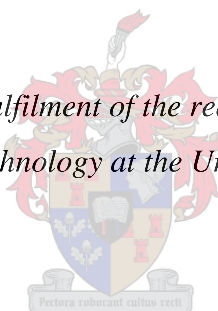
Production of Libraries to Study Biopolymer Metabolism in *Arabidopsis thaliana* and *Tylosema esculentum*

by

Corné Swart



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Abstract

Biopolymers and bio-degradable polymers are of utmost importance to ensure a sustainable economy. Industry depends on raw material, which in many cases are derived from fossil fuels, but in light of looming energy crises and green revolutions attention is being directed at cellulose and starch biopolymers. This study was therefore set forth to investigate novel genetic key elements of cell wall metabolism in *Arabidopsis thaliana* and starch synthesis in an under-utilized southern African crop plant, *Tylosema esculentum.*

In the first section of the study a cDNA library of good quality was constructed from regenerating *A. thaliana* protoplasts as it was expected to be enriching for genes involved in cell wall biosynthesis. Small scale EST sequencing of the library confirmed that a few sequences were similar to genes identified to be highly expressed during protoplast regeneration. The library was to be screened by expression in a microalgae as it is anticipated that cell wall metabolising genes would change the wall structure and visibly alter the colony morphology. An attempt was made at establishing a high-throughput transformation system in the unicellular algae *Chlorella protothecoides* in which the library was proposed to be screened. Conventional microalgal transformation techniques do not appear to be effective in this strain as the study produced no transgenic algae. Alternative studies into a screening system within another species could still lead to the identification of cell wall biosynthetic genes, which was the first objective in the study.

The second objective in the study was to investigate the potential of the orphan crop *T. esculentum* as starch-producing cash-crop in developing southern African countries. In this section of the study a cDNA library of good quality was produced form the tuber of *T. esculentum*. The library was transferred to an expression vector and screened functionally in *E. coli* for the presence of sequences with starch synthase activity. No sequences have been identified yet and screening procedures are still on-going. The starch content in the tuber has also been determined for the first time. The relatively high starch content in combination with low agricultural inputs indicate the potential of the plant as an industrial starch source. Further investigations into the nature of the starch are proposed to identify prospective buyers within the industry.

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Opsomming

Biopolimere en bio-afbreekbare polimere is van kardinale belang om 'n volhoubare ekonomie te ontwikkel. Industriële toepassings maak op die oomblik hoofsaaklik staat op fossielbrandstof verwante bronne, maar met die oog op 'n groen revolusie en energie krissise wat dreig word meer belangstelling getoon in sellulose en stysel biopolimere. Hierdie studie is daarom onderneem om genetiese elemente te identifiseer wat betrokke is by die sintese van die selwand in *Arabidopsis thaliana* en stysel sintese in die suider Afrikaanse gewas *Tylosema esculentum* wat grotendeels onderbenut is.

In die eerste deel van die studie is 'n cDNA biblioteek, van goeie kwaliteit, geskep vanuit *A. thaliana* protoplaste wat besig was om hulle selwande te herbou. Dit word verwag dat die protoplaste gedurende die tydperk aktief besig sal wees om gene uit te druk wat betrokke is by selwandsintese. DNA volgordebepaling het bevestig dat 'n klein aantal volgordes ooreengestem het met gene wat voorheen gevind was om in 'n oormaat uitgedruk te word tydens die herbou van protoplas-selwande. Daar was beoog om die biblioteek in 'n mikroalge uit te druk en sodoende die morfologie op kolonievlak waar te neem vir verandering wat in die selwand meegebring is. Om hierdie rede was die doel om 'n hoë opbrengs transformasie sisteem te ontwikkel in die mikroalge *Chlorella protothecoides.* Algemene mikroalge transformasie tegnieke blyk om nie effektief in die spesie te wees nie aangesien geen transgeniese alge waargeneem is nie. Die ontwikkeling van 'n soortgelyke proses in 'n ander spesie kan steeds lei na die ontdekking van gene betrokke by selwandsintese in *A. thaliana* wat die eerste uitkoms van die projek as geheel was.

Die tweede uitkoms van die projek was om te ondersoek wat die waarskynlikheid was om *T. esculentum* te kommersialiseer as 'n stysel gewas en sodoende 'n inkomste te skep vir arm boere in ontwikkelende lande in suider Afrika. In hierdie gedeelte van die projek was daar 'n goeie cDNA biblioteek geskep uit die knol van *T. esculentum.* Die biblioteek is oorgedra na 'n plasmied waarop dit aktief uitgedruk kon word in *Escherischia coli G6MD2* en daar is gesoek na volgordes wat lei na die sintese van stysel in hierdie bakterieë. Tot op hede is geen sulke volgordes gevind nie, maar die ondersoek gaan steeds voort. Die styselinhoud van die knol is ook vir die eerste keer bepaal in hierdie ondersoek. 'n Styselinhoud wat relatief hoog is en die lae moeite wat geverg word om die gewas te verbou toon dat die plant potensieel het as 'n kommersiële bron van stysel. Verdere ondersoeke in die aard van die stysel word ook voorgestel om toekomstige industriële kopers te identifiseer.

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Table of Contents

Description | Page number

Title 1

Declaration 2

Abstract 3

Opsomming 4

Acknowledgements 5

Table of Contents 6

List of Figures 9

List of Tables 9

Abbreviations 10

Chapter 1: General introduction and literature review 11

1.1 Biopolymers challenges synthetic polymers in an economic tug of war 11

1.2 The plant cell wall, a highly complex resource containing the most abundant biopolymer on earth 12

1.3 Starch, a biopolymer in a booming industry 17

1.4 Development of underutilized crops for economic security in developing countries 20

1.5 Aim of the project 22

Chapter 2: Construction of an *Arabidopsis thaliana* cDNA library enriched for cell wall biosynthesis genes and an investigation into the establishment of a high-throughput transformation system in *Chlorella protothecoides* for screening applications 23

2.1 Introduction 23

2.2 Materials and methods 24

Stellenbosch University http://scholar.sun.ac.za

2.2.1 Plant material and growth conditions 24
2.2.2 Protoplast isolation 24
2.2.3 Protoplast regeneration 24
2.2.4 RNA extraction from regenerating protoplasts 25
2.2.5 Precipitation of RNA 25
2.2.6 Isolation of mRNA 26
2.2.7 Construction of a cDNA library 26
2.2.8 Bacterial culture and maintenance 26
2.2.9 Small scale isolation of plasmid DNA 26
2.2.10 Restriction digests 26
2.2.11 Sequencing of inserts selected at random from the library 27
2.2.12 Large scale isolation of library plasmid DNA 27
2.2.13 Algal strains and cultivation methods 27
2.2.14 Determination of antibiotic sensitivity for *C. protothecoides* 28
2.2.15 Electroporation of *C. protothecoides* 28
2.2.16 Biolistic bombardment of *C. protothecoides* 28
2.2.17 Chemical transformation of *C. protothecoides* 29
2.2.18 Glass bead transformation of *C. protothecoides* 29
2.2.19 *Agrobacterium* mediated transformation of *C. protothecoides* 30

2.3 Results and discussion 31
2.3.1 Protoplast isolation and regeneration 31
2.3.2 Construction of a cDNA library enriched for genes related to cell wall synthesis 32
2.3.3 Analysis of ESTs selected at random from the library 33
2.3.4 Attempts to establish a high-throughput transformation system in *C. protothecoides* 34

2.4 Conclusion 35

Chapter 3: Library construction and screening for the identification of tuber specific starch synthase genes in *Tylosema esculentum* **37**

3.1 Introduction 37

Stellenbosch University http://scholar.sun.ac.za

3.2 Materials and methods 38

3.2.1 *T. esculentum* seed germination and cultivation 38

3.2.2 RNA extraction from tuber material 38

3.2.3 Precipitation of RNA 38

3.2.4 Isolation of mRNA 39

3.2.5 Construction of a cDNA library 39

3.2.6 Bacterial culture and maintenance 39

3.2.7 Small scale isolation of plasmid DNA 39

3.2.8 Restriction digests 40

3.2.9 Large scale isolation of library plasmid DNA 40

3.2.10 Preparation of chemically competent *E. coli* cells 41

3.2.11 Heat shock transformation of chemically competent *E. coli* cells 41

3.2.12 Construction of pBluescript SK(-)::DEST 41

3.2.13 Library transfer reaction 41

3.2.14 Preparation of electrocompetent *E. coli G6MD2*::pACAG cells 42

3.2.15 Electroporation of electrocompetent *E. coli G6MD2*::pACAG cells 42

3.2.16 Screening of expression library 42

3.2.17 Sequencing of plasmid inserts 43

3.2.18 Tuber starch analysis 43

3.3 Results and discussion 43

3.3.1 Construction of a cDNA library from Marama bean tubers 43

3.3.2 Transfer of cDNA library to an expression vector and qualitative analysis of the expression library 44

3.3.3. Screening of the *T. esculentum* tuber expression library 45

3.3.4. Analysis of tuber starch content and composition 46

3.4 Conclusion 47

Chapter 4: General Discussion **48**

References **51**

Stellenbosch University http://scholar.sun.ac.za

List of Figures

Figure 1.1 Primary Cell Wall Structure and Synthesis 14

Figure 1.2 Higher plant starch synthesis 19

Figure 2.1 Filtered protoplast isolation 31

Figure 2.2 Electrophoresis profile of total RNA extracted from *A. thaliana* protoplasts 31

Figure 2.3 Confirmation of partial cell wall regeneration 32

Figure 2.4 Functional distribution of ESTs represented within the library 35

Figure 2.5 Antibiotic sensitivity of *C. protothecoides* 34

Figure 3.1 Electrophoresis profile of total RNA extracted from the tuber of *T. esculentum* 43

Figure 3.2 Iodine vapour staining of *E. coli G6MD2* 45

List of Tables

Table 1.1 Genes identified in cell wall synthesis 15

Table 1.2 Major under-utilized crops species of Africa 21

Table 2.1 *A. thaliana* regenerating protoplast library insert size and sequencing of ESTs 33

Table 3.1 *T. esculentum* primary library insert size 44

Table 3.2 *T. esculentum* expression library insert size 45

Stellenbosch University http://scholar.sun.ac.za

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cfu	colony forming unit
CTAB	Cetyltrimethylammonium bromide
DDPSC	Donald Danforth Plant Science Center
DEPC	Diethylpyrocarbonate
EDTA	Ethylenediaminetetraacetitc acid
EST	Expressed sequence tag
FAO	Food and Agriculture Organisation
G1P	Glucose 1 phosphate
G6P	Glucose 6 phosphate
GDP	Guanosine diphosphate
kb	kilobase
MP	Modified Proteose
PCW	Primary Cell Wall
PEG	Polyethylene glycol
RACE-PCR	Rapid amplification of cDNA ends – polymerase chain reaction
SCW	Secondary Cell Wall
SDS	Sodium dodecyl sulfate
SS	Starch Synthase
T-DNA	Transfer-DNA
TRIS	Trisaminomethane

Chapter 1: General introduction and literature review

1.1 Biopolymers challenge synthetic polymers in an economic tug of war

The modern world has been growing at an astounding rate, reaching a population of 7 billion in 2011 and predicted to reach 9 billion by 2050 (United Nations, 2011). Accompanying this will be an increased demand that consumers will place on natural resources, thereby reducing sustainability, damaging the environment and inducing climate change (Barnosky *et al.,* 2012). Industry currently depends principally on man-made plastics and polymers for production. Properties that favour them above natural polymers are that they are chemically inert, versatile, lightweight, strong, and damage resistant. These traits, however, complicate their disposal and allow them to accumulate in ecosystems, particularly those that are aquatic (Glover, 1993; Jayasekara *et al*., 2005 Moore, 2008). The challenges in disposal are not the only hindrance, as a number of synthesis procedures produce toxic by-products that also need to be managed (Bay *et al*., 2003; Israel *et al*., 2008). Research and development divisions have been emerging in the majority of market-leading companies with the aim of providing sustainable solutions, but financial implications and technological limitations impede the implementation of bio-degradable polymers (Casale, 2008). Comparisons in the value of biopolymers and their non-degradable polymeric counterparts indicate that where there was once a 100 fold difference in production cost, this has now decreased to a more appealing 3 or 4 fold difference (Festel *et al.,* 2005). Biopolymers, backed by numerous government incentives and legislative rulings are gaining a foothold in industry and, with diminishing fossil fuel reserves and ever-increasing crude oil prices, production costs will increasingly favour bio-degradable polymers.

