OVER-EXPRESSING *ARABIDOPSISArabidopsis* MYB TRANSCRIPTION FACTORS IN *Salvia stenophylla* AND SUGARCANE AND DEVELOPMENT OF MICROPROPAGATION PROTOCOL FOR *Salvia repens*

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Thesis presented for the partial fulfilment of the requirements for the degree of Master of Science in the Institute for Plant Biotechnology at the University of Stellenbosch

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December 2015

Declaration

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December 2015

Summary

Biotechnology is an important tool that is used to isolate and characterise genes. It is also used to produce clones that are genetically and phenotypically similar. Many *Arabidopsis thaliana* transcription factors have been isolated and characterised, but many have yet to be fully described. MYB proteins are members of a super-family of multifunctional transcription factors that can also interact with other transcription factors in the control of pathways. To date, more than 126 *AtMYBs* have been identified, but most have not been fully characterised, particularly in terms of the molecular role(s) they play in plants. *Arabidopsis thaliana MYB3*, *MYB6*, *MYB7, MYB8* and *MYB32* have been reported to be negative regulators of general phenylpropanoid metabolism. It has been reported that the five transcription factors mentioned above are likely to negatively regulate flavonoid biosynthesis, even though they may have different target genes. Studies on *AtMYB13, AtMYB14* and *AtMYB15* reported that they are likely regulators of general phenylpropanoid metabolism. The mentioned roles of the eight *AtMYB* transcription factors means that they can be manipulated in order to see what effect they have on primary and secondary metabolites in plants.

The transcription factors ligated into the pUBI510-GRFCA vector were then used to transform sugarcane callus (**Chapter 3**). Sugarcane produces sucrose which makes up 70% of the sugar produced in the world, making sugarcane a commercially important and profitable plant. The sugarcane callus was transformed via particle bombardment. The transcription factors *AtMYB3*, *AtMYB6*, *AtMYB7*, *AtMYB13* and *AtMYB32* were successfully incorporated into the genomic DNA of the sugarcane callus. The data obtained for callus over-expressing *AtMYB3*, *AtMYB13* and *AtMYB32* on solid media and the callus in liquid media were contradictory (i.e callus on solid media producing more sucrose than the wildtype whereas the same transgenic line will poduce less sucrose that the wildtype in liquid media or *vice versa*). However, *AtMYB13* transgenic lines produced more sucrose than the wildtype. Transgenic lines of *AtMYB7* all produced less sucrose as compared to the wildtype both on solid and in liquid media. The transcription factors which resulted in increased production of starch when over-expressed were *AtMYB7* and *AtMYB13*. The data obtained for *AtMYB6* transgenic lines was highly inconsistent in lines grown on same media and across the two media. The effects of these transcription factors

in the overall metabolism of the sugarcane callus, either on MSC_3 solid or liquid media, could not be fully determined from the GC-MS analysis as there was no consistent phenotypic effect between different transgenic lines for any of the MYB transcription factors used.

In **Chapter 4**, a micropropagation strategy was developed and phytochemicals and their biological activities were determined for the medicinal plant *Salvia repens*. *Salvia* plants have been found to be medicinally important due to the secondary metabolites, particularly the essential oils that they produce. The plant extracts have been found to have many biological activities such as antibacterial, anti-inflammatory, antioxidant and anticancer activities. *Salvia repens* was successfully germinated *in vitro*,with 60% germination being achieved in MS media containing $1x10^{-5}$ times diluted smoke water following scarification for 12 min in 75% (v/v) H_2SO_4. Success rates of 100% were achieved in the hardening off process when the seedlings were moved into the greenhouse. Germination of *S. repens ex vitro* was 100% in an autoclaved soil mixture of 1:1 (v/v) sand and vermiculite. Importantly the medicinal value of *S. repens* produced *in vitro* or *ex vitro* was not lost as the GC-MS metabolite analysis showed that the plants produced the chemicals that are medicinally important. Metabolite extracts of *S. repens* were for the first time reported to be active against fungi with MIC values lower than 1 mg/ml over 4-5 d period against four *Fusarium spp.* tested.

Lastly (**Chapter 5**), transcription factors *AtMYB6* and *AtMYB13* were used to trasnform *Salvia stenophylla* via *Agrobacterium*-mediated transformation, in order to determine whether the over-expression of these transcription factors could up-regulate the production of medicinally and commercially important secondary metabolites in *S. stenophylla*. Whilst both *A. tumefaciens* and *A. rhizogenes* strains were utilised for the transformation procedure, transformation was only achieved using *A. rhizogenes* and no transformants could be generated from the *A. tumefaciens*-treated material. Transgenic hairy roots did not produce any of the medicinally important metabolites. The GC-MS analysis of the transgenic root material identified mainly sugars and other primary metabolites.

Opsomming

Biotegnologie is 'n belangrike instrument wat gebruik kan word om gene te isoleer en te karakteriseer. Dit word ook gebruik om klone wat geneties en fenotipies identies is te produseer. Baie *Arabidopsis thaliana* transkripsiefaktore is al geïsoleer en gekarakteriseer, maar baie moet nog volledig beskryf word. *MYB* proteïene is lede van 'n super-familie van multifunksionele transkripsiefaktore wat ook interaksie het met ander transkripsiefaktore tydens die beheer van metaboliese weë. Tot op hede is meer as 126 *AtMYBs* geïdentifiseer, maar die meeste is nie volledig gekarakteriseer nie, veral nie ten opsigtigte van die molekulêre rol(le) wat hulle in plante speel nie. *Arabidopsis thaliana MYB3*, *MYB6*, *MYB7*, *MYB8* en *MYB32* is gevind om negatiewe reguleerders van algemene fenielpropanoied-metabolisme te wees. Daar is ook berig dat dié vyf transkripsiefaktore moontlik flavenoied-biosintese negatief kan reguleer, selfs al kan hulle verskillende teikengene hê. Studies op *AtMYB13*, *AtMYB14* en *AtMYB15* het berig dat hulle waarskynlik reguleerders van algemene fenielpropanoied-metabolisme is. Die genoemde rolle van die agt *AtMYB* transkripsiefaktore beteken dat hulle gemanipuleer kan word om te bepaal watter effek hulle op primêre en sekondêre metaboliete in plante het.

Die transkripsiefaktore, wat in die pUBI510-GRFCA vektor geligeer was, is toe gebruik om suikerriet-kallus te transformeer (**Hoofstuk 3**). Suikerriet vervaardig sukrose wat tot 70% van die suiker wat in die wêreld geproduseer word opmaak. Dít maak suikerriet 'n kommersieel belangrike en winsgewende plant. Die suikerriet-kallus is getransformeer deur middel van partikel-bombardering. Die transkripsiefaktore *AtMYB3*, *AtMYB6*, *AtMYB7*, *AtMYB13* en *AtMYB32* was suksesvol in die DNA van die suikerriet-kallus opgeneem. Data wat verkry was vir kallus wat *AtMYB3*, *AtMYB13* en *AtMYB32* ooruitgedruk het op soliede media en kallus in vloeibare medium was teenstrydig (m.a.w. kallus op soilede media wat meer sukrose as die wildetipe op soliede media geproduseer het, terwyl dieselfde transgeniese lyn minder sukrose as die wildetipe geproduseer het in vloeibare medium, en anders om). Nietemin, het *AtMYB13* transgeniese lyne meer sukrose geproduseer as die wildetipe. Transgeniese lyne van *AtMYB7* het almal minder sukrose geproduseer as die wildetipem op beide soliede en vloiebare media. Die transkriopsiefaktore wat gelei het tot 'n styging in stysel produksie wanneer hulle ooruitgedruk was was *AtMYB7* en *AtMYB13.* Data wat verkry is van die *AtMYB6*

transgeniese lyne was hoogs veranderlik in lyne wat op dieselfde medium gegroie was en oor die twee media. Die effek van hierdie transkripsiefaktore op die algehele metabolisme van die suikerriet-kallus, hetsy op MSC_3 soliede of vloeibaremedia, kon egter nie van die GC-MS analise ten volle bepaal word aangesien daar geen konsekwente fenotipiese effek tussen die verskillende transgeniese lyne vir enige van die gebruikte *MYB* transkripsiefaktore was nie.

In **Hoofstuk 4** was ‘n mikropropagerings strategie ontwikkel. Fitochemikalieë en hul biologiese aktiwiteite was ook bepaal vir die medisinale plant *Salvia repens*. *Salvia* plante is gevind om medisinaal belangrik te wees as gevolg van die sekondêre metaboliete, veral die essensiële olies, wat hulle produseer. Dit is ook bevind dat die plant-ekstrakte baie biologiese aktiwiteite soos anti-bakteriese, anti-inflammatoriese, anti-oksidant en anti-kanker aktiwiteite het. *Salvia repens* is suksesvol ontkiem *in vitro*, met 60% ontkieming wat bereik is in MS media met $1x10^{-5}$ maal verdunde rook-water na insnyding vir 12 min in 75% (v/v) H_2SO_4. Suksessyfers van 100% was behaal in die afhardingsproses wanneer die saailinge na die glashuis verskuif was. Ontkieming van *S. repens ex vitro* was 100% in 'n geoutoklaveerde grondmengsel van 1:1 (v/v) sand en vermikuliet. Gewigtig het die medisinale waarde van *S*. *repens* wat *in vitro* of *ex vitro* geproduseer was nie verlore gegaan nie. Die GC-MS data metaboliete analise het aangetoon dat die plante die medisinaal belangrike chemikalieë geproduseer het. Metaboliet-ekstrakte van *S*. *repens* was vir die eerste keer na berig aktief teen swamme, met MIK waardes laer as 1mg/ml oor ‘n tydperk van 4-5 d, teen vier *Fusarium* spp wat getoets was.

Laastens (**Hoofstuk 5**), transkripsiefaktore *AtMYB6* en *AtMYB13* was gebruik om *Salvia stenophylla* te transformeer deur *Agrobacterium*-bemiddelde transformasie, om sodoende te bepaal of die ooruitdrukking van hierdie transkripsiefaktore die produksie van medisinale en kommersieël-belangrike sekondêre metaboliete in *S*. *stenophylla* kan verhoog. Alhoewel beide *A*. *tumefaciens* en *A*. *rhizogenes* stamme gebruik was vir die transformasie proses, kon transformasie slegs deur die gebruik van *A*. *rhizogenes* bereik word. Geen transformante kon gegenereer word vanuit die *A*. *tumefaciens* behandelde materiaal nie. Transgeniese harigewortels het geen van die medisinaal belangrike metaboliete vervaardig nie. Die GC-MS analise van die transgeniese wortel materiaal het hoofsaaklik suikers en ander primêre metaboliete geïdentifiseer.

Conference contributions from this thesis

Lekgari GLP, Hills PN, Kossmann J and Makunga NP. Chemical profiling and antimicrobial activity of *Salvia repens* plants grown under different conditions. 5th Medical Research Council Research Conference. Medical Research Council Conference Centre, Cape Town, South Africa, 19-20 October 2011 (Oral and Poster)

Acknowledgements

I thank my supervisors Dr Paul Hills, Prof Nox Makunga for their guidance, patience and assistance.

Professor Cathie Martin and Dr Benedicte Lebouteiller of the John Innes Centre in the United Kingdom for donating the *AtMYB* transcription factors that were used in this research.

Southern African Development Committee through its SCARDA project, Botswana College of Agriculture (BCA) and the Institute for Plant Biotechnology (IPB) made this study possible through funding and I am very thankful.

A big thank you to all IPB students for all the help, guidance, frustration and everything else we shared as we were working to achieve all this. Baie dankie, guys.

Dr Christel van de Vyver and Ms Nonzukiso Vellem were ever so helpful when it came to advice on tissue culture. Dr Hannibal Musarurwa and Mr Sandile Ndimande, thank you guys for always listening to my ever unending questions and passing a lot of ideas by you before I could even start on any of the analyses. Zanele for always being there in the lab especially those late nights and those talks we had, they made up my days and made the work less strenuous.

Mama le Papa even though you cannot even comprehend what I am doing, you have always stood by me and held my hand. Lorato lele kana that you are still giving me drove me. Ke a leboga, Ke a lorata Modimo alo segofatse. Moemedi Kebokilwe the love and support that you have shown me made me to love and appreciate you even more. I love you and God bless you for all the endurance and love you continue to give me.

Above all I thank God Almighty, who has taught me to know that it is well even when I hit hitches and that through you everything is possible and indeed it is. You gave me a family, a home that spreads to all ends of the world and that family lifted my spirits throughout this work, The Old Apostolic Church. Bazalwane no Dade, uThixo ani sikelele and continue showing the same love to everyone that you shall receive. To God be the Glory.

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Abbreviations

α	Alpha
ABA	Abscisic acid
ADH1	Alcohol Dehydrogenase 1
AMDIS	Automated mass spectral deconvolution and identification system
AtMYB	*Arabidopsis thaliana* MYB
Att	Phage attachment site
attL	Left phage attachment site
attR	Right phage attachment site
BA	6-Benzyladenine (PGR)
bp	Base pairs (nucleic acid)
BCA	Botswana College of Agriculture
β	Beta
°C	Degrees Celsius
$Ca(NO)_2$	Calcium Nitrate
$CaCl_2$	Calcium Chloride
CaMV35S	Cauliflower Mosaic Virus 35S
ccdb	Control of cell death B gene
cDNA	Complementary DNA
Cm	Centimetre
Cmr	Chloramphenicol resistance selectable marker
COMT	Caffeic-acid-O-methyl transferase
CTAB	Cetyl trimethyl ammonium bromide
CTD	C-terminal domain
2,4-D	2,4-Dichlorophenoxy acetic acid
ddH_2O	Deionised water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ESTs	Expressed Sequence Tags
Ex vitro	Cultivated in natural conditions
$FeCl_3$	Iron (III) Chloride
g/kg	Grams per Kilogram

g/l	Grams per litre
GC-MS	Gas Chromatography Coupled to Mass Spectrometry
GH	Glass House
Gram–	Gram-negative
Gram+	Gram-positive
GRFCA	Gateway® reading frame cassette A
GUS	Glucuronidase
h	Hour
H_2O	Water
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanosulonic acid
HPLC	High-Perfomance Liquid Chromatography
HTH	Helix-turn-helix
IAA	Indole-3-acetic acid (PGR)
In vitro	“in glass”
INT	P-iodonitrotetrazolium
IPB	Institute For Plant Biotechnology
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
kHz	Kilohertz
KOH	Potassium hydroxide
kPa	Kilopascal
λ	Lambda
L	Litre
LB	Left Border
LC-MS	Liquid Chromatography Coupled to Mass Spectrometry
LiCl	Lithium Chloride
LSD	Least significant difference
M	Molar
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium sulfate
MH	Müeller-Hinton
MIC	Minimal Inhibitory Composition
Min	Minute

Ml	Millilitre
mM	milliMolar
$MnSO_4$	Manganese sulfate
MRC	Medical Research Council
mRNA	Messenger Ribonucleic acid
MS	Murashige and Skoog (1962) medium
1/10 MS	1/10 strength MS
1/2 MS	1/2 strength MS
MYB	Myeloblast
NAA	1-naphthalene acetic acid (PGR)
NaCl	Sodium chloride
NIST	National Institute of Standards and Technology
Nm	Nanometre
NMR	Nuclear Magnetic Resonance
*npt*II	neomycinphosphotransferase
OD	Optical density
PCR	Polymerase chain reaction
%	Percent
pH	Measure of acidity and alkalinity
Pty Ltd	Proprietary limited company
PVP	Polyvinylpyrrolidone
RB	Right Border
®	Registered
Ri	Root inducing
RNA	Ribonucleic acid
Rol	Root locus
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
Rubisco	Ribulose-1,5-bisphosphate carboxylase- oxygenase
SADC	Southern African Development Committee
SCF	Skp1-cullin-F-box
Sp	Species (singular)
SPME	Solid-phase micro extraction

Spp	Species (plural)
LEA1	*Late Embryogenesis Abundant1* gene
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA
TC	Tissue Culture
T-DNA	Transfer DNA
TE buffer	Tris-EDTA buffer
TF	Transcription factor
Ti	Tumour inducing
TLC	Thin layer chromatography
TMS	Trimethylsilyl
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
™	Trade mark
U/µl	Units per microlitre
USA	United States of America
µg	Microgram (10-6 g)
µg/ml	Microgram per millilitre
µl	Microliter (10-6 L)
µm	Micromolar (10-6 M)
$\mu mol.m^{-2}.s^{-1}$	Micromole per meter per second
v/v	Volume to volume ratio
VIP1	VirE2-interacting protein
Vir	Virulence
W	Watts
w/v	Mass per volume ratio
WPM	Woody Plant Media
WT	Wildtype
xg	Times gravitational force
YEP	Yeast extract peptone
$ZnSO_4$	Zinc sulfate

CHAPTER 1: General Introduction

Plants have developed mechanisms through which they are able to acquire water and nutrients, defend themselves against herbivory, diseases and abiotic stresses since they are sessile. They are autotrophic and use sunlight to produce carbohydrates and oxygen via photosynthesis to produce primary metabolites (Taiz and Zieger, 2006). Primary metabolites are needed by the plant at all times for normal growth, development, reproduction and generally perform physiological roles within the plant. We benefit from these as they are our food source and provide raw materials for industries.

Plants also produce secondary metabolites which are produced in small quantitiesas compared to primary metabolites. Secondary metabolites are compounds that play an ecological role and ensure survival and fitness of the plant in the ecosystem (Kennedy and Wightman, 2011). Specific secondary metabolites are produced as a response to a particular environmental stimulus (Kennedy and Wightman, 2011). The compounds produced include bacteriocidal and insecticidal compounds, allelochemicals to prevent competition by other plants and herbivory and chemicals such as anthocyanins which attract pollinators and aid in seed dispersal (Kennedy and Wightman, 2011). Secondary metabolites can serve as nutrients, flavourants, colorants, fragrances and medicines to people (Wu and Chappel, 2008).

