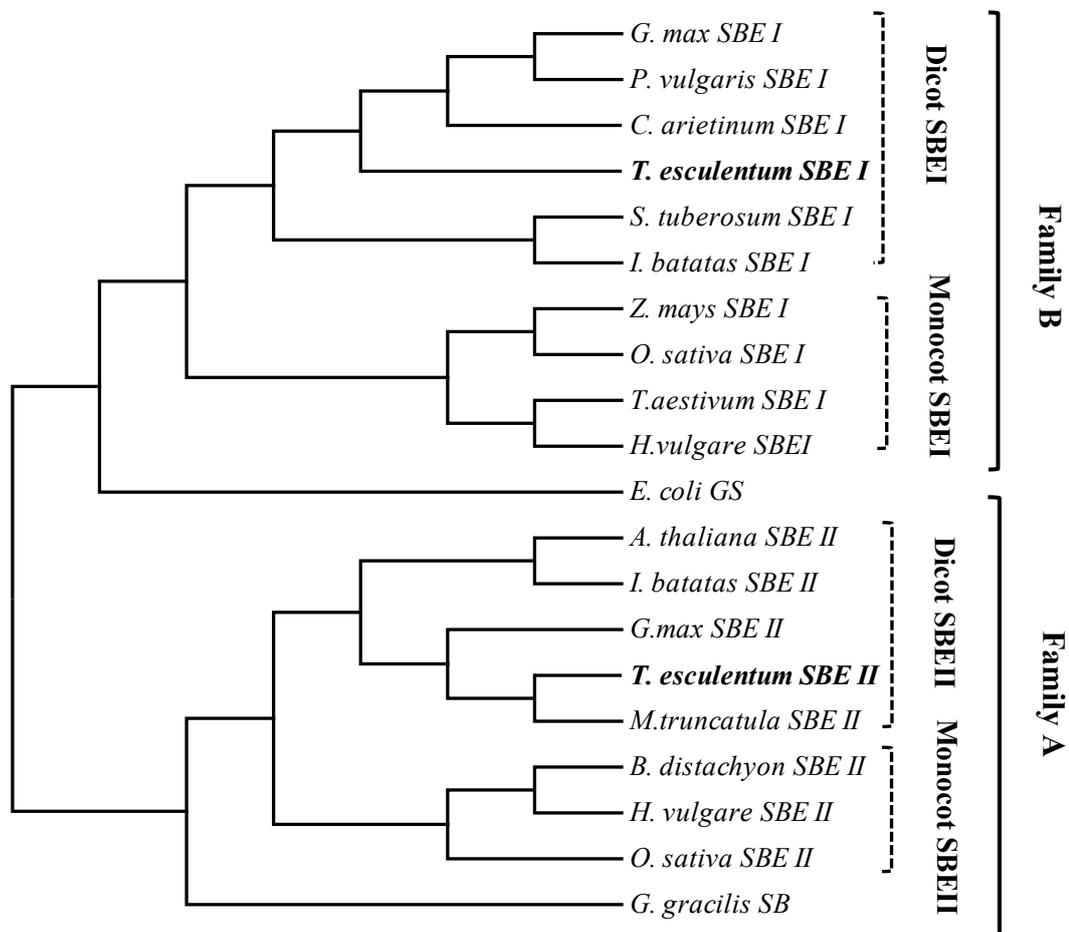


Figure 32: A single phylogenetic tree of SBEs, and Glycogen Branching enzyme of E coli and Starch branching of alga. The amino acid sequences of SBEs were obtained from NCBI GenBank databases, except for SBE I and SBE II from marama. The accession numbers for retrieved sequences are: *C. arietinum* SBE I, XP_004500878.1, *G. max* SBE I, XP_003523080.1, *P. vulgaris* SBE I, BAA82349.1, *I. batatas* SBE I, BAE96954.1, *S. tuberosum* SBE I, CAA70038.1, *Z. mays* SBE I, AAO20100.1, *T. aestivum* SBE I, CAB40980.1, *H. vulgare* SBE I, AAP72268.1, *O. sativa* SBE I, ABL74558.1, *A. thaliana* SBE II, AAB03100.1, *I. batatas* SBE II, BAB40334.1, *G. max* SBE II, XP_003521449.1, *M. truncatula* SBE II, XP_003625770.1, *B. distachyon* SBE II, XP_003575058.1, *H. vulgare* SBE II, CAX51366.1, *O. sativa* SBE II, ACE88265.1, *G. gracilis* SB, AAB97471 and *E. coli*, BAB69770.

4.5. Detection of serine protease inhibitor activity and isolation of serine proteinase coding sequence from *T. esculentum* seeds

4.5.1. Detection of serine protease inhibitor activity by reverse zymogram technology

Detection of serine protease inhibitor activities in mature and green marama bean seeds was carried out using the reverse zymogram technique using gelatin-containing gels. This was carried out by incubating the gelatine-containing SDS PAGE gel after protein separation in a trypsin-containing proteolytic buffer. Addition of trypsin to the proteolytic buffer degrades gelatine and all seed-derived proteins with the exception where serine protease inhibitor activity has blocked trypsin action and inhibitor containing proteins bands are not distained (remaining blue) after de-staining. Reverse zymogram analysis revealed four putative serine inhibitors activities (see Figure 33A and B). All four activities of possible serine protease inhibitors could, however, only be detected in mature seeds and not in maturing green seeds (see Figure 29C).

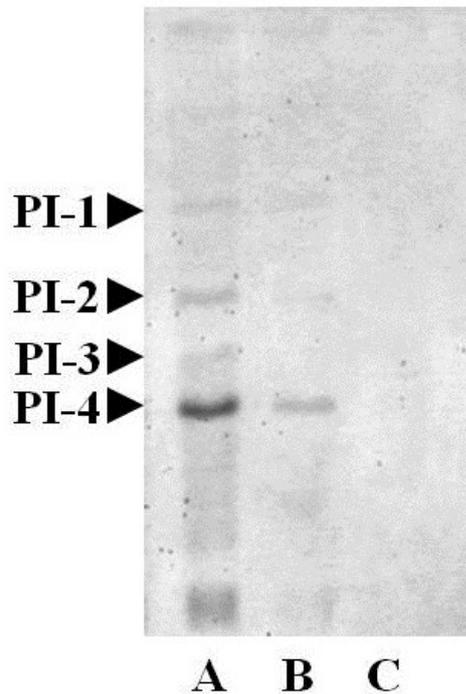


Figure 33: Marama Reverse zymogram indicating location of putative serine protease inhibitor activity indicated by arrows on gelatine containing SDS-PAGE gel after treating the gel with the serine protease trypsin. (A and B) Inhibitors identified in mature seeds with a dilution factor of 1/5 (A) and dilution factor of 1/10 (B) and (C) represent activity of green seeds.

4.5.2. Fluorometric measurement of serine proteinase activity

Total serine protease activity in different legume seeds was determined using the fluorogenic substrate Bz-Arg-MCA (see Figure 34). The control contained 0.2 $\mu\text{g}/\mu\text{L}$ trypsin (EC. 3.4.12.4, TPCCK treated from bovine pancreas), with Bz-Arg-MCA as a substrate (100 μM dissolved in DMSO) and buffer Tris-HCl, pH 7-8. Crude extracts from marama, cowpea and soybean were used to test if they contain serine protease activity.

Analysis showed that trypsin activity was significantly higher ($P < 0.05$) in mature marama bean (2326 ± 356 FU mg^{-1} protein) than maturing green seeds (362 ± 73 FU mg^{-1} protein) indicating less serine protease activity in maturing green seeds. The analyses also showed lower ($P < 0.05$) serine protease activity in both cowpea (877 ± 138 FU mg^{-1} protein) and soybean (381 ± 36 FU mg^{-1} protein). This has shown that mature seeds, in comparison to green seeds or other legume seeds, have higher serine protease activity but also more detectable serine protease inhibitor activity than green marama seeds.

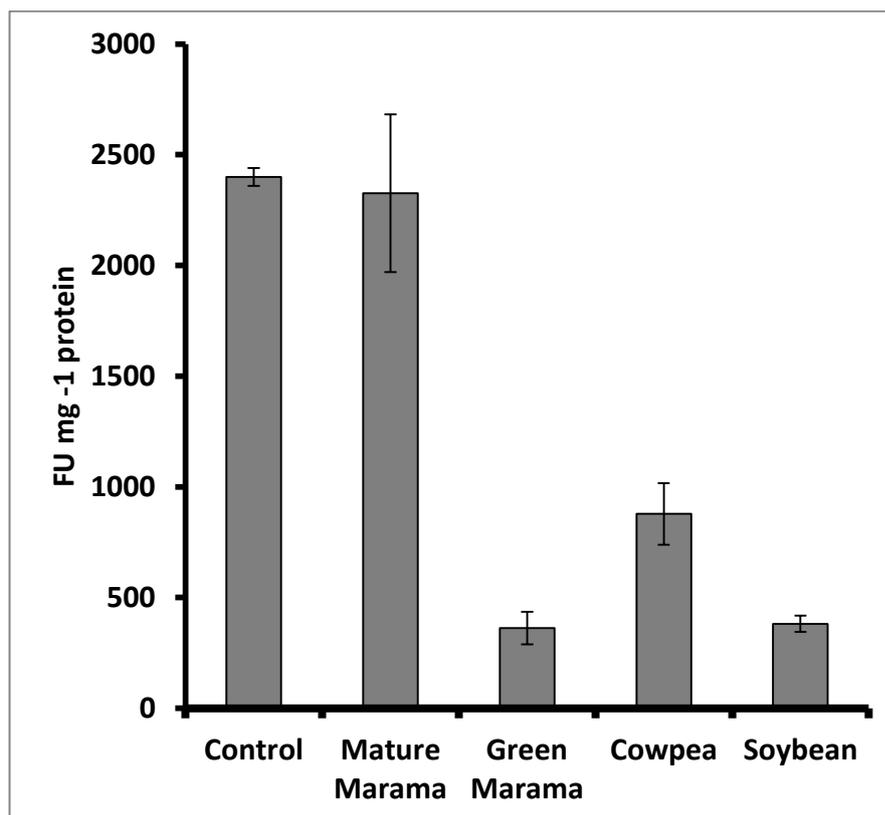


Figure 34: Trypsin activity in crude extracts of marama, cowpea and soybean. Data represent the mean \pm SD of three replicates.

4.5.3. Serine protease inhibitor coding sequence isolation

The degenerate primer combination MBBIF and MBBIR used for PCR amplification of serine protease inhibitor gene in marama bean amplified a band of approximately 500 bp (see Figure 35). The band was recovered from the gel and cloned into the pGEM-T Easy vector. Five positive clones were randomly chosen for restriction analysis and for further sequencing.

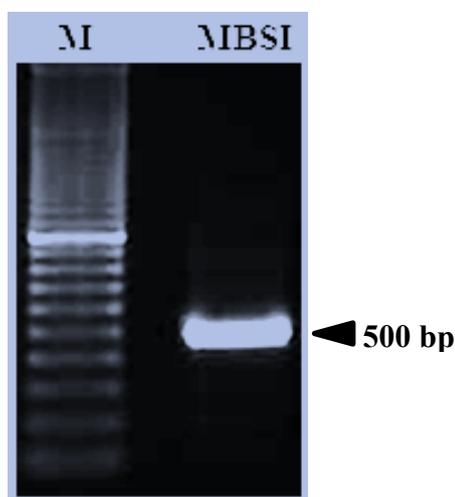


Figure 35: Isolation of a putative serine proteinase inhibitor gene from Marama bean cDNA by RT-PCR.

Restriction analysis showed a uniform restriction profile for all the five clones. All positive clones showed two *EcoRI* fragments (of about 500 and 650 bp) suggesting that there was an internal *EcoRI* site in the cDNA insert of the clones, (see Figure 36).

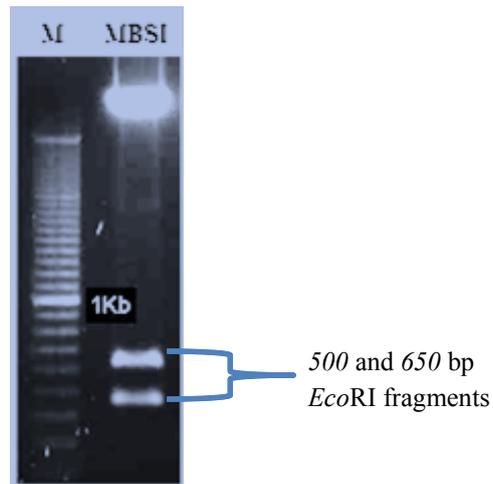


Figure 36: Confirmation of the presence of the cDNA insert released by EcoRI digestion of the pGEM-T Easy plasmid vector and analysed on a 1.5% agarose gel.

4.5.4. Analysis of marama serine protease inhibitor nucleotide sequence

A 495 bp sequence encoding serine protease inhibitor gene from marama seed was obtained after sequencing, (see Figure 37). Similarity searches of marama nucleotide sequences in GenBank entries produced a higher similarity (100%) at the nucleotide level with only the *Cowpea* trypsin Inhibitor (accession number: AY204562).

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5'-
TAAAGCACTAAATCGGAACCCTAAAGGGAGCCCCGATTTAGAGCTTGAC
GGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAG
GAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAAA
CCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTCCATTCGC
CATTACAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCG
CTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTT
GGGTAACGCCAGGGTTTTCCAGTCACGACGTTGTAAAACGACGGCCAG
TGAATTGTAATACGACTCACTATAGGGCGAATTGGGCCCCGACGTCGCATG
CCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAGAATGACAAGAATTA
AGTCTTGTGTCAGAAGATTCAGAAGGTTTCATCAATCACTAGTGAATTCGCGG
CCGCC

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Figure 37: A 495 genomic sequence encoding a mature marama serine protease inhibitor gene.