The responsibility to address environmental difficulties is not exclusively that of industry and academic institutions have been working on similar projects for years in the umbrella field of biotechnology. This can be described as a series of enabling technologies that involve the applications of organisms to manufacturing and service industries in order to achieve environmental sustainability and stability. Biotechnology includes the fields of biochemistry, genetics, molecular biology, biomedical engineering, chemistry and chemical engineering and could, in future, be the foundation of "Green" environmentally friendly technological platforms (Rao, 2010). Another favourable characteristic that most commercially valuable biopolymers share is that they are harvested from plants. As they are perfectly renewable,

plant based biopolymers may strengthen the primary agricultural sector and lead to extensive job creation across all involved industries.

1.2 The plant cell wall, a highly complex resource containing the most abundant biopolymer on earth

Industrial sectors responsible for manufacturing textiles, lumber, thickeners, paper and films, already depend primarily on plant based polymers that are found in the plant cell wall. Composed primarily of the most abundant biopolymer on earth, cellulose, plant cell walls are composed of intricate and complex structures. They are deposited in two separate events, with the primary cell wall (PCW) being synthesised during expansion while, afterwards, the secondary cell wall (SCW) is manufactured within the PCW when cell enlargement ceases (Taylor, 2008; Sánchez-Rodríguez *et al.*, 2010). The PCW, consisting of cellulose, hemicellulose, pectin and glycosylated proteins, is known to be flexible and thin, yet strong, and capable of extension (Baskin, 2005; Sánchez-Rodríguez *et al.*, 2010). SCWs are not present in all cells and, comprised of cellulose, hemicellulose and lignin, they play important roles in providing structural support to tissues that require extreme rigidity and strength (Taylor, 2008).

The majority of externally recognizable plant features are determined by the shape and size of the cell wall (Fry, 2004). Molecular continuity is maintained with the cytoskeleton and plasma membrane, and the physical connection between the plasma membrane and cell wall is determined by responses to changes in osmotic pressure (Szymanski & Cosgrove, 2009). The aforementioned structural roles are complemented with biochemical functions in the regulation of development as numerous surface markers, predicting both developmental patterns and indicating positions, exist within the wall (Knox *et al.*, 1989; Knox, 1990; Pennel & Roberts, 1990; Pien *et al.,* 2001). Furthermore development is aided by the delivery of nutrients to adjacent cells by pathways made possible by the continuous symplastic extension through the plasmodesmata (Cilia & Jackson, 2004). These pathways are also involved in intercellular signalling, whereby RNA molecules, hormones, regulatory proteins and other developmental messengers are delivered to their targets (Oparka, 2004; Kim, 2005). Cell walls are also the site at which biochemical defence pathways are induced by predation and pathogenic infection (Hématy *et al.,* 2009). Responses to bacterial and fungal invasion include, but are not limited to, production of lignin- and protein shells, defensively restructuring the morphology of the wall and the secretion of antimicrobial peptides as a

counter-attack mechanism (Lipka *et al.,* 2005; Field *et al.,* 2006; Hückelhoven, 2007; Underwood & Somerville, 2008).

Because of these complex functions, the cell wall has a specialised structure (Figure 1.1) comprised of intricately weaved polysaccharides similar to a fibreglass- like arrangement (Carpita & Gibeaut, 1993). Hemicelluloses and pectins form the matrix of elaborately arranged polysaccharides that embed the crystalline cellulose rods to construct a strong, yet resilient, wall structure. The major polysaccharide fraction in the wall is represented by cellulose, an unbranched (1,4)-linked β-D-glucan (Taylor, 2008). This is able to form a scaffold, within which a multitude of parallel glucans are arranged to function as a structural backbone with an impressive resistance against enzymatic digestion (McCann & Carpita, 2008). In general each of the cellulose rods contributing to the structural scaffold has a width of 2-4 nm (Thimm *et al.,* 2002).

Several other components are present alongside cellulose and these will be briefly outlined below. The first of these, hemicelluloses, display a high level of similarity to cellulose, but are unable to form microfibrils as a result of branching and modification in the molecular structure of the (1,4)-β-D-linked glycan backbone (Cosgrove, 2005; Scheller & Ulvskov, 2010). Xyloglucan, the most abundant hemicellulose in most species and arabinoxylan, usually present in smaller amounts, have distinct variation within their backbone and are suggested to be involved in the complex networks formed in conjunction with cellulose (Figure 1.1). Theories about the formation of these network have been presented in a number of studies (Cosgrove, 2000; Cosgrove, 2005) and involve ideas such as hemicelluloses binding spontaneously to the cellulose microfibril surfaces, anchoring adjacent units into a scaffold (Fry, 1989; Hayashi, 1989), or that cellulose microfibrils are covered with xyloglucans which are, in turn, bound to other matrix polysaccharides, thereby removing direct linkages between cellulose rods (Talbot & Ray, 1992).

Receiving special attention in recent years, pectins have been described as being possibly the most complex polysaccharides in living systems (Willats *et al.,* 2001, Vincken *et al.,* 2003). Extreme heterogeneity within the group and characteristic domains, with suggested linkages enabled by covalent bonding, are traits that characterise this group of wall polysaccharides (Ridley *et al.,* 2001; Willats *et al.,* 2001, Vincken *et al.,* 2003). Covalent linkages are not only believed to maintain domain positions within pectin molecules, but also to attach pectin to the hemicellulose xyloglucan, thereby entering the network of wall polysaccharides (Rizk *et al.,*

2000; Cumming *et al.,* 2005). Pectic polysaccharides interact with hemicelluloses, but evidence has been presented that they also bind to cellulose, thereby participating in the load-bearing ability of the cell wall (Dick-Pérez *et al.,* 2011, 2012). Pectins assist cell growth by developing into hydrated gels which physically separate microfibrils, allowing them mobility until finally fixing them into place when growth ceases (Stolle-Smits *et al.,* 1999; Morris *et al*., 2000 Cosgrove, 2005). Furthermore they are involved in determining wall thickness and porosity and are responsible for intercellular cell adhesion by the formation of the glue-like middle lamella (Iwai *et al.,* 2001, 2002). The flexibility in the wall is proposed to be aided by neutral pectin polysaccharides which appear to bind to cellulose surfaces (Jones *et al.,* 2003; Zykwinska *et al.,* 2005). The main pectin domains are highlighted in figure 1.1.

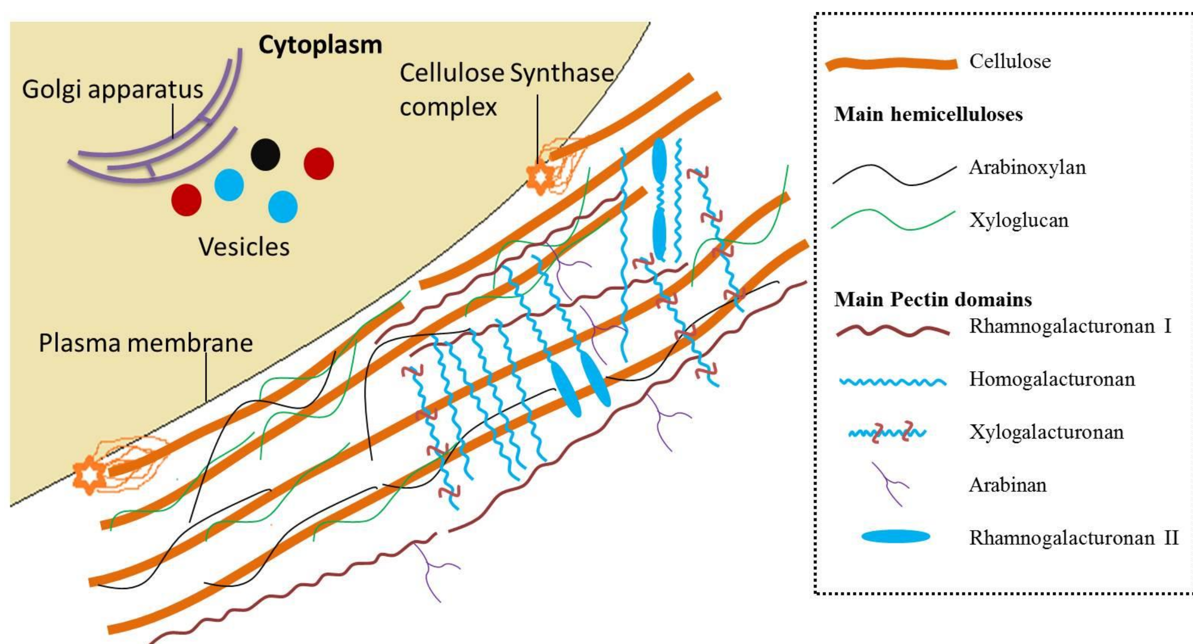

Figure 1.1. Primary Cell Wall Structure and Synthesis. A simplified diagram of the PCW is presented in the figure. Cellulose and hemicellulose interactions are highlighted without pectin on the left-hand side. The right hand side displays the integration of the major polysaccharides that constitute the cell wall. The cellulose synthase complex, membrane bound, synthesises cellulose, whilst the other matrix polysaccharides are manufactured in the Golgi apparatus and transported in vesicles to the cell membrane where they are exported to the cell wall. (modified from Cosgrove 2005)

The synthesis of cellulose and matrix polysaccharides, are executed via distinct pathways. Cellulose in both the PCW and SCW are synthesized by cellulose synthase (CESA) proteins, a group of membrane bound hexameric rosette structures which are functional only when expressed by at least three independent *CESA* genes (Kimura *et al.,* 1999; Burn *et al.,* 2002; Taylor *et al.,* 2003; Burton *et al.,* 2004; Scheible & Pauly, 2004). Matrix polysaccharides originate from the plant's secretory network, with their synthesis localized in the Golgi apparatus, followed by transport to the cell membrane in tiny vesicles that deliver them to the

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wall (Richmond & Somerville, 2000; Hazen *et al.,* 2002). Unlike cellulose which is synthesized by extrusion from the plasma membrane, the matrix polysaccharides can diffuse into the wall to reach their final destination facilitated by the energy gradient generated by cell turgor pressure (Ray, 1967; Proseus & Boyer, 2005).

Table 1.1 Genes identified in cell wall synthesis. A non-exhaustive list of genes identified to have varying levels of involvement in the biosynthesis of the plant cell wall.

