Development of a hairy root bioreactor from *Stevia rebaudiana* to produce steviol glycosides

Zanele Mthembu

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Supervisor: Dr. Shaun Peters
Co-supervisors: Dr. Tapiwa Guzha, Dr. Bianke Loedolff

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SUMMARY

Extracts from the leaves of *Stevia rebaudiana*, a plant native to South America, have been used as natural sweetener for centuries. With the global epidemic of obesity linked to increased prevalence of diabetes, *Stevia* has attracted interest for use as a non-nutritive sweetener (NNS). Unlike currently available NNS which are chemically synthesised (e.g. sucralose), *Stevia* extracts represent naturally occurring NNS with no negative side effects from its use. The sweet-to-taste compounds in *Stevia* are actually due to the accumulation of secondary metabolites in the leaves, specifically two steviol glycosides (SGs, stevioside and rebaudioside A). However, these SGs occur in low concentrations (between 2-4% of total fresh weight) and show variability in plants grown by commercial scale agricultural propagation. The plant also requires high irrigation inputs owing to its sensitivity to even moderate water deficit.

Stevia is currently not a cash crop in South Africa (SA) but there is interest in establishing commercial scale agricultural ventures to establish a *Stevia* economy. SA is also experiencing a concerning rise in the number of new incidences of diabetes amongst its population and recently approved the introduction of a sugar tax that is envisaged to reduce this excessive sugar intake and over time improve the health and well-being of the population. The variable SG yields and the high irrigation inputs required to produce them from the plant are considered major restrictive factors toward establishment of a *Stevia* economy in SA - a naturally water scarce country. Current propagation methods for *Stevia* are both laborious and costly because the seeds are recalcitrant and plants have to be propagated *via* stem cuttings or *in vitro* tissue culture.

Hairy root cultures have been widely used in plants of medicinal importance to obtain high quantities of bioactive secondary metabolites, for use as pharmaceutical drugs. *Agrobacterium rhizogenes* is utilised in this context to induce hairy root formation and a few studies have investigated *Stevia* hairy root cultures but none have reported SG accumulation in these cultures. This study attempted to create *Stevia* hairy root cultures expressing key genes in the SG biosynthesis pathway and accumulating the two sweet SGs, stevioside and rebaudioside A. Additionally, attempts were made to create *Stevia* hairy root cultures overexpressing *UGT74G1* and *UGT76G1* (the two genes responsible for stevioside and rebaudioside A accumulation respectively) with the intention of increasing SG production.. Although we demonstrated that *A. rhizogenes* could be transformed with the plant expression

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constructs and that this transformed *A. rhizogenes* could induce hairy roots from leaf explants, tandem mass spectrometry analyses of root extracts did not identify either stevioside or rebaudioside A. We suspect that the lack of photosynthetic capacity in hairy root cultures resulted in the unavailability of key intermediate substrates for SG biosynthesis that have been proposed to be produced during photosynthesis. However, we are currently investigating if these hairy root cultures could be primed for SG accumulation by growing them in the presence of the proposed intermediate substrates which are available commercially at low cost.

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ABBREVIATIONS

Bert	Bertoni
BRICS	Brazil, Russia, India, China & South Africa
C13	Carbon 13
C19	Carbon 19
CAF	Central Analytical Facility
CaMV35S	Cauliflower mosaic virus 35S promoter
cDNA	Complimentary DNA
CDS	Coding DNA sequence
cm	Centimetre
^{o}C	Degrees Celsius
CPS	*ent*-copalyl diphosphate synthase
DNA	Deoxyribonucleic acid
Dr	Doctor
EFSA	European Food Safety Authority
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
fwd	Forward
g	Gram
GA	Gibberellic acid
GGDP	Geranylgeranyl diphosphate
GRAS	Generally recognized as safe
GTs	Glycosyltransferases
h	Hour
Hyg^{r}	Hygromycin resistance gene
in vitro	" in glass"
IPB	Institute for Plant Biotechnology
KAH	Kaurenoic acid-13-hydroxylase
KO	Kaurenoic oxidase

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KS	Kaurene synthase
kV	Kilovolts
L	Litre
LB	Left border
LB medium	Luria Bertani medium
LC MS/MS	Liquid chromatography tandem mass spectrometry
LR	LR recombination reaction
µF	Microfrequency
µg	Microgram
µl	Microlitre
µmol	Micromoles
MEP	Methylerythritol-4- phosphate pathway
mg/L	Milligrams per litre
min	Minute
ml	Millilitre
mm^2	Millimetre square
MS	Murashige and Skoog (1962) medium
m/z	Mass to charge ratio
NCBI	National Center of Biotechnology Information
n.d	No date
NRF	National Research Foundation
Nos T	Nos terminator
NSS	Non-nutritive sweeteners
NY	New York
Ω	Ohms
OD_{600}	Optical density at 600 nanometers
%	Percent
PAHO	Pan American Health Organisation
PCR	Polymerase chain reaction

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PKU	Phenylketonuria
ppm	Parts per million
p*Ri*	Root inducing plasmid
RB	Right border
rev	Reverse
rpm	revolution per minute
Ri	Root-inducing
RNA	Ribonucleic acid
SA	South Africa
sec	Second
SGs	Steviol glycosides
SSBs	Sugar sweetened beverages
StatsSA	Statistics South Africa
T7	T7 Promoter region
TB	Tuberculosis
TBE	Tris-Borate EDTA buffer
T-DNA	Transfer DNA
Ti	Tumour-inducing
T_R	Right T-DNA region
T_L	Left T-DNA region
UGTs	UDP-glycosyltransferases
USA	United States of America
USAID	United States Agency for International Development
USD	United States Dollar
UV	Ultra violet
vir	Virulence gene
v/v	Volume per volume
WHO	World Health Organisation
w/v	Weight per volume

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w/w	Weight per weight

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1. INTRODUCTION

Stevia rebaudiana (*Stevia*) is a plant native to South America that is now grown globally on a commercial scale for use as a non-nutritive sweetener. The demand for *Stevia* is fuelled by its use as a natural alternative to table sugar (sucrose), which has been linked to the global epidemic status of diabetes (a disease which renders the human body unable to metabolize glucose). *Stevia* extracts were approved for human use by the Food and Drug Administration (FDA) in 2009 and, by the European Union in 2011 (Gardana *et al.,* 2010). China is the leading supplier of *Stevia* plants and their products to the global market (Kinghorn and Soejarto, 1985) and the *Stevia* industry is estimated to be worth USD 565 million annually with significant growth projection in the next five years (Future Market Insights, 2014).

The use of *Stevia* is mainly tied to the occurrence of unique secondary metabolites in its leaves. These are termed steviol glycosides (SGs) and a number are known to accumulate in the plant. However, only two SGs (stevioside and rebaudioside A) are known to impart the sweet-to-taste characteristic that is relevant to their use as alternative sweeteners (Madan *et al.,* 2010). Currently, *Stevia* is not commercially cultivated in SA but our BRICS partners India and China are the major global producers of *Stevia*. However, in 2012, the SA government approved the use of *Stevia* extracts as a natural sugar alternative (Foodstuff South Africa, 2012) and with the planned sugar tax, the SA government is hoping to gradually prevent and cut the high trends of obesity, diabetes and cardiovascular diseases (Manyema *et al.,* 2014).

Seed propagation results in heterogeneous populations and variability in SG content (Sivaram and Mukundan, 2003). Consequently, *Stevia* is propagated by either stem cuttings or tissue culture, and both requires high labour inputs and are limited by the number of clonal individuals obtained from a single plant (Sivaram and Mukandan, 2003). An important factor in *Stevia* conventional cultivation methods such as stem cutting is that the major SGs which impart the sweet taste of *Stevia* extracts (stevioside and rebaudioside A) occur in low and variable amounts approximately 4% of leaf dry weight (Yadav et al., 2011).

In the context of establishing a commercial scale *Stevia* industry in SA, the physiology of the *Stevia* plant poses a natural limitation. Since it is native to tropical climates, its commercial cultivation requires intensive irrigation as it is extremely sensitive to water deficit (Kaushik *et al.,* 2010). *Stevia* plants wilt rapidly under moderate water-deficit and this negatively affects

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the accumulation of SGs (Lemus-Mondaca *et al.,* 2012). The agricultural landscape in SA is typified by its water scarcity and as such any commercial scale venture to propagate *Stevia* would require high irrigation inputs for the successful production of SGs (Ngaka, 2012)

However, there have been considerable efforts in the use of plant tissue cultures as an alternative approach to plant regeneration with significant success (Guruchandran and Sasikumar, 2013; Patel and Shah, 2009; Pande and Gupta, 2013). Despite these substantial efforts for the improvement of *Stevia* propagation there has not been much breakthrough with regards to large-scale production of the SGs. Hairy root bioreactors are an established alternative for the production of secondary metabolites that typically accumulate in relatively low amounts in plant tissue (Mishra and Ranjan, 2008; Flores and Medina-Bolivar, 1995). The advantage of hairy root culture is its ability to grow rapidly in the absence of plant growth regulators and typically produce even more secondary metabolites than the parent plant (Eapen and Mitra, 2001). Since traditional propagation methods and low SG yields in leaf tissue ultimately culminate in relatively low amounts of SG production compared to the input material, hairy root cultures are an attractive alternative. Hairy root cultures have been used in other plants to produce medicinally important compounds such as digoxin from *Digitalis lanata*, quinine and quinidine from *Cinchona spp*, morphine and codeine from *Papaver somniferum* (Saito *et al.,* 1992; Hollman, 1996).

The focus of the study was to produce a *Stevia* hairy root bioreactor system that can reliably produce and accumulate SGs, particularly stevioside and rebaudioside A to be utilised as a natural sugar alternative. Although significant progress has been made in understanding the biological processes involved in the biosynthesis of SGs (Brandle and Telmer, 2007; Yadav and Singh, 2012; Guleria and Yadav, 2013) *Stevia,* a non-model species, remains relatively uncharacterised across the board (Chen *et al.,* 2014). There have been limited biotechnological applications on *Stevia rebaudiana* in SA to actively encourage commercialization. Globally, a few studies have confirmed hairy root induction in *Stevia* utilising *A. rhizogenes* strains, but none have demonstrated SG accumulation in hairy root tissue (Yamazaki *et al*., 1991, Michalec-Warzecha *et al.,* 2016). This study was conducted to develop a feasible and cost effective hairy root bioreactor culture with enhanced SG production.

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1.1 Aims and Objectives of the study

The study is contextualized to the emergent *Stevia* industry in South Africa. We propose that the commercial scale cultivation of *Stevia* in the future will encounter regional specific problems given that South Africa is a water scarce country and *Stevia* plants require intensive irrigation inputs for successful cultivation. Coupled to this is an inherent low and variable SG content in the leaves of *Stevia* (only between 2 - 4% of total fresh weight). We believe that this provides an opportunity to explore new ways for SG production if South Africa is to exploit the future market potential of SG production.

The project thus aimed to investigate the creation of a viable *Stevia* hairy root culture and to determine whether it was able to produce any SGs, and generate transgenic hairy roots overexpressing *UGT74G1* and *UGT76G1* with elevated levels of SGs. To this end we (i) infected leaf explants with various strains of *A. rhizogenes* and a strain containing a binary vector for the overexpression of key SG biosynthetic genes (ii) established if any hairy root cultures expressed key genes from the known SG biosynthetic pathway and (iii) analysed the metabolite profile of hairy root cultures by tandem mass spectrometry to ascertain if they accumulated any SGs.