CHAPTER 5: DISCUSSION

5.1. Physicochemical properties of marama tuber starch

In this study, the physicochemical properties of starch within the tuber of *T. esculentum* are being reported for the first time. To the researcher's knowledge, no study has investigated the starch content and its composition within the tuber of *T. esculentum* and therefore, the starch content, G6P content and amylose content were determined. Isolated starch from marama tuber presented a white colour (Figure 9). Colour is an important criterion in evaluating starch usage as any form of pigmentation on starch will adversely affect its acceptability and that of its products (Galvez and Resurrection, 1992).

It must be remembered that starch is the most important storage material in storage organs (kernels, roots, tubers or stems) of many plant species, and is a major carbohydrate source for human and animal consumption (Ball et al., 1996). According to Bochanikova et al. (2003), starch properties depend on their molecular structures. The main parameters of the starch molecular structure include the amylose and amylopectin ratio, polymerization degree, chain length and distribution (Ball and Morell, 2003). These different parameters determine the characteristics of processing, chemistry and function of starch (Ball, van de Wal and Visser, 1998).

Extensive research has been conducted on cereal, potato, sweet potato and cassava starches due to their ready availability and wide usage in food and non-food applications. However, there is a dearth of information on structure–property relationship among legume starches due to lack of information on their physicochemical properties. Starches of cereals and tubers, identified with suitable physical and chemical properties, are used as thickeners, extenders, stabilizers, gelling agents, dietary calories, and texture modifiers in food formulations (Agunbiade and Longe, 1999). It has also been reported that legume starch pastes are comparatively more viscous than the cereal starches, indicating that they have higher resistance to swelling and rupture than the latter (Tetlow, 2006).

Concentrations of starch in marama tubers were determined using an amylase/amyloglucosidase method (McCleary et al., 1994) which hydrolyses starch to glucose. The starch content in 3 months old marama tubers was approximately 9% of the fresh weight. This compares unfavourably with industrially important starch sources such as potato tuber (15.44% of the fresh weight; Hoover, 2001), cassava roots (18.1-23.4% of the fresh weight, Defloor, Dehing and Delcour, 1998) and maize (65% of the dry weight, Morell and Myers, 2003). It should be considered, however, that the higher starch contents in those species are the result of many years of breeding for increased starch, so it is likely that increases could also be attained in marama bean. As the tuber matures the amount of starch present in marama tubers may also increase and experiments are currently underway to examine this.

Although the reported marama bean tuber starch content is lower than traditional starch crop plants it is worth noting that there is lower agricultural input required for marama bean cultivation. Marama bean is a wild-growing species, which requires little maintenance and the soil in regions where it grows may well be shared with other revenue generating crops. As such it may well be that, despite the lower starch content that is isolated from the tubers may be economically competitive with starches from other sources.

The amylose content for marama tuber starch determined in this study was 35%, higher than that reported for potato (18.7%) (Lloyd et al., 1999) and 19.1% for sweet potato (Collado, Mabesa and Corke, 1999), but within the range (10-38%) of amylose content of native tuber starches (Hoover, 2001). This is also in line with amylose contents for legume starches (32.5-35.6%) reported by Singh, Nakuara, Inouchi and Katsuyoshi, (2008). Amylose and amylopectin content plays an important role in influencing the functional properties of starches. High amylose starches are characterized by their high gelling strength which suggests their usefulness in the production of pasta, sweets, bread and in the coating fried products (Vignaux et al., 2005). High amylose mutants in various species such maize (maize amylose extender) and potato (potato antisense) are produced by decreased branching enzyme, but they generally have reduced starch so are un-economic.

Higher amylose content of marama starches could as well allow it to be used in food applications that often require high amylose gel-forming starches. Nevertheless, the significance of differences in amylose content mentioned above could not be commented on due to the difference in methods and other equipment used by investigators.

Root and tuber starches contain significant amounts of covalently bound monophosphate esters (Blennow, Engelsen, Nielsen, Baunsgaard and Mikkelsen, 2002). In this study the phosphate monoester content on the 6-position of the glucose monomer within marama tuber starch was determined after acid hydrolysis. The level amounts of Glc-6-bound phosphate was lower than that of potato (7.8 -33.5 nM Glu-6-P/ mg starch), cassava (2.5 nM Glu-6-P/ mg starch), and *Maranata arundinacea* (Arrow root) (4.5 nM Glu-6-P/ mg starch) observed by Blennow, Bay-Smidt, Olsen and Møller, (2000). However, the figure obtained in this study is higher than those reported for cereal starches, such as barley and maize (0.0 nM Glu-6-P/ mg starch) (Blennow et al., 2000).

The starch phosphate content has been reported to be influenced by growing conditions and temperature and varies with the botanical origin of the starch (Nielsen et al., 1994). Low starch phosphate content of marama tuber could be influenced by the arid growing condition, higher temperature and low amount of phosphate in the soil.

The presence of phosphate groups in starch is known to confer increased hydration capacity of starch pastes after gelatinization (Blennow et al., 2002) and the starch-phosphate content is correlated to starch-paste peak viscosity and gel-forming capacity (Blennow et al., 2002). For industrial uses, chemical modification of cereal starches in order to incorporate phosphate is a requirement because it prevents crystallization and affects the viscosity of the final product (Ellis et al., 1998). Starch crops with increased phosphate content are therefore desirable because a high natural degree of phosphorylation avoids or reduces expensive and environmentally unfriendly industrial chemical processes.

The heating of a starch-water dispersion under shear above its gelatinization temperature yields starch pastes. The pasting profiles of a starch is an effective method for relating starch functionality with its structural features and access the potential industrial application in products dependent on the viscosity and thickening behaviour of starch. The marama tuber starch pasting temperature was higher than that observed for commercial potato starch. Pasting properties may be influenced by factors such as degree of branching of amylopectin and starch granule structure (Kim, Wiesenborn, Lorenzen and Berglund, 1996). The peak viscosities observed for marama tuber starch was higher than that observed for potato. The peak viscosity is an indicator of water binding capacity and ease with which the starch granules are disintegrated and often correlated with the final quality of the end product (Ragae and Abdel-Aal, 2006). Higher peak viscosity is an indication that more starch has been gelatinized during processing (Suhendro, Kunitz, Mcoonough, Rooney and Waniska, 2000).

The hot paste viscosity of potato starch samples was comparable, with marama tuber starch showing a higher value. A higher hot paste viscosity represents low cooking loss and superior eating quality in food products (Cruz, Abraão, Lemos and Nunes, 2013). The breakdown viscosity of marama tuber starch was higher than that of potato starch. A lower breakdown value suggests the stability of starches under hot conditions. The breakdown viscosity is a result of the disintegration and alignment of gelatinized starch granules due to the continuous shear stress and higher temperature, thus indicating the shear thinning property of starch (Yadav, B.S. Yadav, R.B. and Kumar, 2011).

Marama tuber starch showed higher value of cold paste viscosity or final viscosity. A higher final viscosity relates to the stability of the cooled, cooked starch paste to shear and it is due to the formation of an amylose network resulting in gel structure (Miles, Morris, Orford and Ring, 1985). Setback viscosity is a measure of retrogradation of starch after cooling of the cooked starch paste. As a measure of the degree of re-association during cooling among the starch molecules involving amylose (Charles, 2004), leached from swollen starch granules, it is used as a measure of the gelling ability of starch (Singh et al., 2008). The higher value of setback in marama may be the result of higher amylose content as the linear amylose molecule favours more intermolecular hydrogen bonding than amylopectin, which has a branched structure. Marama tuber starches possible application could, therefore, exist in the food industry as its gelling property is double that of potato starch.

Electron micrographs of marama tuber starch granules (Figure 11) showed that they are round, elliptical and oval presenting a smooth surface and no fracture being observed. The granules had an estimated diameter ranging from 6 -22 μm . These observations are in accordance with previous results obtained for starch granules isolated from legume species such *C. sativa* (Cruz et al., 2013) and *P. sativum* (Wang, Sharp and Copeland, 2011). Starch granule size plays a significant role in influencing the pasting parameters of starches and may have influence on the physicochemical and digestibility properties of starch (Kaur, Sandhu and Lim, 2010). Fine starch granules could be used as fat substitutes in high fat foods (Wang et al., 2011). The variation in the size and shape of starch granules is attributed to the biological origin (Svegmark and Hermansson, 1993). The morphology of starch granules depends on the biochemistry of the chloroplast or amyloplast, as well as physiology of the plant (Badenhuizen, 1969). Few clear links between starch structure and physical properties have been found (exceptions being high amylose and high phosphate). As such, a further, detailed characterisation of chain length and amylose size in marama bean may help to explain the physical properties.

5.2. Isolation of a gene encoding starch biosynthesizing enzyme SSSI from cDNA library constructed from *T. esculentum* tuber

Complementary DNA library construction and analysis is considered as an indispensable tool for functional genomic analysis. Construction of a good cDNA library is the prerequisite for the isolation of full-length cDNA by screening. There are many advantages to construct a cDNA library, for gene cloning.

Firstly, a cDNA library is an array of DNA copies of an mRNA population that are propagated in a cloning vector and usually maintained in *E. coli*. Therefore, cDNA library screening is considered as a useful technique for the isolation of the full-length cDNA from a particular tissue or time in which the gene is highly expressed. Secondly, the cDNA library can be phagemid library or plasmid library. Thirdly, since a cDNA library contains most mRNA molecules expressed in the organs, many different genes can be screened from one cDNA library.

Since starch synthesizing enzymes in one organ may contain many isoforms encoded by different genes, to construct a cDNA library will be a best method to isolate these starch synthesizing enzyme genes. There are three chief aspects that identify the quality of a cDNA library. According to Clareke-Carbon's formula, a good cDNA library should contain at least 1.7×10^5 independent clones to ensure that the 99% low abundance mRNA would be present in the library (Liu et al., 2009). The high recombination efficiency is another index of good quality library (Wu et al., 2007, Al-Taweel, Dilantha Fernando and Brûlé-Babel, 2011). The third aspect is that the average length of inserted cDNA should be no less than 1.0 kb to ensure the integrity of cDNA.

In this study cDNA library was constructed from marama tuber and the primary library, constructed in pENTRTM 222, contained a titer of approximately 1.5×10^6 cfu. This should, therefore, be a good representative as it exceeded the general guideline of 1×10^6 clones which ensures sufficient transcript coverage and good quality library (Ying, 2003).

Therefore, the cDNA library has been used for preliminary screening for starch synthase in the study. The properties of cDNA library are that a good cDNA library should be large enough to contain representatives of all sequences of interest, some of which may be derived from low-abundance mRNAs. The clones in a good cDNA library should contain a minimal number of small cDNA inserts (often defined arbitrarily as 500 bp), whilst most cDNA inserts should be near full-length copies of the mRNA molecules from which they were derived.

The cDNA library constructed from marama tuber in this study is considered as a good cDNA library as it met the properties for a good cDNA library. The titer of the primary cDNA library constructed was approximately 1.5×10^6 cfu. The library has average insert size of approximately 1.5 kb and sizes ranged from 0.4 kb to 3.4 kb, with 90% of the sequences exceeding 1 kb. The average insert size of 1.5 kb matches the theoretical average size of eukaryotic cDNAs (Ohara and Temple, 2001). Therefore, all criteria of a good cDNA library are satisfied, as a good cDNA library should guarantee at least 1×10^6 cfu of primary clones and 87% recombinant clones, and contain an average insert size of at least 1 kb. Starch synthases are GT-B-fold glycosyl- transferases classified within family GT5 in the CAZy database (Coutinho, Stam, Blanc and Henrissat, 2003; <http://www.cazy.org>). They catalyse the addition of glucose from ADP-glucose to the non-reducing end of growing malto-oligosaccharide (MOS) chains exclusively via α -1, 4-glycosidic linkages with retention of the configuration of the transferred sugar (Zeeman et al., 2010). Therefore, Starch synthase genes are responsible for the bulk deposit of the sugars in the starch granules.

Multiple starch synthase genes have been reported in different plant species (Ball and Morell, 2003). Five starch synthase families are recognized in plant species namely GBSS, SSSI, SSSII, SSSIII and SSSIV (Smith, Denyer and Martin, 1997). Among these gene families, none has been characterized in marama. In this study, a new cDNA clone was isolated and characterized from marama tuber cDNA library. Employing cDNA library construction and screening strategies allowed the isolation of the first cDNA which encoded an SSSI from *T. esculentum*. In order to establish the presence of SSS in marama, a cDNA expression library from marama tuber was screened in *E. coli* G6MD2::pACAG cells. In this study, plasmid pACAG was used to complement a mutant of *E. coli* (G6MD2; Schwartz 1966) which carries a chromosomal deletion for all of the *glg* genes.

The mutant was transformed with a plasmid carrying a mutated form of *glgC* with altered allosteric properties (*glgC16*) (Creuzat-Sigal, Latil-Damotte, Cattaneo, Puig, 1972) in order to constitutively supply the SSS with substrate. Cells transformed with the plasmid containing the SSS cDNA gave a heavy blue staining with iodine after growth on glucose containing media, whereas cells transformed with an empty plasmid did not stain at all. The result was in agreement with Kossmann et al. (1999), that bacteria grown on a glucose rich media accumulate linear glucans when an active form of starch synthase is expressed and stain dark blue when exposed to iodine. This was an indication that there was functional starch synthase gene in marama tuber expression library. Sequencing of clones which showed the expression of an active form of starch synthase resulted in a cDNA clone with an insert size of 678 bp.

Alignment of the SSSI protein sequence with that of other SSSI species shows high identity and similarity, especially with potato SSSI reported by Kossmann et al. (1999). Comparison of the predicted amino acid of cDNA in comparison with those of the SSSI proteins from other plant species and glycogen synthases demonstrated similarity in amino acid sequences of the proteins. The marama SSSI protein showed 100% similarity with *S. tuberosum*, 77% similarity with *M. truncatula* SSSI, 75% with *C. arietinum* and *G. max*. These results were also reflected in the phylogenetic relationships inferred from NJ tree constructed from the sequence alignment. The phylogenetic tree shows the proteins were classifiable into two major groups, monocots and dicots. The cloned marama SSSI clustered together with other dicot plants than monocots hence elucidating a monocot/dicot divergence in the evolution of angiosperms. This finding is consistent with the overall genetic differences between dicot and monocots as revealed in the Angiosperm Phylogeny Group classification (APGIII, 2009).