Cellulose synthesis	*CESA* Superfamily	Family of genes encoding cellulose synthase catalytic subunit	Doblin *et al*., 2002; Scheible & Pauly, 2004 Somerville, 2006
	KORRIGAN	β-1,4-glucanase	Lane *et al*., 2001; Szyjanowicz *et al*., 2004
	CYT1	Gene product involved in biosynthesis of GDP-mannose	Lukowitz *et al*., 2001
	PEANUT	Enzymes involved in the biosynthesis of glycosylphosphatidylinositol membrane anchors	Gillmor *et al*., 2005
	KOBIT01	Plant specific gene-unknown function	Pagant *et al*., 2002
	COBRA	Protein essential for microfibril organization	Roudier *et al*., 2005
Hemicellulose synthesis	*CSL* Superfamily	Family of genes possibly encoding Golgi-localized glycan synthases involved in the biosynthesis of these polysaccharides	Richmond & Somerville, 2001; Hazen *et al*., 2002
	FRA8	Involved in xylan biosynthesis, exact function unknown	Baydoun *et al*., 1989; Zhong *et al*., 2005
	FUT1/MUR2	Encodes Xyloglucan-α-fucosyltransferase	Perrin *et al*., 1999; Faik *et al*., 2000; Vanzin *et al*., 2002
	KATAMARI1 (KAM1)/MUR3	Xyloglucan-β-galactosyltransferase	Madson *et al*., 2003
	AtXT1 & AtXT2	α-Xylosyltransferase	Faik *et al*., 2002
	UGE4	Alters cell specific xyloglucan content	Nguema-Ona *et al*., 2006
Pectin synthesis	*QUA1 (GUAT8)*	Homogalacturonan galacturonosyltransferase activity	Ridley *et al*., 2001
	NpGUT1	Pectin biosynthesis	Iwai *et al*., 2002
	PARVUS/GLZ1 (GATL1)	Pectin biosynthesis	Lao *et al*., 2003; Shao *et al*., 2004
	GATL Superfamily	Galacturonosyltransferase-like proteins	Lao *et al*., 2003; Shao *et al*., 2004; Sterling *et al*., 2006
	ARAD1	Arabinan α-1,5-arabinosyltransferase, biosynthesis of arabinan sidechains of RG-1	Harholt *et al*., 2006
	GAUT1 – related Superfamily[γ]	Homogalacturonan galacturonosyltransferase activity	Sterling *et al*., 2006

The question then raised is which genes are involved in producing this dynamic structure of polysaccharides, highly glycosylated proteins and lignin that represent the cell wall? The truth is that question is largely unanswered. Estimates suggest that 10% of the *A. thaliana* genome (approximately 2500 genes) might be involved in cell wall metabolism (McCann & Carpita, 2008). These include in excess of 730 genes encoding putative glycosyltransferases or glycosyl hydrolases and several hundred more thought to be involved in wall biosynthesis or function (Henrissat *et al.,* 2001). In most cases the catalytic activity has been deduced from studying sequence data, however the exact biological roles and enzymatic activities have not been identified for most of these candidate proteins (Fry, 2004). One reason for this is the difficulty encountered when applying traditional biochemical techniques in cell wall studies (Lerouxel *et al*., 2006). More recently, the use of genetic and genomic resources have led to the identification of a number of cell wall biosynthesis genes (Table 1.1). The methods employed have depended on a mixture of forward and reverse genetic approaches combined with subsequent genomic analysis by association and transcriptional profiling.

Although the genetic approaches to gene identification have been somewhat successful, a number of limitations are still apparent. Assigning gene function by forward and reverse genetics depends on the availability of mutants of interest, or the ability to identify candidate genes. In some instances difficulty has been encountered with these methods, as mutants have aberrant phenotypes, deterring growth by inadequate root hair formation, or engender lethality at early stages of development (Favery *et al.,* 2001; Goubet *et al.,* 2003). Mutants utilized in a reverse genetics approach may appear phenotypically unaltered for a number of reasons, for example due to gene redundancy masking the phenotype. Strong evidence to support these statements has been gathered from genomic approaches, as genes belonging to the *CESA, CSL, GAUT1, GAUT7* and *GATL* superfamilies exist in multiple copies with high homology (Richmond & Somerville, 2001; Sterling *et al.,* 2006; Somerville, 2006). The development of new strategies to investigate genes putatively identified to be involved in wall synthesis, or to identify previously overlooked genes is, therefore, essential to expand upon our current knowledge of wall architecture, biosynthesis and regulatory processes (Lerouxel *et al.,* 2006). A better understanding of these key-processes could also contribute to reducing current high costs associated with the production of biofuels from lignocellulosic carbohydrates (da Costa Sousa *et al.,* 2009).

1.3 Starch, a biopolymer in a booming industry

Another versatile glucan-based biopolymer extracted from raw plant material, starch, is utilized extensively in food, feed and non-food based industries (Marz, 2006). Besides being the source of over 80% of the human populations calories (Keeling & Myers, 2010), it is integrally tied to the production of adhesives, agrochemicals, cosmetics, detergents, paper, board, pharmaceuticals, plastics, textiles and renewable energy. According to the European Starch Industry (www.aaf-eu.org/european-starch-industry) the market for starch in 2012 was worth €7.7 Billion in the EU alone. Synthesised in a number of tissues in many species, starch granules are highly diversified in size and physical properties. The primary interest in higher plant starches is due to their economic value and this has motivated studies into the biochemistry, genetics and molecular biology of starch metabolism.

Energy captured by photosynthesis in the chloroplasts leads to production of starch granules which act as storage molecules for energy in a transitory form in leaves, stems and roots of plants (Smith *et al*., 2005), whilst usually maintaining long term reserves in seeds and tubers. Under normal conditions starch granules are semi-crystalline (Zobel, 1988), forming a white powder when extracted and dried. Although the innate heterogeneity in shape and size is remarkable, even amongst starches in the same plant (Kossmann & Lloyd, 2000), those originating from the same organ in a species are generally similar (Jane *et al.,* 1994). Within a starch granule, known for over a century, there are two fractions, amylose and amylopectin (Kossmann & Lloyd, 2000). Amylopectin, which makes up the majority (normally approximately 75%) of the granule, is a branched molecule with a molecular weight estimated to be in the range of 10^7 and 10^9 Daltons (Buléon *et al.,* 1998). Known to be the determining feature in starches crystallinity and granular structure, amylopectin's glucosyl residues extend as chains of between six and an excess of a hundred, linked by α-1,4-bonds (Zeeman *et al*., 2010). Branching within the amylopectin structure is facilitated by the presence of α-1,6 bonds, connecting the α-1,4-linked chains in a highly organized manner (Kossmann & Lloyd, 2000; Zeeman *et al*., 2010). These branching points confer a left-hand helical structure to amylopectin chains. Exact details describing the architecture of amylopectin at molecular level are not clear at this stage (Zeeman *et al.,* 2010), but the branching pattern, frequency of branching points and chain lengths amalgamate to bring forth a dendriform, or treelike structure with a semi-crystalline nature (Hizukuri, 1986; Keeling & Myers, 2010). These clustered chains enable the higher-order structure of starch to exist as concentric rings which have been observed microscopically with the light- and scanning

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electron microscope (Buttrose, 1960; Zeeman *et al.,* 2002; Pilling & Smith 2003). The other 25% of the starch granule is made up of amylose, a smaller molecule composed of glucose units linked by α-1,4-bonds (Buléon *et al.,* 1998). Amylose structure has been reported to be influenced by the presence of a tiny proportion of α-1,6 bonds (Peat *et al.,* 1952; Hizukuri *et al.,* 1981; Buléon *et al*., 1998).

In the primary stages of starch biosynthesis, the key metabolite, ADP-glucose, is generated from a reaction between ATP and G-1-P under the catalysis of ADP-glucose pyrophosphorylase (Espada, 1962; Preiss & Sivak 1998a; Preiss & Sivak 1998b; Preiss, 1999). This reaction, the first committed step in the pathway of starch synthesis, fuels additional reactions catalysed by starch synthases and branching enzymes which produce the final polysaccharide (see Figure 1.2). Higher plant starch synthases are encoded by five classes of genes, GBSS (granule-bound starch synthase) and SSI to SSIV (Ball & Morrel, 2003). Amylose synthesis takes place via GBSS, an enzyme which is closely bound to the starch granule itself (Nelson & Rines, 1962; Ball & Morrel, 2003; Zeeman *et al.,* 2010). The remaining four classes of starch synthases, responsible for amylopectin synthesis are usually partially soluble in the stroma of the plastid and partially associated with the starch granule (Zeeman *et al*., 2010). Analyses of data relating to the biochemical properties of starch synthase isoforms and their genetic phylogeny indicate that they have different specialized functions within amylopectin synthesis, some producing chains of differing lengths within amylopectin and others involved in initiating granule synthesis (Tetlow *et al.,* 2004). Contributing to amylopectin synthesis branching enzymes add branching points to actively extending chains (Nielsen *et al*., 2002). These enzymes are also subdivided into two groups, of which the first group is involved in the transfer of shorter chains to the C6 position of the extending glucosyl moiety and the latter in the transfer of longer chains (Ball & Morrel, 2003; Tetlow *et al*., 2004).

In addition to the two α-glucans, amylose and amylopectin, starch also contains proteins and lipids. The quantity and attributes of these proteins and lipids in combination with the size and structure of amylose molecules are responsible for the differences in starches between species (Takeda *et al.,* 1987; Morrison, 1988; Takeda *et al.,* 1989). Ultimately depending on the application of the starch, crop-plant species are selected based on the physical properties of their raw starch extract. Natural variation has been essential to the successful application of starch economically, but further alterations can be induced by cross-breeding, mutant lines and transgenic approaches (Davis *et al.,* 2003). Biotechnological approaches to date have

included the overproduction of starch, modification in starch regulatory pathways and alterations in starch structure (Zeeman *et al.,* 2010). Starch furthermore can be modified physically or chemically post-harvest and this is often done, to suit specific industrial requirements (Jobling, 2004).