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2. LITERATURE REVIEW

2.1 Increased dietary sugar consumption is associated with increased incidence of insulin resistance

Sugar has historically been associated with human society but in the late 18th century the first mechanized refinery process involving sugarcane vastly improved both the production and accessibility of refined sugar to the human population (Clemens *et al.,* 2015). Currently about 175 million metric tons of refined sugar is consumed annually (The Statista Portal n.d.). Although sugar consumption may not directly cause diabetes especially diabetes Type II, it has been associated with predisposing risk factors such as obesity and lack of exercise (Stuckler *et al.,* 2012; Anton *et al.,* 2010). Obesity is a major global health problem in developing countries which typically must manage the dual burdens of chronic and infectious diseases, and this leads to excessive total healthcare costs (Malik *et al.,* 2010). The primary source of sugar intake that contributes to the obesity epidemic is the sweetened sugar beverages (SSBs). An increased consumption of SSBs and diabetes Type II prevalence have been noted over the last few decades resulting in governments implementing interventions to reduce sugar intake such as sugar taxes (Nielsen and Popkin, 2004).

The World Health Organisation (WHO) recently released the first summative report of the worldwide occurrence of diabetes (WHO, 2016). It is clear that the incidence of diabetes is rising to pandemic proportions and that developing countries (including SA) are already dealing with the challenges of treatment in already strained public health systems. Many developing countries lack sustained public awareness campaigns stimulating healthy lifestyles and this serves only to compound new incidences of non-communicable diseases (like diabetes). The SA government has recently taken steps to address the excessive intake of dietary sugar of the populous by proposing a tax on sugar-sweetened drinks that is envisaged to reduce this excessive sugar intake and over time improve the health and well-being of the population (Blecher, 2015). While this issue has become contentious in terms of whether it will work in the absence of a multipronged strategy to address public health issues, South Africa is not the first country to introduce such a tax and Mexico, France, Hungary (and New York City) have already introduced such sugar taxes Pan American Health Organisation (PAHO, 2015).

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It is in this context that efforts to source alternative sweeteners that have little to no impact on general human health have intensified. These alternative sweeteners are generally defined as non-nutritive (since they do not provide any calories when consumed) and may occur naturally (e.g. *Stevia* extracts) or most often represent chemically synthesized alternatives (e.g. saccharin; Shwide-Slavin *et al.,* 2012.

2.2 A closer view into non-nutritive sweeteners (NNS)

Also known as artificial sweeteners or non-caloric sweeteners, NNS are sugar alternatives that provide sweetness without glycaemic effects in the body (Gardiner *et al.,* 2012). NNS can be up to a thousand times sweeter than sucrose (the most common dietary sugar). The intense sweetness allows for consumption of small portions to give sugar-like sweetness in foods, therefore people with obesity and those suffering from diabetes can enjoy foods and beverages without the risk of adding calories. Among the NNS that have been approved by the FDA, and have been granted a generally recognized as safe (GRAS) status, five are chemically synthesized (aspartame, saccharin, acesulfame K, neotame and sucralose) and one is a natural extract from the plant *Stevia* (Shwide-Slavin *et al.,* 2012).

However, the health advantages and disadvantages of these NNS have been in question since their discovery and introduction (Weihrauch and Deihl, 2004). There are negative side effects associated with NNS and the conflicting evidence by a recent study (Pepino, 2015) that reported metabolic responses to an oral glucose load after sucralose ingestion supporting the idea that NNS as the whole can be metabolically active in the body. The concept of NNS being metabolically inert with no glycaemic responses in the body can no longer hold true (Pepino, 2015), however, with this being said, human studies on natural *Stevia* extracts have shown potential benefits with no recorded negative side effects making it a better choice in the management, treatment and prevention of obesity (Ashwell, 2015; Elnaga and Mohamed, 2016).

2.2.1 Saccharin

Saccharin, was approved before 1958 for general use as an organic non-nutritive sweetener. It is about 200-700 times sweeter than sucrose (Fitch and Keim, 2012). As a synthetic alternative sweetener, saccharin is believed to pass through the body without being metabolized giving off no calories. However, it has been reported to have carcinogenic potential since it caused bladder cancer in rats (Reuber, 1978).

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2.2.2 Aspartame

Aspartame was approved in 1981 and is about 160-220 times sweeter than sucrose. Unlike saccharin, aspartame is metabolized in the body yielding fewer calories than those obtained from the same amount of refined sugar to produce the same sweetness (Tandel, 2011). Further metabolism of aspartame yields aspartic acid, methanol and phenylalanine, therefore it must be used with caution by people with phenylketonuria (PKU) condition because their bodies cannot metabolize phenylalanine to tyrosine (Magnuson *et al.,* 2007).

2.2.3 Acesulfame K

Generally known as acesulfame potassium and approved in 1988, it is about 200 times sweeter than sucrose (Fitch and Keim, 2012). Although it is not metabolized in the body and is excreted unchanged by the kidneys, its natural breakdown over time yields acetoacetamide as a by-product and this is toxic at high doses in the body. Studies (Bandyopadhyay *et al.,* 2008; Karstadt, 2010) have found it to be genotoxic and that it can inhibit fermentation of glucose by intestinal bacteria (Bian *et al.,* 2017).

2.2.4 Sucralose

Sucralose is also poorly metabolized during the digestion process because the body does not recognize it as a carbohydrate. It is made from the sucrose molecule however, three of the hydroxyl groups are replaced by chlorine atoms (Shwide-Slavin *et al.,* 2012), and thus it passes through the body unchanged with relatively small amounts being absorbed in the gastrointestinal tract. Sucralose has been reported to be non-carcinogenic and non genotoxic; however, it has been identified to cause migraines and headaches (Gardiner *et al.,* 2012; Romo-Romo *et al.,* 2016).

2.2.5 Neotame

Neotame is a dipeptide methyl ester derivative that is about 7000-8000 times sweeter than sucrose and highly stable (Tandel, 2011). It was approved in 2002 but has been rarely used. In humans it is rapidly absorbed, and like aspartame it yields aspartic acid and phenylalanine but only small amounts are needed to sweeten foods therefore it does not pose any major threat to people with PKU (Shwide-Slavin *et al.,* 2012).

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2.2.6 S. rebaudiana extract as a natural non-nutritive sweetener

Stevia is known to have been used as a natural sweetener by the Aztecs culture in South America (Kinghorn, 2002). Leaf extracts from *Stevia* are claimed to be about 300 times sweeter than sucrose (Phillips, 1987). In addition to the sweetness intensity, SGs also show thermostability up to 200ºC, and are thus suitable for the use in cooked foods (Lemus-Mondaca, 2012). *Stevia* has been used in applications as a sweetener in food and beverage industries, confectionaries, fruit and milk drinks, delicacies and as dietary supplements (Mehrotra *et al.,* 2014).

The sweet-to-taste effect in the leaves is actually due to natural accumulation of secondary metabolites termed steviol glycosides (SGs). These naturally occurring SGs are non-caloric and it is for this reason alone that *Stevia* is heralded as one of the most important naturally occurring NNS for use as a dietary sugar-substitute (Anton *et al.,* 2010). The SGs have been shown to have no effect on blood glucose and pressure when consumed and, no recorded side effects have been reported in extensive animal model testing (Barriocanal *et al.,* 2008). A human study on toxicity and intake supports its safe use as an NNS (Anton *et al.,* 2010).

2.3 Current *S. rebaudiana* trends and status in sweetener markets

Currently, the global sweetener markets include both caloric (traditional sugar) and non-caloric sweeteners (chemically synthesized and natural) with consumer preferences driving a shift toward an increased demand for natural NNS compounds. Consequently, *Stevia* is considered as the forerunner in terms of its use as an artificial sweetener and there is a definitive interest in cultivating the plant on a commercial scale in order to harvest SGs (Patel and Shah, 2009; Aman *et al.,* 2013).

While the global sweetener market was estimated to be USD 68.1 billion in 2014 and is expected to increase to USD 95.9 billion by 2020, the market share of *Stevia*-based products was estimated at USD 347.0 million in 2014 with a projected increase to reach USD 562.2 million in 2020 (Future Markets Insights, 2014). This was estimated in the context of volume of consumption of *Stevia*. The introduction of *Stevia* sweeteners to the market have been the main focus of the large food and beverage companies (Clos *et al.,* 2008). Most recently (2014), *Stevia* was the focus of media attention when both Coca-Cola and PepsiCo simultaneously announced the launch of new low calorie soda variants which contained *Stevia* extracts, highlighting the emerging importance of *Stevia* in the NNS market.

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However, globally the demand for *Stevia* is localized to the Asia Pacific region (largest consumer) followed by North America, Latin America and Europe, respectively United States Agency for International Development (USAID Market Brief, 2014). In SA, no *Stevia* market exists but on the basis of the increasing global demand and the context of the proposed sugar tax there is an interest in growing *Stevia* on a commercial scale. In this regard a locally based company, (FoodStuff, 2016), that specializes in plant-based extracts for consumer use has outlined a pioneering venture in SA. They plan to conduct a pilot project to develop both the agricultural knowledge and technological ability toward large-scale commercialization of *Stevia*. A number of factors need to be taken into account in order for such ventures to be successful. Given that SA is considered a water scarce country (Sershen *et al.,* 2016; Knox *et al.,* 2010) factors such as climate change, crop management strategies, production techniques and water management should be looked into first in order to cultivate *Stevia* in large scale plantations. This is linked to the natural history of the *Stevia* plant and the intensive inputs required for successful commercial scale growing (Jia, 1984).

Bitter after-taste is one of the major problems associated with SGs and hinders most food and beverage companies wishing to use *Stevia* as a sweetener. An alkaloid iminosugar, steviamine was found to be responsible for the bitter aftertaste (Michalik *et al.,* 2010), and biotechnological techniques aiming to remove this bitter aftertaste are essential and may increase the market figures since some companies are currently reluctant to use *Stevia*.

2.4 The natural history of *S. rebaudiana* leads to problems in commercial scale growing

S. rebaudiana (Bert) Bertoni is a member of the *Asteraceae* family, one of 154 members of the genus *Stevia* and one of the two species to produce steviol glycosides (Madan *et al.,* 2010). It is native to the tropical region of Paraguay, where the indigenous Gaurani Indians have been using it since ancient times as a sweetening agent (Yadav and Guleria, 2012). It also occurs in neighbouring Brazil and Argentina (Soejarto, 2002). While unsuccessful attempts were made in England to establish the crop in 1942 (Lewis, 1992), *Stevia* has been introduced into countries such as Japan, Mexico, United States of America (USA), Indonesia, Tanzania and Canada as a crop (Yadav *et al.,* 2011) and is now extensively cultivated for its SGs outside the native range.

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Stevia occurs naturally in subtropical regions and tropical regions of South America and it can grow best in semi-humid subtropical areas with a temperature of 21-43°C and cannot tolerate extreme cold temperatures below 9°C (Huxley, 1992; Singh and Rao, 2005). Its growth is dependent on existing weather conditions and with five different stages of growth, namely germination and seed establishment, vegetative growth, floral bud initiation, pollination to fertilization and seed growth and maturity (Ramesh *et al.,* 2006). Seeds of *Stevia* are said to be recalcitrant and have a very poor percentage of germination because they are small in size and largely infertile (Singh and Rao, 2005).