Multiple alignment of SSSI amino acid sequences from varieties of plant species indicated that marama SSSI protein sequence contained two conserved regions common to all other known starch synthases and *E. coli* glycogen synthase (van der Leij et al., 1991; Baba et al., 1993). In the first conserved region, highly conserved sequence PSRFEPGLNQLY was present and STGGLRDTVKDFN sequence was present in the second conserved region. These results indicate that the SSSI detected in the present study encodes a functional starch synthase enzyme.

The roles of these conserved regions for enzymatic function are not certain, since the function of SSSI in plants during starch synthesis process remain to be determined (Furukawa, Tagaya, Inouye, Preiss and Fukui, 1990). This is the case because; there is no mutant or transgenic plant with a convincing phenotype on the function of SSSI in starch metabolism that has ever been described in detail (Knight et al., 1998; Li et al., 1999).

Soluble starch synthase I, however, maybe essential for the synthesis of normal amylopectin as reported by Dauvillée et al. (2005) in *Arabidopsis* leaves. The absence of SSI in *Arabidopsis* leads to a deficiency in the number of the shorter glucans of amylopectin assumed to define the A (most outer chains of amylopectin) and B1 chains (inner chains of amylopectin that belong to only one cluster) (Hizukuri, 1986). It has been suggested by Kossmann et al. (1999) that SSSI also contributes to the deposition of transient starch in leaves than the synthesis of storage starch in tubers.

5.3. Isolation and sequence analysis of genomic clones encoding AGPase large and small subunits genes from *T. esculentum*.

Screening of genomic or cDNA library by a DNA probe or antibody, however useful for isolating genes, is tedious, laborious and costly in comparison to the PCR technique. The PCR technique was used to directly amplify *T. esculentum* partial AGPase genes in this study.

To date, *T. esculentum* whole genome has not been sequenced and the potential relationship between starch property and starch biosynthesis enzymes in *T. esculentum* is still unknown. Therefore, it was imperative to isolate and analyse genomic sequences of two gene isolates coding for large and small subunits of AGPase in *T. esculentum*. The two partial isolated genes may have significant genetic effects on starch traits in marama. AGPase in higher plants is thought to be composed of two different classes of subunit, large and small, forming together a heterotetramer (Weber, Heim, Borisjuk and Wobus, 1995). Sequences coding for AGPase small and large subunits have earlier been isolated through PCR based method from several plants such as maize (Bhave, Lawrence, Barton and Hannah 1990), potato (La Cognata, Willmitzer and Muller-Rober, 1995), sugar beet (Muller-Rober, Sonnewald and Willmitzer 1995), wheat (Olive Ellis and Schuch, 1989), barley (Villand et al., 1992), Arabidopsis (Villand, Olsen and Kleczkowski, 1993), sweet potato (Harn Bae, Lee, Min and Liu, 2000), and tomato (Park and Chung, 1998).

Similarly, several genes encoding AGPase have previously been isolated from legume plants such as *C. arietinum* (Singh, Choi, Modi and Okita 2002), *P. sativum* (Burgess, Penton, Dunsmuir and Dooner, 1997) and *Vicia faba* (Weber et al., 1995). The AGPase nucleotide sequences and predicted amino acids obtained exhibited significant homologies with other cloned AGPase proteins from other plant species and more specifically with legumes. Small subunit showed higher homology (86.49-96.40%) than large subunit (74.04-94.17%).

Such homology between different subunits suggests that these two genes might have evolved from a common ancestor, most probably by a gene duplication event (Smith-White and Preiss, 1992). This is in agreement with investigations carried out by Smith-White and Preiss, (1992) when they reported that large subunits from different organisms show low homology to each other, while small subunits from different organisms exhibit high degree of similarity. This result is also consistent with genetic difference between dicot and monocots AGPase large and small subunits that sequences of small subunit reveal a higher homology to each other than larger one and can be further distinguished with respect to their origins from either monocot or dicot plants. (Georgelis, Braun, Shaw and Hannah, 2007).

Therefore, small subunit from a monocot source has been found to be slightly similar to small subunit from another monocot and not from a dicot. The latter, however cannot be said for the large subunit proteins from different organisms which are known to show low homology to each other (Muller-Rober et al., 1995). The isolation of two different groups of genomic DNAs encoding the AGPase enzyme may indicate that in marama, the AGPase enzyme is composed of two distinct subunits as previously shown for maize (Denyer et al., 1996), barley (Thorbjørnsen, Villand, Denyer, Olsen and Smith, 1996) and rice (Sikka et al., 2001).

Although only one type of genomic DNA clone corresponding to the AGPase large and small subunits were isolated, this does not exclude the possibility of isoforms of these genes being present in marama. Previous studies have reported isoforms of small and large subunits of AGPase in plant species such as maize, potato, sweet potato, bean, pulse, chicken pea, pea and *Arabidopsis* (Weber et al., 1995; Müller-Röber et al., 1992; Burgess et al., 1997, Singh et al., 2002;).

Blastp analysis of genomic clones encoding marama AGPase large and small subunit revealed putative conserved domains, the family and ADP-glucose pyrophosphorylase, a subfamily of a very diverse glycosyl transferase family 2 (Marchler-Baurer, 2011). Nucleotidyl transferase family includes a wide range of enzymes which transfer nucleotides onto phosphosugars, while ADP-glucose pyrophosphorylase is involved in the biosynthesis of starch in plants and glycogen in animals respectively. Marchler-Baurer, (2011), indicated that there is considerable homology in a number of highly conserved regions in the sequence of bacterial and plant ADP-glucose pyrophosphorylase subunits. In the present study, a conserved domain, Nucleotidyl transferase domain was found in the deduced peptide sequences of marama AGPase. The deduced peptide sequences of marama AGPase had three highly conserved regions: the Adenosine triphosphate binding site (ATP binding motif), catalytic site and Glucose-1-phosphate binding site (Glc-1-p site). Similar sites have been reported in potato (Frueauf, Ballicora and Preiss, 2001), maize (Georgelis et al., 2007) and common bean (Weber et al., 1995). This suggests that the deduced marama proteins belong to Nucleotidyl transferase family and ADP-glucose pyrophosphorylase sub family of glycosyl transferase 2.

Phylogenetic relationship inferred from NJ trees constructed from sequence alignment indicated that deduced proteins were classifiable into two major clusters, dicots and monocots. This suggested that the sequences and their functions may have diverged among plant AGPase large and small subunits. These results are consistent with overall genetic differences between dicot and monocots as reflected in the Angiosperm Phylogeny Group classification (APGIII 2009), which represents a monocot and dicot divergence in the evolution of angiosperms. Marama AGPase large and small subunits clustered together with other legume species under the dicot cluster, which reflects the difference of starch biosynthesis between two plant lineages.

This implies that marama is evolutionary similar to other legumes in terms of starch biosynthesis pathway. AGPase genes have been employed in efforts to increase starch accumulation in plants. For example, a mutant variant of the AGPase gene from *Escherichia coli* (glgC16) has been used for overexpression in transgenic potatoes (Zeeman et al., 2010). This has resulted in some transgenic potato lines having up to 60% more starch in tubers on a fresh-weight basis compared to control plants (Stark et al., 1992). However, manipulating AGPase alone may not be the most promising strategy for increasing starch contents in plant storage organs (Smith, 2008). There is good evidence that increasing the supply of ATP to the plastid can stimulate the production of ADP-glucose and hence the rate of starch biosynthesis in all organs.

5.4. Isolation and sequence analysis of genomic clones encoding SBEs genes from *T. esculentum*

Starch branching enzymes (SBEs) play a pivotal role in amylopectin biosynthesis by catalysing chain transfer by cleavage of an α -1, 4 linkage following a condensation of an α -1,6 linkage (Hamada et al., 2001). Starch-branching enzymes also play important roles in the synthesis of amylopectin. In the past decades, several isoforms have been isolated from both monocot cereals, such as in cereal grains such as maize (Gao et al., 1997), barley (Sun, Sathish, Ahlandsberg and Jansson, 1998), and wheat (Repellin, Båga and Chibbar, 2001) and in dicot plants including pea (Burton et al., 1995), kidney bean (Hamada et al., 2001), potato (Larsson et al., 1996) and *Arabidopsis* (Fisher, Gao, Kim, Boyer and Gultinan, 1996). A gene encoding starch branching enzyme (SBEI) was firstly isolated from pea embryo (Bhattacharyya, Smith, Ellis, Hedley and Martin, 1990).

In this study genomic DNA encoding SBEs was isolated from marama genomic DNA using degenerate primers which were designed based on conserved domains of SBEIs and SBEIIs. Genomic DNA encoding SBEs have been reported from plants, including rice (Kawasaki Mizuno, Baba and Shimada 1993), maize (Kim et al., 1998), wheat (Båga, Glaze, Mallard and Chibbar, 1999) and apple (Han et al., 2004). Genes encoding the two SBE families have been identified from several plant species (Kawasaki et al., 1993; Burton et al., 1995; Kim et al., 1998; Larsson et al., 1998; Båga et al., 1999). The isolated marama SBEI and SBEII gene clones in this study were similar to those of other plant starch branching enzymes.

On alignment of SBEs sequences from different plant sources including marama SBEI, a catalytic α -amylase conserved domain was found in the amino acid sequences. Motif “AESHDQ” together with other three motifs is highly conserved in all SBEs, and function in the active site of α -amylase and their postulated catalytic groups (Jespersen et al., 1993). A region with the reported consensus sequence P/EQXLPS/NGKF/II/VP is conserved in SBEIIs but not in SBEIs (Burton et al., 1995) and has been inferred that this distinction between the two families is a major reason for their different enzymatic activities (Martin and Smith, 1995). Deduced amino acid sequence encoding SBEII for marama also showed region GDQYLPNGTVI corresponding to the SBEII-specific sequence P/EQXLPS/NGKF/II/VP. Thus, the isolated SBEII for marama belongs to SBEs family A.

The phylogenetic tree revealed that angiosperms SBE sequences are grouped into two super clusters designated, Family A. Family A, which contained SBEIIs and family B which was composed of SBEIs. These results indicated that the ancestor of SBEI and SBEII genes should have duplicated, prior to the divergence of monocots from dicots (Han et al., 2004), with a divergence date between monocots and dicots inferred to be 200 million years based on multiple gene molecular clocks using two landmark events and nucleotide sequences from protein-coding genes of the mitochondrial genome (Laroche, Li and Bousquet, 1995). Furthermore, SBE sequences from monocots were separated from those of dicots in both SBEI and SBEII clusters, resulting in two monophyletic sub clusters within each cluster.

These results suggest that both SBEI and SBEII genes have separated following the divergence of monocots from dicots. These results further support that the isolated SBEs gene fragments belongs to family A and B. Conserved catalytic regions found in the two gene clones encoding SBEs in marama are matching to those found in other plant SBEs. Catalytic properties of the SBEI and SBEII isoforms differ, and it has been concluded that SBEIIs catalyse the formation of amylopectin with shorter branch chains than SBEIs (Martin and Smith, 1995). In-vitro investigations of purified maize SBEI and SBEII, it was demonstrated that SBEI transfers long branches and SBEII short branches during amylopectin synthesis (Guan and Preiss, 1993).

5.5. Detection of serine protease inhibitor activity and isolation of serine proteinase coding sequence from *T. esculentum* seeds.

Proteolytic enzymes and their inhibitors are known to be involved in a wide variety of cellular metabolic functions (Michaud, 1998) ranging from the one control of protein catabolism to the selective degradation of foreign proteins and the bulk hydrolysis of dietary proteins (Wolf, 1992). The implication of proteolytic enzymes in plant-pest and plant-pathogen interactions is well well-known and control strategies based on their inhibition with protease inhibitors have been proposed for the protection of plants against herbivorous pests (Ryan, 1990), pathogenic fungi (Lorito et al., 1994) and parasitic nematodes (Atkinson, Urwin, Hansen and McPherson, 1995).

In humans, proteolytic enzymes are imperative not only in the hydrolysis of dietary proteins, but also in the regulation of various cellular functions and the development of several diseases, including tumor metastasis (Bode and Huber, 2000), rheumatoid arthritis (Oliva et al., 2010), Alzheimer's disease (Eriksson, Janciauskiene and Lannfelt, 1995), emphysema (Chapman, Stone and Vavrin, 1984) pancreatitis (Steer, Meldolesi and Figarella, 1984), and infectious diseases like AIDS (Kaplan and Wanstrom, 1991).

Given the therapeutic potential of protease inhibitors, in this study marama bean seed has been employed as a source for the detection of natural product, specifically the serine protease inhibitor which may have a possible application potential in the cosmetic or pharmaceutical industry to block in particular elastase activity. Assumption that marama bean might be a source for unique serine protease inhibitors is based on the findings that a serine protease inhibitor was identified in marama bean representing about 10.5% of total protein (Starcher, Bryant and Elfant, 1986).

A reverse zymogram technique was used as an effective tool for detection of natural marama serine protease inhibitors activity. Reverse zymogram screening for marama dry and green seeds showed distinctive putative serine protease inhibitor activity on a protease activity gel indicating inhibition of serine protease activity. The bands were not found when green seeds were analysed. However, there is no further evidence so far that these bands correspond to serine protease inhibitors in marama seeds.

This result has shown that mature dry seeds, in comparison to maturing green seeds or other legume seeds, have higher serine protease activity but also more detectable serine protease inhibitor activity than green marama seeds. An increased protease activity has been found at complete development and physiological maturity in soybean seed (Pladys and Vance, 1993). In cowpea, gene expression and maturation from onset of germination to the transformation of the dormant state of the seed to the vigorous metabolic state has been reported to increase protease inhibitory activity in the seed (Gatehouse A.M.R, Gatehouse, J.A and Boulter, 1980).