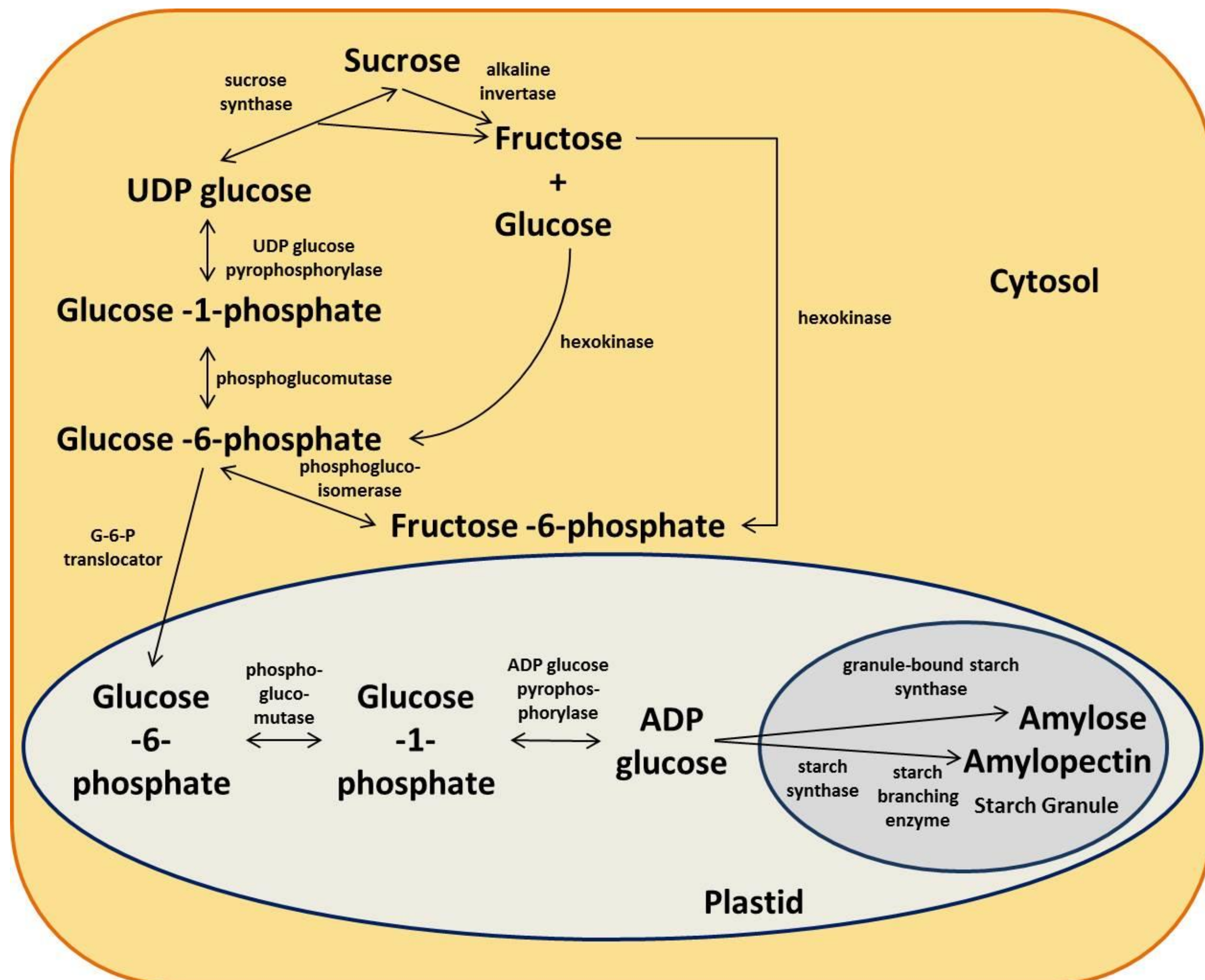

Figure 1.2 Higher plant starch synthesis. A simplified diagram is presented that demonstrates the partitioning of reactions in starch synthesis by their location within the plant cell. (Modified from http://www.jic.ac.uk/STAFF/trevor-wang/images/full/starchpath2.jpg)

Recent analyses by the European Starch Industry have indicated that starch production has increased from 8.7 million tonnes to 10 million tonnes between the period of 2004 and 2011. This growth in the starch sector had been predicted previously (Ellis *et al*., 1998). Although millions of tonnes of starch are harvested annually from wheat, maize and potato in the EU, only 20% of the starch is used in its native form, whilst 23% is modified, and 57% is used as starch-derived sweeteners. Identifying additional sources of starch from under-utilized crops could assist in alleviating poverty by creating new industries.

1.4 Development of underutilized crops for economic security in developing countries

Comparing the contribution of crop plants in the agricultural sector, it has been observed that a number of species have been underutilized or neglected. Dependence on a limited number of crops exists around the world in both developed and developing countries. Globally more than 7000 species are harvested as wild or cultivated plants, but only 150 of these crops are produced on a commercially significant scale (Jaenicke & Höschle-Zeledon, 2006). These statistics have been met with a number of strategic frameworks set forth to promote interest in orphan crops, by organization such as the International Centre for Underutilized Crops, the Global Facilitation Unit for Underutilized Species and the International Plant Genetic Resources Institute (Williams & Haq, 2002; Gündel *et al.,* 2003; Jaenicke & Höschle-Zeledon, 2006). In the most recent framework, the developing regions identified as high-priority for the implementation and improvement of under-utilized crops are Asia, the Pacific and Sub-Saharan Africa (Jaenicke & Höschle-Zeledon, 2006). These are generally seen as the most disadvantaged and are in dire need of solutions to the problems encountered with agricultural security and downstream revenue generating applications of cultivated crops (Naylor *et al*., 2004).

Within these developing regions, significant interest in has been taken in Sub-Saharan Africa, as this represents the region most likely to fail in meeting the first Millenium Development Goal (MDG1), which aims at a 50% reduction in extreme hunger and poverty by 2015 (Bartel, 2009). Asia and Latin America have increased agricultural productivity over the last three decades, whilst similar progress in Africa has been stagnant (Bartel, 2009). Orphan crops are particularly popular in Africa as they are better suited to extreme soil and environmental conditions than major crop species (Tadele, 2009). Taking into account the surface area of cultivation, or their contribution to feeding the population, the main orphan crops in Africa are highlighted in Table 1.2 (Tadele, 2009). Of these, Cassava, a food source for over a billion people worldwide has its highest production in Africa (FAO, 2010; Okogbenin *et al*., 2013; DDPSC, 2011). It is also useful as a cash crop for poor farmers, with transferable applications to industry, producing amongst many others biofuels, animal feed and starch (Okogbenin *et al.,* 2013). The cassava starch industry represents an unlikely success story, one where large scale modern factories now depend upon an under-utilized crop species (Sriroth *et al.,* 2000). This development was in part possible because of the low technology required to extract starch from the cassava roots (Sriroth *et al.,* 2000). Other contributing factors include the high starch content of the roots, the superb resilience of the

plant under suboptimal conditions, and the communal farming methods employed by millions of farmers to cultivate the crops (Cock, 1982; Sriroth *et al.,* 2000).

Considering the traits that make under-utilized crops so favourable the developing regions and the successes that have been reported as a source of income, or feeding of populations, the identification of previously overlooked species should receive more attention (Williams & Haq, 2002; Gündel *et al.,* 2003; Jaenicke & Höschle-Zeledon, 2006). Not only should this be a priority for both developed and developing countries, but research to domesticate these plants, or modify them by breeding or biotechnology should be pursued as well (Naylor *et al.,* 2004). One such tropical orphan crop with unexploited potential in Southern Africa is the Marama bean. Displaying agronomic traits favouring its survival in dry and hot regions, the plant has in recent times received significant interest as a cash crop (Jackson *et al*., 2010) Although primary investigations have revolved around the oleaginous protein rich seeds, the plant also maintains a relatively under-studied subterranean tuber with an unknown starch content (Biesele & Murray, 1983; Müseler, 2005; Jackson *et al*., 2010) Currently no revenue is being generated from the tuber of the plant and local farmers could benefit financially if industrial applications were developed, as in the case of cassava.

Table 1.2 Major under-utilized crop species of Africa. Crops are selected on the basis of their contribution to feeding the population or surface of cultivation (Tadele, 2009).

Botanical Name	Common Name	Type of Crop	Reference
Eleusine coracana	Finger Millet	Cereal	Savitha *et al.,* 2013
Eragrostis tef	Tef	Cereal	Tatham *et al., 1995*
Digitaria exilis	Fonio	Cereal	De Lumen *et al*., 1993
Vignia unguiculata	Cowpea	Legume	Sprent *et al.,* 2010
Vigna subterranea	Bambara groundnut	Legume	Sprent *et al.,* 2010
Lathyrus sativus	Grass pea	Legume	Grela *et al.,* 2010
Manihot esculentum	Cassava	Root	Babaleye, 2005
Dioscorea spp	Yam	Root	Williams & Haq, 2002
Ensete ventricosum	Enset	Trunk/root	Birmeta *et al.,* 2004

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1.5 Aim of the project

The outcome of this project was to shed light on the metabolism of cell wall synthesis and starch synthesis in higher plants. The first aim of the project was to identify genes involved in cell wall synthesis in the model plant *A. thaliana*. The approach was to construct a cDNA library, enriched for cell wall synthesis genes, from regenerating protoplasts. The aforementioned library was then to be screened in a unicellular algae for which a genetic transformation protocol would be established. The second aim of the project was to identify starch synthesis genes from the tuber of the orphan crop *T. esculentum*. The experimental design involved the construction of a tuber-specific cDNA library, which was to be transferred to a bacterial expression vector and screened for starch synthase activity in *E. coli*.

Chapter 2: Construction of an *Arabidopsis thaliana* cDNA library enriched for cell wall biosynthesis genes and an investigation into the establishment of a high-throughput transformation system in *Chlorella protothecoides* for screening applications

2.1 Introduction:

The complexities of synthesising the plant cell wall, the most abundant renewable resource on earth (Pauly & Keegstra, 2008) remains relatively poorly understood. Reasons for this include the difficulty of isolating the structural components (Cosgrove, 2005), as well as problems encountered with a number of forward and reverse genetics approaches as discussed in Chapter 1. Considering that it is estimated that 10% of *A. thaliana* genes are involved in cell wall metabolism, with the function of hundreds of these genes still unknown, the need for novel experimental approaches to identification their roles clearly exists (Lerouxel *et al*., 2006; Carpita & McCann, 2008).

In 2001, Hicks *et al*. evaluated the potential uses of unicellular algae to investigate carbohydrate metabolism in vascular plants. Sharing a number of similarities to higher plants, the algae in the division *Chlorophyta* offer a unique experimental platform. They often contain genomes that are considerably smaller than those in higher plants and are thought to have less genetic redundancy. Due to the lack of developmental complexity (such as organ differentiation) localization of expression patterns could also be eliminated as a reason for overlooking genes. Additionally many species contain a vascular plant-like cell wall, a short life-cycle and simple culturing procedures which further contribute to the value of these organisms as a scientific platform.

In this chapter I aimed to set up a novel screening system designed to identify genes involved in plant cell wall metabolism in a two-staged approach. The first aim of the project was to construct a cDNA library from regenerating *A. thaliana* protoplasts, which would be expected to be enriching for genes involved in cell wall synthesis (Gipman, 2001). The second aim was to develop a high-throughput transformation system in the unicellular algae *Chlorella protothecoides* and evaluate its potential for screening a vascular plant library. Taking into account previous reports which indicate that some unicellular algae display a visibly altered colony morphology when the cell-wall structure is modified (Hicks *et al.,* 2001) and the success in transforming *Chlorella* species (Coll, 2006). It would therefore be hoped that when

higher plant cell wall metabolising genes are expressed in this algae they will alter the cell wall and this in turn will alter colony morphology. Misshapen colonies could then be isolated and the transgene within them identified**.**

2.2 Materials and methods:

2.2.1 Plant material and growth conditions

Arbidopsis thaliana ecotype *Col-O* were grown at 80 µE.ms^{-2}.s^{-1} on peat discs (Jiffy, Efekto) with a 16 hr light and 8 hr dark photoperiod at approximately 25 ºC for 6 weeks.

2.2.2 Protoplast isolation

A. thaliana plants were incubated overnight in a humid dark environment prior to protoplast isolation. Leaves (length: 5cm; width: 2 cm, total weight: 35g) harvested from *A. thaliana* plants had the lower epidermal layer disrupted by rubbing with a fine grit (p600) sandpaper (Endler *et al*., 2006). Protoplasts were released by incubating the leaves in a protoplast digest/wash solution (Sigma Aldrich) with 1.5% w/v Cellulase Onozuka R-10 (from *Trichoderma viride,* Sigma Aldrich) and 0.4% Macerozyme R-10 (from *Trichoderma reesei,* Apollo Scientific) at 30˚C with occasional gentle swirling by hand. Before the addition of leaves the solution was preheated to 55 ˚C to ensure complete solubilisation of enzymes and filtered through a 0.22 µm filter. Incubation was seen as sufficient after approximately 6 hours, when the solution turned green and disappearance of tissue clumps was observed. The solution containing protoplasts was collected and filtered through a 50 µm mesh (Falcon) and the protoplast number and integrity assessed by light-microscopy. Protoplasts were pelleted by centrifugation in a swinging bucket rotor at 400 x *g* for 10 minutes after which the supernatant was removed and the protoplast gently washed in a volume of protoplast wash/digest solution (Sigma Aldrich).