One major limitation to commercial scale growing of *Stevia* is that seed-propagation also results in heterogenous populations and variability in SG content (Nakamura and Tamura, 1985). Therefore, due to the poor seed germination and SG variability, cultivation through seeds is usually not the best approach (Saqib *et al.,* 2015). *In vitro* culture is considered the most efficient way to rapidly mass propagate *Stevia* plants (Sivaram and Mukundan, 2003). However, propagation through cuttings is both labour and cost intensive and still leads to variability on SG content when plants are field grown (Karim *et al.,* 2008).

A second major limitation to large scale cultivation of *Stevia* is the need for consistent supply of water. Plants wilt rapidly under moderate water-deficit and this negatively affects the accumulation of SGs (Lemus-Mondaca *et al*., 2012). Thus, despite *Stevia* being considered as the only source of the SGs used as non-nutritive sweeteners intensive effort has been required to develop 90 varieties of *S. rebaudiana* for cultivation in specific climatic conditions around the world (Ibrahim *et al*., 2008; Singh and Rao, 2005). However, these varieties still require intensive irrigation input and SG yields remain variable and sensitive to climatic conditions.

2.5 What are the steviol glycosides of *S. rebaudiana*?

SGs are secondary metabolites, tetracyclic diterpenoids with a high sweetness intensity, proven to be non-toxic and non-mutagenic (Bondarev *et al.,* 2003). About 8 SGs namely: stevioside, rebaudioside A, B, C, D, E, steviolbioside and dulcoside A accumulates in the leaves of *Stevia* and their concentrations vary widely depending on the genotype and production environment (Brandle *et al.,* 1998). Stevioside and rebaudioside A are two major SGs out of various SGs that are among those that are negatively correlated to each other since according to their biosynthetic relationship stevioside is the substrate for the synthesis of rebaudioside A, hence plants with high rebaudioside A will probably be low in stevioside (Shibata *et al.,* 1991). Despite being non-caloric, non-nutritive steviol glycosides are known

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to be stable in a wide range of pH and heat, and are non-fermentative (Kinghorn and Soejarto, 1985).

2.5.1 Insights into steviol glycosides biosynthesis

The high concentration of SGs found in *Stevia* leaves if compared to other plants organs accounts for the higher intensity of sweetness of *Stevia* leaves (Brandle and Telmer, 2007). In *Stevia*, SGs are synthesized *via* the mevalonate-independent, methylerythritol phosphate pathway (MEP), where the majority of SGs are synthesized through glycosylation reactions that begin with the aglycone steviol and ends with the production of rebaudioside A (Madan *et al.,* 2010). Determination of the subcellular location of several enzymes involved in SG biosynthesis proved the spatial organization of the biosynthesis pathway itself (Humphrey *et al.,* 2006). Kaurene oxidase (KO), an enzyme with dual roles in both gibberellic acid (GA) and SG biosynthesis, was found to be located in the endoplasmic reticulum (ER) (

Figure 2.1).

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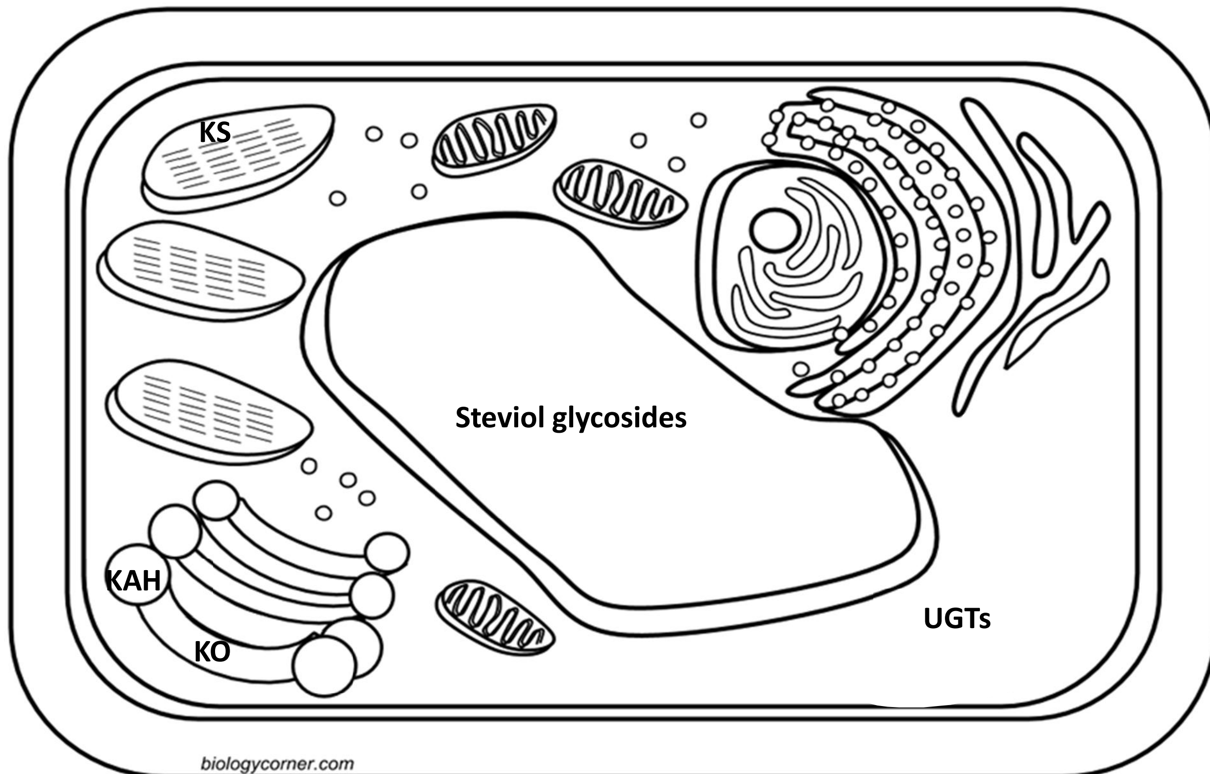

Figure 2.1 Schematic model of a plant cell showing subcellular organization of the enzymes involved in the steviol glycosides biosynthesis pathway. KS (Kaurene synthase); KO (Kaurene oxidase); KAH (Kaurenoic acid 13-hydroxylase; UGT74G1, UGT76G1, UGT85C2 (UDP-glycosyltransferases; Brandle and Telmer, 2007)

Kaurene synthase (KS) was found to be located in the chloroplast stroma (

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Figure 2.1). The reaction intermediate kaurene formed by kaurene synthase then moves out of the stroma through membranes into endoplasmic reticulum and in the presence of KO and kaurenoic 13-hydroxylase (KAH) to form steviol, which is then transported to the cytoplasm for glycosylation by uridine diphosphate glycosyltransferase (UGT) enzymes to produce SGs which are then moved to the vacuole (Humphrey *et al.,* 2006), which is not surprising since the central vacuole of plant cells has been associated with the protection of secondary metabolites from sensitive metabolic processes within the cytosol (Martinoia *et al.,* 2000).

2.5.2 The role of uridine diphosphate glycosyltransferases

Glycosyltransferases (GTs) are ubiquitous in nature and are required for the transfer of sugars from various sugar donors to important biomolecules including glycan, lipids and peptides. They have been presently classified into >80 families and are involved in numerous biological processes such as cell signalling, cell adhesion and carcinogenesis, mostly in humans (Chang *et al.,* 2011)

Uridine diphosphate glucose is the common donor and hydroxylated molecules are acceptors in the GT catalysed reactions, hence the name UGTs for the plant glycosyltransferases (Wang and Hou, 2009). UGTs are said to be region-specific to substrate molecules (Fukuchi-Mizutani *et al.,* 2003; Lim *et al.,* 2003). In plants UGTs are localized in the cytosol and are involved in the biosynthesis of plant secondary metabolites and regulation of plant hormones (Bowles *et al.,* 2006)

2.5.3 The methylerythritol 4-phosphate (MEP) pathway for steviol glycoside biosynthesis

In *Stevia*, SGs are synthesized *via* the plastid localized methylerythritol 4-phosphate pathway (Brandle and Telmer, 2007). Both GA and steviol, like all other diterpenoids, are synthesized from the precursor molecule geranylgeranyl diphosphate (GGDP) by the deoxyxylulose 5-phosphate pathway (Figure 2.2). This is followed by the activity of two cyclases *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS) to produce *ent*-kaurene (Humphrey *et al.,* 2006). The *ent*-kaurene is further oxidized at the C-19 position to form *ent*-

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kaurenoic acid (Brandle *et al.,* 1998), it is at this stage where the GA and SG biosynthesis pathways diverge. The *ent*-kaurenoic acid is hydroxylated in the reaction catalysed by kaurenoic acid 13-hydroxylase at the C-13 position to form steviol. The formation of aglycone steviol is the first committed step of the SG biosynthesis pathway (Kim *et al.,* 1996). Steviol is then glycosylated through a sequential reaction catalysed by UGTs: UGT85C2; UGT74G1 and UGT76G1, whereby these three of four glycosyltransferases have been identified and characterized (Richman *et al.,* 2005). The addition of the C13-glucose to steviol is catalysed by UGT85C2, the C19-glucose by UGT74G1 and the C3 of the glucose at C-13 position by UGT76G1 producing steviolmonoside, stevioside and rebaudioside A