A cDNA gene portion encoding serine protease inhibitor for marama bean seeds has been isolated and showed higher homology to Cowpea Bowman-birk like trypsin protease inhibitor. The obtained high sequence homology matched that reported for *G. max* (98%) and *Glycine soja* (99%) reported by Wang, Chen and Qiu, (2008). To the researcher's knowledge, this is the first fragment of Bowman-birk like trypsin inhibitor for marama identified, allowing ultimately establishing a database for marama serine proteinase inhibitors for phylogenetic analysis and also possibly protein inhibitor's production. A Kunitz type serine protease inhibitor from marama has been isolated and partially characterized by Nadaraja et al. (2009). The inhibitor has been reported to be in large quantities and specific for inhibition of pancreatic and neutrophil elastase. Kunitz inhibitors are considered important for plant defense against insects (Oliva et al., 2010), while Bowman-birk inhibitors have been considered for their anti-tumoral activity.

Trypsin inhibitors of *Peltophorum dubium* and soybean have been reported to induce cell death of human leukemic Jurkat lines by caspases 3 and 8 activation (Troncoso, Biron, Longhi, Retegui and Wolfenstein-Todel, 2007). The inhibitor from the seeds of Chinese black soybean *Glycine max* has been reported to suppress cell proliferation of MCF-7 breast cancer cells and HepG2 hepatoma cells (Ye and Ng, 2009). Peptides derived from *B. rufa* Trypsin Inhibitor have been reported to inhibit the process of tumor cells adhesion to extracellular matrix glycoproteins (Bode and Huber, 2000). Marama bean may as well have potent serine protease inhibitors which could be utilized in pharmaceutical and in human health.

5.6. Unique contribution to the body of knowledge of Science

Until recently, the basic knowledge of the physicochemical and functional properties of marama tuber starch is has not been reported and documented. This study makes known for the first time the physicochemical and functional properties of native tuber starch and makes a possible provision for a new starch source. It is hoped that by further exploring the potential of marama starch as a raw material, it can be applied in various applications in both industry and food processing that will produce high valued products. Although starch and starch biosynthesis have been researched for several decades in other plants, the research of starch biosynthesis especially in marama is almost non-existent. The analysis of starch biosynthesis indicates that some starch properties are controlled by the enzymes involved in starch biosynthesis pathway.

Therefore, to analyze marama starch at the molecular level, it is important to isolate and characterize the genes encoding starch synthesizing enzymes in marama. From these analyses, very useful information on the characterization of marama SSSI, AGPase and SBEs sheds light on the molecular mechanisms which may confer unique properties to marama starch. This thesis research on the isolation and characterization of SSSI, AGPase and SBEs is the first investigation of marama starch at the molecular level. This information contributed to the pool of already isolated and characterized higher plants starch biosynthetic genes, which may assist in underpinning the starch biosynthesis pathway in plant species such as marama bean where it is still not understood. The information on the genes and the enzymes they encode may facilitate further research on starch polymer biosynthesis and starch granule formation in marama. The isolation of starch synthesizing enzyme genes from marama is only the first step in understanding starch biosynthesis in this species.

Serine protease inhibitors from legume seeds have been characterized in recent years. Such studies can provide important tools for understanding important physiological and pathological phenomena leading to a better understanding of the mechanism where proteolysis is involved. The detection of serine protease inhibitors activities in marama seeds provides for the first time fundamental information to facilitate their manipulation as a source of possible development of pharmaceutical products. The isolated serine protease inhibitor gene will broaden the pool of known plant serine protease inhibitor genes with possible potent serine inhibitor which to the researcher's knowledge, neither detection nor characterization of any serine protease inhibitor gene family in marama has been carried.

CHAPTER 6: CONCLUSION

Native marama tuber starch reported in this study presented different physicochemical and functional properties to those previously reported. The relatively high proportion of amylose present in the starch and their interesting functional properties make it a possible industrial source of starch. Native marama tuber starch presented a pasting profile similar to potato starch, but with higher pasting gelatinization and peak viscosity temperature. This makes native marama tuber starch a potential technological alternative to potato starch especially in application needing higher pasting gelatinization.

In the present study, a cDNA library from marama tuber was constructed and screened for starch synthase genes. This is the first report of isolating SSSI gene from marama through cDNA library construction and screening. The SSSI gene in marama may possess specific function in the biosynthesis of starch. The isolation of the cDNA encoding soluble starch synthase will allow for informative studies of the structure and functional relationship of this protein and also for a more detailed examination of its role in starch biosynthesis in marama tuber. Similarly, in this study, the first AGPase gene clones from marama were isolated, characterized and described by comparing them to other plant AGPase gene like sequences. This information will provide useful information for understanding the mechanism of marama starch synthesis and provides a theoretical foundation for regulating the gene to increase starch content and quality.

This study paved a way for further characterization of AGPase in marama in order to establish whether the gene has similar functional structure to already characterized plant AGPase genes. The study also reported for the first time on starch branching enzymes from marama. SBEs could be playing a crucial functional role in amylopectin synthesis in marama which needs to be established. This could provide a foundation for modifying starch from marama through gene regulation. For example, Mutant lines that lack SBEIIb activity in maize and barley induce greater amylose content in comparison with normal starches. Suppression of SBEII gene expression may be as well capable of inducing amylose content of starch in marama. However, this assumption needs to be verified in future studies.

Finally, the detection of serine protease inhibitor activities in marama seeds provide for the first time fundamental information to facilitate their manipulation as a source of potent inhibitors with possible application in pharmaceutical system. At the same time, marama serine protease inhibitor sequence from maturing seeds has been amplified and sequenced from a marama cDNA library although such inhibitor activity was not detected on a zymogram. As a new result, mature seeds had higher serine protease activity in comparison to maturing marama seeds or soybean and cowpea seeds. To the researcher's knowledge, this is the first fragment of serine protease inhibitor sequence from marama identified allowing ultimately establishing a database for marama serine proteinase inhibitors for phylogenetic analysis and also inhibitor protein production.

CHAPTER 7: RECOMMENDATIONS

The potential of marama tuber starch has not been utilized due to lack knowledge of processing technique and product development. Future studies should focus on transforming marama tuber starch into a value added product. In this form the application of such material can be broadened either in food or non-food industries. Investigation into the potential application of marama bean tuber starch as a thickener in food would be prudent in the future. Further information on the characterization of physical, chemical and functional properties of marama bean starches needs to be made available, information which will help in establishing whether it is useful for specific food or industrial utilisation. For example, most commercial companies when examining functional properties in starch compare the characteristics of competitive starches for particular applications. For those characteristics which are unattainable with native starches, the alternative is to look towards some form of value-addition to achieve the desired results. An improved understanding of the production and utilization of this material is vital for domestication, economic growth and viability of marama bean as a tuber crop.

Three mature portions of starch biosynthesis genes (AGPase, SBEs and SSSI) have been cloned from marama. However, amino acid data could not be considered to be the ideal for understanding the properties and structure of the three starch biosynthesis genes in marama.

Therefore, the full-length cloning of these genes in marama would be necessary in order to provide more accurate information on the proteins encoded by them. The cloning work could be carried out by designing primers specific to the three isolated genes using the currently available gene sequences from this study, to clone full cDNA using encoding marama starch biosynthesis by RT-PCR. Thus, it will be prudent to isolate and sequence these starch biosynthetic genes in marama so that additional structural and functional analyses of these genes can be conducted to provide a comprehensive understanding of these genes in marama. The constructed cDNA library needs to be screened further for other starch biosynthesis genes apart from SSSI which may be present in the tuber. This could be achieved by either using a probe or antibody specific for these genes.

It will be prudent in the future to understand the role of starch biosynthetic genes in marama starch synthesis, marama lines with altered starch debranching enzyme activities must be produced using genetic engineering techniques. For instance, marama lines with reduced SSSI activity could be obtained by introduction of the marama cDNA, as characterized in this study. Expression analysis of these genes needs to be established in the future, as this will provide fundamental information to facilitate their manipulation in the development of a marama plant with higher starch content or altered starch functional properties.

Screening of protein extracts from mature and green marama beans for inhibitor activity against the serine proteases chymotrypsin and elastase using reverse zymogram technology and establishment of inhibitor profiles need to be established in future work. It would be interesting to study in the in the future any reason for the higher serine protease activity in mature seeds than in green seeds as reported in this study.

It will be vital to perform protein sequencing to determine the size of proteins visualized by the reverse zymogram experiment for the three serine proteases. This will require performing the zymograms for trypsin, chymotrypsin and elastase under sterile conditions to minimize contamination followed by the reduction of bands of interest and alkylation in order to denature the proteins and hopefully allow subsequent trypsin digestion. A liquid chromatography mass spectrometry (LCMSMS) based peptide sequencing could be performed for subsequent protein identification and size determination.

REFERENCES

- Abel, G. J. W., Springer, F., Willmitzer, L., and Kossmann, J. (1996). Cloning and functional analysis of a cDNA encoding a novel 139 kDa starch synthase from potato (*Solanum tuberosum* L.). *Plant Journal*, 10,981-991.
- Agunbiade, S.O., and Longe, O.G. (1999). The physico-functional characteristics of starches from cowpea (*Vigna unguiculata*), pigeon pea (*Cajanus cajan*) and yam bean (*Sphenostylis stenocarpa*), *Food Chemistry*, 65, 469-474.
- Alisdair, R.F., Willmitzer, L., and Trethewey, R.N. 2002. Sucrose to starch: a transition in molecular plant physiology. *Trends in Plant Science*, 7, 35-41.
- Al-Taweel, K.; Dilantha Fernando, W.G.; Brûlé-Babel, A.L. (2011) Construction and Characterization of a cDNA library from wheat infected with *Fusarium graminearum* *Internatinal Journal of Molecular Science.*, 12, 613–626.
- Ambigaipalan, P., Hoover, R., Donner, E., Liu, Q., Jaiswal, S., Chibbar, R., Nantanga, K. K. M. and Seetharaman, K. (2011). Structure of Faba bean, Black bean and Pinto bean starches at different levels of granule organization and their physicochemical properties. *Food Research International*, 44, 2962-2974.
- APG III. (2009). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG III. *Botanical Journal of the Linnean Society*, 161, 105-121.
- Atkinson, H.J., Urwin, P.E., Hansen, E., and McPherson. M.J. (1995). Designs for engineered resistance to root-parasitic nematodes. *Trends in Biotechnology*, 13, 369-374.

- Baba, T., Nishihara, M., Mizuno, K., Kawasaki, T., Shimada, H., Kobayashi, E., Ohnishi, S., Tanaka, K-I., and Arai, Y. (1993). Identification, cDNA cloning, and gene expression of soluble starchsynthase in rice (*Oryza sativa* L.) immature seeds. *Plant Physiology*, 103, 565-573.
- Badenhuizen, N. P. (1969). The biogenesis of starch granules in higher plants. New York: Appleton Crofts.
- Bae, J. M., Giroux, M., and Hannah, L. (1990). Cloning and characterization of the brittle-2 gene of maize. *Maydica*. 35, 317-322.
- Båga, M., Glaze, S., Mallard, C.S., Chibbar, R.N. (1999). A starch-branching enzyme gene in wheat produces alternatively spliced transcripts. *Plant Molecular Biology*, 40, 1019–1030.
- Ball, S. G., and Morell, M. K. (2003). From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. *Annual Reviews of Plant Biology*, 54, 207-233.
- Ball, S., Guan, H., James, M., Myers, A., Keeling, P., Mouille, G., Buléon, A., Colonna, P., and Preiss, J. (1996). From glycogen to amylopectin: a model for the biogenesis of the plant starch granule. *Cell*, 86, 349-352.
- Ball, S., Marianne, T., Dirick, L., Fresnoy, M., Delrue, B., and Decq, A. A. (1991). *Chlamydomonas reinhardtii* low starch mutant is defective for 3 phosphoglycerate activation and orthophosphate inhibition of ADP-glucose pyrophosphorylase. *Planta*, 185, 17-25.
- Ball, S.G., van de Wal M.H.B.J., and Visser R.G.F. (1998). Progress in understanding the biosynthesis of amylose. *Trends in Plant Science*, 3, 462-467.
- Ballicora, M. A., Laughlin, M. J., Fu, Y. B., Okita, T. W., Barry, G. F., and Preiss, J. (1995). Adenosine 5'-diphosphate-glucose pyrophosphorylase from potato tuber-significance of the N-terminus of the small-subunit for catalytic properties and heat-stability. *Plant Physiology*, 109, 245-251.

- Batey, I.L. (2007). Interpretation of RVA curves. In: The RVA Handbook. Crosbie, G.B., and Ross, A.S, eds. AACC International: St. Paul, MN.
- Beckles, D. M., Smith, A. M., and Rees, T. A. (2001). A cytosolic ADP-glucose pyrophosphorylase is a feature of graminaceous endosperms, but not of other starch-storing organs. *Plant Physiology*, 125, 818-827.
- Bhattacharyya, M.K., Smith, A.M., Ellis, T.H., Hedley, C., and Martin, C. (1990). The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme. *Cell*, 60, 115-122.
- Bhave, M. R., Lawrence, S., Barton, C., and Hannah, L. C. (1990). Identification and molecular characterization of Shrunken-2 cDNA clones of maize. *Plant Cell*, 2, 581-588.
- Birk, Y. (2003). Plant Protease Inhibitors: Significance in Nutrition, Plant Protection, Cancer Prevention and Genetic Engineering. Springer-Verlag, Berlin.
- Birk, Y., Gertler, A., and Khalef, S. (1963). A pure trypsin inhibitor from soya beans. *Biochemistry Journal*, 87, 281-284.
- Blauth, S. L., Kim, K. N., Klucinec, J., Shannon, J. C., Thompson, D. and Gultinan, M. (2002). Identification of mutator insertional mutants of starch-branching enzyme 1 (sbe1) in *Zea mays* L. *Plant Molecular Biology*, 48: 287-297.
- Blennow, A., Bay-Smidt, A.M., Olsen, C.E., and Møller, B.L. (2000). The distribution of covalently bound phosphate in the starch granule in relation to starch crystallinity. *International Journal of Biological. Macromolecules*, 27, 211-218.
- Blennow, A., Engelsen, S.B., Nielsen, T.H., Baunsgaard, L., and Mikkelsen, R. (2002) Starch phosphorylation: a new front line in starch research. *Trends in Plant Science*, 7, 445-450.