Between 1960 and 2010, the world population has increased from 3 billion to 6.8 billion people (http://www.populationmedia.org/issues/population). To date it stands at more than 7.2 billion people and it is estimated that it will have risen to more than 9.5 billion people by the year 2050 (www.worldometers.info/world-population). Considering the growing world population, increased production of plant metabolites, whether primary or secondary, is crucial. Primary metabolites are important as the growing population needs to be fed on maybe even smaller agricultural land as they now occupy more land (Abughlelesha and Lateh, 2013). Ways in which to increase food production without using more land, more fertilisers, using less or no herbicides and insecticides therefore need to be established.

The World Health Organisation has estimated that about 80% of the developing world depends on traditional herbal remedies for their healthcare needs (Jäger and van Staden, 2000). A report by Wu and Chappel (2008) also indicated that nearly two thirds of approved drugs since 1981 are derived from natural secondary metabolites. As the production of secondary metabolites by plants in their natural habitat in the wild is triggered by environmental factors (Kennedy and Wightman, 2011), strategies that can be used to increase their production even when the plant is stressed need to be developed. Biotechnology is an important tool that can be used to manipulate plant metabolism to aid in this quest.

Biotechnology is an applied science and has been defined by the Organisation for Economic Co-operation and Development (1999) as the use of living organisms, their parts, products and models through science and technology applications, to alter living or non-living material to produce goods, services and give knowledge. There are three concepts under which biotechnology can be applied. The concepts are referred to as first, second and third generation biotechnology. The definitions of the three concepts are:

In first-generation biotechnology, organisms are used directly as they are, without being altered; examples include the use of yeast in fermentation and the extraction of pharmaceuticals from plants without any alterations to the plant (McKelvey et al., 2004). Second-generation biotechnology uses more specialized techniques which are not limited to but include tissue culture, micropropagation, identification, characterisation and production of pharmaceuticals and other natural products, embryo manipulation and bioprospecting (McKelvey et al., 2004). The third generation biotechnology, commonly referred to as molecular biology, manipulate processes in living organisms at a higher, precise and targeted level resulting in the organisms producing desired traits which suit mankind (McKelvey et al., 2004). Molecular biology includes genetic engineering whereby metabolites can be altered by way of over-expressing, silencing or knocking out particular gene/s in order to see the effect thereof in the targeted organism/s. Genetic engineering can be used to alter metabolites so that they are produced in an already functional form in order to reduce costs for industries as they would not have to treat the metabolites after harvesting it (Budziszewski et al., 1996).

Chapter 2: Literature review

2.1 Sugarcane

Sugarcane (*Saccharum spp.*) is a monocotyledonous plant of the family Poaceae that grows in tropical and subtropical regions of the world. Its main products are raw sugar, molasses and bagasse (Legendre and Burner, 1994), the most important of these being raw sugar from the sucrose that the plant produces.

Conventional breeding methods have been reported to improve sucrose yield by 1 to 1.5% yearly in Australia (Grof and Campbell, 2001). This is still not sufficient (Grof and Campbell, 2001) and has led to most researchers moving towards genetic engineering to improve sucrose production in sugarcane, using modern gene transfer techniques. Other research areas pertaining to sugarcane are aimed at improving its tolerance to insect and fungal attacks, herbicide resistance and improved fibre quality (Arencebia et al., 1998). This has also been problematic as transformation tends to work very well in dicotyledonous plants but not as efficiently in monocotyledonous plants. However, in 1992, Bower and Birch reported successful transformation of sugarcane using particle bombardment and to date this method is used routinely to transform sugarcane. The other challenge that had to be overcome to transform sugarcane was the lack of a suitable promoter to drive gene expression. Gene promoter elements from rice actin (Grof et al., 1996a), the small unit of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Grof et al., 1996b) and the maize ubiquitin gene (Christensen et al., 1992) have been reported to show some activity in sugarcane. The most efficient of these promoter elements was reported to be the maize ubiquitin promoter (Grof and Campbell, 2001).

Sugarcane has been shown to be a good bioreactor and also a source of renewable energy (Glassop et al., 2010) as it can accumulate compounds to high levels due to its vigorous growth, making it a good plant to use for studying the effects of biosynthetic genes. Research in the Institute for Plant Biotechnology (IPB, Stellenbosch University) is being conducted to identify means by which sucrose accumulation can be enhanced. This has mainly been conducted through manipulating enzymes involved in sucrose synthesis or degradation, or those

involved in the metabolism of other carbohydrates that affect the production and accumulation of sucrose, particularly starch (Table 2.1). To understand and be able to manipulate the accumulation of sucrose, or any other chemical, one has to understand how it is synthesised, compartmentalised and broken down.

Table 2.1 Some of the research conducted by past students on sugarcane in the IPB

Thesis title	**Author**
Influence of hexose-phosphates and carbon cycling in sucrose accumulation in sugarcane spp.	MJ van der Merwe (2005)
Trehalose and carbon partitioning in sugarcane	S Bosch (2005)
Genetic manipulation of the cell wall composition of sugarcane	JPI Bekker (2007)
The analysis and reduction of starch in sugarcane by silencing ADP-glucose pyrophosphorylase and over-expressing β-amylase	SJ Ferreira (2007)
Genetic manipulation of sucrose-storing tissue to produce alternative products	H Nell(2007)
Characterisation of transgenic sugarcane lines with perturbed pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PFP) activity	AL Spracklen (2009)
Analysis of the role of relative nucleotide concentrations on the regulation of carbohydrate metabolism in higher plants	G Boussiengui-Boussiengui (2010)

All of the research described in Table 2.1 involved targeted manipulation of specific enzymes, aimed at increasing the accumulation of the primary metabolite sucrose. However, these projects targeted only specific steps or pathways. The genetic manipulation of genes encoding transcription factors may offer an alternative method, whereby the expression of several genes in a particular biosynthetic pathway may be manipulated.

2.2 *Salvia stenophylla* and *Salvia repens*

Salvia plants are dicotyledonous plants belonging to the Lamiaceae family and are aromatic, herbaceous perennial shrubs. *Salvia stenophylla* has blue flowers and grows in the Southern African countries Namibia, Lesotho, Botswana (Jäger and Van Staden, 2000) and in many provinces of South Africa, particularly in the Eastern Cape, Western Cape, Free State and KwaZulu-Natal (Jäger and Van Staden, 2000; Viljoen et al., 2006; Kamatou et al., 2008). *Salvia repens* grows in the Eastern Cape and Kwazulu-Natal provinces and has flowers which can vary from white to purple or deep blue.

Salvia species are known throughout the world for their medicinal properties. Traditionally, these plants are used to treat respiratory ailments such as influenza, chronic bronchitis and tuberculosis as well as other ailments such as stomach ache, diarrhoea, rash, wounds, fever, headache and gynaecological complaints (Watt and Breye-Brandwijk, 1962; Auge et al., 2003; Scott et al., 2004). The most important secondary metabolites produced by *Salvia* spp. are essential oils. *Salvia stenophylla* contains high concentrations of epi-α-bisabolol (28.9-41% of total essential oils) (Viljoen et al., 2006), which has anti-inflammatory bioactivities (Jäger and Van Staden, 2000; Kamatou et al., 2008). By comparison, chamomile, which is usually the preferred species for isolation of this terpenoid, contains 17% epi-α-bisabolol in relative oil abundance. This terpenoid is used in the aromatherapy and cosmetics industries, making it economically important. Gono-Bwalya (2003) identified δ-3-careen and α-bisabolol at total oil concentrations of 24.9-33.0% in *Salvia repens*. Non-volatile compounds in *Salvia,* especially phenolics, also play an important role in the medicinal properties of the plants. Most of these have been identified as caffeic acid derivatives (Kamatou et al., 2008) and these include, in order of abundance; rosmarinic acid, which is an anti-oxidant (Lu and Foo, 2002); carnosol, which has antioxidant and anticarcinogenic properties, inhibiting the formation of tumors; and carnosic acid, which also displays antioxidant activities (Kamatou et al., 2008).

To date, most of the research conducted on these plants has been concerned with characterising the composition of their essential oils (Jequier et al., 1980; Viljoen et al., 2006). Such research also provided scientific support for the significance of these plants in traditional medicine, concluding that these plants have anti-inflammatory,

anti-cancer, anti-oxidant and anti-mycobacterial activities (Kamatou et al., 2008). In terms of total essential oils, *Salvia stenophylla* contains α-bisabolol (46.5%), limonene (38.1%), δ-3-carene (24.9%), γ-terpinene (20.3%), p-cymene (18.4%) and (E)-nerolidol (53.6%), amongst other compounds (Viljoen et al., 2006) at higher concentrations from different samples of *S. stenophylla* analysed.Kamatou et al. (2005) identified that *Salvia repens* contains β-phellandrene (22.2%), β-caryophellene (12.4%), limonene (9.8%) and camphor (6.9%) at the highest concentrations out of the 55 metabolites they identified in relative abundance. The abundance of these compounds has, however, been shown to vary from environment to environment or locality to locality(Viljoen et al., 2006).

Kamatou et al. (2008) reported that the compounds in *Salvia* species described above have activities against the causal agents of several of the ailments traditionally treated with these medicinal plants. For example, it was discovered that *Salvia* spp. show promising activity against *Mycobacterium tuberculosis,* with Minimal Inhibitory Concentration (MIC) values ranging from 0.03 to 8.00 mg/ml (Kamatou et al., 2007). Due to the traditional uses of these plants and now backed up by scientific evidence, the plants are being harvested for the formal commercial sector and also for informal trade by the local people. This is problematic since whole plants are used (Kamatou et al., 2008) and this may lead to wild populations diminishing and to the plants becoming endangered. It is therefore important to find alternative ways to access the secondary metabolites which accumulate in these plants.

Protocols for the growth of these plants under aseptic conditions have been established. The tissue culture conditions for seed germination and shoot initiation on different explants have been investigated and optimised for the three species *S. africana-lutea* (Makunga et al., 2008), *S. stenophylla* (Musarurwa et al., 2010) and *S. runcinata* (Figlan, 2012). Tissue culture provides axenic material that is suitable for genetic manipulation. *Agrobacterium*-mediated transformation has been shown to be an important tool in genetic manipulation, as it is a natural process where only the T-DNA (transfer DNA) which is flanked by the left and right borders of the Ti (tumour inducing) or Ri (root hair inducing) plasmid of the bacterial genome is inserted into the DNA of the host plant (Georgiev et al., 2008). This thus offers us a convenient

way of using biotechnology to manipulate the genetic and thus chemical composition of these plants in order to up-regulate production of the secondary metabolites.

Biotechnology has, to date, not really been harnessed in the South African *Salvia* species. Hairy root production, due to transformation by *Agrobacterium rhizogenes* and the incorporation of*rol* genes from the Ri plasmid into the plant genome, has been achieved in *S. africana-lutea* (Ramogola, 2009) and *S. runcinata* (Figlan, 2012).The same is also true for *Salvia* species across the world. *Salvia miltiorrhiza* has been tested for susceptibility to transformation by wildtype *A. tumefaciens* containing an empty pCAMBIA2301 expression vector (Yan and Wang, 2007). *Salvia tomentosa* was transformed by Marchev et al. (2011), where the standard transformation of leaf disks using *A. rhizogenes* was compared to a two-phase temporary immersion system for genetic transfer. Khawar et al. (2003) showed susceptibility to transformation by *A. tumefaciens* of the two species *S. sclareae* and *S. pratense*.

However, there have been some targeted studies which aimed to transform *Salvia* species either to produce targeted secondary metabolites or to make the plants more stress tolerant. In our laboratory, secondary metabolite production, particularly terpenoids (which form essential oils), was enhanced by *A. tumefaciens* mediated transformation using *AgGPPS2* gene in *S. stenophylla* (Musarurwa, 2013). Han et al. (2007) transformed *S. miltiorrhiza* successfully using *A. tumefaciens* with the *TaLEA1* gene from wheat which encodes for a late-embryogenesis-abundant protein, making the plant more salt and drought tolerant. The studies mentioned above targeted particular genes within pathways and not a large pool of genes. Transcription factors can therefore be used in order to manipulate an entire pathway instead of particular individual steps within the pathway.

2.3 Transcription factors

Transcription factors are regulatory proteins that modulate the expression of specific groups of genes through sequence-specific DNA-binding and protein-protein interactions (Broun, 2004). They are generally composed of at least two discrete domains, a DNA-binding domain and an activation/repression domain, which operate

together to regulate many physiological and biochemical processes by modulating the rate of transcriptional initiation of target genes (Yanhui et al., 2006).

Some transcription factor families are found in both plants and animals and originated from common ancestral genes while others evolved specifically in plants (Yanagisawa, 2004). An example of such transcription factors is the set of MYB transcription factors. The first identified *MYB* gene was the oncogene *v-Myb* which was derived from the avian myeloblastosis virus (Sanz, 2010), a type of leukaemia (cancer of blood cells), hence the acronym MYB, derived from myeloblastosis. The MYB family of transcription factors is highly restricted in slime moulds, fungi and animals whereas in higher plants it is large (Sanz, 2010). Members of this family are characterised by having a structurally-conserved MYB DNA-binding domain. This domain consists of four imperfect amino acid sequence repeats that typically consist of one to three imperfect repeats (R1, R2, and R3) (Du et al., 2009). Each repeat contains about 52 amino acids (Dubos et al., 2010). Each of these sequence repeats forms three α helices with the second and third of each repeat, building an HTH (helix-turn-helix) structure that has three tryptophan or hydrophobic residues that are regularly spaced and forms a hydrophobic core in the 3D HTH structure (Dubos et al., 2010). The MYB proteins are divided into 4 classes, namely R1R2R3-MYBs, R2R3-MYBs, R1-MYBs and 4R-MYBs (Dubos et al., 2010). Of these classes, the R2R3-MYB class is the largest at 126 identified proteins (Sønderby et al., 2007) and the 4R-MYB the smallest, with only 1 known protein (Dubos et al., 2010).

MYB transcription factors have been reported to be multifunctional (Yanhui et al., 2006) and are involved in the regulation of meristem formation, the cell cycle, control of cellular morphogenesis (Jin and Martin, 1999), but most significantly in the control of secondary metabolite pathways such as the phenylpropanoid pathway (Martin and Paz-Ares, 1997; Jin and Martin, 1999). They can also interact with other transcription factors (Martin and Paz-Ares, 1997) in the control of pathways. Yanhui et al. (2006) looked at the expression of different *Arabidopsis thaliana* MYB transcription factors following different plant hormone and salt stress treatments. Higher expression levels of MYB transcription factors were observed when plants were treated with jasmonic acid and salicylic acid (Yanhui et al., 2006). These are phenolic phytohormones that mediate plant defences against pathogens and insects that limit

damage to plants from biotic stress (Halim et al., 2006); thus suggesting that the MYB transcription factors are highly expressed when plants are exposed to abiotic and biotic stresses.As they can control multiple pathway steps, they have emerged as a powerful tool for the manipulation of complex metabolic pathways in plants (Broun, 2004).

Arabidopsis thaliana MYB transcription factors used in this study were AtMYB3, AtMYB6, AtMYB7, AtMYB8, AtMYB13, AtMYB14, AtMYB15 and AtMYB32. The eight transcription factors were donated by Professor Cathie Martin and Dr Benedicte Lebouteiller of the John Innes Centre in the United Kingdom. The donated transcription factors have been reported to be involved in the phenylpropanoid pathway. *Arabidopsis thaliana* MYB3, AtMYB14 and AtMYB15, which belong to the R2R3 subfamily II of MYB transcription factors, have been reported to enhance the phenylpropanoid pathway (Jin et al., 2000; Martin et al., 2011).The R2R3MYB subfamily IV contains 6 transcription factors, AtMYB3, AtMYB4 (not used in this study), AtMYB6, AtMYB7, AtMYB8 and AtMYB32, which have been reported to be repressors of the phenylpropanoid pathway (Jin et al., 2000;Martin et al., 2011).

Functions of AtMYB transcription factors in the same subfamily have been shown to be redundant. Sanz (2010) reported that *A. thaliana* plants where *AtMYB6* and *AtMYB8* were over-expressed resulted in impaired growth with either germination rate being slowed down or reduced, seedlings emerging and forming only the first leaf and the rosette of the transgenic lines being smaller compared with the wildtype. Changes in expression levels of *AtMYB4* and *AtMYB32* have been reported to result in a similar phenotype where the pollen lost cytoplasm and collapsed (Preston et al., 2004). Bang et al. (2008) reported that expression of *AtMYB3* in *A. thaliana* is induced by abiotic stress and ABA treatment. Jin et al. (2000) reported that when *AtMYB4* was over-expressed in *A. thaliana* it resulted in enhanced sensitivity to UV-B light. A similar response to UV-B light by *A. thaliana* was observed by Sanz (2010) when *AtMYB6* and *AtMYB8* were over-expressed. *AtMYB7* (Fornale et al., 2013) was shown to be induced by salt stress and to play a role in UV protection.

The three transcription factors *AtMYB13*, *AtMYB14* and *AtMYB15* are from subfamily II of the R2R3MYB transcription factors. *A. thaliana* plants where *AtMYB14* (Chen et al., 2014) and *AtMYB15* (Agarwal et al., 2006) genes were knocked out displayed

increased freezing tolerance, even though *AtMYB14*is down-regulated by cold treatment and *AtMYB15* is up-regulated by cold treatment (Chen et al., 2014). *AtMYB13* and *AtMYB15* have been reported to be up-regulated by wounding in *A. thaliana* and also to be involved in ABA-mediated responses to environmental signals (Kirik et al., 1998; Chen et al., 2006). The subfamily II members of the R2R3MYB transcription factors therefore also have overlapping functions.

After looking at the reported functions of the eight transcription factors used in this study, it was expected that the transcription factors from the same subfamily, will result in somewhat semilar metabolite profiles when over-expressed. Consequently, transgenic lines over-expressing R2R3MYB subfamily II transcription factors should result inan increase in secondary metabolites as they positively influence the phenylpropanoid pathway,whereas plants over-expressing R2R3MYB subfamily IV transcription factors are expected to produce less secondary metabolites.

Table 2.2 Known functions of AtMYB transcription factors used in the study.