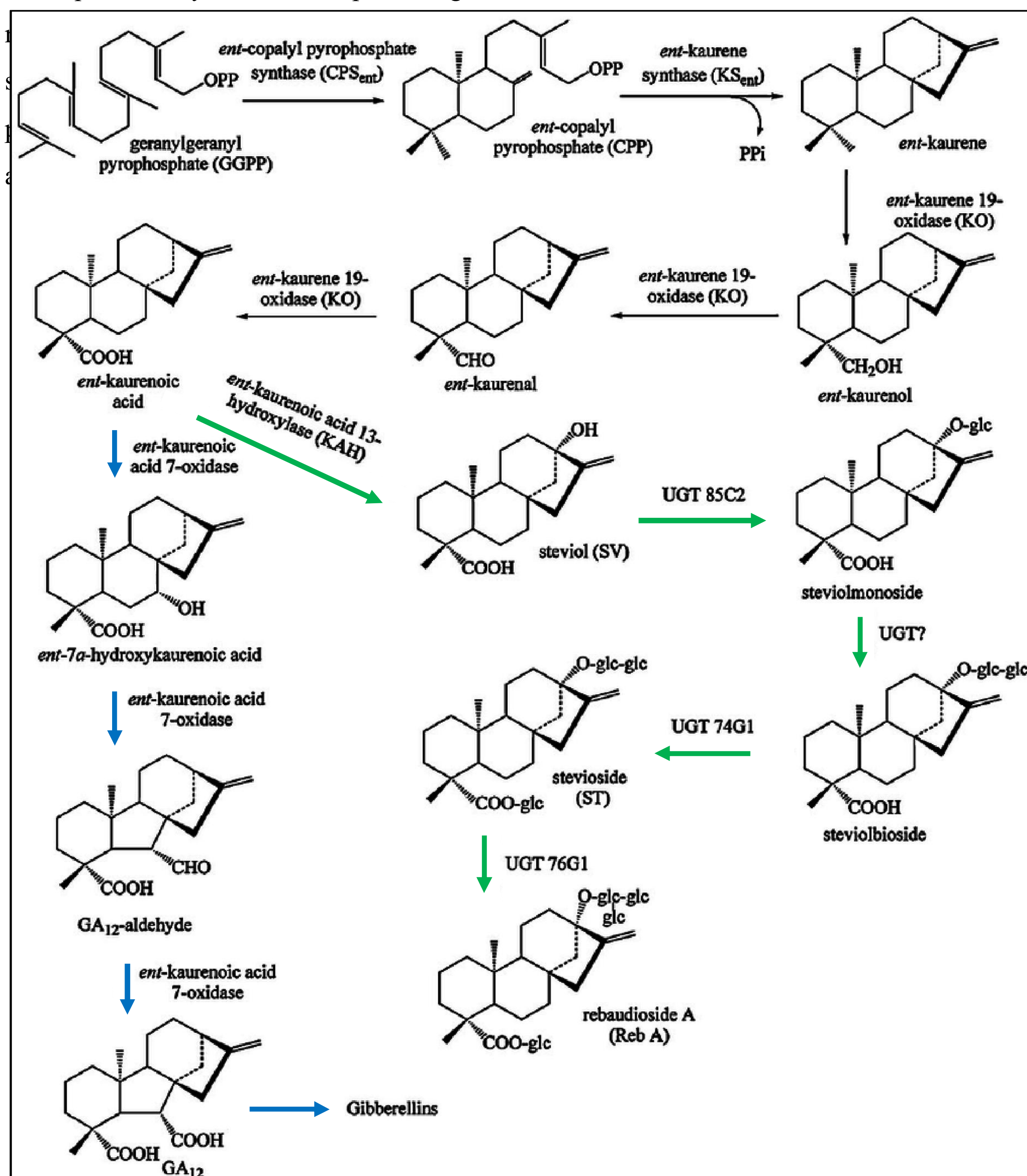

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Figure 2.2: Illustration of steviol glycosides biosynthesis pathway showing the first steps shared with gibberellic acid biosynthesis. Green arrows indicating the steviol glycoside biosynthesis pathway as it diverges from gibberellic acid biosynthesis (Blue arrows)
(Mohamed *et al.,* 2011)

2.6 Pharmacological action and biological activity of steviol glycosides

Despite its sweetening properties *Stevia* contain other nutritional components such as amino acids, minerals, vitamins and phytochemicals (Chu *et al.,* 2000). *Stevia* is also a source of carbohydrates, fibre and proteins, all molecules required for human health maintenance (Sativa *et al.,* 2004; Abou Arab *et al.,* 2010). SGs have been reported to have a number of pharmacological properties for the treatment of certain diseases (Madan *et al.,* 2010). Chen *et al* (2005) stated that *Stevia* has an anti-diabetic activity. *Stevia* leaf extracts have been used for many decades as an anti-diabetic agent in South America because of the significant fact that SGs does not affect glucose metabolism in the body.

According to Goyal *et al.* (2010), *Stevia* has vasodilator activity and in that sense has a positive role in the control of hypertension. *Stevia* has also been reported to act as an inhibitor, and prevents the initiation and promotion of, some tumours (Paul *et al.,* 2012; Yasukawa *et al.,* 2002). Despite the extensive knowledge of the SG biosynthetic pathway and the many biological activities that are attributed to the SG component of *Stevia* extracts, there have been no reports on the use of biotechnology based-strategies to address the problems of low (and variable) SG yields in field grown plants, which form the basis of all commercial scale production of SGs for human consumption.

2.7 Hairy root bioreactors can successfully produce high-value secondary metabolites targeted for human consumption

The extensive use of *Agrobacterium tumefaciens* strains as a principal method (apart from biolistic methodologies) in plant genetic transformation is well described in the literature (Mersereau *et al.,* 1990; Krenek *et al.,* 2015). In this context the disarmed tumour inducing *Ti* plasmid that is naturally associated with *A. tumefaciens* has been recruited as the delivery

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system to stably integrate foreign DNA into the plant genome, thereby creating stable genetic transformants (Rogowsky *et al.,* 1990).

However, *A. rhizogenes* is a soil-borne bacterium that has also been reported to transfer a T-DNA segment of the root inducing plasmid (*Ri*) into the host (Figure 2.3) where it is stably integrated into the cell genome. This plasmid carries genes which disrupt the natural plant hormone homeostasis (primarily auxin and cytokinin metabolism) in the plant cell and leads to the development of hairy roots from the site of infection. Hairy root induction from an *A. rhizogenes* infected plant results in the induced *Agrobacterium* movement towards the plant cells, binding to the surface components of the cell wall, activating the virulence (*vir)* genes, thus transfer and integration of the transfer-DNA (T-DNA) into the plant genome (Zupan and Zambryski, 1997). The infection process is allowed by the genetic information contained in the *Ri* (root-inducing) plasmid (Figure 2.3) carried by *Agrobacterium*. Six to eight genes concentrated on the *vir*- region within the p*Ri* are involved in the DNA transfer. Within the p*Ri*, the genetic information between the right and left T-DNA regions (T_R-DNA and T_L-DNA) is transferred and stably integrated to the plant cell genome.

Auxin biosynthesis and other genes of the T_R section are responsible for increased levels of auxins in the transformants and for opines used by bacteria for feeding (Gartland, 1995). The four genes *rol A*, *B*, *C* and *D* are contained within the T_L-DNA in the p*Ri*, which enhance auxin and cytokinins, formation of hairy roots by transformed tissues (Hong *et al.,* 2006), thus the hairy root phenotype is due to these *rol* genes. The choice of bacterial strain is very important since some plant species are very resistant to infection; however the *LBA9402* known to be hypervirulent.

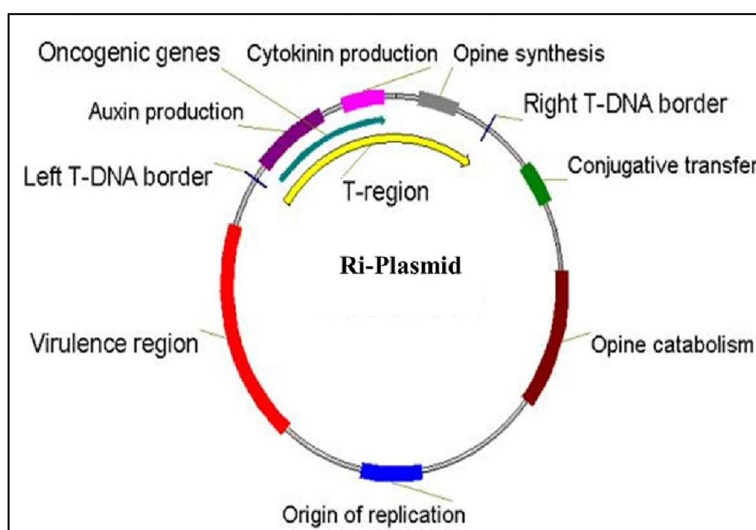

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Figure 2.3: The *Ri* plasmid of *A. rhizogenes.*
From the left border illustrating regions of auxin and cytokinins production, oncogenic genes, opine synthesis to the right border, conjugative transfer region, opine catabolism region, origin of replication and virulence region (Samanthi,2017).

Hairy root cultures are a promising alternative in biotechnology as a method for consistent production of valuable metabolites from plant cells. In recent times, the use of plant hairy roots for the production of various chemicals such as pharmaceuticals, pesticides and flavourings has been explored (Toivonen, 1993; Chandra and Chandra, 2011). Plant hairy roots have proved useful in this regard because they are stably produce metabolites (Payne *et al.*, 1992). They show continuous and active growth in hormone-free media and often produce valuable products at higher levels than the original plant leaves or roots (Flores and Curtis 1992). Hairy root cultures can also be effective in producing large quantities of genetically isogenic disease-free plants through "artificial" seeds that are obtained from organogenesis of hairy roots (Honda *et al.,* 2001). Some examples of the successful use of hairy roots for commercial scale secondary metabolite production include scopolamine, caffeine from *Coffea arabica L,* anthraquinone from *Cassia acutifolia* and ginseng from *Panax ginseng* (Nazif *et al*., 2000; Waller *et al*., 1983; Sarfaraj Hussain *et al*., 2012).

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3. MATERIALS AND METHODS

3.1 Plant growth and propagation

Stevia rebaudiana plants used in this study were purchased from the Builders' Express gardening centre in Stellenbosch and grown and maintained at the Institute for Plant Biotechnology (IPB, Stellenbosch University), in 12 inch pots with a soil mixture of equal proportions; 1:1:1 (w/w) of vermiculite, potting soil and sand. Greenhouse conditions were 16 h light (120-150 µmol/m2): 8 h dark at 22-25°C, 60% relative humidity.

3.2 *Agrobacterium rhizogenes* growth, competent cell preparation and transformation

The *Agrobacterium rhizogenes LBA9402* and *A4T* strains were obtained from the IPB stock and were used to induce transgenic hairy roots in *Stevia*. Both strains were streak-plated on Luria Bertani (LB) agar medium supplemented with rifampicin (50 µg/mL) for 2 days at 28°C. A single colony of each strain was sub-cultured into 50ml liquid LB media at 28°C until final OD_{600}= 0.8-1.0. The culture was then centrifuged (Heraeus™ Multifuge, Thermo Scientific) in a pre-chilled Falcon® tube at 4000 rpm at 4°C for 10 min. The supernatant was discarded and the pellet was suspended in 2.5 ml ice cold water by pipetting and 50 ml ice cold water was added and spun for 15 min at 4000 rpm, after which water was discarded. The pellet was re-suspended in 20 ml cold sterile 10% glycerol and centrifuged for 15 min at 3000 rpm at 4°C and the supernatant was discarded quickly. The pellet was suspended in 0.5 ml of 20% cold sterile glycerol. The cells were aliqouted into 100 µl and were kept in a -80°C freezer for further use.

One microgram of plasmid DNA was electroporated into 100 µl of the competent *A. rhizogenes* strains at 2.47 V, 2000 Ω and 25 µF in 2 mm cuvettes. After electroporation, 900 µl of sterile LB media were added to the cells and incubated with shaking at 28°C for 2 hrs. A volume of 100 µl was spread-plated onto LB agar plates supplemented with the appropriate antibiotics.

3.3 Hairy root culture induction and maintenance

A. rhizogenes grown overnight at 28°C in LB media supplemented with 100 µM acetosyringone and rifampicin 50 mg/L was pelleted by centrifugation at 13 000 rpm for 1 min and re-suspended to an OD_{600} of 0.5 in 1X Murashige and Skoog (MS) liquid media, pH

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5.7. *Stevia* leaves were harvested from the greenhouse maintained plants, rinsed briefly under running tap water, sterilised in 20% (v/v) bleach solution with a drop of Tween-20 for 10 min and rinsed 5 times with sterile water. One millilitre of overnight cultures of *A. rhizogenes* (grown in LB broth at 28ºC) were centrifuged for 5 min at room temperature and the pellets resuspended in 2 ml 1X MS (pH 5.7, 3% sucrose v/v, 100 µM acetosyringone). 10-30 mm^2 leaf sections were infected by incision with a sterile blade inoculated with the bacterial suspension or with 1X MS for the controls.