- Bochanikova, I., Wasserman, L. A., Krivandin, A. V., Fornal, J., Blaszczyk, W., Chernykh, V. Ya., Schiraldi, A., and Yuryev, V.P. (2003). Structure and thermodynamic melting parameters of wheat starches with different amylose content. *Journal of Thermal Analysis and Calorimetry*, 74, 681–695.
- Bode, W., and Huber, R. (2000). Structural basis of the endo-proteinase protein inhibitor interaction. *Biochimica et Biophysica Acta*, 1477, 241-252.
- Bowman, D.E. (1946). Differentiation of soy bean anti-tryptic factors. *Proceedings of the Society for Experimental Biology and Medicine*, 63, 547-550.
- Bozonnet, S., Jensen, M.T., Nielsen, M.M., Aghajari, N., Jensen, M.H., Kramhøft, B., Willemoës, M., Tranier, S., Haser, R., and Svensson, B. (2007). The ‘pair of sugar tongs’ site on the non-catalytic domain C of barley α -amylase participates in substrate binding and activity. *FEBS Journal*, 274, 5055-5067.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248–254.
- Breiteneder, H., and Radauer, C. (2004). A classification of plant food allergens. *Journal of Allergy and Clinical Immunology*, 113,821-830.
- Buléon, A., Colonna, P., Planchot, V., and Ball, S. (1998). Starch granules: structure and biosynthesis. *International Journal Biological Macromolecules*, 23, 85-112.
- Burgess, D., Penton, A., Dunsmuir, P., and Dooner, H. (1997). Molecular cloning and characterization of ADP-glucose pyrophosphorylase cDNA clones isolated from pea cotyledons. *Plant Molecular Biology*, 33,431-444.
- Burrell, M. M. (2003). Starch: the need for improved quality or quantity-an overview. *Journal of Experimental Botany*, 54, 451-456.

- Burton, R., Jenner, H., Carrangis, L., Fahy, B., Fincher, G., Hylton, C., Laurie, D., Parker, M., Waite, D., and van Wegen, S. (2002). Starch granule initiation and growth are altered in barley mutants that lack isoamylase activity. *Plant Journal*, 31, 97-112.
- Burton, R.A., Bewley, J.D., Smith, A.M., Bhattacharyya, M.K., Tatge, H., Ring, S., Bull, V., Hamilton, W.D.O., and Martin, C. (1995). Starch branching enzymes belonging to distinct enzyme families are differentially expressed during pea embryo development. *Plant Journal*, 7, 3-15.
- Busi, M., Palapoli, N., Valdez, H. A., Fornasari, M. S., Wayllace, N. Z., Gomez Casati, D. F., Parisi, G., and Ugalde, R. A. (2008). Functional and structural characterization of the catalytic domain of the starch synthase III from *Arabidopsis thaliana*. *Proteins*, 70, 31-40.
- Cao, H., Imparl-Radosevich, J., Guan, H., Keeling, P. L., James, M. G., and Myers, A. M. (1999). Identification of the soluble starch synthase activities of maize endosperm. *Plant Physiology*, 120: 205-215.
- Castro, S., Silveira, P., Coutinho, A. P., and Figueiredo, E. (2005). Systematic studies in *Tylosema* (Leguminosae). *Botanical Journal of the Linnean Society*, 147, 99-115.
- Chapman, H.A., Stone, O.L., and Vavrin, Z. (1984). Degradation of fibrin and elastin by intact human alveolar macrophages in vitro. Characterization of a plasminogen activator and its role in matrix degradation. *Journal of Clinical Investigations*, 73(3), 806-815.
- Charles, A. L. (2004). Some physical and chemical properties of starch isolates of cassava genotypes. *Starch/Stärke*, 56, 413-418.

- Chingwaru, W., Majinda, R.T., Yeboah, S.O., Jackson, J.C., Kapewangolo, P.T., Kandawa-Schulz, M., and Cencic¹, A. (2011). *Tylosema esculentum* (Marama) Tuber and Bean Extracts Are Strong Antiviral Agents against Rotavirus Infection. *Evidence-Based Complementary and Alternative Medicine*, 2011, 1-11.
- Chiotelli, E., and Le Meste, M. (2002). Effect of small and large wheat starch granules on thermo-mechanical behavior of starch. *Cereal Chemistry*, 79, 286-293.
- Clark, J. R., Robertson, M., and Ainsworth, C. C. (1991). Nucleotide sequence of a wheat (*Triticum aestivum* L.) cDNA clone encoding the waxy protein. *Plant Molecular Biology*, 16, 1099-1101
- Close, T.J., Christmann, J.L., and Rodriguez, R.L. (1983). M13 bacteriophage and pUC plasmids containing DNA inserts but still capable of beta-galactosidase alpha complementation. *Gene*, 23 (2), 131-136.
- Coetzer, L.A., and Ross, J.H., (1977). *Tylosema* (Caesalpinaceae). *Flora Southern Africa*, 16, 61-64.
- Coetzer, L.A., Robbertse, P.J., and Grobbelaar, N. (1983). Heterostylism in the Genus *Tylosema* in Southern-Africa. *South African Journal of Botany*, 2, 248.
- Collado, L. S., Mabesa, R. C., and Corke, H. (1999). Genetic variation in the physical properties of sweet potato starch. *Journal of Agriculture, Food and Chemistry*, 47, 4195-4201.
- Commuri, P. D., and Keeling, P. L. (2001). Chain-length specificities of maize starch synthase I enzyme: studies of glucan affinity and catalytic properties. *Plant Journal*, 25, 475-486.
- Copeland, L., and Preiss, J. (1981). Purification of spinach Leaf ADP-glucose pyrophosphorylase. *Plant Physiology*, 68 (5), 996-1001.

- Coutinho, P.M., Stam, M., Blanc, E., and Henrissat, B. (2003). Why so many carbohydrate-active enzymes related genes in plants? *Trends in Plant Science*, 8, 563-565.
- Creuzat-Sigal, N., Latil-Damotte, M., Cattaneo, J., and Puig, J. (1972). Genetic analysis and biochemical characterization of mutants impairing glycogen metabolism in *Escherichia coli* K-12. In: Piras R, Pontis HG (eds) Biochemistry of the glycoside linkage. Academic Press, New York, pp 647-680.
- Cross, J. M., Clancy, M., Shaw, J. R., Greene, T. W., Schmidt, R. R., Okita, T. W., and Hannah C. (2004) Both subunits of ADP-glucose pyrophosphorylase are regulatory. *Plant Physiology*, 135,137-144
- Cruz, B.R., Abraão, A.S., Lemos, A.M., and Nunes, F.M. (2013). Chemical composition and functional properties of native chestnut starch (*Castanea sativa* Mill) *Carbohydrate Polymers*. 94, 594-602.
- Dauvillée, D., Dumez, S., Wattebled, F., Roldán, I., Planchot, V., Berbezy, P., Colonna, P., Vyas, D., Chatterjee, M., Ball, S., Mérida, A., and D'Hulst, C. (2005). Soluble starch synthase I: a major determinant for the synthesis of amylopectin in *Arabidopsis thaliana* leaves. *Plant Journal*, 43, 398-412.
- Defloor, I., Dehing, I., and Delcour, G. (1998). Physicochemical properties of cassava starch. *Stärke*, 50, 58-64.
- Delatte T., Umhang M., Trevisan M., Eicke S., Thorneycroft D., Smith S. M., Zeeman S. C. (2006). Evidence for distinct mechanisms of starch granule breakdown in plants. *Journal of Biological Chemistry*, 281, 12050–12059.
- Delatte, T., Trevisan, M., Parker, M. L., and Zeeman, S.C. (2005). *Arabidopsis* mutants *Atisa1* and *Atisa2* have identical phenotypes and lack the same multimeric isoamylase, which influences the branch point distribution of amylopectin during starch synthesis. *Plant Journal*, 41, 815-830.

- Denyer, K., Dunlap, F., Thorbjornsen, T., Keeling, P., and Smith, A. M. (1996). The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra plastidial. *Plant Physiology*, 112,779-785.
- Denyer, K., Johnson, P., Zeeman, S.C., and Smith, A.M. (2001). The control of amylose synthesis. *Journal of Plant Physiology*, 158,479–487.
- Denyer, K., Waite, D., Edwards, A., Martin, C. and Smith, A. M. (1999). Interaction with amylopectin influences the ability of granule-bound starch synthase I to elongate malto-oligosaccharides. *Biochemical Journal*, 342,647-653.
- Denyer, K., Waite, D., Motawia, S., Møller, B.L., and Smith, A.M. (1999b). Granule bound starch synthase I in isolated starch granules elongates malto oligosaccharides processively. *Biochemistry Journal*, 340,183–191.
- Dexter, J. E., Preston, K. R., Martin, D. G., and Gander, E. J. (1994). The effects of protein-content and starch damage on the physical dough properties and bread-making quality of canadian durum-wheat. *Journal of Cereal Science*, 20,139-151.
- Dian, W., Jiang, H., and Wu, P. (2005). Evolution and expression analysis of starch synthase III and IV in rice. *Journal of Experimental Botany*, 56,623-632.
- Doane, W. M. (1994). Opportunities and challenges for new industrial uses of starch. *Cereal Foods Worlds*, 39 (8), 556-563.
- Edwards, A., Borthakur, A., Bornemann, S., Venail, J., Denyer, K., Waite, D., Fulton, D., Smith A., and Martin C. (1999). Specificity of starch synthase isoforms from potato. *European Journal of Biochemistry*, 266,724-736.
- Edwards, A., Marshall, J., and Sidebottom, C. (1995). Biochemical and molecular characterization of novel starch synthase from potato-tubers. *Plant Journal*, 8, 283-294.

- Ellis, R.P., Cochrane, M.P., Dale, M.F.B., Duffus., C.M., Lynn, A., Morrison., I.M., Prentice., R.D.M, Swanston., J.S. and Tiller, S.A. (1998). Starch production and industrial use. *Journal of the Science of Food and Agriculture*, 77,289-311.
- Eriksson, S., Janciauskiene, S., and Lannfelt, L. (1995). Alpha 1-antichymotrypsin regulates Alzheimer beta-amyloid peptide fibril formation. *Proceedings of the National Academy Science*, 92(6), 2313-2317.
- FAOSTAT - Food and Agriculture Organization of the United Nations. (2007). Retrieved from <http://faostat.fao.org/default.aspx>.
- Fisher, D. K., Boyer, C. D., and Hannah, L. C. (1993). Starch branching enzyme II from maize endosperm. *Plant Physiology*, 102(3), 1045-1046.
- Fisher, D.K., Gao, M., Kim, K.N., Boyer, C.D., and Gultinan, M.J. (1996). Two closely related cDNAs encoding starch branching enzyme from *Arabidopsis thaliana*. *Plant Molecular Biology*, 30, 97–108.
- Frances, H., and Bligh, J. (1999) Genetic Manipulation of Starch Biosynthesis: Progress and Potential. *Biotechnology and Genetic Engineering Reviews*, 16 (1), 177-202.
- Francis, C.M., and Campbell, M.C., (2003). New High Quality Oil Seed Crops for Temperate and Tropical Australia. Rural Industries Research and Development Corporation. RIRDC Publication No 03/045 RIRDC Project No UWA-47A.
- Frohberg, C. (2008). Genetically modified plants which synthesize a starch having increased swelling power. *International Patent application* No, WO/2008/017518.
- Frueauf, J.B., Ballicora, M.A., and Preiss, J. (2001). Aspartate residue 142 is important for catalysis by ADP-glucose pyrophosphorylase from *Escherichia coli*. *Journal of Biological Chemistry*, 276: 46319–46325.

- Fu, Y., Ballicora, M. A., Leykam, J. F., and Preiss, J. (1998). Mechanism of reductive activation of potato tuber ADP-glucose pyrophosphorylase. *Journal of Biological Chemistry*, 273, 25045-25052.
- Fujita, N., Satoh, R., Hayashi, A., Kodama, M., Itoh, R., Aihara, S., and Nakamura, Y. (2011). Starch biosynthesis in rice endosperm requires the presence of either starch synthase I or IIIa. *Journal of Experimental Botany*, 62(14): 4819-4831.
- Fujita, N., Yoshida, M., Asakura, N., Ohdan, T., Miyao, A., Hirochika, H., and Nakamura, Y. (2006). Function and characterization of starch synthase I using mutants in rice. *Plant Physiology*, 140, 1070-1084.
- Fulton, D. C., Stettler M., Mettler T., Vaughan C. K., Li J., Francisco P., Gil M., Reinhold H., Eicke S., Messerli G., Dorken G., Halliday K., Smith A. M., Smith S. M., Zeeman S. C. (2008). β -AMYLASE4, a noncatalytic protein required for starch breakdown, acts upstream of three active β -amylases in *Arabidopsis* chloroplasts. *Plant Cell*, 20, 1040–1058.
- Fulton, D.C., Edwards, A., Pilling, E., Robinson, H.L., Fahy, B., Seale, R., Kato, L., Donald, A.M., Geigenberger, P., Martin, C., and Smith, A.M. (2002). Role of granule-bound starch synthase in determination of amylopectin structure and starch granule morphology in potato. *Journal of Biological Chemistry*, 277, 10834–10841.
- Furukawa, K., Tagaya, M., Inouye, M., Preiss, J., and Fukui, T. (1990). Identification of lysine 15 at the active site in *Escherichia coli* glycogen synthase. *Journal of Biological Chemistry*, 265, 2086-2090.
- Furukawa, K., Tagaya, M., Tanizawa, K., and Fukui, T. (1993). Role of the conserved Lys-X-Gly-Gly sequence at the ADP-glucose-binding site in *Escherichia coli* glycogen synthase. *Journal of Biological Chemistry*, 268, 23837-23842.