AtMYB TF	Function	Reference
AtMYB3	Repressor of the phenylpropanoid pathway, possibly specifically anthocyanin synthesis.	Wang et al. (2010)
	Functions in abiotic stress signalling, in concert with CTD (C-terminal domain) phosphatase-like CPL1.	Bang et al. (2008)
AtMYB6	Repressors of the phenylpropanoid pathway, and perhaps anthocyanins specifically.	Wang et al. (2010)
AtMYB7	Encodes transcriptional repressors of the phenylpropanoid pathway.	Dubos et al. (2010)
AtMYB8	Reported to be repressors of the phenylpropanoid pathway.	Sanz (2010)
AtMYB13	Involved in ABA-mediated responses to environmental signals.	Dubos et al. (2010)
AtMYB14	Freezing tolerance in *Arabidopsis* by affecting expression of CBF genes.	Chen et al. (2013)

AtMYB TF	Function	Reference
	The*AtMYB14* gene was down-regulated by cold treatment.	
AtMYB15	Involved in cold stress tolerance, over expression reduces tolerance and silencing results in more tolerance to cold by its activation or repression of CBF genes.	Dubos et al. (2010)
	Involved in ABA-mediated responses to environmental signals and drought tolerance.	Yanhui et al. (2006)
	Direct regulator of the *Arabidopsis* shikimate pathway in response to wounding.	Dubos et al. (2010)
AtMYB32	Encodes transcriptional repressor. Required for normal pollen development in *Arabidopsis thaliana.*	Dubos et al. (2010) Preston et al. (2004)
	Repress the *COMT* (caffeic acid-O-methyl transferase, key enzyme in lignin biosynthesis) gene in *Arabidopsis.*	Du et al. (2009)

2.4 Plant breeding: Conventional breeding versus biotechnology

Cultivation of crops as a food source is one of the oldest human professions (Jauhar, 2006) and the very first step that was taken to domesticate crops is the earliest form of plant breeding (Snape, 2004). Plant breeding,selecting plants with desirable traits whether by quantity or quality, is not a new phenomenon. Even before the dawn of genetics, people could select for crops that are resistant to drought, that have high yields and can survive most stresses (Harlander, 2002) by just selecting the seed from the desired plant so that it can be ploughed in the next season. The poor performing plants were selected off the gene pool and the desired plant's generations were continued, this, however, was not enough as people moved on to

cross breed crops in order to genetically pass the desired traits. This is what is now referred to as conventional breeding.

Conventional breeding methodologies became scientific after the rediscovery of Mendel's laws (Snape, 2004). What is needed most by any plant breeder is genetic variation, as the more varied the gene pool is the more the choice to interbreed and obtain good quality plants (Harlander, 2002). However, this is a lengthy procedure and it can take many years to develop a cultivar that can be taken out into the field and be adopted for commercialisation (Harlander, 2002). This is mainly because plant breeding is a matter of 'crossing the best with the best and hoping for the best' (Snape, 2004). This led to scientists having to come up with a faster and more targeted way of breeding referred to as third generation biotechnology or genetic engineering.

In genetic engineering, genes are isolated and characterised in order to determine the particular gene that gives the desired trait. The gene of interest is then directly manipulated into the genome of the host plant, transforming its genetic make-up. This is done in the shortest time as a genetically transformed plant can be commercialised in a year or two (Harlander, 2002). Thus genetic engineering is just an advance of conventional plant breeding that can cut costs as it is more targeted and takes a short time (Snape, 2004).

2.5 Secondary Metabolites

Secondary metabolites are natural chemicals that are produced by plants. They are non-nutritive (Craig, 1996) but are needed by plants for purposes such as disease and pathogen defence and control. Studies have shown that secondary metabolites are important in human health. This is because they display different biological activities such as anti-oxidant, anti-inflammatory, anti-cancer and anti-bacterial activities. Most of these biochemicals are ingested in food such as fruits, vegetables and whole grains (Craig, 1996).

However, some important phytochemicals, especially ones in traditional medicinal plants, are not readily available to people as they are not separated and purified in their working active form. Ways in which these phytochemicals can be extracted, purified and their biological activities assayed from plants have to be devised in order

for the chemicals to be put through clinical trials before being distributed for consumption.

2.5.1 Metabolite extraction

Phytochemical extraction techniques follow a more or less standard protocol. A typical phytochemical extraction procedure starts with the disruption of plant tissue and cells by homogenisation in order to release the chemicals (Dai and Mumper, 2010). This is mainly done using a pestle and mortar under liquid nitrogen, althoughplant tissues can also be air driedin a well aerated room, usually at room temperature,after which the tissue is crushed using pestle and mortar.

Actual extraction then follows where the homogenised plant tissue is immersed in a solvent. Solvents are commonly used for extractions because they are readily available, inexpensive and easy to use (Dai and Mumper, 2010). Solvents, just like secondary metabolites, differ in polarity, so different solvents can be used depending on the kind of secondary metabolites that are targeted for extraction (Gupta et al. 2012). The three polarity strengths of solvents are polar, medium-polar and non-polar. Polar solvents extract polar chemicals and non-poplar solvents extract polar chemicals (Gupta et al. 2012). Examples of polar solvents include methanol, ethanol and water, medium-polar solvents examples are ethyl acetate, acetone and dichloromethane and non-polar solvents include toluene, chloroform and hexane. Therefore different solvents can be mixed for extraction or they can be used in sequence in the same sample material, depending on their polarity in order to extract both polar and non-polar solvents. Metabolite extraction also depends on temperature and extraction time as some metabolites like polyphenolics are easily hydrolised and oxidized at high temperatures and long extraction times resulting in decreased yield (Dai and Mumper, 2010).

Different extraction methods can be adapted to aid extraction efficiency. The most simple and easy methods to use are maceration, hydro-distillation using steam and soxhlet extraction (Handa et al., 2008). In maceration, the homogenised plant sample is soaked in a solvent in a closed container and it is left at room temperature. A sonicator can be used to shake the contents so as to enhance the extraction of compounds. The solvent is then decanted and filtered to remove debris. During

hydro-distillation the plant sample, which can be dry or wet, is placed in a flask and immersed in water. The flask is then connected to a condenser and heated. The distillate is collected in a tube that is connected to the condenser. A rotary evaporator can be used for this process. Hydro-distillation is useful for extraction of volatile phytochemicals such as essential oils (Doughari, 2012). For soxhlet extraction, the homogenised plant sample is placed in a cellulose thimble in an extraction chamber. The chamber is then placed on top of a collection flask which is placed beneath a condenser. A solvent of choice is then added to the sample which gets heated up under reflux. The condensed solvent with extracts is collected in the flask underneath. In addition to the methods described above there are other extraction methods that can be used, such as sublimation, percolation and ultrasound-assisted extraction (Handa et al., 2008).

2.5.2 Metabolite analysis

Different techniques that are based on separation of metabolites have been developed and are routinely used to identify and quantify metabolites. There is no single technique which can be used to characterise all metabolites in a plant extract (Kopka et al., 2004). Simple and advanced chromatography based techniques such as thin layer chromatography (TLC), gas chromatography coupled to mass spectrometry (GC-MS), Liquid chromatography coupled to mass spectrometry (LC-MS),high-performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy (NMR)are commonly used to characterise metabolites in plant extracts (Kopka et al., 2004).

2.5.2.1 Thin Layer Chromatography (TLC)

Thin Layer Chromatography is a simple, fast and reproducible chromatographic technique (Marston and Hostettmann, 1999). TLC can be used to determine optimal solvents to extract particular metabolites. The solvents can then be used to extract metabolites for further analysis like metabolite identification using GC-MS and for use in biological activity assays in order to determine MICs against pathogens like fungi and bacteria.

2.5.2.2 Gas Chromatography-Mass Spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry distinguishes itself among other types of chromatography because of its excellent separation capacity, and its ability to generate a mass spectrum for each compound, allowing separation and detection of many metabolites (Glassop et al., 2007). Additionally, putative metabolite identification is simplified when standardized retention indices and mass spectra are registered in a database (Sakamoto et al., 2010). Non-volatile compounds have to be chemically derivitised before being analysed so as to make them volatile (Kopka et al., 2004), but this is not necessary for volatile compounds.

2.6 Biological activity of plant extracts

Activity of plant extracts against disease causing agents such as bacteria and fungi can be determined using biological activity assays. A simple and efficient microtitre plate assay has been developed by Eloff (1998). The assay has been adapted to assess the antimicrobial activity of plant extracts against different bacterial and fungal strains looking at minimum inhibitory concentration (MIC). Extracts with MIC values below 1 mg/ml are worth noting (Van Vuuren, 2008) as they indicate that the extracts have high anti-microbial activity.

The Eloff (1998) assay determines the growth or inhibition of growth of the microbes measured in round-bottomed 96-well microtitre plates with the addition of the bacterial growth indicator, p-iodonitrotetrazolium (INT [Sigma]) salts. INT serves as an electron acceptor that is reduced from a colourless compound to a red coloured formazan product by biologically active cells, within 10-60 min (Eloff, 1998; Gabrielson et al., 2002). This reaction can be visually seen by the colour change as less INT will be reduced when the bacteria is killed resulting in reduced colour intensity as compared to when the bacteria is not killed.

2.7 Plant transformation techniques

2.7.1 Biolistics

This is a technique whereby the gene is transferred to the plant material at high speed by adapting a ‘shotgun’ technique. The gene of interest ligated to the plant expression vector is adhered to tungsten or gold metal microprojectiles which are 1-3

μm diameter in size. The mixture is then bombarded into the plant material with the hope that some of the plasmid will penetrate the cells of the plant material and be incorporated into the genomic DNA of the plant (Sanford, 1999).

The technique has proven to be very important in sugarcane transformation, as first illustrated by Bower and Birch (1992). This is because *Agrobacterium*-mediated transformation resulted in a low success rate and the transformed calli in most cases did not result in transgenic plants when regenerated (Potrykus, 1991). Although other transformation techniques such as electroporation and polyethylene glycol treatments did allow regeneration of rice and maize plants which are monocotyledonous, the techniques were limited as plant regeneration proved to encounter severe difficulties (Bower and Birch, 1992). The same techniques were tested for sugarcane but although the calli was successfully transformed, plant regeneration was never achieved (Bower and Birch, 1992). Biolistics has been proven (Franks and Birch, 1991) to produce large numbers of transgenic sugarcane calli and is a simple and efficient method which results in the transformed calli readily regenerating plants. After bombardment, the callus undergoes a series of selection steps on media containing (an) appropriate antibiotic(s) to select for transformants.

2.7.2 *Agrobacterium*-mediated transformation

Agrobacterium is a common soil gram-negative bacterium which causes crown gall disease or hairy roots in plants by transferring a segment of DNA, referred to as the T-DNA region, from the plasmid which the *Agrobacterium* harbours (Gelvin, 2003). *Agrobacterium tumefaciens* carries the tumor inducing (Ti) plasmid, whereas the hairy root inducing (Ri) plasmid is found in *Agrobacterium rhizogenes*. Wounding in plants induces the release of phenolic compounds called acetosyringone. Acetosyringone is then detected by *Agrobacterium* and this leads to the bacteria infecting the plant at the wound site. This results in the infected cells multiplying at a very high rate due to hormones, auxin and cytokinin that the host produces due to the presence of the *Agrobacterium.* This makes it an easy tool for plant genetic transformation. *Agrobacterium*-mediated transformation was initially believed to work successfully only on dicotyledonous plants as *Agrobacterium tumefaciens* naturally only infects these plants (de la Riva et al., 1998), but studies have shown that it can

also work in monocotyledonous plants such as rice although usually at a much lower level of efficiency (Grof and Campbell, 2001). The genetic material that is introduced into the plants during transfromation is called T-DNA which is located on either the Ti or Ri plasmid (Gasserand Fraley, 1989; Gelvin, 2003). Before plasmids can be used for transformation, they are disarmed by replacing the T-DNA region of the Ti or Ri plasmid with a gene of interest (Gasser and Fraley, 1989).

A series of steps are involved in the infection of the plant. The genes involved in these steps have been identified. *Agrobacterium* firstly attaches to the host cells through bacterium and host cell ligand-receptor interaction that occurs due to phenolics, mainly acetosyringone, which it perceives (Gelvin, 2003). The attachment leads to the virulence (Vir) proteins in the bacteria being activated. Virulence proteinsare responsible for the excision of the T-DNA region from the plasmid and its delivery into the host plant's cell (Gasser and Fraley, 1989). The T-DNA region is flanked by borders that are 25 bp long. These borders are referred to as the left border (LB) and right border (RB). The sequences of the LB and RB borders are the targets for the border specific endonucleases that are responsible for processing the T-DNA from the Ti plasmid. The Vir proteins that have been named are VirA, VirB, VirC, VirD, VirE, VirF and VirG. First the VirA protein perceives the acetosyringone at the wounded area of the plant, acting as the first step of signal perception and transduction leading to the transphosphorylation of the VirG protein (Gelvin, 2003). The VirA/VirG complex activity then activates the other Vir proteins. Next, VirD1/D2 generate a mobile copy of the T-DNA, which gets cleaved by VirD2 and coated by the single stranded DNA binding protein VirE and is transported from the bacterium to the host plant cell by the VirB/VirD4 protein complex (Gelvin, 2003). The VirE coat protein protects the T-DNA from being degraded by both the bacterial and plant cell nucleases (Gelvin, 2003).

In the plant cell, the VirE2-interacting protein (VIP1) and the bacterial functional homolog (VirE3), interacts with VirE2 coating the T-DNA, making the VirE2 to adapt to the host plant's karyopherin alpha (Tzfira and Citovsky, 2006), which mediates the import of VirE2 into the host cell nucleus (Lacroix et al., 2004). Upon reaching the nucleus, VirF interacts with VIP1 and ASK1 (homolog of SKP1 protein in the SCF (Skp1-cullin-F-box) complex, which identifies the proteins for degradation (Tzfira and

Citovsky, 2006), resulting in proteolytic degradation of the coating around the T-DNA so that it can integrate with the host's DNA. After the integration, the T-DNA then becomes a part of the infected cell's DNA, thus the T-DNA is replicated as the plant's genomic DNA divides resulting in multiple copies. The genes carried by the T-DNA are thus expressed by the plant cell.

2.8 AIMS AND OBJECTIVES

Since many MYB transcription factors have been shown to play important roles in plant metabolism, several MYB transcription factor genes, donated by Professor Cathie Martin and Dr Benedicte Lebouteiller (John Innes Centre in the United Kingdom) were transformed into sugarcane and *Salvia* plants. This study therefore was aimed at over-expressing the *Arabidopsis thaliana MYB3, MYB6, MYB7, MYB8, MYB13, MYB14, MYB15* and *MYB32* transcription factor-encoding genes in sugarcane and *S. stenophylla*, using particle bombardment and *Agrobacterium*-mediated transformation respectively, in order to:

1. Determine how the different transcription factors will affect primary and secondary metabolism in sugarcane.

2. Up-regulate production of medicinal terpenes and phenols in *Salvia* plants.

3. Determine whether transcription factors from the dicotyledonous plant *A. thaliana* will affect monocotyledonous (*Saccharum* sp.) and dicotyledonous (*Salvia spp.*) plants differently when heterologously over-expressed.

CHAPTER 3: Over-expressing *Arabidopsis* MYB transcription factors in sugarcane callus

3.1 Introduction

3.1.1 Sugarcane and economics

Sugarcane (*Saccharum* spp.) is a commercially important plant in South Africa. Of the 100 sugarcane industries in the world, South Africa ranks as number 15 in terms of production and revenue. The sugarcane industry in South Africa brings in revenue of, on average, R12 billion per annum and employs almost a million people either directly or indirectly (www.sasa.org.za). This makes it a very important plant to the livelihood of people and thus research to find ways in which sugarcane can be improved in terms of yield and product increment need to be harnessed.

3.1.2 Metabolites in sugarcane

Sugarcane is a known bioreactor and has been estimated to be capable of storing 25% of its fresh weight as sucrose (Wang et al. 2005) making it ideal for studies on accumulation of metabolites other than sucrose. Research on sugarcane is based on primary metabolites as they are the ones that are economically important. The most important primary metabolite in sugarcane is sucrose, which is targeted for increased production. However, secondary metabolites in sugarcane have not been well studied. Secondary metabolites are produced depending on the conditions that a plant is exposed to at a given time, thus they are important for the plant-environment interactions. Secondary metabolites affect the production of primary metabolites as they are mainly involved in protection of the plant against stresses, whether biotic like pathogens and abiotic like drought and salt stresses. A stressed plant is likely to produce a lower yield and this may affect the accumulation of the desired compounds.

There are only a limited number of studies that have looked at the effect of secondary metabolite accumulation in sugarcane. These include work by Wahid and Ghazanfar (2006) who demonstrated that the accumulation of soluble phenolics, anthocyanins and flavones lead to enhanced salt-stress tolerance. Franca et al.

(2001) used sugarcane ESTs (Expressed Sequence Tags) to predict the profile of isoprenoid and phenylpropanoid metabolism in sugarcane and identified the branches of secondary metabolism activated during tissue-specific stages of development and the adaptive response of sugarcane to agents of biotic and abiotic stress. Yang et al. (2010) optimised the production of phenolic metabolites in sugarcane and used them as a spray to show that they are active against *Ralstonia solanacearum*, a bacterium that cause wilting in tomato, showing the efficacy of these metabolites against biotic stress. Other research areas pertaining to sugarcane are aimed at improving its tolerance to insect and fungal attacks, herbicide resistance and improved fibre quality (Arencebia et al., 1998).

3.1.3 Accumulation of secondary metabolites in sugarcane

Metabolites can be up-regulated or down-regulated if genes that activate or repress their production are either over-expressed or shut down respectively. For the purpose of altering metabolites, especially secondary metabolites which are not as well studied in sugarcane compared to primary metabolites, a method that can act on single or multiple gene pathways might prove to be ideal. *Arabidopsis thaliana* MYB transcription factors therefore offer this breakthrough as they have been shown to be able to work on single or multiple gene pathways independently or in conjunction with other transcription factors (Martin and Paz-Ares, 1997). The *AtMYB* transcription factors can either up-regulate or down-regulate accumulation of secondary metabolites especially in the phenylpropanoid and flavone pathways.