The explants were co-infected in the dark at 28ºC for 2 days on 1X MS solid media and subsequently transferred to 1X MS (3% w/v sucrose) medium supplemented with 100 µg/L cefotaxime to remove the bacteria from the explants and grown in the dark at 25ºC. Explants were sub-cultured every 2 weeks or as necessary if the bacterial growth persisted.

Sub-culturing of roots approximately 1 cm long was done by excision and separation from the explants and was transferred onto fresh 1X MS liquid media (pH 5.7, 3% w/v sucrose) medium with or without 20 µg/ml hygromycin selection to distinguish between hairy roots transformed with the *Ri* T-DNA and those doubly transformed with the *Ti* and *Ri* plasmid DNA and further incubated in the dark at 25ºC. Three weeks after growth on solid media the hairy roots were sub-cultured into liquid 1X MS (pH 5,7 3% w/v sucrose) and grown with shaking (90 rpm) in either constant dark or light/dark cycle (same as greenhouse conditions) growth rooms. Half the growth media was replaced with fresh media weekly.

3.4 Confirmation of hairy root induction and steviol glycoside biosynthesis gene expression analysis

Crude genomic DNA extractions were carried out on hairy root tissue according to a modified Edwards' DNA extraction protocol (Lu, 2011) and 100 ng of DNA was utilised in all PCR reactions.

For gene expression, total RNA was extracted from hairy roots grown for 4 weeks under either dark and light conditions and leaf tissue from a *Stevia* plant as a control using the RNeasy® Plant Mini Kit (Qiagen, Whitehead Scientific, South Africa), following the manufacturer's instruction.

The cDNA synthesis was done using the Promega M-MLV Reverse Transcriptase RNase H Minus, Point Mutant Kit in a reverse transcription reaction. Using the primers designed

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according to *rol* B, *rol* C gene and steviol glycoside biosynthesis genes (Table 3.1) with the following thermocycling conditions: initial denaturation temperature 95ºC, 2 min, denaturation 95ºC, 50 sec; annealing 58ºC, 50 sec; extension 72ºC, 30 sec; final extension 72ºC, 2 min and holding 10ºC indefinitely for 25 cycles. Amplification products were then visualized under UV light on 2% agarose gel on TBE buffer stained with Pronosafe nucleic acid stain (0.005% v/v).

3.5 Amplification and cloning of the *UGT74G1* & *UGT76G1* genes into pMDC32 for constitutive expression in *Stevia* hairy root bioreactors

Primers to amplify full length coding DNA sequences (CDS) amplicons for *S. rebaudiana* *UGT74G1* (accession number: AY345982.1) and *UGT76G1* (accession number: AY345974.1) were designed based on sequence information obtained from the Nucleotide Database of National Centre of Biotechnology Information (NCBI). All primers used in this study were produced and supplied by Inqaba Biotech.

Total leaf RNA was extracted from fresh leaves of *S. rebaudiana* the RNeasy® Plant Mini Kit (Qiagen, Whitehead Scientific, South Africa), following the manufacturer's instruction. The synthesis of complimentary DNA (cDNA) was done using 1 µg total RNA, oligo dT_{18} primer and a recombinant M-MuLV Reverse Transcriptase using a Thermo Scientific RevertAid First strand cDNA synthesis kit, following the manufacturer's instructions.

The high-fidelity, Q5 DNA polymerase (New England Biolabs) was used to amplify the two CDS amplicons following the manufacturer's instruction with gene specific primers (Table 1) with the following thermocycling conditions: initial denaturation temperature 98ºC, 30 sec; denaturation 98ºC, 10 sec; annealing 60ºC, 30 sec; extension 72ºC, 45 sec; for 35 cycles; and a final extension 72ºC, 2 min. All DNA/RNA electrophoresis and visualization throughout this study was conducted on 1% agarose TBE gels stained with Pronosafe 0.005% (v/v; Conda, South Africa) and visualized under UV light.

Single discrete amplicons of *UGT74G1* and *UGT76G1* were column-purified with the Promega Wizard® Plus SV Mini-prep DNA Purification system, A-tailed with the Promega GoTaq DNA polymerase and cloned into the pCR8/GW/TOPO vector system (Invitrogen) and transformed into One Shot® TOP10 *E. coli* (Thermo Fischer) chemically-competent cells.

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Colony PCR was done to determine insert orientation using the gene specific cloning primers and the T7 promoter (Table 1) primers. A single bacterial colony was selected using toothpicks and was briefly dipped into a 20µl PCR mixture under the following thermocycling conditions: initial denaturation temperature 95ºC, 2 min; denaturation 95ºC, 50 sec; annealing 60ºC, 50 sec; extension 72ºC, 30 sec; final extension 72ºC, 2 min and holding 10ºC indefinitely. After confirmation, *pCR8/UGT74G1* and *pCR8/ UGT76G1* entry vectors were isolated from the One Shot® TOP10 cells using the Promega mini-prep kit standard protocol and sequenced at the Central Analytical Facility (Stellenbosch University) to confirm orientation and validate the fidelity of the amplification process. The genes were sub-cloned into the Gateway destination vector pMDC32 using a conventional LR clonase reaction and transformed into chemically-competent *E. coli* OMNIMAX cells (Invitrogen Gateway® LR Clonase®). The pMDC32 is a binary plant vector whose T-DNA region contains dual constitutive expression versions (CaMV35S) and *Nos* terminator.

Again the independent presence and orientation of the two genes in PMDC32 were confirmed *via* PCR with a combination of gene specific primers and vector specific primers: $UGT74G1_{(fwd)}$ and $UGT74G1_{(rev)}$; $UGT76G1_{(fwd)}$ and $UGT76G1_{(rev)}$; $UGT74G1_{(fwd)}$ and $Nos\ T_{(rev)}$; $UGT76G1_{(fwd)}$ and $Nos\ T_{(rev)}$; $pMDC32_{(fwd)}$ and $UGT74G1_{(rev)}$; $pMDC32_{(fwd)}$ and $UGT76G1_{(rev)}$

Table 1: Primers for *UGT74G1* and *UGT76G1* gene amplification and construct confirmation

Gene	Primer sequence5'→3' (forward/reverse)
UGT74G1 (GI: AY345982.1)	ATGGCGGAACAACAAAAG/ TTAAGCCTTAATTAGCTCACTTACAA
UGT76G1 (GI: AY345974.1)	ATGGAAAATAAAACGGAGACC/ TTACAACGATGAAATGTAAGAAACTA
Nos T	AAGACCGGCAACAGGATTG
pMDC32	AGAGGATCCCCGGCTACC
T7	AATACGACTCACTATAGG

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Table 2: Primers for identification of hairy root cultures and SG gene expression

Gene	Primer sequence 5'→3'(forward/reverse)	Amplicon length (bp)
Actin2	CGCCATCCTCCGTCTTGATCTTGC/ CCGTTCGGCGGTGGTGGTAA	111
rol B	GCACTTTCTGCATCTTCTTCG/ CCTGCATTTCCAGAAACGAT	383
rol C	GCACTCCTCACCAACCTTCC/ ATGCCTCACCAACTCACCA	586
Kaurene synthase (KS; GI: AF097310.1)	ACCAAAGAACGGATCCAAAAACTG/ AGACACTCAGGGAAACAAGGC	125
Kaurenoic oxidase (KO; GI: AY995178.1	AGCTATGAGACAAGCATTGGGA/ CGACGTCAATTGCACCCATC	128
Kaurenoic acid 13-hydroxylase (KAH)	AACTCTGGCACTCCTACGTG/ CAAAACGGTCGCCAAACAAC	119
*UGT85C2 (*AY345978.1)	CATCGGGCCCACATTGTCTA/ CTCTGATTGGGATGCTCGCT	99
UGT74G1(GI: AY345982.1)	ACAGTAACACCACCACCACC/ GACCCAACTTGTTTGAATGTTTCC	274
UGT76G1(GI: AY345974.1)	TTCACACCAACTTCAACAAACCC/ ATGCGTTCGTCTTGTGGGTC	107

3.6 Extraction and LC-MS/MS analysis of steviol glycosides from hairy roots and *Stevia* leaves

Steviol glycoside extractions were conducted using the method described by Routaboul *et al*., (2006) with minor modifications. Hairy roots harvested from dark and light conditions and *Stevia* leaves were freeze-dried overnight. One hundred milligrams of each of the hairy roots and *Stevia* leaves were ground into a fine powder using the Retsch mill and metabolites extracted in 2 ml acetonitrile/water (75:25; v/v) for 5 minutes at 4ºC before sonication on ice

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for 20 minutes. Paracetamol (5 ppm) were added to each sample as an internal standard. Following sonication, centrifugation was done for 5 minutes at 15000 rpm and the supernatant was kept at 4°C overnight. The remaining plant material was used for further metabolite extraction using 1ml acetonitrile/water (75:25; v/v) overnight at 4°C.The extract was centrifuged and the second supernatant kept at 4°C. The two extracts (supernatants) were combined together and were evaporated in vacuum and dry residue re-dissolved in 500 µl 50% methanol. The extracts were aliqouted in 200 µl vials for LC-MS/MS analyses.

LC-MS/MS analyses were performed with a Waters Synapt G2 quadrupole time-of-flight mass spectrometer (Waters Corporation, Milford, MA, USA) equipped with a Waters Acquity UPLC. Samples were separated on a Waters UPLC BEH C18 column (2.1 x 100 mm; 3.5 µm) at a flow rate of 0.3 ml/min at 35°C. Solvent A consisted of 0.1% acetic acid in water and solvent B was 0.1% acetic acid in acetonitrile. The mobile phase gradient was from 0% to 60% solvent A over 5 min, maintained for 2 min at 60% solvent A before the column was re-equilibrated to the initial conditions. Electrospray ionization was applied in the negative mode and the scan range was from m/z 150 to 1500. The capillary voltage was set a 2.5 kV, the cone voltage was 15 V, the source temperature 120°C and the desolvation temperature was 275°C. The desolvation gas and cone gas flows were 650 L/h and 50 L/h, respectively. Metabolite quantification (where applicable) and fold changes were conducted against a series of standard flavonoids (stevioside and rebaudioside A at a concentration of 1 mg/ml), and metabolite recovery was monitored with the internal standard (paracetamol, 0.1 mg ml^{-1}). Metabolites were monitored using their deprotonated quasi-molecular ions and quantified or identified (where possible) with the TargetLynx application manager (Waters MassLynx V4.1V software).

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4. RESULTS

4.1 Induction of hairy root cultures utilizing *A. rhizogenes* on *Stevia* leaf explants

Since SG accumulation is strongly linked with photosynthetic vegetative tissue *Stevia* leaves were chosen as the ideal explants for hairy root induction. Two strains of *A. rhizogenes* (*A4T* and *LBA9402*) were preliminarily evaluated for their capacity to initiate *Stevia* hairy root culture. Following inoculation of the leaf explants (Figure 4.1 A), hairy root formations absent in the control (Figure 4.1 B), emerged within 20 days of infection with either *A. rhizogenes A4T* (Figure 4.1 C) or *LBA 9402* (Figure 4.1 D). Two to three weeks after inoculation with both strains separately, qualitatively strain *LBA9402* produced more hairy roots per explant (Figure 4.1 D) than *A4T* (Figure 4.1 C) and the hairy roots from strain *LBA9402* appeared to grow with more vigour (Figure 4.1 E-F). Consequently, only strain *LBA9402* was utilised for the rest of the study.