- Galvez, F. C. F., and Resurrection, A. V. A. (1992). Reliability of the focus group technique in determining the quality characteristics of mung bean (*Vigna radiata* (L.) Wikzec) noodles. *Journal of Sensory Studies*, 7(4), 315.
- Gao, M., Fisher, D. K., Kim, K. N., Shannon, J. C., and Gultinan, M. J. (1997). Independent genetic control of maize starch-branching enzymes IIa and IIb. *Plant Physiology*, 114, 69-78.
- Gao, M., Wanat, J., Stinard, P. S., James, M. G., and Myers, A. M. (1998). Characterization of dull1, a maize gene coding for a novel starch synthase. *Plant Cell*, 10, 399-412.
- Gatehouse, A.M.R., Gatehouse, J.A., and Boulter, D. (1980). Isolation and characterization of trypsin inhibitors from cowpea (*Vigna unguiculata*). *Phytochemistry*, 19,751-756.
- Geigenberger, P. (2011). Regulation of starch biosynthesis in response to a fluctuating environment. *Plant Physiology*, 155(4), 1566-1577.
- Geigenberger, P., Stamme, C., Tjaden, J., Schulz A, Quick, P.W., Betsche, T., Kersting, H.J., and Neushaus, H.E. (2001). Tuber physiology and properties of starch from tubers of transgenic potato plants with altered plastidic adenylate transporter activity. *Plant Physiology*, 125, 1667-1678.
- Georgelis, N., Braun, E.L., Shaw, J.R., and Hannah, L.C. (2007). The two AGPase sub-units evolve at different rates in angiosperms, yet they are equally sensitive to activity-altering amino acid changes when expressed in bacteria. *The Plant Cell*, 19 1458-1472.
- Gerard, C., Colonna, P., Buleon, A., and Planchot, V. (2001). Amylolysis of maize mutant starches. *Journal of the Science and food Agriculture*, 1281-1287.
- Gibson T.S., Solan, V.A., and McCleary, B.V. (1997). A procedure to measure amylose in cereal starches and flours with concanavalin *American Journal of Cereal Science*. 25, 111-119.

- Guan H. P., and Preiss J. (1993). Differentiation of the properties of the branching isozymes from maize (*Zea mays*). *Plant Physiology*, 102, 1269-1273.
- Gujaska E, Reinhard WD, Khan K. 1994. Physicochemical properties of field pea, pinto and navy bean starches. *Journal of Food Science*, 59,634-646.
- Hamada, S., Ito, H., Hiraga, S., Inagaki, K., Nozaki, K., Isono, N., Yoshimoto, Y., Takeda, Y., and Matsui, H. (2002). Differential characteristics and subcellular localization of two starch-branching enzyme isoforms encoded by a single gene in *Phaseolus vulgaris* L. *Journal of Biological Chemistry*, 277, 16538-16546.
- Hamada, S., Nozaki, K., Ito H., Yoshimoto, Y., Yoshida, H., Hirage, S., Onodera, S., Honma, M., Takeda, Y., and Matsui, H. (2001). Two starch-branching enzyme isoforms occur in different fractions of developing seeds of kidney bean. *Biochemistry Journal*, 359, 23-34.
- Han, Y., Xu, M., Liu, X., Yan, C., Korban, S.S., Chen, X., Gu, M. (2004). Genes coding for starch branching enzymes are major contributors to starch viscosity characteristics in waxy rice (*Oryza sativa* L.). *Plant Science*, 166, 357-364.
- Hanashiro, I., Itoh, K., Kuratomi, Y., Yamazaki, M., Igarashi, T., Matsugasako, J., and Takeda, Y. (2008). Granule-bound starch synthase I is responsible for biosynthesis of extra-long unit chains of amylopectin in rice. *Plant Cell Physiology*, 49:925–33
- Harn, C., Knight, M., Ramakrishnan, A., Guan, H., Keeling, P. L. Wasserman, B. P. (1998). Isolation and characterization of the zSSIIa and zSSIIb starch synthase cDNA clones from maize endosperm. *Plant Molecular Biology*, 37, 639-649.

- Harn, C.H., Bae, J.M., Lee, S.S., Min, S.R. and Liu, J.R. (2000) Presence of multiple cDNAs encoding an isoform of ADP-glucose pyrophosphorylase large subunit from sweet potato and characterization of expression levels. *Plant Cell Physiology*, 41, 1235-1242.
- Hartley, M. L., Tshamekeng, E., and Thomas, S. M. (2002). Functional heterostyly in *Tylosema esculentum* (Caesalpinioideae), *Annals of Botany*, 89, 67-76.
- Hartley, J.L., Temple, G.F., and Brasch, M.A. (2000). DNA cloning using in vitro site specific recombination. *Genome Research*, 10, 1788-1795.
- Hizukuri, S. (1986). Polymodal distribution of the chain lengths of amylopectins, and its significance. *Carbohydrate Research*, 147,342–347.
- Hocman, G. (1992). Chemoprevention of cancer: Protease inhibitors. *International Journal of Biochemistry*, 24, 1365-1375.
- Holse, M., Husted, S., and Hansen, Å. (2010). Chemical composition of marama bean (*Tylosema esculentum*) a wild African bean with unexploited potential. *Journal of Food Composition and Analysis*, 23 (6), 648–657.
- Hoover, R. (2001). Composition, molecular structure, and physico-chemical properties of tuber and root starches: a review. *Carbohydrate Polymer*, 45, 253-267.
- Hoover, R., and Ratnayake, W. S. (2002). Starch characteristics of black bean, chick pea, lentil, navy bean and pinto bean cultivars grown in Canada. *Food Chemistry*, 78(4), 489-98.
- Hussain, H., Mant, A., Seale, R., Zeeman, S.C., Hinchliffe, E., Edwards, A., Bornemann, S., Smith, A.M., Martin, C., and Bustos, R. (2003). Three isoforms of isoamylase contribute different catalytic properties for the debranching of potato glucans. *Plant Cell* 15:133–149.
- International Starch Market Index. (2012). Retrieved from <http://www.starch.dk/isi/market/index>.

- James, M.G., Denyer, K., and Myers, A.M. (2003). Starch synthesis in the cereal endosperm. *Current Opinion in Plant Biology*, 6, 215–222.
- Jane, J., Chen, Y. Y., Lee, L. F., McPherson, A. E., Wong, K. S., Radosavljevic, M., and Kasemsuwan, T. (1999). Effects of amylopectin branch chain length and amylose content on the gelatinization and pasting properties of starch. *Cereal Chemistry*, 76, 629-637.
- Jane, J-L., Kasemsuwan, T., Leas, S., Zobel H., Robyt, J.F. (1994). Anthology of starch granule morphology by scanning electron microscopy. *Starch-Stärke* 46:121-129.
- Jenkins, P.J., and Donald, A. M. (1995). The influence of amylose on starch granule structure. *International Journal Biology Macromolecules*, 17, 315-321.
- Jenkins, P.J., Cameron, R.E., and Donald, A.M. (1993). A universal feature in the structure of starch granules from different botanical sources. *Starch-Stärke* 45,417- 420.
- Jeon, J.S., Ryoo, N., Hahn, T.R., Walia, H., and Nakamura. Y. (2010). Starch biosynthesis in cereal endosperm. *Plant Physiology and Biochemistry*, 48 (6), 383-392.
- Jespersen, H. M., MacGregor, E. A., Henrissat, B., Sierks, M. R. and Svensson, B. (1993). Starch- and glycogen-debranching and branching enzymes: prediction of structural features of the catalytic (β/α)₈-barrel domain and evolutionary relationship to other amylolytic enzymes. *Journal of Proteins Chemistry*, 12,791-805.
- Kaplan, A.H., and Swanstrom, R. (1991). Human immunodeficiency virus type 1 Gag proteins are processed in two cellular compartments. *Proceedings of the National Academy Science*, 88(10), 4528-4532.
- Karim, A. A., Toon, L. C., Lee, V. P. L., Ong, W. Y., Fazilah, A., and Noda, T. (2007). Effects of phosphorus contents on the gelatinization and retrogradation of potato starch. *Journal of Food Science*, 72, 132-138.

- Kaur, M., Sandhu, K. S., and Lim, S., T. (2010). Microstructure, physicochemical properties and in vitro digestibility of starches from different Indian lentil (*Lens culinaris*) cultivars. *Carbohydrate Polymers*, 79, 349-355.
- Kawasaki, T., Mizuno, K., Baba, T., Shimada, H., (1993). Molecular analysis of the gene encoding a rice starch branching enzyme. *Molecular Genetics*, 237, 10-16.
- Keeling, P.L., and Myers, A.M. (2010). Biochemistry and genetics of starch synthesis. *Annual Review of Food Science and Technology*, 1, 271-303.
- Khurana, H.K., and Kanawjia, S.K. (2007). Recent trends in development of fermented milks. *Current Nutrition and Food Science*, 3, 91–108.
- Kim, K. N., Fisher, D., Gao, M., and Gultinan, M. (1998). Genomic organization and promoter activity of the maize starch branching enzyme I gene. *Gene*, 216, 233-243.
- Kim, Y. S., Wiesenborn, D. P., Lorenzen, J. H., and Berglund, P. (1996). Suitability of edible and potato starched for starch noodles. *Cereal Chemistry*, 73(3), 302-308.
- Kleczkowski, L.A., Villand, P., Luthi, E., Olsen, a-A., and Preiss, J. (1993). Insensitivity of barley endosperm ADP-glucose pyrophosphorylase to 3 phosphoglycerate and orthophosphate regulation. *Plant Physiology*, 101, 179-186.
- Knight, M. E., Harn, C., Lilley, C. E. R., Guan, H., Singletary, G. W., MuForster, C., Wasserman, B. P., and Keeling P. L. (1998). Molecular cloning of starch synthase I from maize (W64) endosperm and expression in *Escherichia coli*. *Plant Journal*, 14, 613-622
- Kossmann, J., Abel, G. J. W., Springer, F., Lloyd, J. R., and Willmitzer, L. (1999). Cloning and functional analysis or a cDNA encoding a starch synthase from potato (*Solanum tuberosum* L.) that is predominantly expressed in leaf tissue. *Planta*, 208, 503-511.

- Kuriki, T., Stewart D. C., and Preiss, J. (1997). Construction chimeric enzymes out of maize endosperm branching enzyme I and II: activity and properties. *Journal of Biological Chemistry*, 272, 28999-29004.
- La Cognata, U., Willmitzer, L., and Muller-Rober, B. (1995). Molecular cloning and characterization of novel isoforms of potato ADP-glucose pyrophosphorylase. *Molecular and General Genetics*, 246, 538-548.
- Laroche, J., Li, P., and Bousquet, J. (1995). Mitochondrial DNA and monocot-dicot divergence time. *Molecular Biology and Evolution*, 12(6), 1151-1156.
- Larsson, C. T., Hofvander, P., Khoshnoodi, J., Ek, B., Rask, L., and Larsson, H. (1996). Three isoforms of starch synthase and two isoforms of starch branching enzyme are present in potato tuber starch. *Plant Science*, 117, 9-16.
- Lawton, J.W. (2004). Uses of native starch. In: Encyclopedia of Grain Science, Wrigley, C., Corke, H., and Walker, C.E. Elsevier Ltd: Oxford.
- Leterrier, M., Holappa, L.D., Broglie, K.E., and Beckles, D.M. (2008). Cloning, characterization and comparative analysis of a starch synthase IV gene in wheat: functional and evolutionary implications. *BMC Plant Biology*, 8, 98-107.
- Li Z., Mouille, G., Kosar-Hashemi, B., Rahman, S., Clarke, B., Gale, K. R., Appels, R., and Morell, M. K. (2000). The structure and expression of the wheat starch synthase III gene. Motifs in the expressed gene define the lineage of the starch synthase III gene family. *Plant Physiology*, 123,613-624
- Li, Y.P.; Xia, R.X.; Wang, H.; Li, X.S.; Liu, Y.Q.; Wei, Z.J.; Lu, C. and Xiang, Z.H. (2009). Construction of a full-length cDNA Library from Chinese oak silkworm pupa and identification of a KK-42-binding protein gene in relation to pupa-diapause termination. *Internatinal Journal of Biological Science*, 5, 451-457.

- Li, Z., Rahman, S., Kosar-Hashemi, B., Mouille, G., Appels, R., and Morell, M. K. (1999). Cloning and characterization of a gene encoding wheat starch synthase I. *Theoretical Applied Genetics*, 98, 1208-1216.
- Lin, T. P., Casper, T., Somaville, C., and Preiss, J. A. (1988). Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L) lacking ADP-glucose pyrophosphorylase activity. *Plant Physiology*, 86, 1131-1135.
- Liu, F., Makhmoudova, A., Lee, E. A., Wait, R., Emes, M. J., and Tetlow, I. J. (2009). The amylose extender mutant of maize conditions novel protein protein interactions between starch biosynthetic enzymes in amyloplasts. *Journal of Experimental Botany*, 60, 4423-4440.
- Lloyd, J.R., Springer, F., Buleon, A., Muller-Rober, B., Willmitzer, L., and Kossmann, J. (1999). The influence of alterations in ADP-glucose pyrophosphorylase activities on starch structure and composition in potato tubers. *Planta*, 209, 230-238.
- Lorito, M., Broadway, R.M., Hayes, C. K., Woo, S. L., Noviello, C., Williams, D. L., and Harman, G. E. (1994). Proteinase inhibitors from plants as a novel class of fungicides. *Molecular Plant-Microbe Interactions*, 7, 525-527.
- Marchler-Bauer, A. (2011) CD-Search: glycosyl transferases protein domain annotations on the fly. *Nucleic Acids Research*, 32, W327-W331.
- Martin, C., and Smith, A. M. (1995). Starch biosynthesis. *Plant Cell*, 7, 971-985.
- Mauro, D.J. (1996). An update on Starch. *Cereal Foods World*, 41, 776-778.
- Mayers, A. M., Morell, M. K., James, M. G., and Ball, S. (2000). Recent progress toward understanding biosynthesis of amylopectin crystal. *Plant physiology*. 122, 989-998.
- McCleary, B.V., Solah, V., and Gibson, T.S. (1994). Quantitative measurement of total starch in cereal flours and products. *Journal of Cereal Science*, 20, 51-58.