2.2.3 Protoplast regeneration

Washed protoplasts were collected by centrifugation in a swinging bucket rotor at 400 x *g* for 10 minutes and, after aspiration of the supernatant, re-suspended in B5 (Sigma Aldrich) regeneration media (0.4M glucose; 3.1 g/l B5 salts; 0.112 % w/v B5 vitamins; pH 5.8; 1.0 mg/l 2,4-Dichlorophenoxyacetic acid; 0.15 mg/l 6-Benzylaminopurine) before being incubated in the dark for 3 or 6 hours. After incubation the regenerating protoplasts were observed by light microscopy and harvested by centrifugation at 400 x *g* for 10 minutes.

2.2.4 RNA extraction from regenerating protoplasts

RNA was extracted by means of a CTAB RNA extraction procedure. 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. RNA extractions were performed by the addition of 10 ml CTAB buffer to approximately 1.42 x 10^7 protoplasts immediately after centrifugation and removal of the supernatant. The samples were vigorously mixed by vortexing for 30 seconds and separated into microcentrifuge tubes. The samples were then incubated at 65 °C for 30 minutes, and vortexed for 30 seconds at 5 minute intervals. Centrifugation was performed at 16 000 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 precipitated by the addition of LiCl to the collected supernatant at a final concentration of 2 M and incubation overnight at 4 °C. The sample was centrifuged at 16 000 x *g* for 1 hour at 4 °C, the supernatant removed and the pellet washed with 70% ethanol. This was centrifuged at 16 000 x *g* for ten minutes at 4 °C, and the ethanol removed. The resulting RNA pellet was allowed to dry for 30 minutes before 30 µl of DEPC-treated dH_20 was used to re-suspend it.

2.2.5 Precipitation of RNA

The RNA concentration in each sample was determined spectrophotometrically. The samples were combined into a 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. 2.5 volumes of ethanol were mixed gently with the solution before being incubated at -80 °C overnight. The mixture was centrifuged at 16 000 x *g* at 4 °C for one hour, the supernatant removed and the pellet washed with 70% (v/v) ethanol. RNA was recovered by centrifugation for 30 minutes at 16 000 x *g* and 4 °C. Following removal of the supernatant the pellet was centrifuged for 20 minutes at 16 000 x *g* at 4 °C and the remaining ethanol was also removed. RNA was re-suspended in DEPC-treated dH_20 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 RNA

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2.2.6 Isolation of mRNA

mRNA was isolated from the total RNA using a commercially available kit (GenElute^{TM} mRNA miniprep kit, Sigma Aldrich). All procedures were performed according to manufacturer's protocol.

2.2.7 Construction of a cDNA library

The cDNA library was constructed with approximately 1 µg of starting mRNA. Library construction was accomplished by using the CloneMiner ^{TM} II cDNA library kit (Invitrogen). All procedures were performed according to the manufacturer's protocol.

2.2.8 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).

2.2.9 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 200µl of 50 mM TRIS-HCl pH 8.0; 10 mM EDTA; 0.1 g/l RNAse A by vortexing. 200 µl of 200 mM NaOH; 1% (w/v) SDS was added and the tube mixed gently by inversion followed by the addition of 200 µl 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 16 000 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 16 000 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.

2.2.10 Restriction digests

DNA was digested by restriction enzymes *Bsr*G I, *Xho*I and *Nco*I (Fermentas) according to the manufacturer's instructions.

2.2.11 Sequencing of inserts selected at random from the library

Plasmid DNA was sequenced by the Central Analytical Facility of the University of Stellenbosch.

2.2.12 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 dH_20 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*. 1 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 was 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).

2.2.13 Algal strain and cultivation methods:

Cultures of *Chlorella protothecoides* were maintained on solid modified proteose (MP) media (2.94 mM $NaNO_3$; 0.17 mM $CaCl_2.2H_2O$; 0.3 mM $MgSO_4.7H_2O$; 0.43 mM K_2HPO_4; 1.29 mM KH_2PO_4; 0.43 mM NaCl; 1 g/l peptone powder; casein 0.5 g/l and Bacto agar 12 g/l) in a controlled environment (16:8 day:night cycle, 50 $\mu E.ms^{-2}.s^{-1}$ light and 25 °C). Subculturing was performed once a month to maintain axenic cultures. Liquid cultures were

routinely initiated by means of a pregrowth culture and growth monitored by cell count with an improved Neubauer Brightline haemocytomer.

2.2.14 Determination of antibiotic sensitivity for C. protothecoides

Solid MP media and stock solutions of antibiotics and herbicides were prepared. Solid MP media was supplemented with either antibiotics or herbicides ranging from 5 µg/ml to 1000 µg/ml final concentration. Liquid cultures in the logarithmic growth phase were harvested and plated onto SMP media and monitored for growth. Antibiotic selection was determined by the lowest minimum inhibitory concentration required to inhibit algal growth completely.

2.2.15 Electroporation of C. protothecoides

Electroporation procedures were performed as described by Chow and Tung (1999) and Chen *et al* (2001). Cultures were grown in liquid MP media to a density of 1 x 10^7 cells/ml (early stationary phase), collected by centrifugation at 2000 x *g* for 2 minutes and re-suspended in an equal volume of washing media (0.2M Mannitol; 0.2M Sorbitol). Cells were incubated on ice for an hour, collected by centrifugation at 2000 x *g* at 4 ˚C for 2 minutes and taken up in electroporation buffer (0.08 M KCl; 0.005 M $CaCl_2$; 0.01 M HEPES-KOH, pH 7.0; 0.2 M Mannitol; 0.2 M Sorbitol; Chow and Tung, 1999). To an aliquot of 40 µl (4 x 10^6 cells), 0.5-1.0 µg of pCAMBIA2301 (www.cambia.org) was added prior to electroporation. An addition of 2.5 µg of herring sperm DNA was also tested. Electroporation of samples were done in 0.2 cm electro-cuvettes at 1500, 1800 and 2000 V/cm (25 µF; 200 Ohm) and plated onto selective (35 µg/ml of G418) and non-selective solid MP media. Plates were monitored for 3-4 weeks.

2.2.16 Biolistic bombardment of C. protothecoides

Biolistic bombardment was performed similar to a method used successfully for *Chlamydomonas reinhardtii* transformation by Boynton *et* al (1988). Liquid cultures were grown to reach an approximate density of 5 x 10^6 cells/ml, which were then plated onto sterile cellophane discs (A.A. Packaging Limited, UK) on top of solid MP media. The algae were allowed to form a lawn over a period of 7 days in preparation for biolistic bombardment. 5 mg of tungsten powder (0.7 µm per particle, Bio-Rad) was sterilised in 400 µl of ethanol, washed three times with sterile dH_2O and re-suspended in 50 µl of Milli-Q water (MQ) (Millipore). Approximately 10µg of pCAMBIA 2301 (1 µg/µl) was precipitated onto the tungsten by the concurrent addition of 50 µl $CaCl_2$ (2.5 M) and 20 µl of Spermidine

(0.1 M) during incubation on ice. A custom built gene gun was utilized for the bombardment procedure. Before use the chamber was disinfected with 70% (v/v) ethanol, the helium cylinder outlet pressure set to 1000 kPa and the solenoid timer adjusted to 0.05 seconds. From the precipitation mixture a volume of 100 µl of supernatant was aspirated before re-suspension, followed by the transfer of 5 µl into the centre of the support grid of a sterile syringe filter holder (stainless steel, 13 mm diameter). The MP media, covered with a lawn of algae was placed in the central circle of the gene gun before the door was closed. A vacuum was created within the compartment and the tungsten was fired at the lawn of algae when the pressure reached 80 kPa. The cellophane discs were carefully removed, each washed with a 1 ml of liquid MP media, and plated thinly onto selective (35 µg/ml of G418) and non-selective plates. Plates were monitored for the presence of growth for 3-4 weeks.

2.2.17 Chemical transformation C. protothecoides

A chemical transformation method for yeast (Chen *et al.*, 1992) was tested on *C. protothecoides*. Cultures were harvested in the logarithmic phase (excess of 1 x 10^7 cells/ml) by centrifugation at 2000 x *g* for 2 minutes at room temperature. For each millilitre of centrifuged culture, the pellet was re-suspended in 100 µl of One-step buffer (8% w/v polyethylene glycol (PEG) 4000; 100 mM dithiothreitol (DTT); 1 µg pCAMBIA 2301; 50 µg herring sperm carrier DNA). After vigorous vortexing the cell mixture was incubated at 45˚C for 30 minutes before being plated directly onto non-selective and selective (35 µg/ml G418) solid MP media. Plate growth was monitored for 3-4 weeks.

2.2.18 Glass bead transformation of C. protothecoides

Glass bead agitation transformation of *C. protothecoides* was performed by means of a procedure routinely used for *Chlamydomonas reinhardtii* (Kindle, 1990). Cells were cultured (1 x 10^6 cells/ml) before 100 ml was centrifuged for 2 minutes at room temperature at 2000 x *g*. The pellet was taken up in 2 ml of fresh liquid MP media and incubated with gentle shaking (100 rpm) for 2 hours at room temperature. Transformations were performed by adding 300 µl of cells to microfuge tubes containing 300 mg of sterile glass beads (425-600 µm, acid washed, Sigma Aldrich), 100 µl of 20 % w/v PEG 4000, 1-2 µg of plasmid DNA (pCAMBIA 2301, pBK-CMV; Agilent Technologies, or pEmuKN; Franks & Birch, 1991) and vortexing at maximum speed for 30 seconds. The transformation mixture was diluted into liquid MP media, cultured overnight, plated thinly onto selective (35 µg/ml G418) and non-selective solid MP media, and growth monitored for 3-4 weeks.

2.2.19 Agrobacterium mediated transformation of C. protothecoides

Cells of *C. protothecoides* were utilized in a transformation procedure in a manner similar to that used by Kathiresan & Sarada (2009) on *Haematococcus pluvialis* and by Pratheesh *et al*. (2012) on *C. reinhardtii.* A total of 1 x 10^6 cells were spread over solid MP plates and allowed to develop a lawn of cellular growth for a week. *Agrobacterium tumefaciens* strains EHA 105 (L,L succinamopine type, A *vir* helper, also maintains a supervirulant Ti plasmid, a T-DNA deletion derivative of pTiBo542; Hood *et al*., 1986) and LBA 4404 (Octopine type with A *vir* helper maintaining the disarmed pAL4404 Ti plasmid, a derivative of pTiAch5 with a T-DNA deletion; Hoekema *et al*., 1983) with pCAMBIA 2301 were cultured at 28 °C overnight with shaking in liquid YEP media (10 g/l Peptone powder; 10 g/l Yeast extract powder; 5 g/l NaCl) with appropriate antibiotics. When the *Agrobacterium* cultures reached an OD_{600} of 0.5, the bacteria were collected by centrifugation at 2800 x *g* for 10 minutes. The pellet was washed with sterile dH_20, centrifuged again for 10 minutes at 2800 x *g* before the washing step was repeated. After the final centrifugation step, the *Agrobacterium* cells were taken up in 3 ml of liquid MP before additions of acetosyringone to final concentrations of 0 µM, 100 µM and 250 µM. 200 µl of the *Agrobacterium* solution was spread onto each MP plate with *C. protothecoides* and co-cultivated in the dark for 48 hours at 25 °C. Liquid MP media (600 µg/ml Cefotaxime) was used to wash the co-cultivated cultures from the solid media, and allowed to shake for one hour. The algae were pelleted by centrifugation at 100 x *g* for 5 minutes, washed 3 times with dH_20 containing 600 µg/ml cefotaxime and plated thinly onto selective (35 µg/ml G418) and non-selective solid MP media (600 µg/ml cefotaxime). Growth and possible recurring *Agrobacterium* infections were monitored for 3-4 weeks.