Hairy root cultures are typically cultivated under constant dark conditions but since photosynthetically active tissue appears to be a pre-requisite for SG accumulation(Modi *et al.,* 2016) we decided to investigate whether the light conditions would impact hairy root growth and the accumulation of SGs in our cultures. All initial liquid cultures of the hairy roots were conducted in the dark for 4 weeks. Fifty percent of the cultures were then transferred into long day conditions (16 h light/ 8 h dark) and unsurprisingly they developed from the typical yellow-brown colour of dark-grown roots (Figure 4.1 G) to a vibrant green hue (Figure 4.1 H)

To determine if hairy roots were transgenic for the *Ri* plasmid DNA, semi-quantitative PCR was conducted on cDNA from hairy roots grown for 4 weeks in liquid culture. Two known and well characterised hairy root *Ri* plasmid-specific genes (*rol B* and *rol C*) were shown to be present and expressed in hairy root extracts but absent in leaf tissue, confirming the transgenic nature of the hairy roots (

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Figure 4.2).

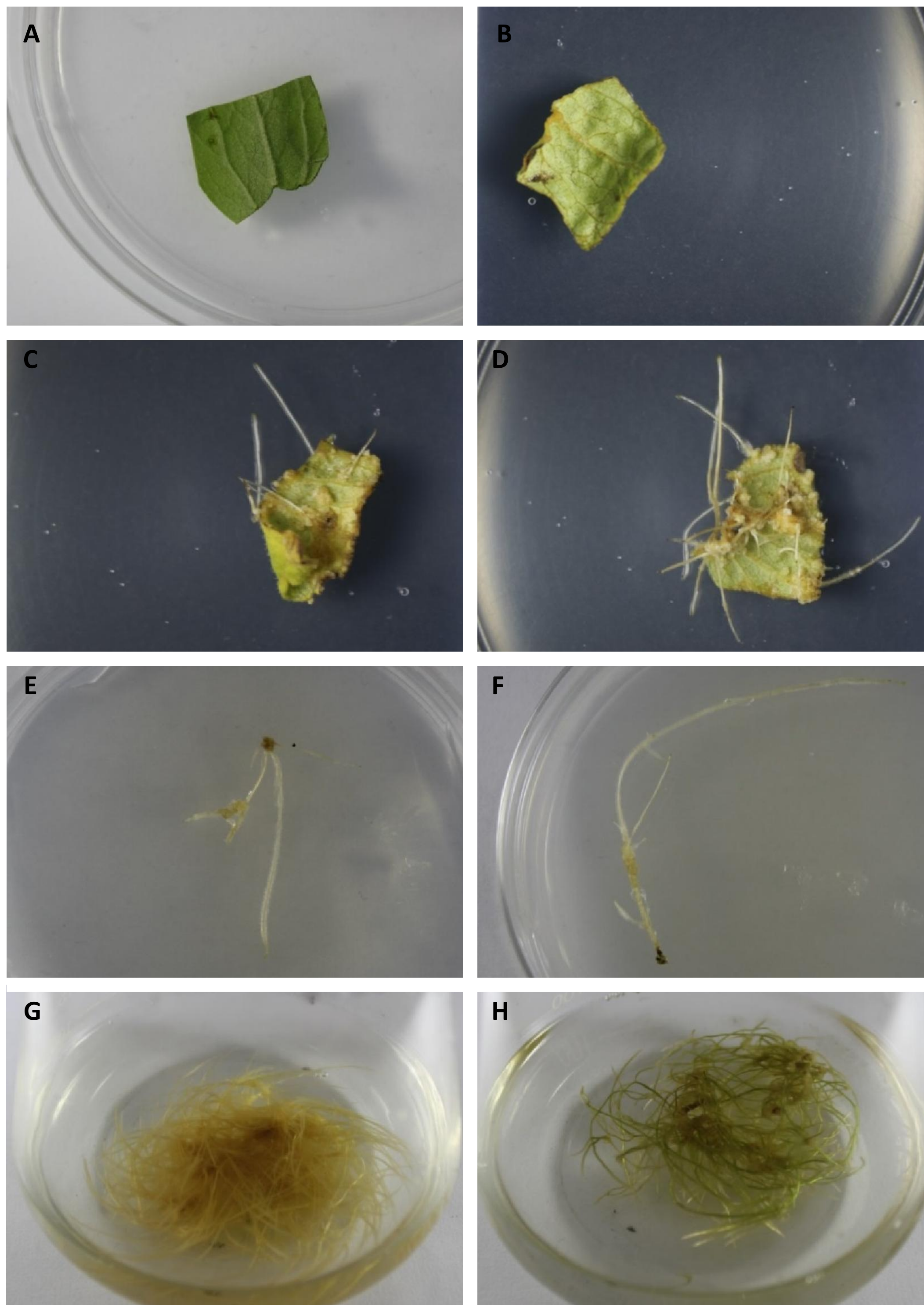

Figure 4.1: Sequential stages of hairy root induction in *Stevia rebaudiana* using *A rhizogenes* strains *A4T* and *LBA9402* on leaf explants

A, newly inoculated leaf explant at day 0; B, control *Stevia* explants 20 days post- inoculation with 1X MS; C & D, *Stevia* explants 20 days post infection with *A4T* and *LBA9402* respectively; E & F, hairy roots infected with *A4T* and *LBA9402* 10 days after excision from mother explants; G & H, hairy roots from *LBA9402* after 4

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weeks in liquid media under dark or light conditions respectively. Hairy root inductions were attempted in multiple independent experiments and a minimum of 50 explants were used per strain or light condition

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Since SG production is light-dependent it follows that SG gene expression is potentially dependent on the light and photosynthetic status of the hairy root cultures. Using sqRT-PCR, we additionally managed to show that for the most part, SG biosynthesis genes are expressed to similar levels in hairy root tissue grown under constant dark or long day conditions (

Figure 4.2). These expression levels are also comparable to those in leaf tissue which is known to accumulate the highest levels of SGs in *Stevia* (Brandle and Telmer, 2007).

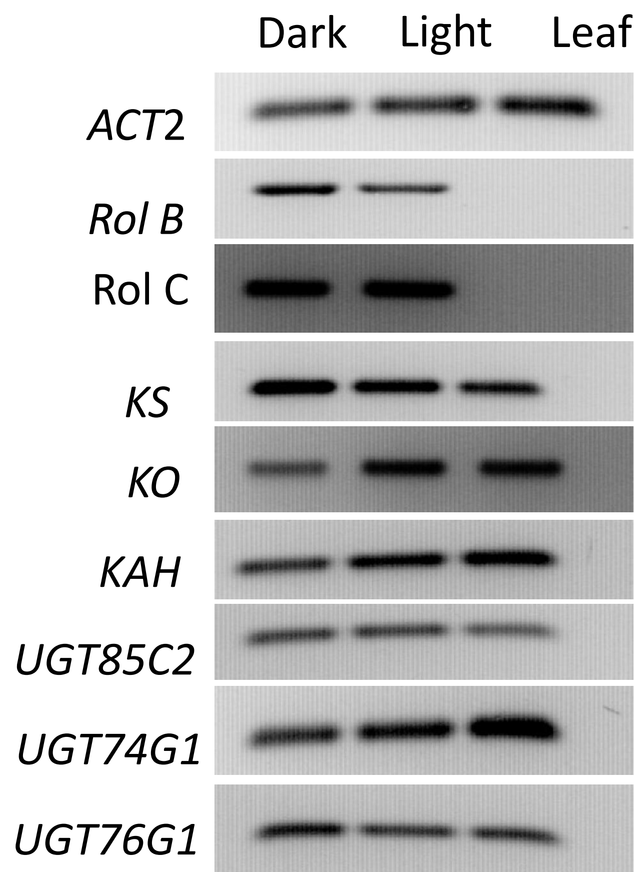

Figure 4.2: SG biosynthesis gene expression in hairy root cultures grown in dark or light conditions as determined by sqRT-PCR.
PCR was performed with gene specific primers for each gene for the predetermined linear range cycle number (25). cDNA was generated from total RNA extracted from a pool of 5 independent hairy root lines per growth

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condition. *ACT2* (Actin2); *rol B* & *rol C* (oncogenic genes from the pRi); *KS* (Kaurene synthase*);* *KO* (Kaurene oxidase); *KAH* (Kaurenoic acid 13-hydroxylase); *UGT85C2*, *UGT74G1* & *UGT76G1* (UDP-glycosyltransferases.

4.2 Generation of transgenic hairy root cultures overexpressing *UGT74G1* and *UGT76G1*

An objective of the research was to produce transgenic hairy root cultures that constitutively express the 2 final genes, UGT71G1 and UGT76G1 in the steviol glycoside biosynthesis pathway with the intention of increasing stevioside and rebaudioside A accumulation in these cultures.

4.2.1 UGT gene isolation and sub-cloning into pMDC32, a plant expression vector

To this end, the full length CDS regions of both *UGT74G1* and *UGT76G1* were amplified from *Stevia* leaf extract-based cDNA utilizing a high-fidelity DNA polymerase and subsequently cloned into the Gateway technology entry vector pCR8/GW/TOPO. Positive transformants containing the genes of interest were identified *via* colony PCR utilizing the gene specific primers originally utilized to amplify the genes from *Stevia* (Figure 4.3). pCR8/GW/TOPO cloning is bi-directional and as such two additional primer combinations were utilized to confirm the genes were inserted into the entry vector in the forward sense, a requisite for successful gene expression upon recombination into the destination vector. Only clones with a forward sense insertion will result in successful PCR amplification with the gene-specific forward primer and the T7 primer, and not with the gene-specific reverse and T7 primer combination (ThermoFisher Scientific, pCR8 product information booklet).

After insert orientation and gene sequence fidelity was confirmed *via* PCR and subsequent plasmid DNA sequencing, respectively, the 2 genes were sub-cloned into the destination vector pMDC32, *via* LR clonase technology, to generate pMDC32/UGT7XG1 expression vectors (whereby X stands for either 4 or 6). Again positive transformants were identified *via* PCR with gene specific primers and a combination of gene and vector specific primers to ensure correct orientation (

Figure **4.4**).

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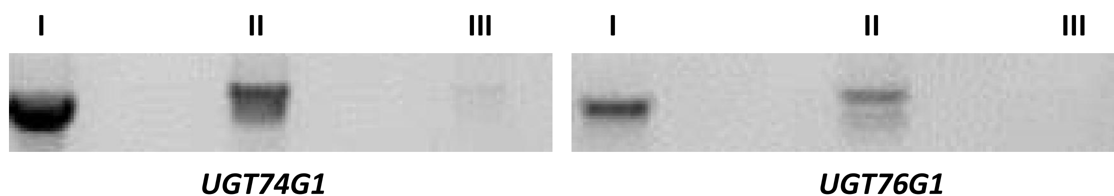

Figure 4.3: PCR confirmation of *UGT* gene insertion and insert orientation in the entry vector pCR8/GW/TOPO
PCR was conducted with either gene-specific full length CDS primers (insertion) or a combination of a gene specific primer with the T7 promoter specific primer. I, gene specific primers; II, forward sense primers; III, reverse sense primers.