- Michaud, D. (1998). Gel electrophoresis of proteolytic enzymes, *Analytica Chimica Acta*, 372, 173-185.
- Miles, M. J., Morris, V. J., Orford, P. D., and Ring, S. G. (1985). The role of amylose and amylopectin in the gelation and retrogradation of starch. *Carbohydrate Research*, 135, 271-281.
- Mitchell, R.A.C., Keys, A.J., Madgwick, P.J., Parry, M.A.J., and Lawlor, D.W., (2005). Adaptation of photosynthesis in marama bean *Tylosema esculentum* (Burchell A.Schreiber) to a high temperature, high radiation, drought-prone environment. *Plant Physiology and Biochemistry*, 43, 969-976.
- Monaghan, B.G., and Halloran, G.M. (1996). RAPD variation within and between natural populations of morama [*Tylosema esculentum* (Burchell) Schreiber] in southern Africa. *South African Journal of Botany*, 62, 287-291.
- Morell, M. K., Bloom, M., Knowles, V., and Preiss, J. (1987). Subunit structure of spinach leaf ADP-glucose pyrophosphorylase. *Plant Physiology*, 85, 182-187.
- Morell, M. K., Kosar-Hashemi, B., Cmiel, M., Samuel, M. S., Chandler, P., Rahman, S., Buleon, A., Batey, I. L. and Li, Z. Y. (2003). Barley *sex6* mutants lack starch synthase IIa activity and contain a starch with novel properties. *Plant Journal*, 34, 172-184.
- Morell, M.K., and Myers, A.M. (2005). Towards the rational design of cereal starches. *Current Opinion in Plant Biology*, 8, 204-210.
- Mosele, M.M., Hansen, Å.S., Hansen, M., Schulz, A., and Martens, H.J. (2011). Proximate composition, histochemical analysis and ultra-structural localisation of nutrients in immature and mature seeds of marama bean (*Tylosema esculentum*) an underutilised legume. *Food Chemistry*, 127, 1555–1561.
- Mosolov, V.V., and Valueva, T.A. (2005). Proteinase inhibitors and their function in plants: a review. *Applied Biochemistry and Microbiology*, 41, 227-246.

- Mu, C., Harn, C., Ko, Y. T., Singletary, G. W., Keeling, P. L., and Wasserman, B. P. (1994). Association of a 76-kDa polypeptide with soluble starch synthase I activity in maize (cv73) endosperm. *Plant Journal*, 6, 151-159.
- Mu-Forster, C., Huang, R., Powers, J. R., Harriman, R. W., Knight, M., Singletary, G. W., Keeling, P. L., and Wasserman, B. P. (1996). Physical association of starch biosynthetic enzymes with starch granules of maize endosperm: granule-associated forms of starch synthase I and starch branching enzyme II. *Plant Physiology*, 111: 821-829.
- Muller-Rober, B., Sonnewald, U., and Willmitzer, L. (1992). Inhibition of the ADP glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber protein genes. *EMBO Journal*, 11, 1229-1238.
- Munyikwa, T.R.I., Langeveld, S., Jacobsen, E., Visser, R.G.F. (1997). Cassava starch Biosynthesis: New avenues for modifying starch quantity and quality. *Euphytica*, 96, 65-75.
- Murphy, P. (2000). Starch. In: Phillips, G.O., Williams, P.A., editors. Handbook of hydrocolloids. Abington, England: Woodhead Publishing.
- Nadaraja, D., Weintraub, S.T., Hakala, K.W., Sherman, N.E., and Starcher, B. (2010). Isolation and partial sequence of a Kunitz- type elastase specific inhibitor from marama bean (*T. esculentum*). *Journal of Enzyme Inhibition and Medicinal Chemistry*, 25 (3), 377-382.
- Nakamura Y, Takahashi JI, Sakurai A, Inaba Y, Suzuki E, Nihei, S., Fujiwara, S., Tsuzuki, M., Miyashita, H., Ikemoto, H., Kawachi, M., Sekiguchi, H., and Kurano, N. (2005). Some cyanobacteria synthesize semiamylopectin type polyglucans instead of glycogen. *Plant Cell Physiology*, 46, 539-545.
- Nakamura, T., Yamamori, M., Hirano, H., and Hidaka, S. (1993). Identification of three *Wx* proteins in wheat (*Triticum aestivum* L.). *Biochemical Genetics*. 31, 75-86.

- Nakamura, Y. (2002). Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. *Plant Cell Physiology*, 43,718-725.
- Nepolo, E., Takundwa. M., Chimwamurombe. M., Cullis. C. A. and Kunert. K. (2009). A review of geographical distribution of marama bean [*Tylosema esculentum* (Burchell) Schreiber] and genetic diversity in the Namibian germplasm. *Afr. J. Biotechnology*, Vol. 8 (10), 2088-2093.
- Nielsen, T.H., Baunsgaard, L., and Blennow, A. (2002). Intermediary glucan structures formed during starch granule biosynthesis are enriched in short side chains, a dynamic pulse labeling approach. *Journal of Biological Chemistry*, 277, 20249-20255.
- Nielsen, T.H., Wischmann, B., Enevoldsen, K., and Møller, B.L. (1994). Starch phosphorylation in potato tubers proceeds concurrently with de novo biosynthesis of starch. *Plant Physiology*, 105, 111-117.
- Norioka, S., and Ikenaka, T. (1983). Amino acid sequence of trypsin chymotrypsin inhibitors (AI, AII, BI and BII) from peanut (*Arachis hypogaea*): a discussion on the molecular evolution of legume Bowman-Birk type inhibitors. *Journal of Biochemistry*, 94, 589–599.
- Oh, N. H., Seib, P. A., Ward, A. B., and Deyoe, C. W. (1985). Noodles. IV. Influence of flour protein, extraction rate, particle-size, and starch damage on the quality characteristics of dry noodles. *Cereal Chemistry*, 62,441-446.
- Ohara, O., Temple, G. (2001). Directional cDNA library construction assisted by the in vitro recombination reaction. *Nucleic Acids Research*, 29, 22-30.
- Okita, T. W., Nakata, P. A., Anderson, J. M., Sowokinos, J., Morell, M., and Preiss, J. (1990). The subunit structure of potato tuber ADP-glucose pyrophosphorylase. *Plant Physiology*, 93,785-790.

- Oliva, M.L., and Sampaio, U.M. (2008). Bauhinia Kunitz-type proteinase inhibitors: structural characteristics and biological properties. *Biological Chemistry*, 389, 1007-1013.
- Oliva, M.L.V., Silva, M.C.C., Sallai, R.C., Brito, M.V., and Sampaio, M.U. (2010). A novel sub-classification for Kunitz proteinase inhibitors from leguminous seeds *Biochimie*, 92, 1667-1673.
- Olive, M.R., Ellis, R.J., and Schuch, W.W. (1989). Isolation and nucleotide sequences of cDNA clones encoding ADPglucose pyrophosphorylase polypeptides from wheat leaf and endosperm. *Plant Molecular Biology*, 12, 525-538.
- Patron, N.J., and Keeling, P.J. (2005). Common evolutionary origin of starch biosynthetic enzymes in green and red algae. *Journal of Phycology*, 41, 1131–1141.
- Pilling, E., and Smith, A.M. (2003). Growth ring formation in the starch granules of potato tubers. *Plant Physiology*, 132, 365-371.
- Pladys, D., and Vance, C.P. (1993). Proteolysis during development and senescence of effective and plant gene-controlled ineffective Alfalfa nodule. *Plant Physiology*, 103,379-384.
- Powell, A.M., (1987). Marama bean (*Tylosema esculentum* Fabaceae) seed crop in Texas. *Economic Botany*, 94, 41.
- Preiss, J. (1988). Biosynthesis of starch and its regulation. *The Biochemistry of Plants*, 14,181-254.
- Preiss, J. (1991) Biology and molecular biology of starch synthesis and its regulation. *Oxford Surveys of Plant Molecular and Cell Biology*, 7, 59-114.
- Ragae, S., and Abdel-Aal, E.-S. M. (2006). Pasting properties of starch and protein in selected cereals and quality of their food products. *Food Chemistry*, 95, 9 18.

- Rahman, S., Kosar-Hashemi, B., Sameul, M. S., Hill A., Abbott, D. C., Skerritt, J. H., Preiss, J., Appels, R., and Morrel M. K. (1995). The major proteins of wheat endosperm starch granules. *Australian Journal of Plant Physiology*, 22, 793-803.
- Ral, J.P., Colleoni, C., Wattedled, F., Dauvillee, D., Nempont, C., Deschamps, P., Liz, Z., Morell, M.K., Chibbar, R., Purton, S., D'Hulst, C., and Ball, S.G. (2006). Circadian clock regulation of starch metabolism establishes GBSSI as a major contributor to amylopectin synthesis in *Chlamydomonas reinhardtii*. *Plant Physiology*, 142, 305-317.
- Regierer, B., Fernie, A.R., Springer, F., Perez-Melis, A., Leisse, A., Koehl, K., Willmitzer, L., Geigenberger, P., and Kossmann, J. (2002). Starch content and yield increase as a result of altering adenylate pools in transgenic plants. *Nature Biotechnology*, 20, 1256-1260.
- Regina, A., Bird, D., Topping, D., Bowden, S., Freeman, J., Barsby, T., Kosar Hashemi, B., Li, Z., Rahman, S., and Morell, M. (2006). High amylose wheat generated by RNA-interference improves indices of large bowel health in rats. *Proceedings of the National Academy of Science*, 103, 3546-3551.
- Repellin, A., Båga, M., and Chibbar, R.N. (2001) Characterization of a cDNA encoding a type I starch branching enzyme produced in developing wheat (*Triticum aestivum* L.) kernels. *Journal of Plant Physiology*, 158, 91-100.
- Ridout, M.J., Parker, M.L., Hedley, C.L., Bogracheva, T.Y., and Morris, V.J. (2003). Atomic force microscopy of pea starch granules: granule architecture of wild type parent, r and rb single mutants, and the rrb double mutant. *Carbohydrate Research*, 338, 2135-2147.
- Roldan, I., Wattedled, F., Lucas, M. M., Delvalle, D., Planchot, V., Jimenez, S., Perez, R., Ball, S., D'Hulst, C., and Merida, A. (2007). The phenotype of soluble starch synthase IV defective mutants of *Arabidopsis thaliana* suggests a novel function of elongation enzymes in the control of starch granule formation. *The Plant Journal*, 49, 492-504.

- Ryan, C. A. (1990). Proteinase inhibitors in plants: Genes for improving defences against insects and pathogens. *Annual Review of Phytopathology*, 28, 425-449.
- Ryan, C.A. (1990). Protease inhibitors in plants: genes for improving defenses against insects and pathogens. *Annual Review of Phytopathology*, 28, 425-449.
- Saito, M., Konda, M., Vrinten, P., Nakamura, K., Nakamura, T. (2004). Molecular comparison of waxy null alleles in common wheat and identification of a unique null allele. *Theoretical Applied Genetics*, 108, 1205–1211.
- Sambrook, J., and Russell, D. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sambrook, J., Fritsch E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2 ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanger, F., Nicklen, S., and Chase, A.R. (1977). DNA Sequencing with Chain Terminating Inhibitors. *Proceedings of the National Academy of Sciences*, 74(12), 5463-5468.
- Schwall, G.P., Safford, R., Westcott, R.J., Jeffcoat, R., Tayal, A., and Shi, Y.K., Gidley, M.J., and Jobbling, S.A. (2000). Production of very-high amylose potato starch by inhibition of SBE A and B. *Nature Biotechnology*, 18, 551-554.
- Schwartz, M. (1966). Location of the maltose A and maltose B loci on the genetic map of *Escherichia coli*. *Journal of Bacteriology*, 92, 1083-1089.
- Shapter, F.M., Egger, P., Lee, L.S., Henry, R.J. (2009). Variation in granule-bound starch synthase I (GBSSI) loci amongst Australian wild cereal relatives (Poaceae). *Journal of Cereal Science*, 49, 4–11.

- Shigechi, H., Koh, J., Fujita, Y., Matsumoto, T., Bito, Y., Ueda, M., Satoh, E., Fukuda, H., and Kondo, A. (2004). Direct production of ethanol from raw corn starch via fermentation by use of a novel surface-engineered yeast strain codisplaying glucoamylase and alpha-amylase. *Applied Environmental Microbiology*, 70, 5037-5040.
- Sikka, V.K., Choi, S.B., Kavakli, I.H., Sakulsingharoj, C., Gupta, S., Ito H., and Okita, T. (2001). Subcellular compartmentation and allosteric regulation of the rice endosperm ADP-glucose pyrophosphorylase. *Plant Science*, 161, 461-468.
- Singh, S., Choi, S.B., Modi, M.K. and Okita, T.W. (2002) Isolation and characterization of cDNA clones encoding ADP-glucose pyrophosphorylase (AGPase) large and small subunits from chickpea (*Cicer arietinum* L.). *Phytochemistry*, 59, 261-268.
- Smith, A.M. (2008). Prospects for increasing starch and sucrose yields for bioethanol production. *Plant Journal*, 54, 546-558.
- Smith, A.M., Denyer, K., and Martin, C. (1997). The synthesis of the starch granule. *Annual Review of Plant Physiology and Plant Molecular Biology*, 48, 67-87
- Smith, A.M., Denyer, K., and Martin, C.R. (1995). What controls the amount and structure of starch in storage organs? *Plant Physiology*, 107,673-677.
- Smith-White, B.J., and Preiss, J. (1992). Comparison of proteins of ADP-glucose pyrophosphorylase from diverse sources. *Journal of Molecular Biology*, 34,449-464.
- Solovyev, V., Kosarev, P., Seledsov, I., and Vorobyev, D. (2006). Automatic annotation of eukaryotic genes, pseudogenes and promoters. *Genome Biology*, 7, 1-10.
- Staheli, J.P., Boyce, R., Kovarik, D., and Rose, T.M. (2011). CODEHOP PCR and CODEHOP PCR Primer Design. *Methods in Molecular Biology*, 687, 57-73.