3.1.4 *Arabidopsis thaliana* MYB transcription factors

Arabidopsis thaliana transcription factor genes *AtMYB3, AtMYB6,AtMYB7*, *AtMYB8*, *AtMYB13, AtMYB14, AtMYB15 and AtMYB32*, were over-expressed in sugarcane. The transcription factors AtMYB13, AtMYB14 and AtMYB15 have been reported to enhance the phenylpropanoid pathway (Martin et al., 2011). The remaining transcription factors, MYB3, AtMYB6, AtMYB7, AtMYB8 and AtMYB32, are negative regulators of general phenylpropanoid metabolism (Jin et al., 2000).

It is completely unknown whether these eight transcription factors play any role in the production of carbohydrates. As sugarcane is economically important in South Africa due to its production of sucrose and is readily available in our laboratory, we aimed

to test the effects of these transcription factors on overall metabolite production by over-expressing them in sugarcane. This was a blue-sky research approach, aimed at determining whether over-expression of the transcription factors would have any potentially commercially valuable effect(s) on primary and/or secondary metabolism in sugarcane. For the primary metabolites we focused on sucrose and starch, as these are important in the sugarcane industry.

3.2 Materials and Methods

3.2.1 Plant material and culture

Sugarcane 88H callus was grown in continuous culture. It was cultured under asceptic conditions on autoclaved MSC_3 medium (4.43 g/l MS [Murashige and Skoog, 1962] salts [Highveld Biological, South Africa] supplemented with 20 g/l sucrose, 0.5 g/l casein [Sigma Life Science]a nd 3 mg/l 2,4-D, adjusted to pH 6 using KOH and solidified with 2.2 g/l Gelrite). Calli were maintained at 28°C in the dark and sub-cultured every 2 weeks. All media used for this study were autoclaved at 121°C for 15 min at 103 kPa and then poured upon cooling into 25 ml Greiner petri dishes.

3.2.2 Construction of the plant expression vectors containing MYB transcription factors

3.2.2.1 Amplification and isolation of *E. coli* plasmids containing transcription factors

Arabidopsis thaliana transcription factor genes *AtMYB3, AtMYB6, AtMYB13, AtMYB14, AtMYB15 and AtMYB32*were donated already cloned in the pDONR207 Gateway®-compatible vector, whilst *AtMYB7* and *AtMYB8* were cloned in the pDONR201 Gateway®-compatible vector. The lyophilized plasmids were re-suspended in 10 µl sterile milliQ water, after which 1 µl of each was mobilised into 20 µl *E. coli* DH5α electro-competent cells, prepared according to the BIO-RAD protocol, via electroporation using a BIO-RAD Gene Pulse X cell-electroporator using the preset *E. coli* bacterial protocol. Immediately following electroporation, 1 ml SOC (20 g/l tryptone, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) media was added to the cells.

The transformed *E. coli* cells were then incubated for 1 h at 37°C before being plated out on LB (10 g/l tryptone, 10 g/l NaCl, 5 g/l yeast extract powder and 15 g/l bacteriological agar) plates containing 50 µg/ml gentamycin (*AtMYB3*, *AtMYB6*, *AtMYB13*, *AtMYB14*, *AtMYB15* and *AtMYB32)* or 50µg/ml kanamycin (*AtMYB7* and *AtMYB8)* for selection and incubated at 37°C for 16 h. Single colonies were picked for each plasmid containing the transcription factor and grown overnight in the shaker at 37°C in 10 ml LB medium containing 50 µg/ml of the appropriate antibiotic. Plasmid DNA was isolated using the Zyppy™ plasmid mini-prep alkaline lysis kit (Zymo Research). After quantifying the isolated plasmid DNA from the donor plasmids using the Nanodrop® (ND 1000 V3.6.0) spectrophotometer, the gene inserts were verified by electrophoresis on a 1% (w/v) agarose gel. The plasmid DNA was then recombined into Gateway®-compatible plant expression vector plasmids (destination vectors, Section 3.2.3) to get expression clones.

3.2.2.2 Construction of pUBI510-AtMYB Gateway® destination vectors

The expression vector pUBI510, containing the maize ubiquitin promoter (Figure 3.3B) can be used to transform sugarcane callus via particle bombardment. The vector has an ampicillin resistance gene for bacterial selection and is used in conjunction with the helper plasmid pEmuKN, which is a selection vector conferring geneticin resistance in plants for callus selection. The pUBI510 vector was converted into a Gateway® destination vector by ligating the 1711 bp Gateway®reading frame cassette A (GRFCA, Figure 3.3A), into the *Sma*I site of pUBI510 following the manufacturer's protocol (Invitrogen), to generate pUBI510-GRFCA (Figure 3.3C). This procedure was kindly performed by Dr Nelius Swart (IPB).

3.2.2.3 Mobilisation of transcription factors into the plant expression vectors

Gateway® vector recombination, following the manufacturer's protocol (Invitrogen, 2008), was used to transfer the transcription factor genes into the plant expression vectors. Firstly, the donor vectors containing the *AtMYB* entry clones and the plant Gateway®cloning vectors were diluted to a concentration of 150 ng/µl, then 1 µl of each was added to a reaction containing 6 µl TE buffer at pH 8 (10 mM Tris[2-amino-2-[hydroxymethyl]-1,3-propanediol]-Cl and 1mM EDTA [Ethylenediaminetetraacetic acid]), 2 µl of LR clonase (Invitrogen) was added to a final volume of 10 µl. The

reaction mixture was vortexed and then incubated at 25°C for 2 h. After incubation, the reaction was stopped by the addition of 1 µl Proteinase K (2 µg/µl), followed by incubation at 37°C for 10 min. Next, 1 µl of the reaction was used to transform 20 µl of electro-competent *E. coli* DH5α strain cells by electroporation. Immediately following electroporation, 1 ml of SOC media was added and the tube was incubated for 1 h at 37°C. An optimisation step was then carried by adding the 1 ml SOC solution containing the transformed *E. coli* to 9 ml of liquid LB media containing the appropriate antibiotic to obtain a final volume of 10 ml. Following overnight incubation of this culture in a shaker at 37°C, the bacterial suspension was diluted 1000x with liquid LB containing the appropriate antibiotics, after which 100 µl of the diluted bacterial suspension was plated out on LB agar media 50 µg/ml ampicillin (pUBI510-GRFCA).

Positive and negative controls were included. For the negative control, the LR Clonase II enzyme was not added to the reaction whereas for the positive control, pENTR™-GUS, as supplied by the manufacturer, was added instead of the entry vector.

3.2.2.4 Verification of gene insertion in plant transformationvectors

Following transformation, single colonies were inoculated into 10 ml LB liquid medium containing the appropriate antibiotic for selection and incubated overnight at 37°C. Plasmid DNA was then isolated following an alkaline lysis mini-prep protocol (http://biology.unt.edu/ayre-lab-plant-physiology-biochemistry-and-signaling/ayre-lab-protocols), after which the DNA was purified using a Wizard® DNA Clean-Up System (Promega). The isolated plasmid DNA was then used for restriction digests, sequencing and PCR, to verify that the transcription factors were correctly inserted into the vectors. For restriction digests and plasmid maps, only results for *AtMYB6* and *AtMYB14* are presented and PCR results are presented for all the eight transcription factors used, including the positive control.

3.2.2.5 Restriction Digests

For all restriction digests, 1 µg plasmid DNA was digested as recommended by the enzyme manufacturer, Fermentas Life Sciences. Details of the restriction endonucleases used to test the various constructs are presented in Table 3.1. The

reactions were incubated in a water bath at 37°C for 1 h. Following digestion, 4 µl of loading buffer (30% [v/v] Glycerol, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol) was added to the reactions which were then electrophoresed on a 1% (w/v) agarose TBE (Tris 89 mM, 89 mM Boric Acid and 2 mM EDTA, pH 8) gel stained with ethidium bromide.

Table 3.1 Expected band sizes from single and double restriction digests of pUBI510 containing the *AtMYB* genes *AtMYB6* and *AtMYB14*

Vector	*Eco*RI	*Hind*III	*Bam*HI	*Eco*RI+*Hind*III	*Eco*RI+*Sal*I	*Eco*RI+*Bam*HI
pUBI510-*AtMYB6*	6388bp	3976bp 2412bp	5713bp 675bp	3384bp 2412bp 592bp	3985bp 1404bp 999bp	5394bp 675bp 319bp
pUBI510-*AtMYB14*	6240bp	3974bp 2266bp	6240bp	3384bp 2266bp 590bp	3985bp 1404bp 445bp 406bp	5394bp 846bp

3.2.2.6 PCR reactions

The PCR reactions were performed using gene-specific primer pairs (Table 3.2). Dream*Taq* ™ DNA Polymerase (Fermentas Life Sciences) was used according to the manufacturer's instructions. The PCR reaction conditions were an initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, different annealing temperatures depending on the primers (Table 3.2), extension at 72°C for 1 min and a final extension at 72°C for 7 min, as recommended by the manufacturer.

Table 3.2 PCR primer sequences, annealing temperatures and expected product size for the different transcription factors.

Gene	Primer sequences (5' - 3')		Annealing temperature	Band size
AtMYB3	GCTCACATGAACAAAGGAGCTTGGAC	Forward	58°C	685bp
	TCCGACAACACAAACACACCGCATC	Reverse		
AtMYB6	ACGGTGAAGGTTGTTGGCGTTC	Forward	58°C	612bp
	ACAGTGACAAACGCGCCTCC	Reverse		
AtMYB7	CCTTGCTGCGAGAAAGAACACATGAAC	Forward	58°C	598bp
	TCTGGTTTTGCCATGGTGGACTGA	Reverse		
AtMYB8	GCTCACATGAACAAAGGAGCATGGACT	Forward	59°C	580bp
	TCGGACTGTACCCGAGTGTTAGGT	Reverse		
AtMYB13	TGGGGAGAAGACCATGCTGTGA	Forward	56°C	637bp
	TCTAGTGAGCCCTCCCAATTCTCT	Reverse		
AtMYB14	GGGAAGAGCACCATGTTGTGAG	Forward	55°C	624bp
	TCGATCAATCCTTCCCAATCCTCTATC	Reverse		
AtMYB15	GGCGAGCCCTCCCTAAGCAA	Forward	60°C	722bp
	CCCGCCGGTTCTAGCCAATACA	Reverse		
AtMYB32	GTGAGAAAGACCACACAAACAA	Forward	58°C	795bp
	CCAAAGTGCTAAAATCCAAACCC	Reverse		
GUS-POSITIVE CONTROL	GACGCGTCCGATCACCTGCG	Forward	60°C	461bp
	TCATTGTTTGCCTCCCTGCTGC	Reverse		

3.2.3 Sugarcane transformation

3.2.3.1 Transformation and selection of transformants

Sugarcane callus was transformed with *AtMYB* transcription factors contained in the pUBI plant expression vector as prepared in 3.2.2.3. The IPB sugarcane particle bombardment transformation protocol was used. Briefly, 5 µg/µl of pUBI510 with

insert and 5 µg/µl of the helper plasmid pEmuKN (a selection plasmid containing the *nptII* [*neomycinphosphotransferase*] gene coding for genetecin resistance) were precipitated onto 0.1 g of 0.7 µm diameter sterile tungsten particles (GradeM-10, Bio-Rad) using 20 µl of 0.1 M spermidine and 50 µl of 2.5 M sterile $CaCl_2$, which are added at the same time whilst vortexing. The mixture was then allowed to stand on ice for 5 min; whereafter 100 µl of the supernatant liquid was removed. The remainder was thoroughly mixed and 5 µl used to bombard sugarcane callus that had been placed in a circle with a diameter of 2.5 cm onto MSC_3 medium supplemented with 0.2 M sorbitol and 0.2 M mannitol, 4 h prior to bombardment. The calli was placed 12 cm away from the gene gun barrel and bombardment was done at a pressure of 1000 kPa of helium cylinder gas and vacuum of 80 kPa in the gene gun chamber. After 3 h the bombarded callus was placed on standard MSC_3medium without antibiotics for 3 days and then transferred to MSC_3medium containing 45 µg/l geneticin (Roche) for selection. The callus was sub-cultured onto new selection medium every 2 weeks.

3.2.3.2 Molecular analysis: Isolation of genomic DNA

Sugarcane genomic DNA was extracted using a modified CTAB protocol (White et al., 2008). Immediately prior to extraction, 3% (v/v) β-mercaptoethanol was added to autoclaved extraction buffer (2% [w/v] CTAB; 2% [w/v] polyvinylpyrrolidone [PVP] K-40; 25 mM EDTA; 100mM Tris-HCl, pH 8.0; 2 M NaCl; 0.5g/l spermidine) dissolved in double distilled water and the extraction buffer heated to 65°C on a heating block. Callus material from putative transformants (callus that survived selection after bombardment) was homogenized in liquid nitrogen using a clean mortar and pestle and 100 mg of the homogenized material used for extraction. The homogenized material was added to 1.2 ml heated extraction buffer, and immediately mixed by vortexing. The tubes were then returned to the heating block and heated at 65°C for 30 min, with shaking every 5 min. The tubes were centrifuged for 5 min at maximum speed (13000 *xg*) in a bench-top microcentrifuge. The supernatant was transferred into a new 2 ml microfugetube and an equal volume of chloroform-isoamylalcohol (24:1 v/v) added. Samples were vortexed for 30 s and centrifuged again at 13000 *xg*for 5 min. The aqueous phase was transferred into a new tube and the chloroform-isoamylalcohol extraction repeated. Genomic DNA was precipitated from the

supernatant in a fresh, sterile tube by adding 600 µl isopropanol and mixing by inverting the tube 2-3 times. The tubes were then incubated for 10 min at -20°C, after which they were centrifuged at 13000 *xg* for 5 min and the supernatant discarded, retaining the DNA pellet. The pellet was then washed in 1 ml 70% (v/v) ethanol, flicking the pellet into the solution and allowing the tube to stand for 30 min. The tube was centrifuged at 13000 *xg* for 2 min and the supernatant discarded. The tubes were opened and left to dry at room temperature, whereafter the pellet was re-suspended in 50 µl TE buffer (pH 8). The DNA concentration was determined using a Nanodrop® spectrophotometer (ND 1000 V3.6.0 by NanoDrop Technologies) and then stored at -20°C until analysis.

3.2.3.3 Verification of transformation using PCR and restriction digests on genomic DNA

The isolated genomic DNA was analysed to check for incorporation of the transgenes. This was done using the PromegaGoTaq® PCR kit using the Green GoTaq® Reaction Buffer, following the manufacturer's protocol. Isolated DNA (1 µl of 100 ng/µl) was used for PCR analysis, using the primers listed in Table 3.1 for the transcription factors and the GUS-positive control. The PCR conditions were as described in Table 3.2. Further analyses were done for *AtMYB8*, *AtMYB14* and *AtMYB15*. Gene specific primers in combination with plasmid specific primer CamVR 5'AGG GTT TCT TAT ATG CTC AAC3' were again used for PCR. For the gene specific primers in the three TFs, the PCR product was then digested with restriction enzymes (Table 3.3) to distinguish them from the sugarcane orthologues that were amplified by the same primers in the sugarcane wildtype callus. The PCR product was aligned to ESTs of sugarcane from the Gene Index-DFCI website (http://compbio.dfci.harvard.edu/tgi/) before digestion. The restriction digests were performed as in Section 3.2.4.1.

Table 3.3 Expected band sizes from restriction digests of PCR products amplified with gene specific primers from genomic DNA of putative transgenic sugarcane callusfor *AtMYB8, AtMYB14* and *AtMYB15* transcription factors.

AtMYB transcription factor	*BamHI*	*Hind*III
pUBI510-AtMYB8	-	263bp 317bp
pUBI510-AtMYB14	624bp	251bp 373bp
pCambia2200-AtMYB15	722 bp	-

-: The restriction digest was not used for the particular*AtMYB* transcription factor.

3.2.3.4 RT-PCR to verify transgene expression

Reverse transcriptase PCR (RT-PCR) was carried out in the calli that had survived on selection medium including for *AtMYB6,AtMYB7*, *AtMYB8*, *AtMYB14*, *AtMYB15* and *AtMYB32*. Firstly, RNA was isolated from 100-200 mg of sugarcane callus following the CTAB method as optimised by White et al. (2008) and described in 4.2.3.1 above. The only differences were that all RNA centrifugation steps were carried out at 4°C for 15 min and the pellet precipitated overnight at 4°C in 1/3 volume of 8 M LiCl instead. Precipitated RNA was collected by centrifugation at 13000 *xg* for 1 h. The RNA pellet was re-suspended in ddH_2O instead of TE buffer after being washed with ethanol. The RNA concentration was quantified using the Nanodrop® and RNA stored at -80°C until analysis.

The Fermentas Revertaid™ First Strand cDNA Synthesis kit was used to construct complementary DNA from the isolated RNA to check for gene expression. This was done following the manufacturers protocol. In short, 1 µg RNA, 1 µl oligo (dT)18 primer,4 µl 5X reaction buffer, 1 µl of the 20 U/µl Ribolock RNase inhibitor, 2 µl (10 mM) dNTP mix and 1 µl RevertAid M-MuLV Reverse Transcriptase (200 U/µl) and nuclease free water to a total volume of 20 µl, were added to a 1.5 ml microfuge tube. The mixture was incubated at 42°C for 1h and the reaction was terminated by heating the mixture for 5 min at 70°C. The concentration of the cDNA was quantified using the Nanodrop® spectrophotometer.

To check for expression of the *MYB* transcription factor transgenes, cDNA was analysed via RT-PCR. The Fermentas Revertaid™ First Strand cDNA Synthesis kit was used to construct first strand complementary DNA from the isolated RNA to check for gene expression. This was done following the manufacturers protocol and concentration of the cDNA was quantified using the Nanodrop. The PCR amplification utilised the same PCR settings as used for the genomic DNA to confirm transgenesis. Actin was used as the house keeping gene (forward primer 5'TCA CAC TTT CTA CAA TGA GCT3'; reverse primer 5'GAT ATC CAC ATC ACA CTT CAT3', annealing temperature 49°C).