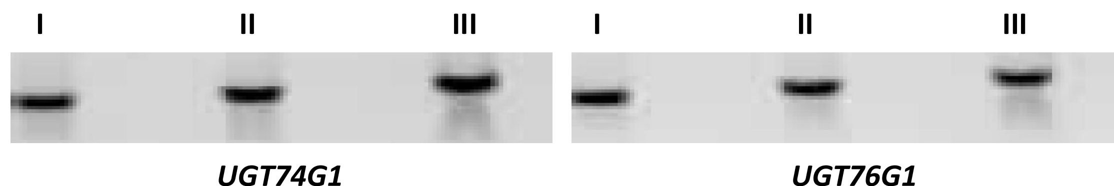

Figure 4.4: PCR-based identification and confirmation of the pMDC32/UGT7XG1 expression vectors in *E. coli*.
Recombinants were identified utilizing gene specific primers and gene insert orientation confirmed *via* a combination of vector and insert specific primers. I, gene specific primer; II vector forward and gene reverse primers; III, gene forward and vector reverse primers.

4.2.2 Independent transformation of A. rhizogenes LBA9402 with pMDC32/UGT7XG1

Subsequent to the generation of the two plant expression vectors harbouring the UGT genes, competent *A. rhizogenes LBA9402 (*section 4.2.1; previously determined to be the better strain for hairy root induction; Figure 4.1 D & F) was independently transformed with both vectors and the successful uptake of the binary vectors dually confirmed, firstly with PCR amplification (Figure 4.5). Gene specific primers were utilised to identify successful transformants and it is apparent the binary vectors were successfully introduced into the bacterium. Additionally, the transformants were screened on LB growth media supplemented with and without a combination of antibiotics (Figure 4.6). Strain *LBA9402* showed endogenous resistance to rifampicin (50 µg/ml) and the binary vector introduces resistance to kanamycin (50 µg/ml). As expected the successfully transformed colonies showed resistance to growth on selection with both rifampicin and kanamycin and were used for further work downstream.

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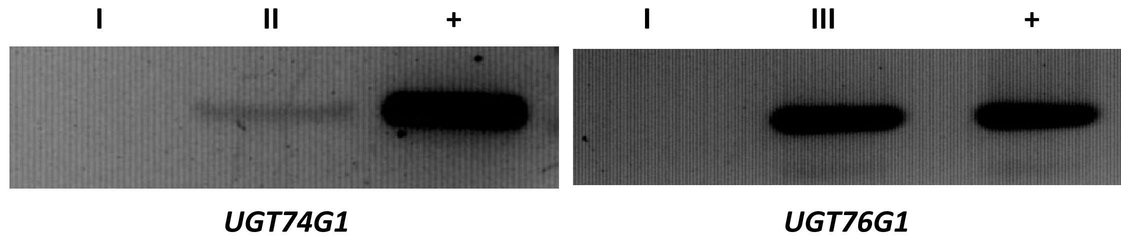

Figure 4.5: pMDC32/UGT74G1 (II) and pMDC32/UGT76G1 (III) expression vectors were successfully transformed into *A. rhizogenes LBA9402* as confirmed *via* PCR.
PCR with gene specific primers was performed on plasmid DNA isolated from overnight cultures of *A. rhizogenes* independently transformed with the two expression vectors. I, untransformed control; II, pMDC32/*UGT74G1*; III, pMDC32/*UGT76G1*; +, positive control.

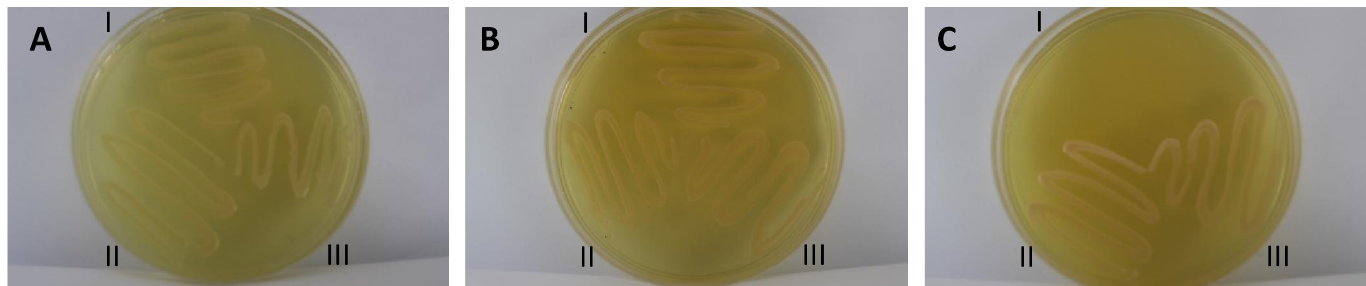

Figure 4.6: ***A. rhizogenes LBA9402*** **harbouring pMDC32/7XG1 expression constructs is resistant to selection on kanamycin.**
Untransformed bacteria (I), bacteria transformed with either pMDC32/UGT74G1(II) or pMDC32/UGT76G1 (III) was grown on LB media with either no antibiotic (A), with rifampicin (50 µg/ml) only (B) or on rifampicin and kanamycin (50 µg/ml) (C)

4.2.3 Co-transformation of Stevia with Ri and Ti for the production of transgenic hairy roots constitutively expressing UGT74G1 and UGT76G1.

Stevia explants were infected with *A. rhizogenes LBA9402* harbouring either pMDC32/*UGT74G1* or pMDC32/*UGT76G1* and allowed to grow and produce hairy root cultures. Four weeks after the emergence of hairy roots the cultures were transferred to liquid media and genomic DNA was extracted to determine if the hairy roots were transgenic for both *Ri* and *Ti* T-DNA. PCR analysis revealed that the *Ri* T-DNA was inserted into these cultures as confirmed by the amplification of the *rol C* gene (Figure 4.7).

However, using the vector forward and gene specific primers for the pMDC32/*UGT7XG1* construct PCR showed the *Ti* T-DNA was not successfully integrated into the hairy root genome (Figure 4.7).

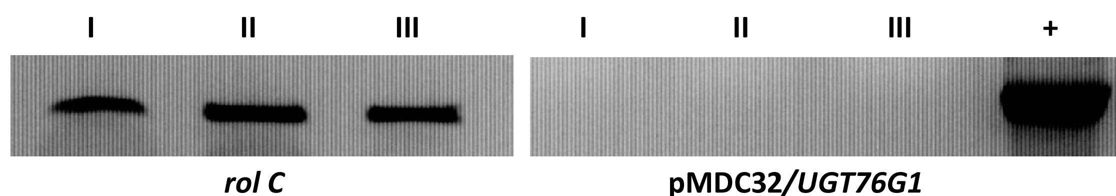

Figure 4.7: Representative image of identification of hairy roots from explants infected with *A. rhizogenes LBA9402* carrying a binary expression vector
PCR was conducted on genomic DNA from 3 representative hairy root lines (I, II & III) with *rol C* and pMDC32/*UGT76G1* construct specific primers. +, positive control plasmid DNA

Furthermore, these hairy roots were unable to survive in liquid media with hygromycin, visibly changing colour and eventually dying (Figure 4.8 B), confirming a failure to co-transform and simultaneously induce hairy root formation. This result was consistent across multiple hairy root lines and regardless of which of the two binary vectors was used. At this

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point the research focus was shifted solely onto the hairy root cultures induced *via* the classic approach.

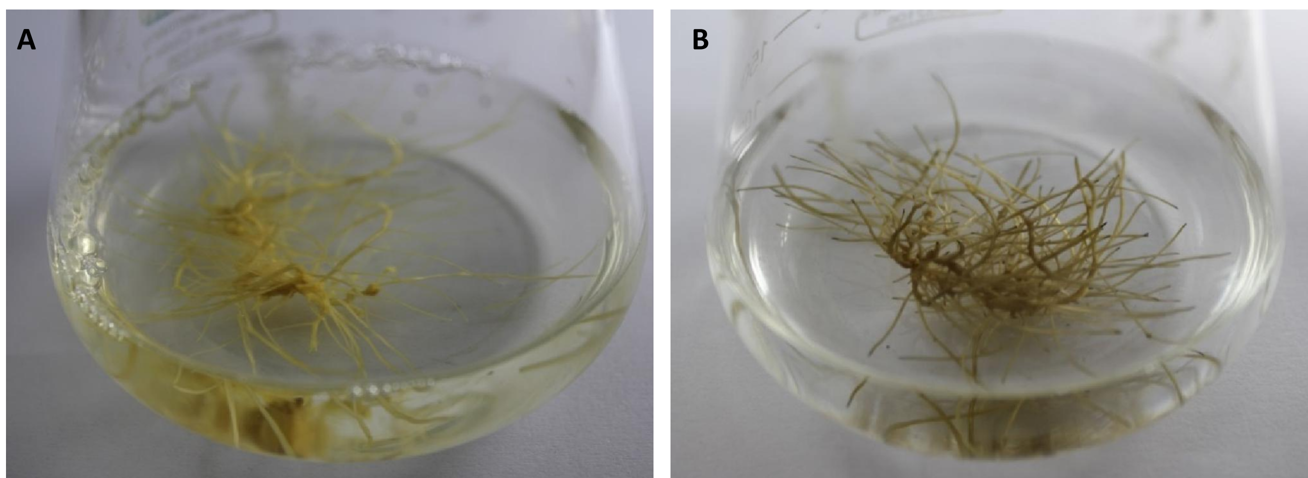

Figure 4.8: Hairy root cultures co-transformed with pMDC32/*UGT7XG1* are not resistant to hygromycin selection
Four-week-old hairy roots were grown in liquid 1X MS media. A, hairy root culture pre-hygromycin selection. B, hairy root culture in hygromycin selection for approximately 1 week under constant dark conditions.

4.3 Identification of SGs accumulating in *Stevia* hairy root cultures *via* an LC-MS/MS approach

Total metabolites were extracted from hairy root cultures (both light and dark) and leaf material to determine steviol glycoside accumulation *via* LC-MS/MS analyses. The mass spectrum (m/z ratio) of each peak, correlating to commercial standards (stevioside and rebaudioside A), were extracted from the total ion chromatogram to confirm the identity of either stevioside or rebaudioside A in hairy root and leaf metabolite extracts (Figure 4.9). Leaf extracts accumulated both stevioside and rebaudioside A (Figure 4.9 A). When compared to the commercial standards (Figure 4.9 D). Hairy root extracts, from either light or dark controlled conditions, did not accumulate either of these compounds (Figure 4.9 B & C).