2.3 Results and discussion:

2.3.1 Protoplast isolation and regeneration

Approximately 2.84 x 10^7 protoplasts were isolated from *A. thaliana* rosette leaves which appeared to be generally intact as confirmed by microscopic investigation (Fig. 2.1).

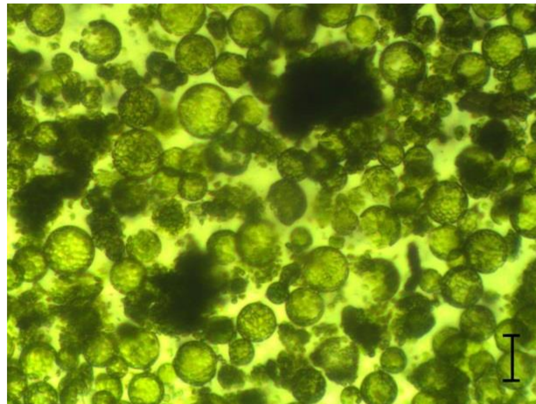

Figure 2.1 Filtered protoplast isolation. Filtered *A. thaliana* protoplasts visualized at 100x magnification. Bar represents 50 µm.

The protoplast isolation was separated into two equal volumes of B5 regeneration media and allowed to partially regenerate their cell walls for 3 or 6 hours respectively before the total RNA was extracted (Fig. 2.2). These time points were selected as the expression of known cell wall biosynthetic genes start 3 hours after hydrolytic enzymes have been removed and peak when regeneration has taken place for 6 hours (Gipman, 2001). Microscopic investigation into deposition of cell wall components confirmed that partial cell wall regeneration after a period of 6 hours took place (Fig. 2.3).

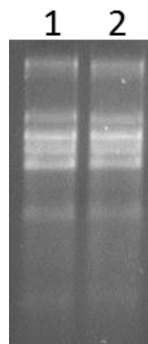

Figure 2.2 Electrophoresis profile of total RNA extracted from *A. thaliana* protoplasts. RNA extracted from protoplasts (1) after 3 hours of regeneration and (2) after 6 hours of regeneration was separated by non-denaturing agarose gel electrophoresis (1% w/v).

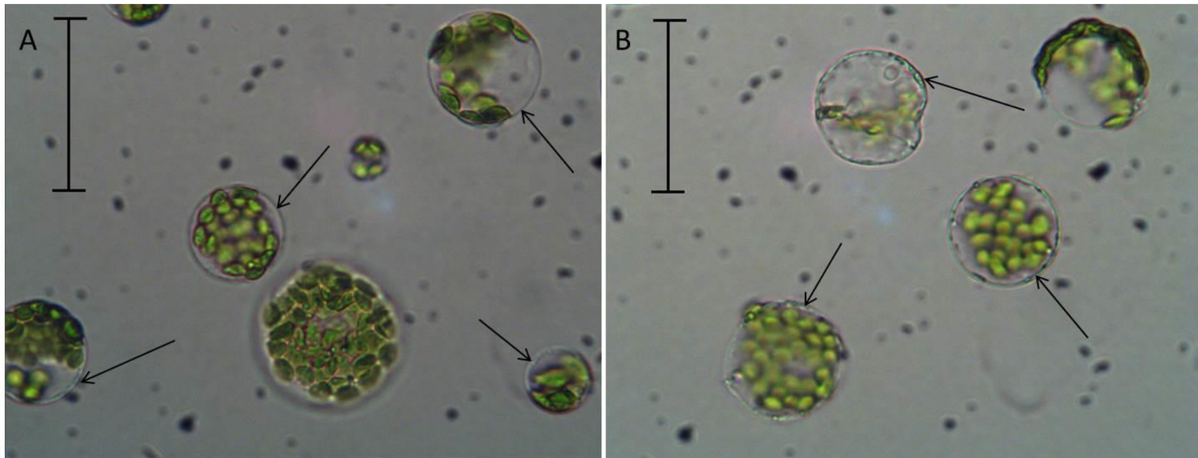

Figure 2.3 Confirmation of partial cell wall regeneration. *A. thaliana* protoplasts visualized at 400x magnification. A. Protoplasts in B5 regeneration media after 0 Hrs of regeneration. B. Protoplasts in B5 regeneration media after 6 Hrs of regeneration. Arrows highlight the cell membrane where the deposition of cell wall components can be seen. Bars represent 50 µm each.

2.3.2 Construction of a cDNA library enriched for genes related to cell wall synthesis

A cDNA library from regenerating protoplasts was constructed in pENTR™ 222 with the CloneMiner™ II cDNA library kit. The library contained a titer of approximately 1.07 x 10^6 cfu which exceeded the general guideline of 1 x 10^6 clones. It is therefore expected that the library is representative as the number of clones should ensure sufficient transcript coverage. The library was qualitatively analysed by selecting 22 random clones, digesting the pENTR™ 222 entry plasmid with *Bsr*G I and consequently separating the fragments by agarose gel electrophoresis.

The average insert size (Table 2.1) was determined by comparison to a known DNA ladder (λ DNA/*Pst*I digest, Sigma Aldrich). Sizes were maintained within the range of 0.4 and 2.4 kb, and the average insert size was found to be 1.16 kb (Table 2.1). This is close to the theoretical average size of eukaryotic cDNA's which has been estimated as 1.35 kb (Xu *et al*., 2006). Each plasmid contained an insert indicating a recombination rate of 100% and approximately 59% of the sequences exceeded 1 kb.

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Table 2.1 ***A. thaliana*** **regenerating protoplast library insert size and sequencing of ESTs.** Average insert size of 22 randomly selected clones determined by digestion of plasmid DNA with *Bsr*G I and separation by agarose gel electrophoresis. Samples were also sequenced from the M13 forward primer to discern the identity of inserts by Blastn analysis. Predicted size of full-length cDNA is also presented.

Clone	Insert size (kb)	EST	Full-length cDNA (kb)	Accession Number
1	1.30	Glutamate dehydrogenase 2	1.52	NM_001125711.1
2	0.90	Peptidase S24/S26A/S26B/S26C family protein	0.87	NM_104138.4
3	1.35	C2H2 zinc finger protein FZF	1.54	NM_128011.4
4	0.85	HSP20 family protein	0.72	NM_128504.3
5	1.70	Ribosome biogenesis co-factor	1.66	NM_102901.3
6	0.80	Putative zinc finger (AN1-Like) family protein	0.95	NM_113740.5
7	1.60	Putative glucosyltransferase	1.76	NM_119575.1
8	1.95	Global transcription factor group e8	3.17	NM_001203056.1
9	1.65	Polyadenylate binding protein RBP45B	1.57	NM_101037.3
10	1.20	Arginine decarboxylase 2	2.77	NM_202955.1
11	0.40	Class 1 heat shock protein	0.81	NM_100614.2
12	1.05	Elongation factor EF-2	2.96	NM_179487.1
13	0.65	Uncharacterized protein	0.16	NM_001126022.1
14	0.95	Glutathione S-transferase TAU 11	1.05	NM_105661.4
15	1.40	Heat shock transcription factor 4	1.78	NM_119862.3
16	2.40	Nitrate transporter 1.1	2.14	NM_101083.3
17	1.45	Heat shock protein 70B	2.33	NM_101471.2
18	0.75	Ribosomal protein s21e	0.60	NM_122652.6
19	1.05	Unknown protein (NC domain containing related)	1.08	NM_111138.3
20	0.45	Hypothetical protein	0.74	AK221826.1
21	0.96	Unknown protein, response to low sulfur 2	0.56	NM_122375.2
22	1.00	Gram domain containing protein	1.11	NM_121323.3

2.3.3 Analysis of ESTs selected at random from the library

22 Clones selected at random from the library were sequenced from the M13 Forward primer and the sequences obtained used in Blastn homology searches on NCBI (www.ncbi.nlm.nih.gov). The ESTs identified were compared to loci upregulated during cell wall regeneration of rice (Sharma *et al*., 2011) and cotton (Yang *et al*., 2008) protoplasts, as well as the proteins isolated from the apoplastic region of regenerating *A. thaliana* protoplasts

(Kwon *et al*., 2005). The functional distributions of this small scale EST survey are displayed in Figure 2.4 and Table 2.1.

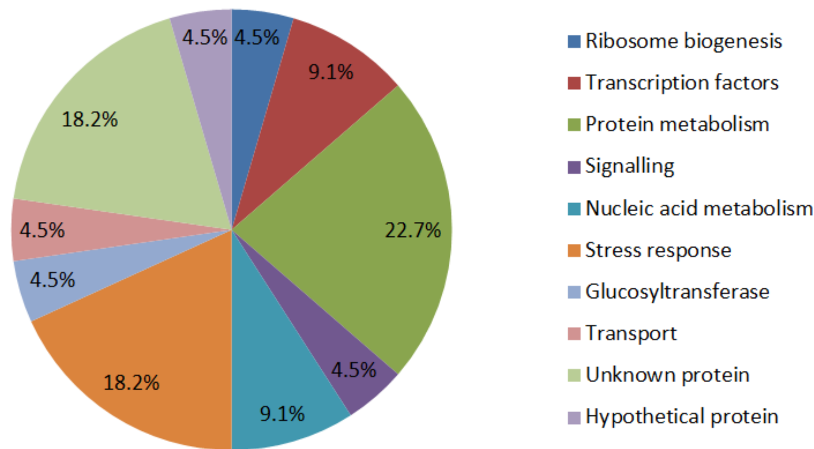

Figure 2.4 Functional distribution of ESTs represented within the library. Clustered representation of functions of 22 ESTs isolated from the library.

During cell wall reconstruction a number of genes are up-regulated (Sharma *et al*., 2011) and the groups highly up-regulated were compared to the ESTs obtained from the library. It was found that the majority (68%) of the ESTs in the library were in functional groups that represented the largest groupings of up-regulated genes during regeneration of the cell wall of protoplasts (Sharma *et al*., 2011). A large proportion of the ESTs also represent unknown, uncharacterized and hypothetical proteins, which has also been observed in other studies (Yang *et al.,* 2008; Sharma *et al.,* 2011). Sequences coding for a protein belonging to the HSP20 family (NM_128504.3) and a putative glucosyl transferase (NM_119575.1) from the library were also found to be highly expressed in rice protoplasts after 12 hours of regeneration (Sharma *et al*., 2011). Glutamate dehydrogenase (NM_001125711.1) a protein found to be loosely associated with the cell wall of *A. thaliana* protoplasts after 1 and 3 hours of regeneration (Kwon *et al.,* 2005) was also present amongst the library ESTs. This small number of sequenced clones indicate that the library is possibly representative of regenerating protoplasts and would, therefore, likely contain a higher proportion of cell wall biosynthetic genes as has been demonstrated previously (Gipman, 2001).