3.2.4 Regeneration of plants

Some of the transgenic callus in the dark was placed on MS medium constituting of 4.43 g/l MS (Murashige and Skoog, 1962) salts (Highveld Biological, South Africa) supplemented with 20 g/l sucrose, 0.5 g/l casein (Sigma Life Science) and 2.2 g/l Gelrite at pH 6, to regenerate plants. The plates were grown at 25°C under a 16 h light/8 h dark photo-period, with light provided by cool, white fluorescent tubes (50 µmol photons.m^{-2}.s^{-1}, 58W Osram L fluorescent tubes). Plantlets that regenerated from calli and had rooted were then moved into Magenta culture bottles (~12 cm x 4.5 cm dimensions) to allow for further growth. After 2 months the plants were removed from the bottles and taken to the glass house. The roots of the tissue cultured plants were washed in sterile dH_2O to remove agar and each plantlet was placed into a 20 cm pot (Calibre Plastic Pty Ltd) containing autoclaved soil mixture (1:1 [v/v] soil and vermiculite (Double Grow Potting Soil, Durbanville, South Africa)). The plants were acclimatised by covering them with sealed plastic bags for a week to maintain humidity and then puncturing holes in the plastic bags while still covering the plants to gradually reduce the humidity. After a further week, the plastic bags were removed.

3.2.5 Suspension cultures

After verifying transformation and expression, callus suspension cultures were established in order to compare the effect of the MYBs on callus on solid medium and liquid medium looking at phytochemicals. For each transgenic callus line as well as the wildtype, 1 g of callus was weighed, crushed gently with a sterile spatula and

placed in 50 ml MSC_3 liquid medium in sterilised 250 ml Erlenmeyer flasks. The flasks were shaken at 180 rpm in the dark at 25-27°C to initiate callus suspension culture. The medium volume was increased to 100 ml after four days and to 150 ml after 14 days. The suspensions were then left to grow for a month with 50 ml of the suspension being removed and replaced every week to avoid accumulation of phenolics, whereafter samples were harvested by centrifugation at 4000 rpm at room temperature for 10 min and analysed for carbohydrates (Hotter, 1997).

3.2.6 Phytochemical analysis

3.2.6.1 Extraction and analysis of carbohydrates

To extract soluble sugars, 100 mg callus samples were ground to a fine powder in liquid nitrogen. Extractions were carried out overnight at 80°C in 2 ml microfuge tubes using 70% (v/v) ethanol: 30% buffer (100 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanosulonic acid]; 20 mM $MgCl_2$). The tubes were then centrifuged at 13 000 *xg* for 15 min and supernatant transferred to a clean microfugetube and retained for quantification of glucose, fructose and sucrose. Three replicates were used for quantification of carbohydrates.

The remaining pellet from the sugar extraction was then washed four times with 1 ml 80% ethanol, where after each wash it was centrifuged at 13 000 *xg* for 10 min and the supernatant discarded. The pellet was then vacuum-dried in the Genevac® EZ-2 Personal Evaporator. Samples of 0.05 g of the pellet were then transferred into fresh microfuge tubes and 400 µl 0.2 M KOH added. The samples were incubated at 95°C for 1 h to dissolve the starch, following which 70 µl of 1 M acetic acid was added to neutralise the KOH. The tube was centrifuged for 10 min at 13000 *xg* and the supernatant transferred to a new microfuge tube. The starch was then digested to glucose by adding 1 µl 10 U/ml Amyloglucosidase dissolved in 50 mM sodium acetate (pH 4.5) to 20 µl of the extract and samples incubated at 37°C for 2 h.

The glucose, fructose and sucrose (by analysing glucose concentration) contents were determined using the sugar enzyme assay method described by Bergmeyer and Bernt (1974). The assay was conducted in round-bottomed 96 well plates (Greiner) at a wavelength of 340 nm using a BMG LABTEC FLUOstar Optima spectrophotometer.

3.2.6.2 Extraction and analysis of phytochemicals using thin layer chromatography

In order to isolate metabolites for TLC, 100 mg callus material for each clone (only *AtMYB3* clones were used) was ground to a fine powder in liquid nitrogen. Methanol, acetone, dichloromethane, chloroform and hexane were used for single extraction and combinations of methanol and acetone, methanol and chloroform and methanol and dichloromethane at equal volumes were used. Extraction was carried out in a sonicator (Bransonic 220; 50/60 kHz) for 40 min, after which samples were filtered through Whatman's filter paper into a previously weighed glass beaker and extraction was repeated. The extracts were then dried, weighed and re-suspended to a concentration of 50 mg/ml and 10µl of this applied to an aluminium-silica TLC paper (Merck, Kieselgel 60 F254). The metabolites were then separated in a TLC tank using petroleum ether:formic acid:toluene:ethyl acetate:chloroform (3:0.6:4:4:2) as the mobile phase. Metabolites were visualised under UV light at a wavelength of 340nm and then sprayed with anisaldehyde in order to be stained for easy visualisation (135 ml absolute ethanol, 5 ml concentrated sulfuric acid, 1.5 ml glacial acetic acid and 3.7 ml of p-anisaldehyde) and developed in the oven at 100°C for 5 min.

3.2.6.3 Metabolite extraction and GC-MS analysis

The GC-MS analysis was done as described by Glassop et al. (2007) but only derivatising samples with Trimethylsilyl(Chlorotrimethylsilazane: Hexamethylsilazane:Pyridine at 1:3:9 v/v ratios [TMS]). The data was analysed with the AMDIS (Automated Mass Spectral Deconvolution and Identification System) programme, which was linked to the NIST library, to identify and quantify the metabolites in all the extracts. For analysis, 75 µl of a 50 mg/ml methanolic calli extract was used. Ribitol (Sigma) was added (1 µl from a 2 mg/ml stock) to each extract as an internal standard. The samples were dried to completion under vacuum, after which methoxyamine hydrochloride (40 µl from 30 mg/ml in pyridine stock) was added and samples incubated at 37°C for 2 h with shaking at 300 rpm. Samples were then derivitised using TMS. The peak area of the putatively idetified metabolites was didvided by the peak area of ribitol (internal standard) and then the resulting value was divided by the fresh mass of the extracted tissue. The calculations were done in order to normalise the metabolites to the internal standard

and resulted in response values for the metabolites per g of fresh mass relative to the internal standard (in %) (Roessner et al. 2001a). In order to confirm the metabolites identified, shikimic acid (Sigma-Aldrich), D-(+)-maltose monohydrate (Sigma-Aldrich), D-(-)-fructose (Sigma-Aldrich), caffeic acid (Sigma-Aldrich) and sucrose (Sigma-Aldrich) were used as authentic standards.

3.3 Results and Discussion

3.3.1 Restriction Analysis

The vector constructs containing the transcription factors were all cleaved at the expected sites for both the single and double restriction digests (Figure 3.1). This suggested successful cloning of the transcription factors into the plant expression vector, pUBI510-GRFCA.

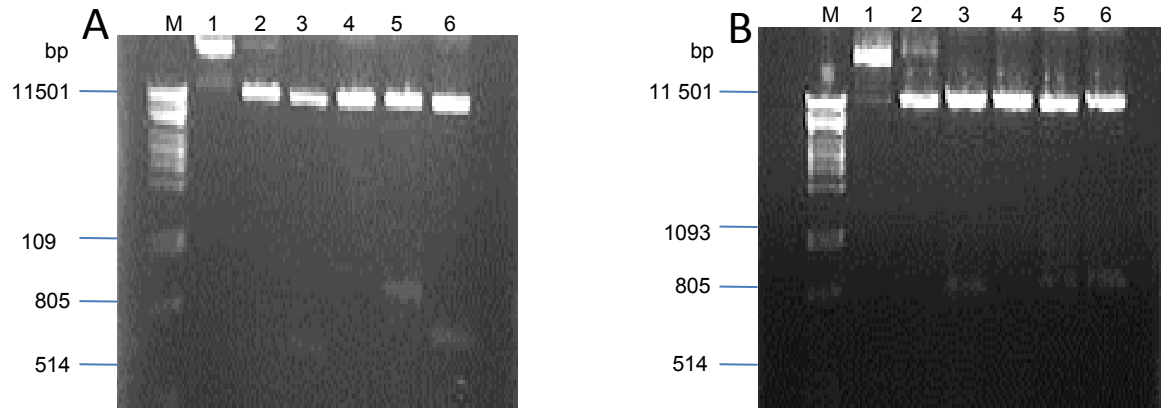

Figure 3.1 1% agarose gel image showing different bands obtained for pUBI510-AtMYB6(A)and pUBI510-AtMYB14(B). M-λ/PstI molecular weight marker, (1-intact isolated plasmid DNA, 2-*Eco*RI, 3-*Hin*dIII, 4-*Bam*HI, 5-*Eco*RI+*Sal*I, 6-*Eco*RI+*Hin*dIII and 7-*Eco*RI+*Bam*HI).

3.3.2 PCR Analysis

All eight *A. thaliana* MYB transcription factors used in the study were successfully cloned into the plant expression vector. Gene-specific primers were used to verify this using PCR and all generated fragments of the expected sizes (Figure 3.2).

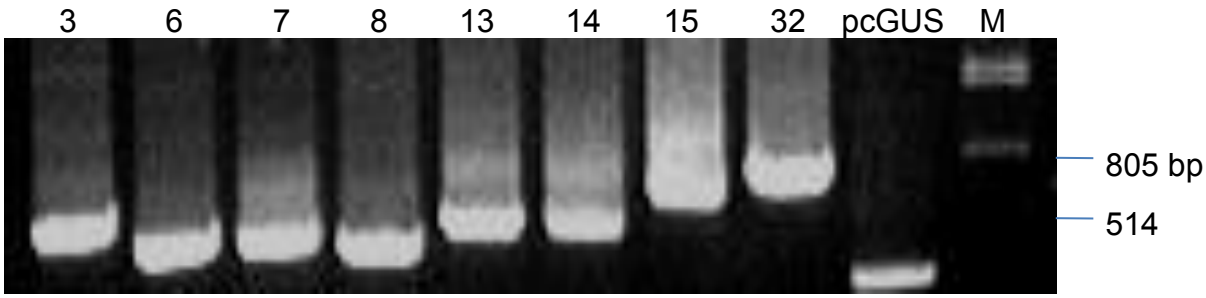

Figure 3.2 pUBI510-AtMYB constructs ready for transformation. The numbers represent the *AtMYB* transcription factors and the band sizes are as shown in Table 3.2

As confirmed by the restriction digests (Figure 3.1) and PCR (Figure 3.2), the *AtMYB* transcription factors were ligated into the plant expression vector successfully as represented in Figure 3.3.

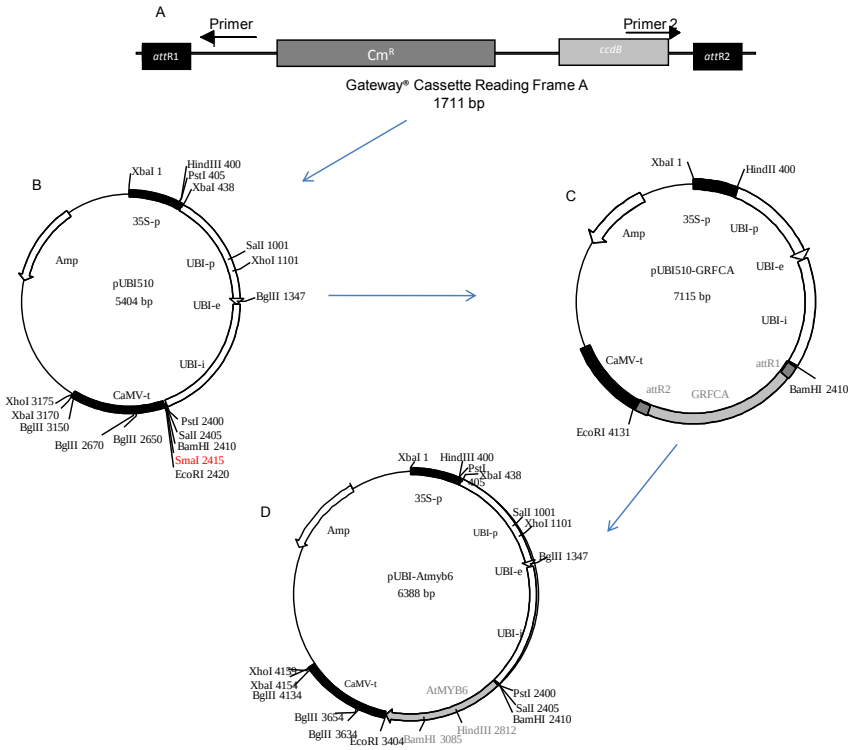

Figure 3.3 Vector constructs. The Gateway® reading frame cassette frame A - GRFCA (A) which was ligated into the pUBI510 expression vector (B), the pUBI510-GRFCA construct (C) and (D) the pUBI510-AtMYB vector used for bombardment, using *AtMYB6* as an example.

3.3.3 Sugarcane transformation and selection of transformants

Bombarded sugarcane callus (Figure 3.4A) survived on geneticin selection medium, resulting in new callus formation (Figure 3.4B). The callus was then bulked up for analysis (Figure 3.4C) to verify transformation, after which plants were regenerated (Figure 3.4D, E and F) and also used to establish suspension cultures (Figure 3.4 G). The regenerated transgenic plantlets started todie in bottles (Figure 3.4F). The surviving plantlets were transferred to the glasshouse but none survived beyond two weeks.

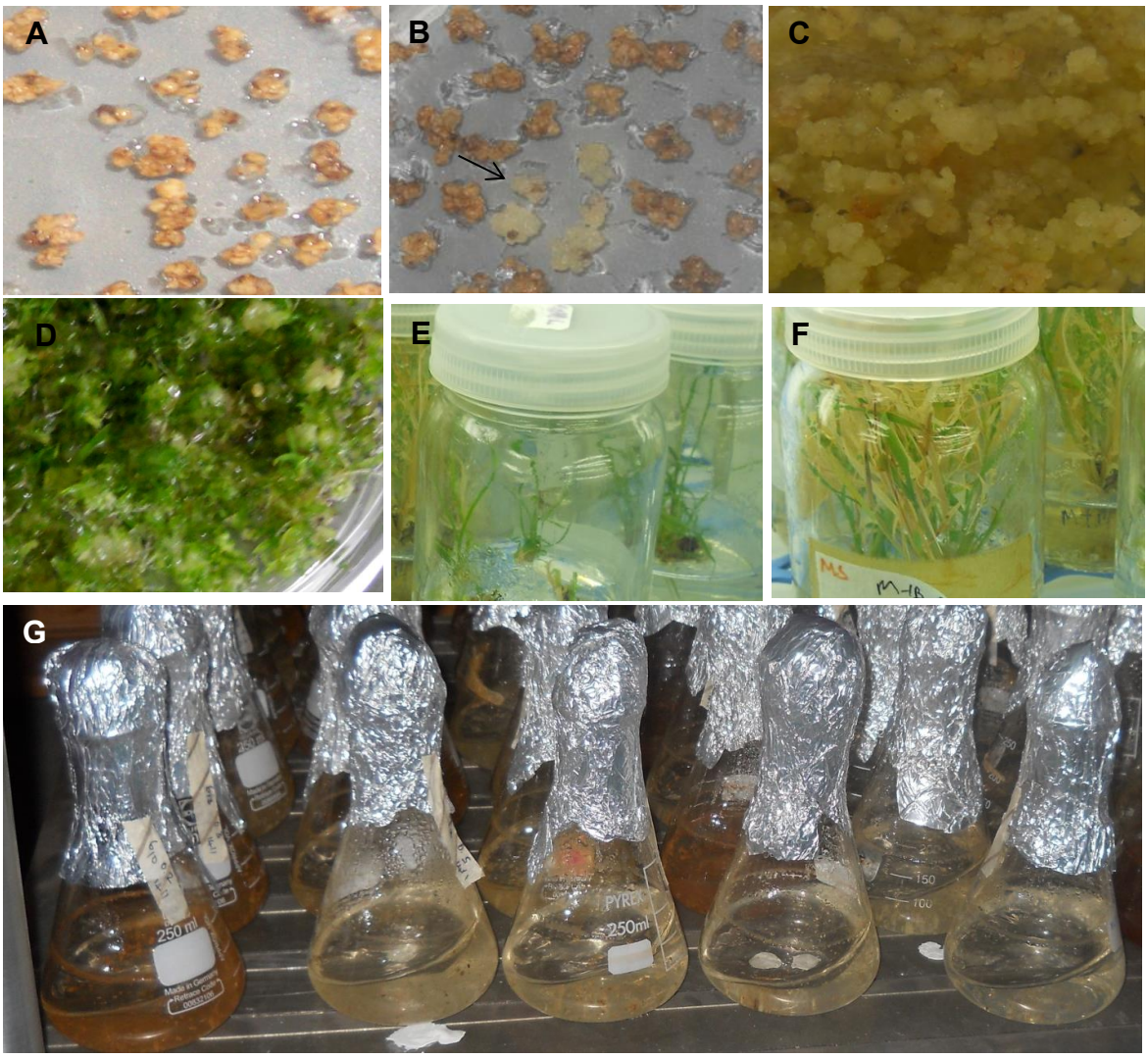

Figure 3.4 A-Bombarded callus undergoing selection, B-callus surviving on selection medium and with new callus growth. Arrow indicates a putative transformant, C-transgenic callus growing on selection medium, D-Plantlets being regenerated from transgenic callus, E-transgenic plants that have rooted, F-transgenic plants ready to be transferred to glasshouse (as evident, most of the regenerated plants died off), G-suspension cultures of transgenic calli.

The regeneration of sugarcane plantlets from transgenic callus for *AtMYB3*, *AtMYB6*, *AtMYB7* and *AtMYB32* was slow compared to the wildtype and there was no difference in plant regeneration between the wildtype callus and callus where *AtMYB13* was over-expressed (data was not recorded). The plants wilted, dried up and died for all subfamily IV *AtMYB* transcription factors. Sugarcane transgenic plantlets where *AtMYB13* was over-expressed were healthier (more green and not dying in bottles) than the other transgenic lines *in vitro*. However, the *AtMYB13* transgenic plants did not survive the hardening off process.