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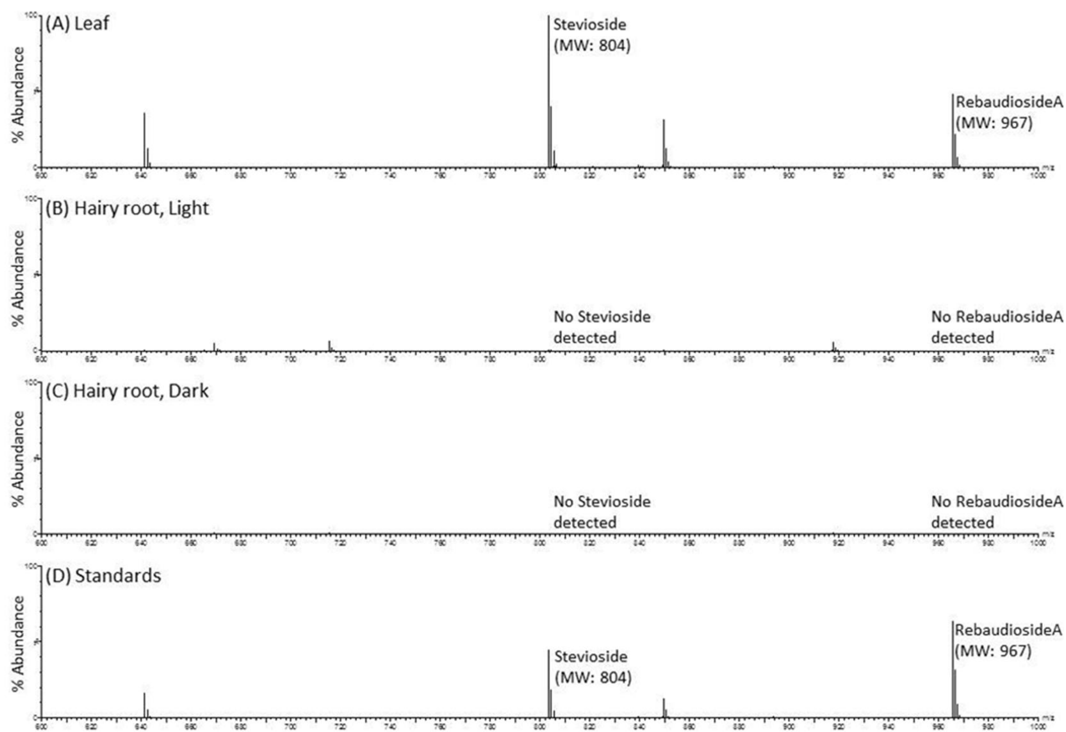

Figure 4.9: Mass spectrum isolated from total ion chromatogram of *Stevia* extracts.
(A) leaf, (B) hairy roots maintained in the light and (C) hairy roots maintained in the dark as compared to (D) commercial standards.

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5. DISCUSSION

The most recent statistics around the global and domestic (SA context) incidence of diabetes paint a bleak picture, firmly placing the condition as a serious global problem linked to dietary lifestyle choices/preferences. The first ever comprehensive global report on diabetes released by the World Health Organization in 2016 clearly indicates that new incidences of diabetes are increasing significantly in developing countries (Roglic, 2016). This has implications in terms of the management and treatment on already strained public healthcare systems. Interestingly, Statistics SA released their mortality report in 2017 which investigated the leading causes of mortality in the SA population (Statistics South Africa; StatsSA, 2014). A very surprising finding was that tuberculosis (TB) and diabetes are the leading causal factors of mortality and in the Western Cape, diabetes accounts for more deaths than TB (StatsSA, 2014).

While diabetes is a multifactorial non-communicable disease, there is evidence linking its onset to obesity. Obesity itself has been clearly linked to dietary and lifestyle preferences, and increased intake of dietary sugars e.g. sucrose (Golay and Ybarra, 2005; Eckel *et al.,* 2011). Consequently, the South African government has promulgated legislation which will in future see a tax being applied to consumer products which contain dietary sugars in an attempt to help reduce excessive sugar intake in the South African population and, improve the general health and well-being of the population (Manyema *et al.,* 2014).

A long standing solution to the problem of excessive dietary sugar intake is the inclusion of non-nutritive sweeteners (NNS) into the human diet, as a substitute to normal sugar. The key feature of NNS is their inability to be metabolized by the human body (Romo-Romo *et al.,* 2016). Thus their consumption still imparts the sweet-to-taste characteristic of dietary sugar but does not affect sugar metabolism within the body. However, only a small number of chemically synthesized sweeteners (sucralose, aspartame, saccharin, acesulfame-K, neotame, and advantame) are approved to be used in food. Additionally two naturally derived NNS (steviol glycosides and Luo Han Guo extract) are generally recognized as safe and carry endorsements for use in food by the FDA (FDA, n.d) and the European Food Safety Authority (EFSA, 2010). It is within this context that this thesis project was conducted and was particularly focused on the context of the use of *Stevia* as a source of NNS.

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Leaf extracts of *Stevia* contain the high-value steviol glycosides (SGs) stevioside and rebaudioside A, NNS that are claimed to be up to 300 times sweeter than sucrose (Phillips, 1987). However, commercial cultivation of the plant occurs primarily *via* tissue culture protocols and stem cuttings of plants resulting in a high labour cost. The SG yields are also very low and variable (between 2 - 4% total fresh mass) and the *Stevia* plant requires intensive irrigation inputs for successful commercial scale SG harvests. The development of such commercial scale agricultural cultivation of *Stevia* has been proposed for SA to develop a domestic supply market (FoodStuff South Africa, 2016) and make *Stevia* products more economically accessible to the general public. However, the problems associated with its cultivation are considered to be major factors restricting the development of this economy.

Our work sought to use *A. rhizogenes* to infect *Stevia* leaf explants in order to induce hairy root development for SG production. Our experiments resulted in the formation of hairy roots (Figure 4.1) and subsequent sub-culturing procedures yielded hairy root cultures which were able to proliferate on solid and, in liquid growth media (Figure 4.1) The production of high value plant secondary metabolites from hairy root bioreactors is well reported (Nazif *et al*., 2000; Waller *et al.,* 1983; Sarfaraj Hussain *et al.,* 2102). However, such approaches are often hampered by (i) low yields of the desired products and (ii) the required scalability for the industrial commercial-scale production of these products (Kim *et al.,* 1996). This is due to the poor understanding of secondary metabolite biosynthesis pathways and common technological processes involved in extraction and accumulation of these compounds in large scale (Brandle and Telmer, 2007). Many secondary metabolites are products of very complicated biosynthetic networks, and their synthesis is triggered by several factors, therefore, the mechanism or enzymes involved for a specific secondary metabolite should be fully understood.

The SG biosynthetic pathway in *Stevia* is only partially resolved (Brandle and Telmer, 2007). In that study it was reported that key steps in SG biosynthesis are localized to plastids (the MEP pathway) starting with the initial formation of the precursor steviol. Further, these primary steps in the synthesis of SGs rely on the formation of *ent*-kaurenoic acid which is known to occur only in plastids. The only steps which occur in the cytoplasm are proposed to be the final stage of glycosylation which yields the SGs stevioside and rebaudioside A. A role for photosynthesis in the biosynthesis of SGs has been explicitly demonstrated (Ladygin *et al.,* 2008). Those authors examined the biosynthesis of SGs in *Stevia* leaves and callus tissue,

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demonstrating that SG accumulation was invariably linked to the presence of chlorophyll and light.

With this in mind and since hairy root normally cultivated in the dark (Saito *et al.,* 1992), we thus conducted experiments where hairy root cultures were propagated under both light and dark conditions and examined the transcript abundance of key SG biosynthetic genes (

Figure 4.2). From these analyses we could clearly confirm integration of the respective root inducing genes (*rol B* and *rol C*) from the *Ri* plasmid of *A. rhizogenes* when compared to leaf explants (

Figure 4.2). However, transcripts of six key SG biosynthetic genes showed similar abundance in hairy root cultures grown in the dark and the light when compared to leaf explants (

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Figure 4.2). These results were surprising given the reports linking photosynthetically active tissues to SG biosynthesis and perhaps there are post-transcriptional processes that drive SG accumulation.

Following on from these analyses, hairy root cultures both grown in the dark and in the light were subjected to an acetonitrile based-extraction to recover secondary metabolites. These extracts were then subjected to tandem mass spectrometry analyses (LC-MS/MS) to determine (i) if the extraction procedure was valid and (ii) if our hairy root cultures were able to accumulate SGs, given that key biosynthetic genes were being expressed. When compared against commercially available pure standards for both stevioside and rebaudioside A, we could clearly detect the presence of both these compounds in leaf extracts. However, we consistently could not detect either compound in extracts from hairy root cultures. The reasons for these observations are unclear, however, in the study linking photosynthesis to SG accumulation it was found that both *Stevia* callus cultures grown in the light and the etiolated *in vitro* regenerants (plants from the callus) exhibited chlorophyll pigments one order of magnitude lower than that in leaves of the intact plants that had not been *in vitro* cultured (Ladygin *et al.*, 2008). We propose that while key SG biosynthetic genes are clearly being expressed in our hairy root cultures, the actual abundance of key substrate intermediates required for SG accumulation may be too low to facilitate the biochemical reactions leading up to accumulation of stevioside and rebaudioside A. Since we were unable to obtained pure intermediate substrates as commercial standards, our analyses did not include determining if any SG biochemical intermediates were present in our extracts.

Our study sought to develop a transgenic hairy root culture where *Agrobacterium rhizogenes* strains would be used to create hairy root cultures and additionally generate hairy roots overexpressing the two key genes involved in the last stages of SG biosynthesis (*viz. UGT74G1* making stevioside and *UGT76G1* making rebaudioside A). These respective genes were amplified from leaf cDNA preparations, cloned into the binary vector pMDC32 (dual CaMV 35S promoter, Curtis and Grossnikalus, 2004), and transformed into *A. rhizogenes* (*LBA9402*). PCR analysis confirmed the presence of the pMDC32 constructs with the target UGT genes within the bacteria (Figure 4.5 and we demonstrated that *A. rhizogenes LBA9402* transformed with the respective constructs grew on media containing the antibiotic kanamycin contrary to untransformed strains (Figure 4.6).

Presently only two studies have confirmed hairy root induction of *S. rebaudiana* transformed with *A. rhizogenes* (Yamazaki *et al.,* 1991; Michalec-Warzecha *et al.,* 2016). However, none have created hairy roots with *A. rhizogenes* strains that also carry binary vectors for plant transformation. Indeed, we consider this approach novel as to our knowledge (following extensive literature review searches), we believe that the use of *A. rhizogenes* for potential hairy roots development and simultaneous genetic transformation of *Stevia* has never been reported and is still potentially a significant approach to increasing SG production. It should be noted that the precedence exists for simultaneous hairy root induction and genetic transformation with an additional binary vector (Rana et al., 2017).

Conclusion and final outlook

Our work attempted to create a transgenic hairy root bioreactor from *Stevia* leaf explants, by transforming *A. rhizogenes* with binary plasmids normally used for *A. tumefaciens* plant genetic transformation methodologies. We showed that *A. rhizogenes* could be transformed stably with these plasmids and that the transformed strain could subsequently induce hairy root cultures. However, we did not detect pMDC32/*UGT74G1* and pMDC32/*UGT76G1* specific genomic DNA in any of the hairy root cultures. Although the untransformed hairy roots clearly have comparable levels of critical genes in SG biosynthesis, they do not accumulate any of the SGs we had standards for (stevioside and rebaudioside A) and have linked this to the possible low concentration of biochemical substrate intermediates that are normally associated with photosynthesis.

In this regard, future work making use of these hairy roots is suggested as follows:

1. Since the overarching aim of the study was to produce SGs in a non-agricultural system, the hairy roots could be propagated under light conditions until they develop leaves. This system could then be analysed to determine if stevioside and rebaudioside A are present. For the duration of the study all light grown hairy root cultures did not develop shoots.
2. One could consider using the substrate intermediates which are produced by photosynthesis as a means of priming hairy roots to then complete SG biosynthesis leading to stevioside and rebaudioside A accumulation.
3. An attempt could be made to generate transgenic *Stevia* plants transformed with the pMDC32 vector system first, and then induce hairy roots from the recovered

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transgenics. These cultures could then be inserted into the approaches described in points 1 and 2 above.

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