2.3.4 Attempts to establish a high-throughput transformation system in C. protothecoides

A range of antibiotics were tested to determine their potential as a selective agent for transformation studies in *C. protothecoides*. It was determined that 35 µg/ml of G418 was the minimum concentration required to completely inhibit algal growth (Fig 2.5 B).

Transformation studies based on an agrobacterium-mediated approach required a lack of sensitivity to cefotaxime (Kathiresan & Sarada, 2009) and it was observed that *C. protothecoides* was insensitive to cefotaxime at concentrations of 1mg/ml (Fig 2.5 D).

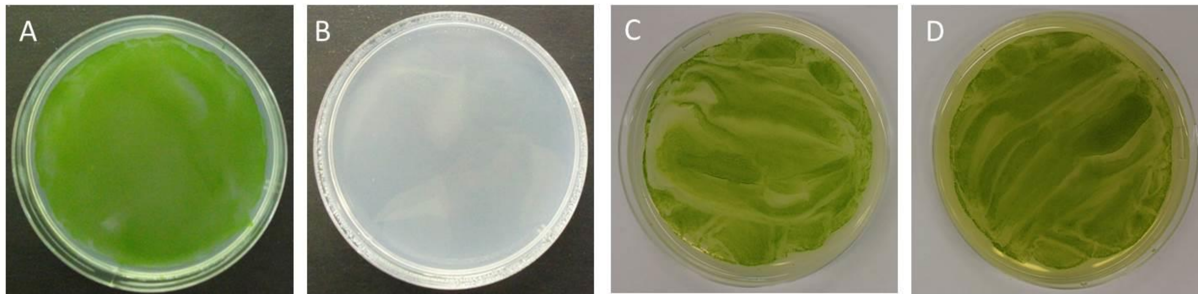

Figure 2.5 Antibiotic sensitivity of *C. protothecoides*. Liquid cultures of *C. protothecoides* spread onto solid MP media with: A. No antibiotics; B. 35 µg/ml G418; C. No antibiotics; D. 1 mg/ml cefotaxime.

Being the most widely used promoter in plant transformation and successfully driving expression in a number of unicellular algae, the CaMV 35S was the promoter of choice in this study (Brown *et al.,* 1991; Jarvis and Brown, 1991; Maruyama *et al.,* 1994; El-Sheekh, 1999; Hawkins & Nakamura, 1999; Kim *et al.,* 2002). The pCAMBIA2301 vector which confers resistance to G418 by means of expression of NPTII (neomycin phosphotransferase II) under the CaMV35S (Cauliflower Mosaic Virus) promoter was selected for transformation procedures. Unfortunately, despite numerous attempts none of the transformation approaches attempted yielded a single transgenic *C. protothecoides* colony. Controls on non-selective media, displaying expected growth, confirmed that procedures were non-lethal. Previous reports have indicated that the algae is transformable by means of the glass-bead transformation procedure (Donald Danforth Plant Science Center, unpublished results). To test if the problem was due to a lack of expression using the CaMV35S promoter I additionally utilised the plasmid pBK-CMV (where expression of the *NPTII* gene is under control of the eukaryotic SV40 promoter that is active in *Chlamydomonas reinhardtii*; Butanaev, 1994) as well as pEmuKN (Where *NPTII* expression is driven by the synthetically modified Adh1 promoter; Franks & Birch, 1991). These were utilized in glass bead transformation attempts on *C. protothecoides,* however, no transformed colonies were observed with the use of these additional transformation vectors either.

2.4 Conclusion:

This section of the study was aimed at the identification of cell wall biosynthetic genes, which were not obtained as significant difficulties were encountered in the development of a

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suitable screening system. The study did result in the production of a good cDNA library which appears to be enriched for genes expressing actively during the regeneration of protoplasts. The current approaches taken to transform *C. protothecoides* have not been successful and this system could be replaced by the use of another strain of *Chlorella* in which an established transformation procedure exists.

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Chapter 3: Library construction and screening for the identification of tuber-specific starch synthase genes in *Tylosema esculentum*

3.1 Introduction

Tylosema esculentum, the Braaiboontjie or Marama bean, is an underutilized crop that occurs natively in the arid and semiarid regions of Namibia, South Africa and Botswana. This perennial species of plant is drought-tolerant and a popular nutrient source for both wild animals and indigenous human populations. Exhibiting annual prostrate runners, which grow well upon dry and sandy soil, the plant belongs to the legume family and bears seedpods containing normally between one and three edible seeds (Keith & Renew, 1975; Müseler, 2005). A subterranean tuber, capable of storing up to 90% (w/v) water maintains the plant during winter months when the runners die off (Keith & Renew, 1975). The seeds, rich in both lipids and proteins, have been studied thoroughly and represent the main economic revenue generated by this crop. For the interest of this project, the focus will be shifted to the tuber that remains largely unstudied (Biesele & Murray, 1983, Müseler, 2005; Jackson *et al.*, 2010). The *T. esculentum* tuber, at the young age of 5 months, has a nutritional content as follows: water, 92.1%; ash (unknown mineral content), 0.42%; carbohydrate, 4.38%; fat, 0.14%; and protein 2.1% (Biesele & Murray, 1983). The composition might change through the aging process as it has been reported that the tuber becomes more fibrous and difficult to chew (Keith & Renew, 1975). Literature also mentions that the tuber starch content is high (Percy *et al.*, 2010), but to my knowledge no studies have been presented on the starch content and composition.

Considering the success of one underutilized crop, cassava, that developed into an internationally competitive starch industry in Thailand (Sriroth *et al.*, 2000), the opportunity exists to investigate the potential of *T. esculentum* as a cash crop in Sub-Saharan Africa. In order to help in developing this, more knowledge is required about the starch in the tuber and the molecular biology of its synthesis. The study set forth in this chapter was performed to identify novel genes involved in starch synthesis in the tuber of *T. esculentum*. The approach taken was to construct (1) a tuber-specific cDNA library from mRNA isolated from the legume, *T. esculentum,* and (2) to screen the aforementioned library to identify genes involved in starch synthesis.

3.2 Materials and methods:

3.2.1 T. esculentum seed germination and cultivation

T. esculentum seeds were a gift of Emmanuel Nepolo (University of Namibia). Seeds were placed in a mixture of 50% sand and 50% potting soil and allowed to grow for approximately three months in a glasshouse between September and December.

3.2.2 RNA extraction from tuber material

T. esculentum tubers were removed from the soil, sliced into smaller discs, and immediately frozen in liquid nitrogen. Material was ground into a fine powder with a mortar and pestle and stored at -80 °C. RNA was extracted by means of a modified CTAB RNA extraction procedure. 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. RNA extractions were performed by the addition of 1.5 ml CTAB buffer to approximately 200 mg of ground material. Mixing was accomplished by vortexing vigorously for 30 seconds. The samples were then incubated at 65 °C for 30 minutes, and vortexed for 30 seconds at 5 minute intervals. Centrifugation was performed at 16 000 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. Precipitation by the addition of LiCl to the collected supernatant to a final concentration of 2 M and incubation overnight at 4 °C followed. The sample was centrifuged at 16 000 x *g* for 1 hour at 4 °C, the supernatant removed and the pellet washed with 70% ethanol. This was centrifuged at 16 000 x *g* for ten minutes at 4 °C, and the ethanol removed. The resulting RNA pellet was allowed to dry for 30 minutes before 30 µl of DEPC-treated dH_20 was used to re-suspend it.

3.2.3 Precipitation of RNA

The RNA concentration in each sample was determined spectrophotometrically. The 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. 2.5 volumes of ethanol were mixed gently with the solution before being incubated at -80 °C overnight. The mixture

was centrifuged at 16 000 x g at 4 °C for one hour, the supernatant removed and the pellet washed with 70% (v/v) ethanol. RNA was recovered by centrifugation for 30 minutes at 16 000 x *g* and 4 °C. Following removal of the supernatant the pellet was centrifuged for 20 minutes at 16 000 x *g* at 4 °C and the remaining ethanol was also removed. RNA was re-suspended in DEPC-treated dH_20 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 RNA

3.2.4 Isolation of mRNA

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.

3.2.5 Construction of a cDNA library

The *T. esculentum* cDNA library was constructed with approximately 1 µg of starting mRNA. Library construction was accomplished by using the CloneMiner ™ II cDNA library kit (Invitrogen). All procedures were performed according to the manufacturer's protocol.

3.2.6 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 DAPA (diaminopimelate) to a final concentration of 0.01% (w/v).

3.2.7 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 200µl of 50 mM TRIS-Cl pH 8.0; 10 mM EDTA; 0.1 g/l RNAse A by vortexing. 200 µl of 200 mM NaOH; 1% (w/v) SDS was added and the tube mixed gently by inversion followed by the addition of 200 µl 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 16 000 x *g* for 10 minutes. The supernatant was transferred to a tube containing 0.7 volumes of isopropanol and

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mixed by inversion. This was left at room temperature for 5 minutes and then centrifuged at 16 000 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.

3.2.8 Restriction digests

DNA was digested by restriction enzymes, *Eco*RV, *Pvu*II and *Mlu*I (Fermentas) according to the manufacturer's instructions.

3.2.9 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 dH_20 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*. 1 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 volume of 8 M LiCl and 2.5 volume of ethanol was 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).

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3.2.10 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 $MgSO_4$; pH 7.5) and grown to an OD_{600} 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 $CaCl_2.2H_20$; 20 mM $MnCl_2.4H_20$; 10 mM $MgCl_2.6H_20$; 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 $CaCl_2.2H_20$; 20 mM $MnCl_2.4H_20$; 10 mM $MgCl_2.6H_20$; 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 OD_{600} of between 1.0-1.5. Aliquots of 50 µl of re-suspended cells were frozen in liquid nitrogen and stored at -80 °C.

3.2.11 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. 350 µl of LB media was added to the cells and incubated for one hour at 37 °C. The cells were plated onto solid LB media containing appropriate antibiotics.

3.2.12 Construction of pBluescript SK(-)::DEST

The Gateway® Cassette was ligated into the *Eco*RV site of pBluescript SK(-) in sense orientation with respect to the Lac promoter, to generate the pBluescript SK(-)::DEST Gateway® compatible expression vector.

3.2.13 Library transfer reaction

A sample of the library plasmid DNA was treated with RNase A (Fermentas) at 37 °C for 30 minutes and an aliquot diluted to 25 ng/ul, after which the manufacturer's instructions were followed to transfer the library by means of an LR Library Transfer Reaction (Invitrogen).

3.2.14 Preparation of electrocompetent E. coli G6MD2 ::pACAG cells

The plasmid pACAG (Kossmann et al. 1999), a pACYC-184 deriviative 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. 2 ml of this was added to 250 ml LB and grown to an OD_{600} 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 µl were frozen in liquid nitrogen and stored at -80˚C.

3.2.15 Electroporation of electrocompetent E. coli G6MD2::pACAG cells

Electrocompetent cells were thawed on ice and 20 µl mixed with approximately 1 µl (100 ng) of expression library plasmid DNA. The cell mixture was transferred a 0.2 cm pre-chilled electroporation cuvette and electroporated (Capacitance, 25 µF; Resistance, 200 ohm; 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 $MgSO_4$; 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 µg/ml chloramphenicol; 50 µg/ml ampicillin; 0.01% w/v DAPA; 20 mM glucose) and grown overnight at 37 °C.