There is some literature, even though not specific to death of transgenic plants where the *AtMYB* transcription factors resulted in deformation of plant tissues slow and reduced germination and also plants being smaller that the wildtype.Sanz(2010) reported that when *AtMYB6* and *AtMY8* transcription factors were over-expressed in *A. thaliana,* seed germination and overall plant growth were altered in comparison to the control. The plants which over-expressed *AtMYB6* resulted in some seedlings not growing after producing only one true leaf (Sanz, 2010). The over-expressed lines also had a small rosette and shorter roots compared to the wildtype (Sanz, 2010). *A. thaliana MYB13* has been reported to result in hook structures in the *A. thaliana* inflorescence (Kirik et al., 1998). The plants where *AtMYB13* was over-expressed resulted in a reversed order of first flowers and axillary buds (Kirik et al., 1998). AtMYB transcription factors in the same subfamily have been reported to be functionally redundant (Sanz, 2010); it is therefore possible that the over-expression of *AtMYB3*, *AtMY6*, *AtMYB7* and *AtMYB32* resulted in the plants dying as they are in the same subfamily IV.

It is possible that over-expressing transcription factors from *A. thaliana* (a dicotyledonous plant) in sugarcane (a monocotyledonous plant) resulted not in just malformation but in actual plant death. Unfortunately there was insufficient time to try other methods such as changing the environment that the transgenic plants were grown in *in vitro* (media, temperature settings and light/dark photoperiod setting) and in the greenhouse. As most of the plantlets started to die *in vitro* (subfamily IV), there was not enough plantlet material to test for metabolite accumulation. Therefore callus cultures on solid and in liquid media, even though not ideal, were the only material available to test for metabolite accumulation.

3.3.4 Molecular analysis

3.3.4.1 Confirmation of transformation

The PCR results of genomic DNA of the bombarded callus after selection showed that two (*AtMYB3*and *AtMYB13*) out of the eight transcription factors were successfully incorporated into the DNA of the sugarcane callus (Figure 3.5). Although gene specific primers designed against the *Arabidopsis thaliana* MYB sequences were used for *AtMYB6, AtMYB7, AtMYB8*, *AtMYB14* and *AtMYB15* the sugarcane callus wildtype showed a band of the correct size for each of the five transcription factors, suggesting amplification from the endogenous sugarcane *MYB* genes in these cases. Consequently it was impossible to distinguish between transgenic and non-transgenic callus for these six TFs by PCR from genomic DNA only.

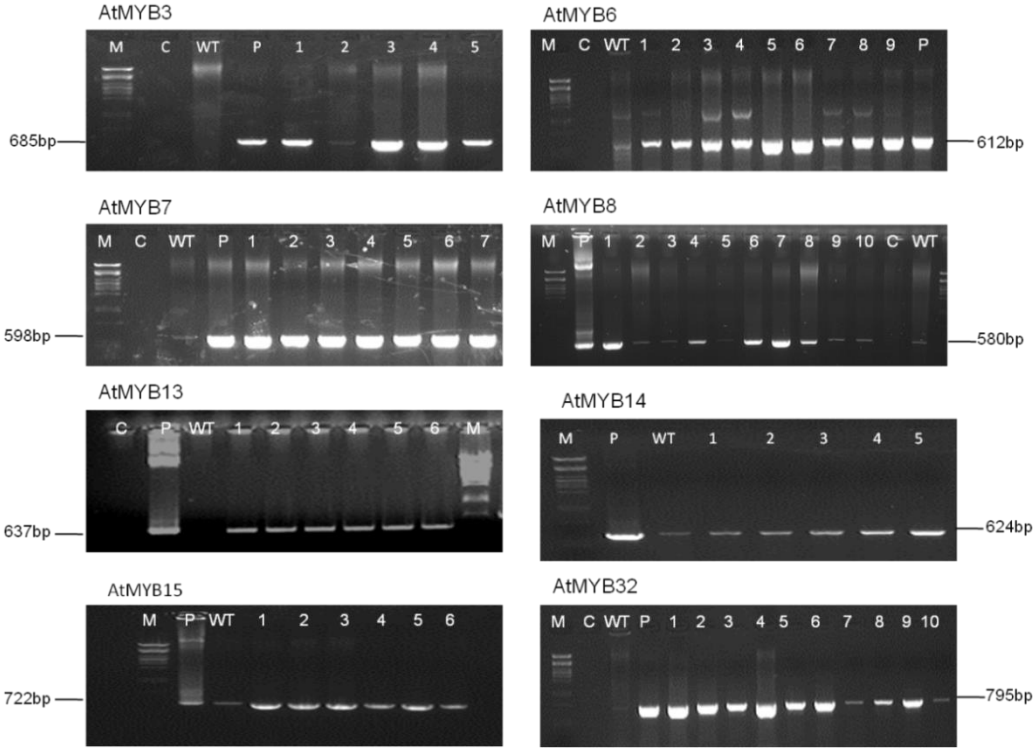

Figure 3.5 Confirming the transformation of sugarcane callus genomic DNA with the *AtMYB* transcription factors using gene specific primers. M-λ/PstI molecular weight marker; C-water as a negative control; P-Plasmid containing the corresponding *AtMYB* transcription factor gene as positive control; WT- wildtype; numbers represent the different regenerated sugarcane transgenic lines.

To further verify the presence of a gene similar to the TFs in wildtype sugarcane, wildtype sugarcane callus genomic DNA was subjected to PCR using gene specific primers for all the eight transcription factors and the positive control. The results clearly depict that the sugarcane wildtype has a gene that is somewhat similar to the sequences of *AtMYB8*, *AtMYB14* and *AtMYB15,* but the same could not be said for *AtMYB6* and *AtMYB7* (Figure 3.6), even though there was amplification in the transgenic sugarcane callus genomic DNA.

Figure 3.6 *AtMYB* specific primers on sugarcane wildtype callus genomic DNA. M - λ/PstI marker; numbers represent amplification using gene specific primer pairs for the corresponding AtMYB transcription factor. GUSpc - positive GUS control as provided in the Gateway® kit. Out of the eight TFs, only three had a gene being amplified by the respective TF specific primers.

3.3.4.2 Further analysis of *AtMYB8*, *AtMYB14* and *AtMYB15* bombarded callus

The plasmid reverse primer CAmVR 5'AGG GTT TCT TAT ATG CTC AAC3' and a gene specific forward primer were further used for PCR to distinguish between the transgenes and the endogenous genes (Figure 3.7A). Restriction digests were also carried out (Figure 3.7B, C and D).

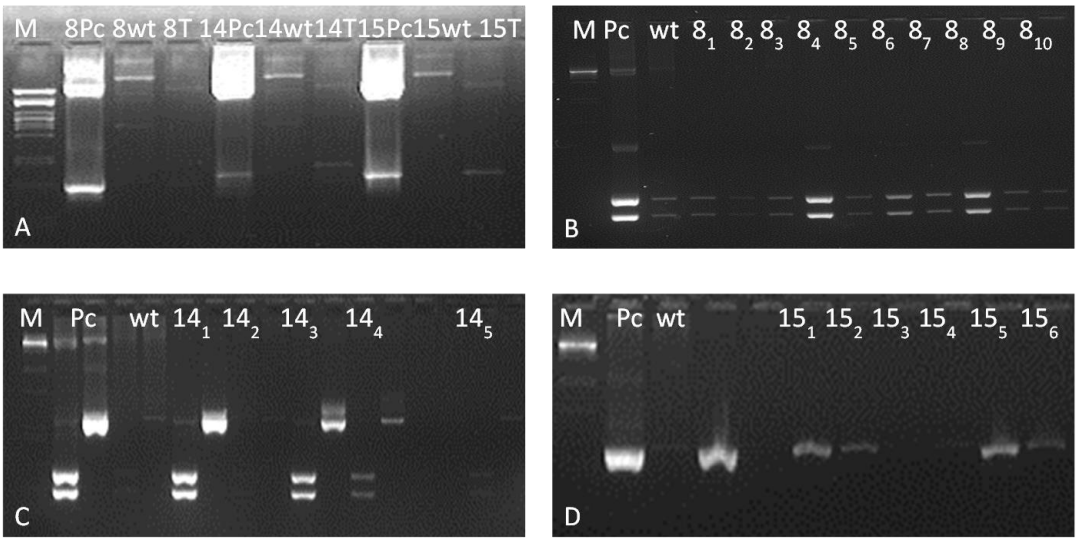

Figure 3.7 A-plasmid CAMvr and gene forward primers (8Pc-AtMYB8 plasmid as positive control, 8wt-wildtype with CAMvr and AtMYB8 primer, 8T-pUBI-AtMYB8 transgenic line and the same is true for 14 and 15). B-restriction digests on gene specific primers PCR product of the WT and pUBI-AtMYB8 (Pc-pUBI-AtMYB8 plasmid as positive control, wt-sugarcane wildtype and numbers represent the different pUBI-AtMYB8 transgenic lines). C-restriction digests on gene specific primers PCR product of the WT and pUBI-AtMYB14 (Pc-pUBI-AtMYB14 plasmid as positive control, wt-sugarcane wildtype and numbers represent the different pUBI-AtMYB14 transgenic lines). D-restriction digests on gene specific primers PCR product of the WT and pUBI-AtMYB15 (Pc-pUBI-AtMYB15 plasmid as positive control, wt-sugarcane wildtype and numbers represent the different pUBI-AtMY15 transgenic lines). M-λ*Pst*I.

The PCR and restriction digests results, however, were still not able to separate the wildtype from the callus that survived selection for the three transcription factors under study. The band sizes obtained for both the PCR and restrictive digests for the wildtype and the transgenic sugarcane callus genomic DNA were similar. Further tests using RT-PCR were conducted to determine whether gene expression could be used to tell the transgenics and non-transgenics apart, as the transgenics could be expected to be expressed at considerably higher levels than the endogenous genes.

3.3.4.3 RT-PCR: Checking for gene expression in cDNA

Gene specific primers were then used for RT-PCR to assess if the transcription factors were not just incorporated into the genomic DNA but that they were expressed. The five transcription factors *AtMYB3*, *AtMYB6*, *AtMYB7*, *AtMYB13* and *AtMYB32* are indeed expressed in the sugarcane callus. For the three transcription

factors *AtMYB8*, *AtMYB14* and *AtMYB15*, a similar gene was also expressed in the wildtype.

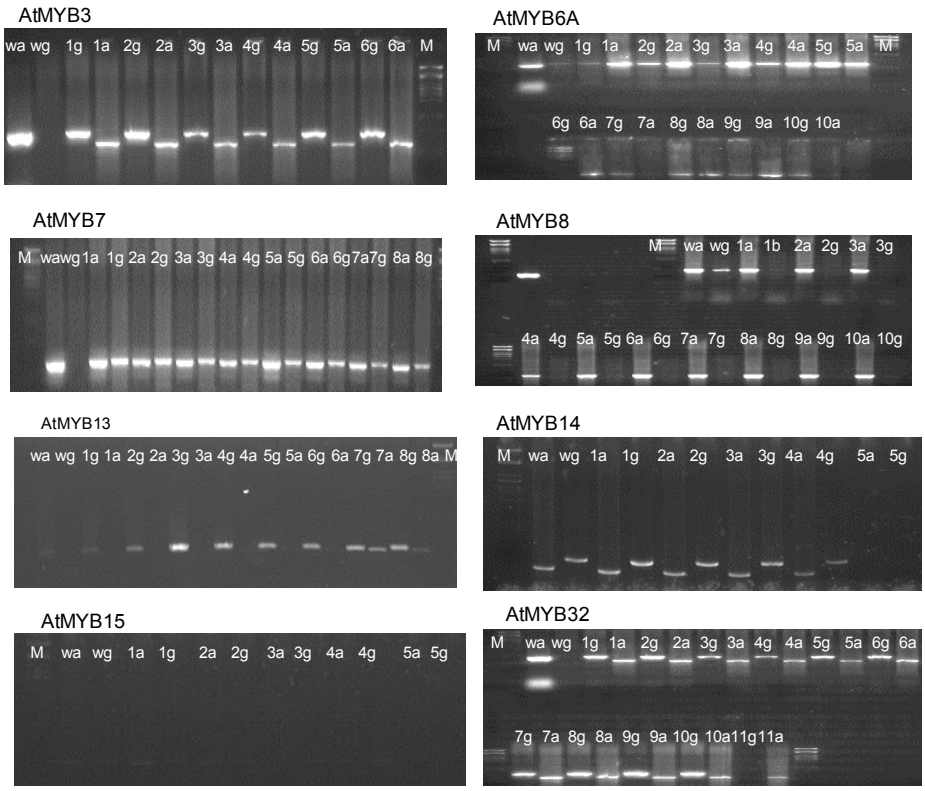

Figure 3.8 Expression of *AtMYB* transcription factor genes in cDNA of sugarcane transgenic callus with actin as the housekeeping gene. The numbers represent the clone for each *AtMYB* gene with a and g representing where actin and the transgene were loaded respectively.M - λ*Pst*I and w - wildtype.

It can thus be concluded that the wildtype sugarcane contained a gene that is similar in sequence to *AtMYB8*, *AtMYB14* and *AtMYB15* which is even expressed to the same level as the putative transgenic callus over-expressing the three *AtMYB* transcription factors. Further studies would have to be carried out to determine if the callus that was bombarded with the three transcription factors and survived selection is transgenic or not looking at levels of expression using quantitative methods such as quantitative PCR. The three *AtMYB* transcription factors were therefore left out for further analysis in this thesis as it was still not verified that they are incorporated into the sugarcane callus DNA.

3.3.5 Phytochemical analysis

3.3.5.1 Extraction and analysis of carbohydrates

Sucrose, glucose, fructose and starch were quantified in calli grown both as solid and suspension culture systems, using an enzyme assay for sugar quantification developed by Bergmeyer and Bernt (1974). It was expected that transgenic lines that contained more starch than the wildtype would contain less sucrose and that the ones that contained less starch would contain more sucrose than the wildtype. Starch only occurs in small amounts in sugarcane, but is, nevertheless, an unwanted product because it reduces the amount of sucrose that can be crystallized from molasses (Ferreira et al., 2008). High starch content in sugarcane leads to high costs in sugarcane milling factories as a bacterial α-amylase has to be added to hydrolyse starch during sucrose extraction. In order to reduce costs and also increase the quality of sucrose produced, it is ideal to produce sugarcane that contains low starch concentrations. Therefore the sugar content (Table 3.4) will be discussed in relation to starch content (Table 3.5) in sugarcane callus both on solid medium and suspension cultures.

Table 3.4 Content of the three soluble sugars in sugarcane transgenic callus. C-callus on solid medium and S-callus suspension cultures. Values within a column that share letters are not statistically different at the 95% confidence level. All values are represented as µmol/g FW.

Clone	Glucose-c	Fructose-c	Sucrose-c	Glucose-s	Fructose-s	Sucrose-s
WT	3.41a ± 0.28	1.84a ± 0.22	8.75a ± 0.64	36.85a ± 1.16	9.13a ± 0.89	20.55b ± 0.76
3_1	4.56a ± 0.70	12.81c ± 1.18	15.03b ± 1.61	28.58d ± 2.08	13.33ab ± 1.06	10.97a ± 0.58
3_3	21.17c ± 0.44	16.01d ± 0.39	47.20c ± 0.20	40.42a ± 0.82	17.52bc ± 0.65	12.22a ± 0.84
3_4	8.01b ± 0.51	5.09b ± 0.62	14.96b ± 0.79	22.71c ± 1.15	19.36c ± 3.49	69.00c ± 3.96
3_5	8.10b ± 1.14	2.85a ± 0.58	13.97b ± 0.57	13.13b ± 1.52	13.22ab ± 0.48	9.60a ± 0.65
Clone	**Glucose-c**	**Fructose-c**	**Sucrose-c**	**Glucose-s**	**Fructose-s**	**Sucrose-s**
WT	2.42ab ± 0.25	3.94a ± 0.38	16.43a ± 2.35	35.09d ± 0.51	34.39c ± 4.42	12.73c ± 0.45
6_1	5.69c ± 0.02	2.11b ± 0.36	18.93a ± 1.60	20.04ab ± 0.70	16.12ab ± 2.37	3.82a ± 0.39
6_2	2.68ab ± 0.38	4.59a ± 0.53	4.89c ± 0.54	22.02bc ± 0.80	23.52a ± 2.05	9.1b ± 0.63
6_8	2.82ab ± 0.31	5.05a ± 0.93	20.17ab ± 1.14	24.71c ± 0.25	18.36a ± 3.40	21.86d ± 1.16
6_9	9.09d ± 0.92	8.27c ± 0.54	23.91b ± 1.28	36.94d ± 2.04	35.97c ± 2.28	8.78b ± 0.66
6_11	3.57b ± 0.41	0.67b ± 0.11	18.09a ± 1.48	18.41a ± 1.26	9.51b ± 0.22	5.54a ± 0.58
6_12	1.95a ± 0.47	3.91a ± 0.23	20.35ab ± 1.36	13.7e ± 0.87	18.97a ± 0.71	42.52e ± 1.54
Clone	**Glucose-c**	**Fructose-c**	**Sucrose-c**	**Glucose-s**	**Fructose-s**	**Sucrose-s**
WT	3.76a ± 0.29	4.61ac ± 0.70	12.88d ± 1.29	36.44b ± 0.78	32.13b ± 3.23	7.54ab ± 1.31
7_1	13.43b ± 0.91	5.40abc ± 1.18	8.01a ± 1.05	38.52b ± 4.08	50.07c ± 1.89	3.66c ± 0.38
7_4	3.63a ± 0.42	6.73ab ± 0.68	4.42b ± 0.45	23.60a ± 0.96	34.52b ± 0.93	6.98ab ± 0.38
7_6	3.82a ± 0.64	7.82b ± 0.37	7.47ab ± 1.10	70.73c ± 2.63	53.93c ± 1.10	9.13b ± 0.83
7_8	4.80a ± 0.45	3.86c ± 0.64	1.31c ± 0.35	24.53a ± 0.58	23.79a ± 0.29	6.99ab ± 0.27
7_9	4.62a ± 0.44	7.09ab ± 1.03	9.01a ± 1.26	22.6a ± 0.80	20.48a ± 1.14	5.99a ± 0.46
Clone	**Glucose-c**	**Fructose-c**	**Sucrose-c**	**Glucose-s**	**Fructose-s**	**Sucrose-s**
WT	3.92a ± 0.45	3.06c ± 0.33	17.30a ± 1.55	31.09b ± 0.18	32.73a ± 0.19	3.53b ± 1.07
13_1	4.83a ± 0.79	10.55ab ± 1.21	42.59b ± 2.72	17.57a ± 1.12	29.45d ± 0.74	8.90a ± 1.81
13_2	17.29c ± 0.80	13.70b ± 1.67	9.95a ± 1.19	29.47b ± 1.72	32.69a ± 0.84	7.57ab ± 0.69
13_7	6.79b ± 0.11	7.11a ± 0.90	16.69a ± 0.95	21.78c ± 0.55	17.66c ± 1.09	8.11a ± 1.95
13_8	7.89b ± 0.24	38.40d ± 1.62	43.32b ± 8.77	16.06a ± 0.70	14.07b ± 0.51	19.05c ± 0.94
Clone	**Glucose-c**	**Fructose-c**	**Sucrose-c**	**Glucose-s**	**Fructose-s**	**Sucrose-s**
WT	1.68a ± 0.35	4.51a ± 0.91	7.8bc ± 0.29	29.91c ± 1.07	10.83a ±	21.72b ± 0.77
32_4	2.09ab ± 0.50	9.30bc ± 2.50	6.66b ± 2.04	8.19a ± 1.52	15.53a ± 2.44	43.65c ± 3.66
32_5	5.18cd ± 1.21	14.52d ± 1.81	35.68d ± 3.54	10.32a ± 1.50	9.25a ± 1.34	12.14a ± 2.08
32_6	4.40bc ± 0.86	5.92ab ± 0.88	12.31ac ± 1.03	16.47b ± 1.91	14.14a ± 1.53	26.70b ± 2.19
32_7	7.11de ± 0.88	1.21a ± 0.35	13.36a ± 1.05	11.87ab ± 1.28	11.22a ± 7.75	10.30a ± 0.75
32_10	9.75e ± 1.04	11.67cd ± 1.78	17.05a ± 0.42	25.48c ± 2.16	7.64a ± 3.18	13.62a ± 2.27