- Starcher, B., Bryant, L., and Elfant, M. (1986). Protease inhibitors of the marama bean. *Advances in Experimental Medicine and Biology*, 199,429–438.
- Stark, D. M., Timmermann, K. P., Barry, G. F. Preiss, J., and Kishore, G. M. (1992) Regulation of the amount of starch in plant tissues by ADP-glucose pyrophosphorylase. *Science*, 258, 287-292.
- Steer, M.L., Meldolesi, J., and Figarella, C. (1984). Pancreatitis. The role of lysosomes. *Digestive Diseases and Sciences*, 10, 934-938.
- Suhendro, E. L., Kunetz, C. F., Mcoonough, C. M., Rooney, L. W., and Waniska, R. D. (2000). Cooking characteristics and quality of noodles from food sorghum. *Cereal Chemistry*, 77, 96-100.
- Sun, C., Sathinsh, P., Ahlandsberg, S., and Jansson, C. (1998). The two genes encoding starch-branching enzymes IIa and IIb are differentially expressed in barley. *Plant Physiology*, 118, 37-49.
- SuperScript™ plasmid system with gateway™ technology for cDNA synthesis and cloning. (Instruction manual). (2010). Invitrogen life technologies. Cat. Series 18248
- Svegmark, K., & Hermansson, A. M. (1993). Microstructure and rheological properties of composites of potato starch granules and amylose: a comparison of observed and predicted structure. *Food Structure*, 12, 181–193.
- Szydlowski, N., Ragel, P., Hennen-Bierwagen, T. A., Planchot, V., Myers, A. M., Mérida, A., D'Hulst, C., and Wattedled, F. (2011). Integrated functions among multiple starch synthases determine both amylopectin chain length and branch linkage location in Arabidopsis leaf starch. *Journal of Experimental Botany*, 62(13), 4547-4559.
- Takeda, T., Guan, H. P., and Preiss, J. (1993). Branching of amylose by the branching isoenzymes of maize endosperm. *Carbohydrate Research*, 240, 253-263.

- Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731-2739.
- Tanaka, A.S., Sampaio, M.U., Marangoni, S., Marangoni, S., de Oliveira, B., Novello, J.C., Oliva, M.K., Finke, E., and Sampaio, C.A. (1997). Purification and primary structure determination of a Bowman–Birk trypsin inhibitor from *Torresea cearensis* seeds. *Biological Chemistry*, 378, 273–281.
- Tatge, H., Marshall, J., Martin, C., Edwards, E.A., and Smith, A.M. (1999). Evidence that amylose synthesis occurs within the matrix of the starch granule in potato tubers. *Plant Cell Environment*, 22, 543-550.
- Tester, R. F., and Morrison, W. R. (1990). Swelling and gelatinization of cereal starches: Effects of amylopectin, amylose, and lipids. *Cereal Chemistry*, 67, 551-557.
- Tetlow, I. J. (2011). Starch biosynthesis in developing seeds. *Seed Science Research* 21, 5-32.
- Tetlow, I. J., Davies, E. J., Vardy, K. A., Bowsher, C. G., Burrell, M. M., and Emes, M. J. (2003). Subcellular localization of ADP-glucose pyrophosphorylase in developing wheat endosperm and analysis of the properties of a plastidial isoform. *Journal of Experimental Botany*. 54, 715-725.
- Tetlow, I. J., Wait, R., Lu, Z. X., Akkasaeng, R., Bowsher, C. G., Esposito, S., Kosar-Hashemi, B., Morell, M. K., and Emes, M. J. (2004). Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein-protein interactions. *Plant Cell*, 16: 694-708.
- Thomson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTA W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673-4680.

- Thorbjørnsen, T., Villand, P., Denyer, K., Olsen, O. A., and Smith, A. M., (1996). Distinct isoforms of ADP glucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. *Plant Journal*, 10, 243-250.
- Tiessen, A., Nerlich, A., Faix, B., Hümmer, C., Fox, S., Denyer, K., Weber, H., Weschke, W., and Geigenberger, P. (2011). Sub-cellular analysis of starch metabolism in developing barley seeds using a non-aqueous fractionation method. *Journal of Experimental Botany*, 63(5), 2071-2087.
- Tiessen, A., Prescha, K., Branscheid, A., Palacios, N., McKibbin, R., Halford, N. G., and Geigenberger, P. (2003). Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. *Plant Journal*, 35: 490-500.
- Tjaden, J., Mohlmann, T., Kampfenkel, K., Henrichs, G., and Neuhaus, H.E. (1998). Altered plastidic ATP/ADP-transporter activity influences potato (*Solanum tuberosum* L.) tuber morphology, yield and composition of tuber starch. *Plant Journal*, 16, 531-540.
- Tomlinson, K., and Denyer, K. (2003). Starch synthesis in cereal grains. *Advances in Botanical Research*, 40, 1-61.
- Travlos, I.S., Economou, G., and Karamanos, A. J. (2007). Effects of heat and soil texture on seed germination and seedling emergence of marama bean, *Tylosema esculentum* (Burch.) A. Schreib. *Journal of Food Agriculture & Environment*, 5, 153-156.
- Troncoso, M.F., Biron, V.A., Longhi, S.A., Retegui, L.A., and Wolfenstein-Todel, C. (2007). *Peltophorum dubium* and soybean Kunitz-type trypsin inhibitors induce human Jurkat cell apoptosis. *International Immunopharmacology*, 7, 625-636.
- Turk, B. (2006). Targeting proteases: successes, failures and future prospects. *Nature Reviews Drug Discovery*, 5, 785-799.

- Utsumi, Y., and Nakamura, Y. (2006). Structural and enzymatic characterization of the isoamylase1 homooligomer and the isoamylase1-isoamylase2 hetro oligomer from rice endosperm. *Planta*, 225, 75–87.
- van de Wal, M., D’Hulst, C., Vincken, J.P., Buléon, A., Visser, R., and Ball, S. (1998). Amylose is synthesized in vitro by extension of and cleavage from amylopectin. *Journal of Biological Chemistry*, 273, 22232–22540.
- van den Koornhuise, N., Libessart, N., Delrue, B., Zabawinski, C., Decq, A., Iglesias, A., Preiss, J., and Ball, S. J. (1996) Control of starch composition and structure through substrate supply in the monocellular Alga *Chlamydomonas reinhardtii*. *Biological Chemistry*, 271, 16281-16287
- van der Leij, F.R., Visser, R.G.F., Ponsteln, AS., Jacobsen, E., and Feenstra, W.J. (1991). Sequence of the structural gene for granule- bound starch synthase of potato (*Solanum tuberosum L.*) and evidence for a single point deletion in the *amf* allele. *General Genetics*, 228, 240-248.
- van der Maesen, L.J.G., (2006). *Tylosema esculentum* (Burch.) A. Schreib. In: Brink,M., Belay, G. (Eds.), PROTA 1: Cereals and pulses/Céréales et légumes secs. PROTA, Wageningen, Netherlands.
- Vignaux, N., Doehlert, D. C., Elias, E. M., McMullen, M. S., Grant, L. A., and Kianian, S. (2005). Quality of spaghetti made from full and partial waxy durum wheat. *Cereal Chemistry*, 82, 93-100.
- Villand, P., Aalen, R., Olsen, O-A., Lonneborg, A., Luthi, E., and Kleczkowski, L.A. (1992). PCR-amplification and sequence of cDNA clones for the small and large subunits of ADP-glucose pyrophosphorylase from barley tissues. *Plant Molecular Biology*, 19, 381-389.
- Villand, P., Olsen, O.A., and Kleczkowski, L.A. (1993). Molecular characterization of multiple cDNA clones for ADP-glucose pyrophosphorylase from *Arabidopsis thaliana*. *Plant Molecular Biology*, 23, 1279-1284.

- Viñarta, S.C., Molina, O.E., Figueroa, L.I.C., and Fariña, J.I. (2006). A further insight into the applications of exopolysaccharides from *Sclerotium rolfsii*. *Food Hydrocolloids*, 20, 619–629.
- Vrinten, P. L., and Nakamura, T. (2000). Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. *Plant Physiology*, 122, 255-263.
- Vrinten, P., Nakamura, T., and Yamamori, M. (1999). Molecular characterization of waxy mutations in wheat. *Molecular and General Genetics*, 261(3), 463-71.
- Wang, S., Sharp, P., and Copeland, L. (2011). Structural and functional properties of starches from field peas. *Food Chemistry*, 126, 1546-1552.
- Wang, Y., Chen, X., and Qiu, L. (2008). Novel alleles among soybean Bowman-Birk proteinase inhibitor gene families. *Science in China. Series C, Life Sciences/Chinese Academy of Sciences*, 51(8), 687-692.
- Wattebled, F., Dong, Y., Dumez, S., Delvallé, D., Planchot, R., Berbezy, B., Vyas, D., Colonna, P., Chatterjee, M., Ball, S., and D’Hulst, C. (2005). Mutants of *Arabidopsis* lacking a chloroplastic isoamylase accumulate phytyglycogen and an abnormal form of amylopectin. *Plant Physiology*, 138, 184–195.
- Weber, H., Heim, U., Borisjuk, L., and Wobus, U. (1995). Cell-type specific, coordinate expression of two ADPglucose pyrophosphorylase genes in relation to starch biosynthesis during seed development in *Vicia faba* L. *Planta*, 195,352-361.
- Weder, J. K. P., and Kaiser, K.P. (1995). Fluorogenic substrates for hydrolase detection following electrophoresis. *Journal of Chromatography*, 698, 181-201.
- Wehmeyer, A.S., Lee, R.B., and Whiting, M. (1969). The nutrient composition and dietary importance of some vegetable foods eaten by the !Kung Bushmen. *South African Medical Journal* 43, 1529-1530.

- Wilkesman, J., and Kurz, L. (2009). Protease analysis by zymography: a review on techniques and patents. *Recent Patents on Biotechnology*, 3,175-184.
- Wolf, D.H. (1992). Proteases as biological regulators. Introductory remarks, *Experientia*, 48, 117-118.
- Wong, K. S., Kubo, A., Jane, J. L., Harada, K., Satoh, H., and Nakamura, Y. (2003). Structures and properties of amylopectin and phytoglycogen in the endosperm of sugary-1 mutants of rice. *Journal of Cereal Science*, 37, 139-149.
- Wu, H.L.; Wan, Q.H.; Fang, S.G.; Zhang, L.Y.; Xia, J.S.; Zhong, Z.Y. (2007) Construction of a cDNA library of blood of *Elaphurus davidianus*. *Acta Theriol. Sin.* 27, 380–384.
- Wunderlin, R., Larsen, K., and Larsen, S. S. (1981). Cercideae. In: Polhill, R. M., and Raven, P. H. (Eds.), Kew: Royal Botanic Gardens. *Advances in legume systematics*, 1, 107-116.
- Yadav, B.S., Yadav, R.B., and Kumar, M. (2011). Suitability of pigeon pea and rice starches and their blends for noodle making. *LWT Food Science and Technology*, 44, 1415-1421.
- Yan L., Bhave, M., Fairclough, R., Konik, C., Rahman, S., and Appels, R. (2000). The genes encoding granule-bound starch synthase at the waxy loci of the A, B, and D progenitors of common wheat. *Genome*, 43: 264-272.
- Yandeu-Nelson, M. D., Laurens, L., Shi, Z., Xia, H., Smith, A. M., and Guiltinan, M. J. (2011). Starch-branching enzyme IIa is required for proper diurnal cycling of starch in leaves of maize. *Plant Physiology*, 156 (2), 479-490.
- Ye, X., and Ng, T.B. (2009). A trypsin-chymotrypsin inhibitor with anti-proliferative activity from small glossy black soybeans. *Planta Medica*, 75, 550-556.
- Ying, S-Y. (2003). Generation of cDNA Libraries: Methods and Protocols. *Methods in Molecular Biology*, Volume, 221.

- Zeeman, S.C., Kossmann, J., and Smith, A.M. (2010). Starch: Its Metabolism, Evolution, and Biotechnological Modification in Plants. *Annual Review of Plant Biology*, 61, 209-234.
- Zeeman, S.C., Smith, S.M., and Smith, A.M. (2002). The priming of amylose synthesis in Arabidopsis leaves. *Plant Physiology*, 128, 1069-1076.
- Zhang, L., Hausler, R., Greiten, C., Hajiresaei, M-R., Haferkamp I, Neuhaus, H.E., Flugge, U.I., and Ludewig, F. (2008). Overriding the co-limiting import of carbon and energy into tuber amyloplasts increases the starch content and of transgenic potato plants. *Plant Biotechnology Journal*, 6, 453-464.
- Zhang, X., Colleoni, C., Ratushna, V., Sirghie-Colleoni, M., James, M. G., and Myers, A. M. (2004). Molecular characterization demonstrates that the *Zea mays* gene sugary2 codes for the starch synthase isoform SSIIa. *Plant Molecular Biology*, 54, 865-879.