3.2.16 Screening of expression library

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 until colonies. Blue colonies were selected and the plasmids extracted on small scale. The isolated plasmids were diluted 10^{-1} and electroporated into *E. coli G6MD2::pACAG* cells 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.

3.2.17 Sequencing of plasmid inserts

Plasmid DNA was sequenced by the Central Analytical Facility of the University of Stellenbosch.

3.2.18 Tuber starch analysis

Tuber starch was analysed using a commercially available kit (Megazyme) based on the selective precipitation of amylopectin using Concanavalin a, according to the manufacturer's instructions.

3.3 Results and discussion

3.3.1 Construction of a cDNA library from Marama bean tubers

Total RNA of good quality (Fig. 3.1) from which mRNA was obtained, was extracted from the tuber of *T.esculentum.* The CloneMiner^{TM} II cDNA library kit (Invitrogen) was then utilized to construct a tuber-specific cDNA library from the *T. esculentum* mRNA.

Figure 3.1 Electrophoresis profile of total RNA extracted from the tuber of *T. esculentum*. RNA extracted from the tuber was separated by non-denaturing agarose gel electrophoresis (1% w/v).

The primary library, constructed in pENTR^{TM} 222, contained a titer of approximately 1.5 x 10^6 cfu. This should, therefore, be representative as it exceeds the general guideline of 1 x 10^6 clones which ensures sufficient transcript coverage. Qualitative analysis of the library was performed by isolating plasmid DNA from 20 colonies at random and consequent digestion with *Mlu*I. The plasmid digests were separated by agarose gel electrophoresis and the fragment sizes (Table 3.1) determined by comparison to a known DNA ladder (λ DNA/PstI digest, Sigma Aldrich)

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Table 3.1 ***T. esculentum*** **primary library insert size.** Insert sizes were estimated following *Mlu*I digests of plasmid DNA and separation by agarose gel electrophoresis.

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

The average insert size was found to be approximately 1.5 kb and sizes ranged from 0.4 kb to 3.4 kb, with 90% of the sequences exceeding 1 kb (Table 3.1). Each of the isolated plasmids contained an insert indicating that the recombination rate was close to 100%. The average insert size of 1.5 kb matches the theoretical average size of eukaryotic cDNA's (Xu *et al.,* 2006).

3.3.2 Transfer of cDNA library to an expression vector and qualitative analysis of the expression library

A Gateway® compatible vector was constructed in pBluescript SK(-). The Gateway® cassette was ligated in sense orientation with regard to the Lac promoter, and the orientation confirmed by sequencing. The library was transferred from pENTR™ 222 to pBluescript SK(-)::DEST by means of a LR library transfer reaction to generate a bacterial primary expression library for functional screening. The final expression library was produced from the plasmids contained within 1 x 10^6 colonies from the primary expression library. 20 Colonies from the expression library were selected at random and their plasmid DNA digested with *Pvu*II. The digested plasmid DNA was separated by agarose gel electrophoresis and the average insert size (Table 3.2) calculated by comparison to a known DNA ladder (λ DNA/PstI digest, Sigma Aldrich). Analysis of the fragments indicated that 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. Recombination rate was close to 100%.

Table 3.2 *T. esculentum* expression library insert size. Insert sizes were estimated following *Pvu*II digests of plasmid DNA and separation by agarose gel electrophoresis.

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

3.3.3 Screening of the T. esculentum tuber expression library

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 (Schwartz, 1966). The bacteria was 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 (Figure 3.1) when exposed to iodine (Kossmann *et al.,* 1999).

Figure 3.2 Iodine vapour staining of *E. coli G6MD2.* *E. coli G6MD2*::pACAG cells stain a pale golden yellow in the presence of iodine vapours, whilst *E. coli G6MD2*::pACAG expressing *Solanum tuberosum* soluble starch synthase I stain dark blue.

In excess of a 100 000 clones from the *T. esculentum* library were screened in *E. coli G6MD2*::pACAG cells and yielded only golden staining colonies. No functional starch synthase was identified from the tuber expression library during the screening procedure. The presence of starch in the tuber (Section 3.3.4.) indicates that a starch synthase should be expressed there. It is possible that the library represents a transcript profile in which starch synthase cDNA's are lowly expressed. In addition to this the cDNA sequences of starch synthases in *A. thaliana* (NM_001203449.1; NM_110984.2; NM_101044.3; NM_117934.4; www.ncbi.nlm.nih.gov) exceed a length of approximately 2.35 kb and it is reasonable to assume that they might be of similar size in *T. esculentum.* With only a fifth of the sequences in the expression library being larger than 2.35 kb, it is possible be that no full length cDNA starch synthase sequence is present. The expression of a correctly folded eukaryotic protein in *E. coli* is often a difficulty encountered, but starch synthases from *Solanum tuberosum* and *A. thaliana* have been shown to be functional in this system before (Kossmann *et al.,* 1999; Szydlowski *et al.,* 2009). Additional screening reactions are on-going to isolate a starch synthase coding sequence. Starch synthase cDNAs could also be identified by designing degenerate PCR primers, specific to highly conserved regions of the starch synthase genes. An alternative procedure to identify the gene coding for starch synthase is also being explored. This involves the isolation and purification of a starch synthase enzyme from the tuber material which can be used in a peptide sequencing reaction. The sequence data obtained herewith can be used to design starch synthase gene specific primers for 5' RACE-PCR and 3' RACE PCR. In principle, next generation sequencing could also be used to identify the sequence by analysis of the entire library.

3.3.4 Analysis of tuber starch content and composition

To our knowledge, no study has investigated the starch content and its composition within the tuber of *T. esculentum*. The starch content, G6P content and amylose content were determined (Table 3.3).

Table 3.3 *T.esculentum* tuber starch composition. Starch content, G6P content and amylose content of the Marama bean 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 given plus standard deviation.

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In comparison to industrially important starch sources, the *T. esculentum* tuber with a starch content of approximately 9% is lower than that of potato tuber (15.44% w/w; USDA), cassava roots (18.1-23.4% w/w, 6 months; Sriroth *et al.*, 1999) and cereal seeds (65% w/w, maize, Li *et al.*, 2011).

Although Marama bean tubers have lower starch content than traditional starch crop plants it is important to consider the low agricultural input required for their cultivation. They grow wild, need little maintenance and the soil area can be shared with other revenue generating crops. The tuber at this stage has no economic value, but studies on the starch properties could possibly identify market segments where it would be of value. A possible application could exist in the food industry, as the viscosity indicates that it has double the gelling property of potato starch (Pers. Comm., Dr. James Lloyd, Stellenbosch University). More studies will need to be performed on the nature of the starch before its potential as an orphan crop in Southern Africa can be realized.

3.5. Conclusion

This study aimed at identifying genes involved in starch synthesis in the tuber of *T. esculentum*. Although this was not achieved a tuber-specific library of good quality was produced, transferred into an expression vector and a screening procedure was established for the identification of a functional starch synthase. Unfortunately this approach did not confirm the presence of a starch synthase within the expression library.

Investigations into the properties of the tuber confirmed the presence of starch. The average starch content, G6P content and amylose content of the tuber was determined and this data will likely lead to additional studies into the nature of the tuber starch and its potential industrial applications.

Chapter 4: General Discussion

Biopolymers are of great importance to industry, and investigations into their metabolism could identify aspects that could further contribute to their economic value. Therefore the anticipated outcomes of the project as a whole were to identify genes involved in biopolymer synthesis in higher plants, with specific focus on cell wall synthesis in *A. thaliana* and starch synthesis in *T. esculentum*. The study did not result in the identification of any novel genes, but was successful in producing data that provides a fundamental structure for the future continuation of such projects.

Great advances have been made in understanding the complex process of wall synthesis, but it is believed that the majority of genes that participate in these activities remain unknown (Lerouxel *et al.,* 2006; Carpita & McCann, 2008). This verifies the importance in developing new experimental platforms wall-metabolism studies. In the first experimental chapter (See Chapter 2) the potential of a new system to investigate cell wall biosynthesis was explored. A cDNA library was constructed from regenerating protoplasts, as it was expected to enrich for cell wall-related genes (Gipman, 2001). The cDNA library was found to be of good quality and a few ESTs with sequence similarity to genes previously discovered in protoplast regeneration studies confirmed that it was representing a population of sequences that are expected to be present during wall regeneration. The difficulty was encountered in establishing a screening method for the produced library.

Unicellular algae offer a unique opportunity to investigate carbohydrate metabolism in vascular plants (Hicks *et al*., 2001) and therefore it was decided to screen the library in a microalgal species. Screening of the library was to be performed by transferring the library into *C. protothecoides* by transformation and studying colony morphology for apparent changes induced by the expression of higher plant genes. The development of a high-throughput transformation procedure was thus essential. Most of the conventional approaches previously found to be successful in producing transgenic microalgae were tested on *C. protothecoides* and no transformants were obtained. The effect of promoter influence on transformation was also tested by introducing the same transgene under the control of different promoters into *C. protothecoides.* Considering these findings experiments are underway to develop a screening method in another strain of unicellular algae with an established transformation system. It is expected that an established screening system in combination with the produced cDNA library will lead to the identification of genes involved

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in cell wall metabolism and possibly contribute to a better understanding of cell wall synthesis in higher plants.

In the second experimental chapter (See Chapter 3) an investigation was performed into the potential of commercialization of the southern African under-utilized crop plant, *T. esculentum*. This section of the project was aimed at the identification of genes involved in the synthesis of tuber starch. The study did produce a tuber-specific cDNA library of good quality from *T. esculentum.* Additionally a screening procedure used in previous identification of a starch synthase gene from *S. tuberosum* (Kossmann *et al*., 1999) was further developed to be used in conjunction with the commercially available Gateway® (Invitrogen) system which was essential for library screening purposes. Despite screening more than 100 000 plasmids, none were found that led to starch synthase activity. The library could perhaps be sequenced using next generation technology in order to find not just starch synthases, but other starch metabolic genes as well. Such a sequencing project would also provide genetic resources for scientists producing a genetic map for Marama bean.

The data generated in the analysis of the tuber starch are the first known information about *T. esculentum* tuber starch content and composition and indicates that it has potential to be a starch crop. Low agricultural input and relatively high yields of tuber starch from this crop might lead to its development as a revenue generating crop for poor farmers in developing regions of southern Africa. Further research endeavours are now directed at studying the nature of the Marama bean tuber starch to identify industries that would serve as prospective buyers. The possibility of a use in the food industries has been suggested due to its high viscosity, with gelling properties approximately twice that of potato starch (Pers. Comm., Dr. James Lloyd, University of Stellenbosch). More studies are bound to follow soon, as the interest of the scientific community especially those working on orphan crops are redirected to the potential value of *T. esculentum* as a starch producing cash-crop.

Essentially the data, experimental components and procedures developed in this study will provide a stable platform for continued studies on the metabolism of biopolymer production in higher plants. To identify the initially proposed cell wall biosynthetic genes from the regenerating protoplast library now depends on the successful establishment of a screening system in an alternative transformable microalgae which then needs to be optimized for high yields of transgenic colonies. The identification of sequences coding for functional starch synthases are still underway by additional rounds of screening within the established system.

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Both of the produced cDNA libraries in addition to their current on-going studies, are also in the process of being utilized in studies beyond the scope of their initial proposed use.

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