Transgenic lines of *AtMYB3* on solid medium all contained statistically more sucrose compared to the wildtype. However, in the liquid medium, *AtMYB3* lines contained less sucrose than the wildtype. Looking at starch content, *AtMYB3* transgenic lines contained statistically more starch than the wildtype on solid medium. In liquid medium, *AtMYB3* lines contained starch at wildtype levels. There was no consistency in sucrose and starch content in *AtMYB6* transgenic lines on solid and in liquid media. Transgenic lines of *AtMYB7* on solid and in liquid media all had less sucrose than the wildtype. Starch content of *AtMYB7* on solid and in liquid medium was higher than the wildtype starch content for all the transgenic lines. It can thus be concluded that over-expressing *AtMYB7* in sugarcane reduces the production of sucrose and increases the production of starch.

The levels of sucrose produced by *AtMYB13* sugarcane callus on solid medium were inconsistent. In liquid culture all the *AtMYB13* transgenic lines had more sucrose than the wildtype. The starch content on solid and in liquid media for *AtMYB13* lines was higher than that of the wildtype. In *AtMYB32,* transgenic lines on solid media contained more sucrose than the wildtype. In liquid media *AtMYB32* lines generally contained less sucrose than the wildtype. The measured starch content in *AtMYB32* transgenic lines was inconsistent in solid media, but in liquid culture, *AtMYB32* lines had more starch than the wildtype.

Even though aim of the study was to determine the effect of the AtMYB transcription factors on the overall metabolites of sugarcane, since sugarcane is commercially important as a source of sucrose, sucrose content and starch content are discussed separately from the other metabolites. It was expected that transgenic lines which produced more starch would result in less sucrose production and vice versa. Starch is an undesirable product in sugarcane as its production competes with sucrose production (Ferreira et al., 2008). In industry it is ideal and cost effective to have sugarcane plants which produce very low starch and higher sucrose concentrations as this saves costs of having to degrade starch in order to produce pure sucrose. On the other hand, sucrose content can determine starch content because if there is an abundant supply of sucrose more carbon partitioning from sucrose in the cytosol can lead to more starch being stored in the plastid. Thus the transcription factors which resulted in transgenics containing high levels of sucrose and high levels of starch as

compared to the wildtype cannot be ignored, although high starch concentrations are a disadvantage in sugarcane refinery industries as this will mean adding more enzymes such as β- amylase to breakdown the starch to reduce its content and this is expensive.

Table 3.5 Content of the starch in sugarcane transgenic callus transformed with *AtMYBs* 3,6,7,13 and 32. The numbers represent the transgenic lines for each *AtMYB*.C-callus on solid media and S-callus suspension cultures. Letters show statistical difference at 95% confidence limit within a column. Measured in µmol/gFW

clone	starch-c	starch-s
WT	0.09a ± 0.01	0.19a ± 0.018
3_1	0.27b ± 0.06	0.22a ± 0.047
3_3	0.10a ± 0.029	0.71b ± 0.058
3_4	0.03a ± 0.003	0.21a ± 0.046
3_5	0.35b ± 0.058	0.23a ± 0.03
clone	**starch-c**	**starch-s**
WT	0.27a ± 0.012	0.20ab ± 0.05
6_1	0.13b ± 0.017	0.25bc ± 0.019
6_2	0.84c ± 0.058	0.14a ± 0.006
6_8	0.27a ± 0.02	0.17a ± 0.021
6_9	0.22ab ± 0.018	0.29cd ± 0.028
6_11	0.30a ± 0.037	0.19ab ± 0.011
6_12	0.74c ± 0.091	0.36d ± 0.029
clone	**starch-c**	**starch-s**
WT	0.02a ± 0.0043	0.05a ± 0.007
7_1	0.15bc ± 0.029	0.33cd ± 0.059
7_4	0.37e ± 0.058	0.15b ± 0.016
7_6	0.04ab ± 0.009	0.12ab ± 0.013
7_8	0.21cd ± 0.028	0.37d ± 0.028
7_9	0.28de ± 0.05	0.25c ± 0.023
clone	**starch-c**	**starch-s**
WT	0.25b ± 0.021	0.20ab ±0.012
13_1	0.85a ± 0.074	0.20ab ± 0.016
13_2	0.87a ± 0.12	0.11a ± 0.014
13_7	0.46b ± 0.048	0.30b ± 0.036
13_8	0.71a ± 0.063	0.53c ± 0.09
clone	**starch-c**	**starch-s**
WT	0.11ab ± 0.018	0.19c ±0.018
32_4	0.15ab ±0.008	0.73a ± .16
32_5	0.58b ±0.092	0.60ab ± 0.076
32_6	0.02a ± 0.0031	0.44bc ± 0.034
32_7	0.55b ±0.43	0.67ab ± 0.096
32_10	0.16ab ± 0.026	0.72a ± 0.047

3.3.5.2 Metabolite extraction and GC-MS analysis

In order to determine which solvent extracted the greatest number of metabolites from the sugarcane callus, methanol, acetone, chloroform, dichloromethane and hexane were used singularly and in combination (methanol+ acetone and methanol

+ dichloromethane) (Figure 3.9). Sugarcane transgenic callus over-expressing *AtMYB3* was used for TLC analysis.

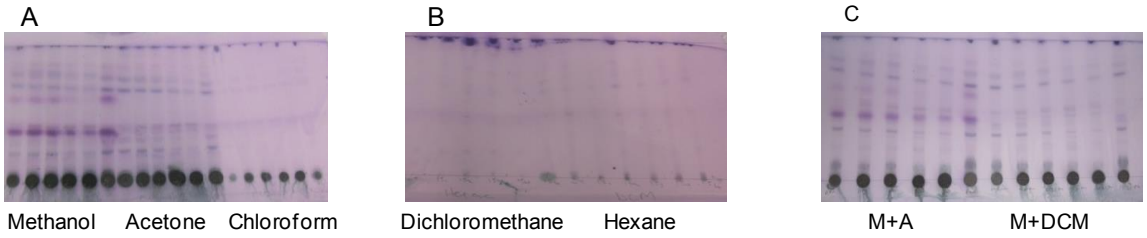

Figure 3.9 Extracts of sugarcane callus transformed with *AtMYB3* sampling for efficient metabolite extraction solvent A-methanol, acetone and chloroform extracts, B-dichloromethane and hexane extracts and C-solvents in combination (M+A - methanol + acetone; M+DCM - methanol + dichloromethane extracts)

Methanol and acetone extracted the most metabolites at 9 bands each. Both appeared to extract an identical set of metabolites, and similar results were observed when these solvents were used in combination. Methanol extracts were therefore derivatised and sent for GC-MS analysis.

The identified metabolites from the GC-MS datafrom callus placed on solid media and also in liquid suspension culture were mainly sugars (Appendix 1 and 2). Some amino acids were also identified, but these were not detected in most of the clones in the GC-MS data. The GC-MS data was inconsistent as some metabolites could only be identified in one of the transgenic lines when the same *AtMYB* was over-expressed and some metabolites could only be identified in either solid or liquid callus media. In general, the GC-MS data was inconclusive and could not be used to further determine how over-expressing *AtMYB* transcription factors affect metabolite content in sugarcane calli.

3.4 Conclusion

Five *AtMYB* transcription factors (*AtMYB3*, *AtMYB6*, *AtMYB7*, *AtMYB13* and *AtMYB32*) were successfully shown to be integrated into the genomic DNA of the sugarcane callus and expressed. The other three *AtMYB* genes (*AtMYB8*, *AtMYB14* and *AtMYB15*) could not be verified, even though the callus survived in the selection media. Sugarcane transgenic plantlets were successfully regenerated from the transgenic callus in tissue culture but did not survive the hardening-off process in the

glasshouse. As a result, no tests were conducted on sugarcane transgenic plant lines; the testing for metabolite accumulation could only be carried out on callus lines grown on solid and liquid cultures.

The AtMYB that gave consistent results for both solid and liquid cultured calli was *AtMYB7* where the transgenics produced less sucrose and more starch as compared to the wildtype. The other transcription factors, *AtMYB3*, *AtMYB13* and *AtMYB32,* resulted in either the solid or liquid media producing more sucrose and the other producing less in comparison to the wildtype. This inconsistency across the culturing media means that it is difficult to make an overall statement in regard to sucrose production in these transgenics. For starch content it appears that most of the transcription factors produced more starch that the wildtype on solid and in liquid culture. Transgenic lines of *AtMYB6,* on the other hand, resulted in varied sucrose and starch contents both in solid and liquid cultures and therefore no general conclusion can be reached as to the effect of the transgene in the production of starch and sucrose.

The interest in sugarcane is to produce sugarcane plants which produces more sucrose than the wildtype but preferably less starch, or at least same levels of starch,as the wildtype. *AtMYB13* could potentially be studied further, as although the data for sucrose production was inconsistent, two of the 4 transgenic lines tested produced more sucrose on solid culture and all the transgenic lines produced more sucrose in liquid culture. For other studies where an investigator wishes to increase the production of starch, *AtMYB7* could be tested. For secondary metabolite data, the GC-MS data was inconclusive for both sugarcane calli on solid and liquid medium.

Chapter 4: *Salvia repens* (Burch. Ex Benth): Micropropagation, analysis of phytochemicals and antimicrobial activity

4.1 Introduction

Salvia repens (Burch. Ex Benth) (Lamiaceae), commonly known as 'Kruipsalie' in Afrikaans or 'Mosisili-wa-loti' in South Sotho (Viljoen et al., 2006) is one of the 30 or so *Salvia*species indigenous to Southern Africa. This species belongs to the same species complex as *Salvia stenophylla*. It is indigenous to the Limpopo, Gauteng, KwaZulu-Natal, Free State, Eastern Cape and Western Cape provinces of South Africa and Lesotho. Like all *Salvias*, it is an aromatic, herbaceous perennial shrub. The name *repens* means creeping, as the plant arises from a creeping rhizome (Viljoen et al., 2006). The flowers vary from white/pale to blue-purple and are maroon at maturity (Viljoen et al., 2006).

Salvia species are known throughout the world for their medicinal properties. Traditionally, these plants are used to treat respiratory problems such as influenza, chronic bronchitis and tuberculosis, as well as other ailments such as stomach ache, diarrhoea, rash, wounds, fever, headache and gynaecological complaints (Watt and Breye-Brandwijk, 1962; Auge et al., 2003).

Research on these plants has to date mainly been concerned with characterising the composition of their essential oils, which are the main biologically-active compounds in *Salvia*, and the biological activities of the plant extracts (Jequier et al., 1980; Kamatou et al., 2005; 2006; 2008; Viljoen et al., 2006). Such research also provided scientific support for the significance of these plants in traditional medicine, concluding that these plants have anti-inflammatory, anti-cancer, anti-oxidant, and anti-mycobacterial activities (Kamatou et al., 2008*). Salvia repens* contains β-phellandrene (22.2%), β-caryophellene (12.4%), limonene (9.8%) and camphor (6.9%) in high concentrations (Kamatou et al., 2005). Concentrations of δ-3-carene and α-bisabolol were determined at 24.9% and 33.0% respectively by Gono-Bwalya (2003) in *S. repens*. The abundance of, and composition of these compounds have, however, been shown to vary from locality to locality and seasonally (Viljoen et al., 2006). Differences in essential oils of *S. repens*, *S. stenophylla* and *S. runcinata,*

depending on where the plant samples were collected, have been reported by Viljoen et al. (2006).

Non-volatile compounds in *Salvia*, especially phenolics, also play an important role in the medicinal properties of the plants. Most of these have been identified as caffeic acid derivatives (Kamatou et al., 2008) and include, in order of abundance; rosmarinic acid, which is an anti-oxidant (Lu and Foo, 2002); carnosol which is an anti-oxidant and anti-carcinogenic, inhibiting formation of tumours; and carnosic acid, which also displays anti-oxidant activities (Kamatou et al., 2008).

Salvia repens is harvested from the wild for both the formal commercial sector and for informal trade by the local people. This is problematic, since whole plants are harvested for use (Kamatou et al., 2008) and this may lead to wild populations diminishing. This problem is worsened as most of the natural habitat is being taken over by urbanisation and agriculture. Unpredictable climatic patterns also pose a threat to the future populations of these plants. It is therefore important to find alternative ways to access the secondary metabolites which accumulate in these plants, to be able to supply the market demand without posing a threat to the continued existence of the species.

A possible solution is tissue culture, which is less time consuming than conventional farming methods and allows for a huge number of plants to be established from very little plant material. With tissue culture a large number of plants can be produced in small space with plants maintaining their genotype as they are propagated in a controlled uniform manner (Matu et al., 2006). This supplies quality material to the formal commercial medicinal plants sector whilst at the same time removing pressure from wild populations, thus preserving biodiversity.

In vitro micropropagation and *ex vitro* acclimatisation methods have been established for four species indigenous to Southern Africa: *S. chamelaeagnea* (Huang and van Staden, 2002), *S. africana-lutea* (Makunga and van Staden, 2008), *S. stenophylla* (Musarurwa et al., 2010) and *S. runcinata* (Figlan, 2012). Huang and van Staden (2002) propagated *S. chamelaeagnea* using callus from leaves and stem explants for shoot multiplication. The remainder were micropropagated by optimising seed germination conditions, followed by culture induction, shoot initiation in different

hormonal combinations and finally acclimatisation, for all the three plant species. These only represent 4 out of about 30 species of *Salvia* indigenous to Southern Africa (Jäger and Van Staden, 2000; Kamatou et al., 2005), indicating that there is much work still to be done on *Salvia* plants, especially considering that they have been used for centuries by the locals as medicine and have to be preserved for future generations.

The aims of this study was therefore to establish an alternative viable germination strategy and to produce large numbers of shoots and phenotypically and genetically stable plants which retain their medicinal properties in order to remove pressure from the wildplants.

4.2 Materials and Methods

4.2.1 Media and growth conditions

For all the experiments, MS media was prepared at either full strength (MS), half strength (1/2 MS) or one-tenth strength (1/10 MS); where full strength MS medium was made up as follows: 4.43 g/l MS salts (Highveld Biological, South Africa) supplemented with 30 g/l sucrose, 0.1 g/l myo-inositol (Fluka Analytical) and 10 g/l agar-agar powder (Biolab, South Africa) at pH 5.7-5.8 and autoclaved at 121°C for 15 min at 103 kPa. All cultures were maintained at 25°C in a 16 h light/8 h dark photoperiod, with light provided by cool, white fluorescent tubes (50 µmolphotons. $m^{-2}.s^{-1}$, 58W Osram L fluorescent tubes). All tissue culture manipulations were conducted in a laminar flow cabinet using sterile equipment.

4.2.2 Germination of *Salvia repens* seeds

Salvia repens seeds were purchased from Plant World Seeds in the United Kingdom in February of 2011 (http://www.plant-world-seeds.com). Upon arrival, the seeds were immediately set out for germination under different treatments.

Seeds were firstly scarified in 50% (v/v) sulfuric acid for 2 min, 6 min or 12 min and washed three times with sterile distilled water, whilst un-scarified seeds were used as the control. All the seeds were then treated for any fungal spores by soaking them for 10 min in a mixture of 0.2% (w/v) Dithane® WG-45 solution (Mancozeb 750 g/kg active ingredient; Efekto, South Africa) and 0.2% (w/v) Folicur® (Tebuconazole 250

g/l active ingredient; Bayer AG, Germany), after which they were washed three times with sterile distilled water. They were further sterilised in 1.5% (w/v) sodium hypochlorite (bleach) for 10 min, washed three times with sterile distilled water and blotted dry on sterile tissue paper.

After treatment, 100 seeds for each treatment were placed in 100 cm x 2 cm petri dishes from BD Falcon™ (10 seeds per petri dish) containing 25 ml of media. Two different media were used; 1/10 MS (Murashige and Skoog, 1962) media and 1/10 MS media containing smoke water (purchased from Kirstenbosch Botanical Gardens; Western Cape, South Africa). The smoke water solution was prepared according to the Baxter et al. (1994) method where the smoke solution is filter sterilised (0.2 µm) and sold as a 10^{-5} (v/v) concentrated solution. Out of the hundred seeds used per treatment, 50 seeds were placed in the dark and the remaining 50 seeds in a 16 h light/8 h dark photoperiod at 25°C, with light provided by cool, white fluorescent tubes (50 µmol photons.m^{-2}.s^{-1}, 58W Osram L fluorescent tubes). Germination was recorded every two days for 30 d. Seed germination in the dark condition was discontinued as the germination rate from the first data collected was very low. The experiment was repeated twice for seeds germinated under light conditions. Emergence of a 0.5–1 cm radicle was regarded as a sign of seed germination. Once seeds had germinated and the seedlings had developed their first pair of true leaves and roots, they were transferred to 1/2 strength MS media in culture jars (~12 cm x 4.5 cm) and left to grow to provide material for subsequent experiments.

4.2.3 Shoot multiplication and culture induction

Two types of explants, leaf squares (1 cm x 1 cm) and 1 cm long nodal explants with two axillary buds were used to induce shoot growth. The explants were obtained from sterile 2 months old plants grown in culture jars from the seed germination experiment. The explants were placed on media in petri dishes containing auxins and cytokinins, either singly or in combination (Table 4.1). Twenty-five explants were used for each treatment, with 5 explants per petri dish. Experiments were carried out under the light conditions described above (Section 4.2.2) and results (callus production, number of shoots produced and number of roots produced per explant) were recorded after 32 d. The experiment was repeated twice.

Table 4.1 The different hormone treatments used for shoot multiplication of *S. repens* explants.

Treatment	Concentration (µM)
MS	Control
BA	4.4 8.9
IAA	2.9 11.4
BA:IAA	4.4:2.9 8.9:11.4
NAA	2.7 10.7
BA:NAA	4.4:2.7 8.9:10.7
BA:2,4D	8.9:6.8

The hormonal concentrations were guided by Musarurwa et al. (2010). After collection of the data, the plantlets that had developed from the explants were pooled together, irrespective of hormonal treatment, and transferred to small culture jars containing 25 ml 1/2 strength MS medium. These were placed under the standard light conditions and were sub-cultured into new media every 4 weeks for continuous culture.

4.2.4 Acclimatisation of tissue culture plants in the glasshouse

After plantlets that were grown in culture jars from the shoot induction experiment had developed roots, 20 rooted plantlets were moved into the glasshouse (Department of Botany and Zoology, Stellenbosch University, minimum to maximum temperatures of 18 to 30°C). Soil mixture of 1:1 (v/v) sand and vermiculite (Double Grow Potting Soil, Durbanville, South Africa) was sterilised by autoclaving. The roots of the tissue cultured plants were then washed in sterile distilled water to remove agar and each plantlet was placed into a pot (20 cm pots, Calibre Plastic) containing the autoclaved soil mixture. The plants were acclimatised by covering them with plastic bags for a week to maintain humidity and then puncturing holes in the plastic bags, while still covering the plants, to gradually reduce the humidity. After a further week, the plastic bags were removed. The plants were left for a month with hand

watering every week and number of plants that survived and acclimatised was recorded.

4.2.5 Data Analysis

Seed germination, shoot and callus induction data were analysed using the Statistica version 10 software (StatSoft, Inc, Tulsa, USA). The data collected was converted to percentages then arcsine transformed before being analysed for variance using ANOVA. For the germination data, repeated measures ANOVA was used followed by one-way ANOVA to check for statistical differences between treatments. Data recorded on day 32 of the shoot multiplication and culture induction experiment was subjected to one-way ANOVA to check for statistical differences between hormonal treatments. For callus production, a repeated measure ANOVA was used to compare callus formation in the two types of explants (1 cm x 1 cm cut leaves and 1cm long nodal explants with two auxillary buds) used. For all data analysis, the Fisher Least Significant Difference (Fisher LSD) test was carried out as a *post hoc* test to determine statistically different means. A 95% confidence limit was used for all statistical tests.

4.2.6 Phytochemical analysis

4.2.6.1 Plant metabolite extraction

Salvia repens plants grown *in vitro* and *ex vitro* were used for metabolite analysis either for volatiles (foliage tissue) or non-volatiles (whole plants). For non-volatiles, whole plants were homogenised in liquid nitrogen using a pre-chilled mortar and pestle prior to metabolite extraction. Metabolites were then extracted from 1 g homogenised plant material (placed in 15 mm x 150 mm glass tubes covered with aluminium foil to reduce evaporation) twice,for periods of 40 min in the sonicator (Bransonic 220; 50/60 kHz). The solvents used for extraction were 4 ml methanol, acetone, dichloromethane and chloroform (Merck, South Africa). The extracts were filtered through Whatman® no.1 filter paper into a previously weighed beaker and then the solvents were dried to completion at room temperature in the fume hood. The beakers containing dried extracts were then weighed to determine the amount of extract. Finally, the dried extracts were re-suspended in their respective solvents to a total concentration of 50 mg/ml and used for analyses.

4.2.6.2 TLC analysis

The plant extracts were diluted to a concentration of 50 mg/ml in respective solvents and 20 µl of each was applied on aluminium:silica (Merck, Kieselgel 60 F254) TLC paper. The TLC paper with plant extracts was developed in a glass tank using petroleum ether:formic acid:toluene:ethyl acetate:chloroform (3:0.6:4:4:2 v/v) as the mobile phase. The TLC plates were sprayed with anisaldehyde (prepared with 135 ml absolute ethanol, 5 ml concentrated sulfuric acid, 1.5 ml glacial acetic acid and 3.7 ml of p-anisaldehyde) and developed in an oven at 100°C for 5 min for visualisation.

4.2.6.3 GC-MS analysis

4.2.6.3.1 Volatiles

For volatiles, 400 mg leaves from tissue culture and glasshouse plants of *S. repens* was used for GC-MS. The protocol was as described by Musarurwa et al. (2010), using Headspace-SPME GC-MS. Volatile compounds produced in tissue culture plantlets and glasshouse plants were compared using HS-SPME of the leaflets with Supelco SPME fibres (DVB/ Carboxen/PDMS, StableFlex [Supelco]). Gas chromatography was performed using a Waters GCT Premier AS 2000 instrument coupled to a mass spectrometer, equipped with an HP5 column (30 m, 0.25 mmID, 0.25 µm film thickness). Temperatures were set at 250°C for both the injection (split injection ratio of 1:5) and the ion source and volatiles were extracted at 80°C for 15 min. Helium was used as the carrier gas (1 ml/min). The data was analysed using MassLynx™ software and the NIST (National Institute of Standards and Technology) library was used to identify the compounds. The identified metabolites were confirmed using camphene, (1R)-(+)-camphor, b-caryophyllene, (1R)-(+)-α-pinene, (-)-α-bisabolol (Sigma–Aldrich; Steinheim Germany) and (+)-3-carene; R-(+)-limonene (Fluka, Sigma–Aldrich) as authentic standards. The experiment was done three times.

4.2.6.3.2 Non-volatiles

Methanol, acetone, dichloromethane and chloroform plant extracts from both tissue culture and glasshouse plants were used. For each sample, 75 µl of a 50 mg/ml concentration was used. The Glassop et al. (2007) GC-MS protocol was used as in 3.2.6.3.

4.2.7 Biological activity assays

The plant extracts were used for anti-bacterial and anti-fungal assays comparing activities of tissue cultured and glasshouse plants. Only the methanol and acetone extracts were used for this experiment. The Eloff (1998) serial dilution micro-titre plate anti-bacterial assay was adapted for both anti-bacterial and anti-fungal assays in order to determine the minimum inhibitory concentration (MIC) values for the plant extracts against the microorganisms.

4.2.7.1 Bacterial growth and anti-bacterial assay

Gram negative strains *Escherichia coli* (ATCC 11775) and *Klebsiella pneumonia* (ATCC 13883) and Gram positive strains *Staphyllococcus aureus* (ATCC 12600) and *Bacillus subtilis* (ATCC 6051) bacteria were tested using the plant extracts. The bacteria were kindly donated by Professor Johannes van Staden from the University of KwaZulu-Natal and kept in glycerol stocks at -80°C. Bacteria were firstly inoculated onto Müeller-Hinton (MH) (Flika Biochemika, Spain) agar in sterile petri dishes (Falcon, 90 mm x 15 mm) and grown overnight at 37°C. Single colonies were picked from streaked plates and grown overnight in an orbital shaker at 200 rpm at 37°C in MH broth. A spectrophotometer was used to adjust the bacterial suspension to OD_{625}= 0.4 - 0.6 using sterile MH broth.

Plant extracts were diluted to a concentration of 50 mg/ml and used to test against bacterial growth. Streptomycin (10 mg/ml) was used as a positive control and the negative controls were sterile distilled water, bacteria-free MH broth and acetone or methanol(which were also used as re-suspension solvents). All the media and equipment used was either autoclaved or filter sterilised. Firstly, 100 µl of sterile water was added to all the 96 wells in the micro-titre plate. Then 100 µl of plant

extract was added to create a two-fold serial dilution down the wells of the plate as illustrated in Figure 4.1 below.

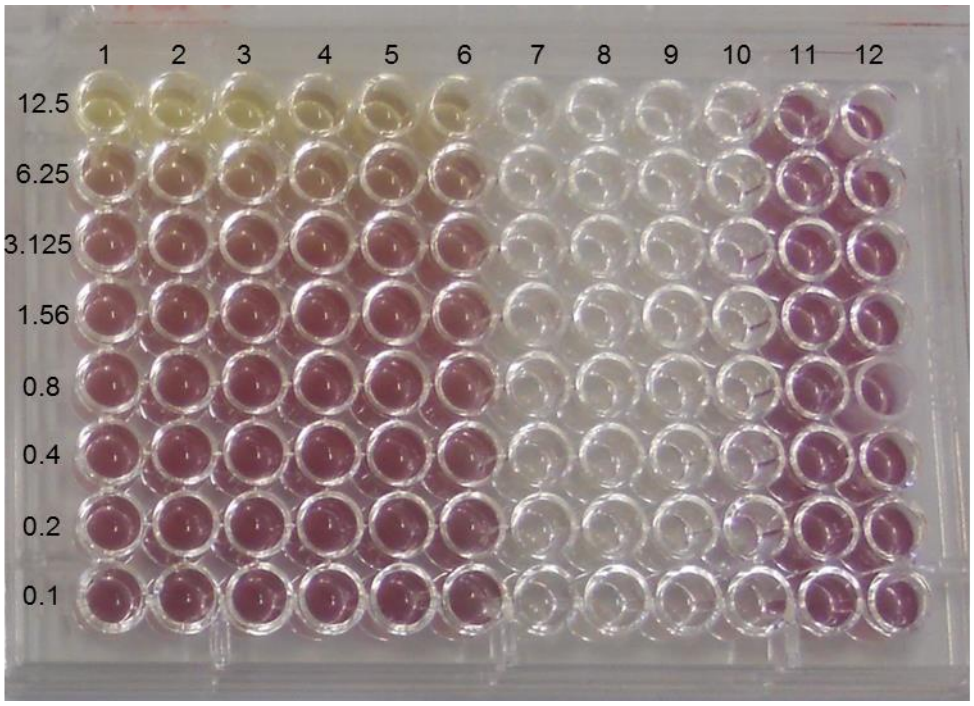

Figure 4.1 Anti-bacterial assay following Eloff (1998) microtitre plate assay. The numbers on the side represent the total concentration of plant extracts down the plate wells after serial dilutions.

The arrangement of the plant extracts and test bacteria was as follows in the 96 well plate;

> Wells 1-3: *Escherichia coli* + plant extract, 4-6:*Bacillus subtilis* + plant extract, 7-9: streptomycin + *E. coli*, 10: sterile MH broth, 11: MH broth + *E. coli* in four wells and *B. subtilis* in the remaining 4 wells and 12: Methanol/Acetone (serially diluted) + *E. coli*in four wells and *B. subtilis* in the remaining 4 wells. For streptomycin the concentration of serial dilutions down the wells were 2.5, 1.25, 0.625, 0.32, 0.16, 0.08, 0.04 and 0.02 all in mg/ml. The same was done on a different plate but now using *Klebsiella pnuemoniae* and *Staphylococcus aureus*.

4.2.7.2 Fungal growth and anti-fungal assay

Fusarium species*F. verticillioides* (MRC 826), *F. subglutinans* (MRC 0115) and *F.proliferatum* (MRC6908) were used as test species for anti-fungal analysis. The three fungi strains were kindly donated by Mrs Lindy Rose (Department of Plant Pathology, Stellenbosch University). Fungal hyphae were scraped from donated plates using a sterile scalpel into 100 ml autoclaved Armstrong medium (20 g sucrose, 0.4 g $MgSO_4.7H_2O$, 1.6 g KCl, 1.1 g (KH_2PO_4), 5.9 g $Ca(NO)_2$, 20 µl $FeCl_3$ [10mg/ml], 20 µl $MnSO_4$ [10mg/ml], 20 µl $ZnSO_4$ [10mg/ml]) made up to 1 l with sterile deionised water) in a 250 ml Erlenmeyer flask. The inoculum was then incubated in a shaker at 25°C at 100 rpm for 4-5 d. The fungal spore suspension was then filtered into a 50 ml sterile falcon centrifuge tube through two layers of sterile cheesecloth and centrifuged at 3500 *g* for 10 min and the supernatant was removed. The conidia were re-suspended and washed twice with de-ionised water with centrifugation between washes. The spores were then re-suspended in 250-500 ml sterile distilled water and spore concentration was determined using a haemocytometer and diluted to a concentration of $2x10^6$ conidia/ml. Tween® 20 surfactant (polyoxyethylene 20-sorbitan monolaurate; Merck, Germany) was added to the conidial suspension before inoculation at a rate of three drops per litre.

To test the plant extracts against fungi, plant extracts were diluted to a final concentration of 10 mg/ml in their respective solvents used for extraction (methanol/acetone) and the same method was used as for anti-bacterial assay but usingamphoterocin B (1 mg/ml), which is used against human pathogens, as a positive control. The negative controls used were sterile distilled water, sterile Armstrong medium and respective solvents used for extraction. The 96 well plate serial dilutions and assay was carried out as illustrated in Figure 4.2 below.

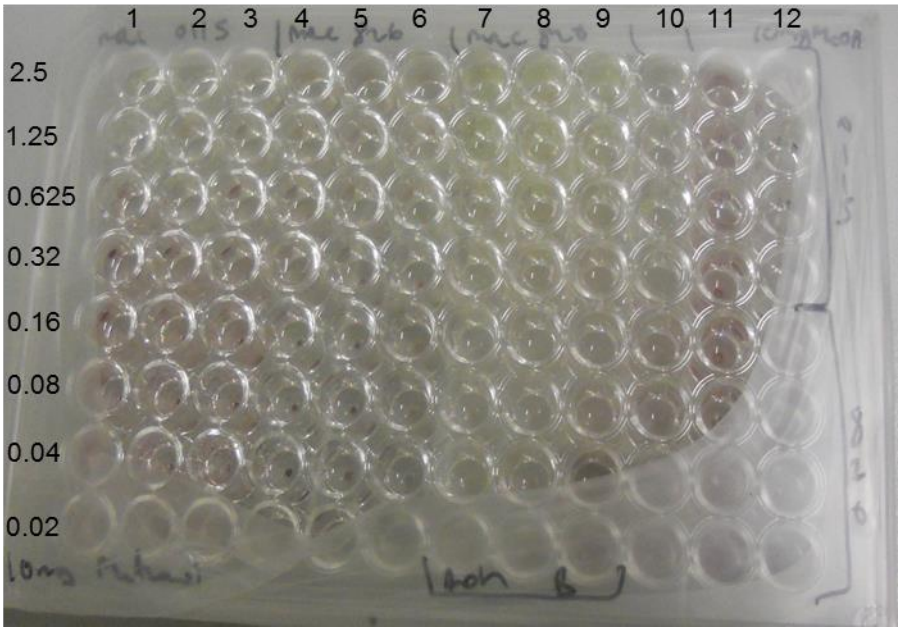

Figure 4.2 Anti-fungal assay following Eloff (1998) microtitre plate assay. The numbers on the side represent the total concentration of plant extracts down the plate wells after serial dilutions.

The arrangement of the plant extracts and test fungi was as follows in the 96 well plate;

> Wells 1-3: *F. subglutinans* (MRC 0115)+ plant extract, 4-6: *F. verticillioides* (MRC 826)+ plant extract, 7-9: Amphoterocin B + *F. verticillioides* (MRC 826), 10: sterile Armstrong medium, 11: Armstrong Medium + *F. subglutinans* (MRC 0115)in four wells and *F. verticillioides* (MRC 826)in the remaining 4 wells and 12: Methanol/Acetone (serially diluted)+ *F. subglutinans* (MRC 0115)in four wells and *F. verticillioides* (MRC 826)in the remaining 4 wells. The concentrations of Amphoterocin B down the wells were 0.25, 0.125, 0.0625, 0.031, 0.002, 0.008 and 0.004 in mg/ml.

For both biological assays, the plates were covered with Parafilm® M (American National CanTM, USA) and incubated at 37°C for 18 h. After incubation,40 µl of 10 mg/ml p-iodonitrotetrazolium chloride (INT) (Sigma, USA) was added and the plates incubated for 1 h at 37°C to detect the growth of bacteria or fungibefore data collection. MIC values were recorded in the wells where no growth was observed (indicated by non-appearance of the pink colour) as defined by Eloff (1998). The anti-bacterial assay MIC values were recorded once off after incubation and for the anti-fungal assay the MIC values were recorded over a period of 5 d. The test was

done three times for both biological assays and data represented as averages of the three tests.

4.3 Results and Discussion

4.3.1 Germination of *Salvia repens* seeds

Many medicinal plants seeds readily germinate in their native environment but not under cultivation or laboratory conditions (Nadjafi et al., 2006). Seeds may not easily germinate due to the seed coat being too hard for water and gas permeability, the embryo being immature, the presence of germination inhibitors, mechanical causes or environmental conditions such as light and temperature (Stidham et al., 1980). Chemical scarification breaks physical seed dormancy by corroding the seed coat, making it easy for the embryo to interact with the germination medium, allowing water and gas permeability and also removing physical constraints for the embryo (Stidham et al., 1980). Even though it is well documented that smoke enhances germination, the molecular signalling mechanisms responsible for smoke derived germination are not yet clearly understood (Soós et al., 2010).

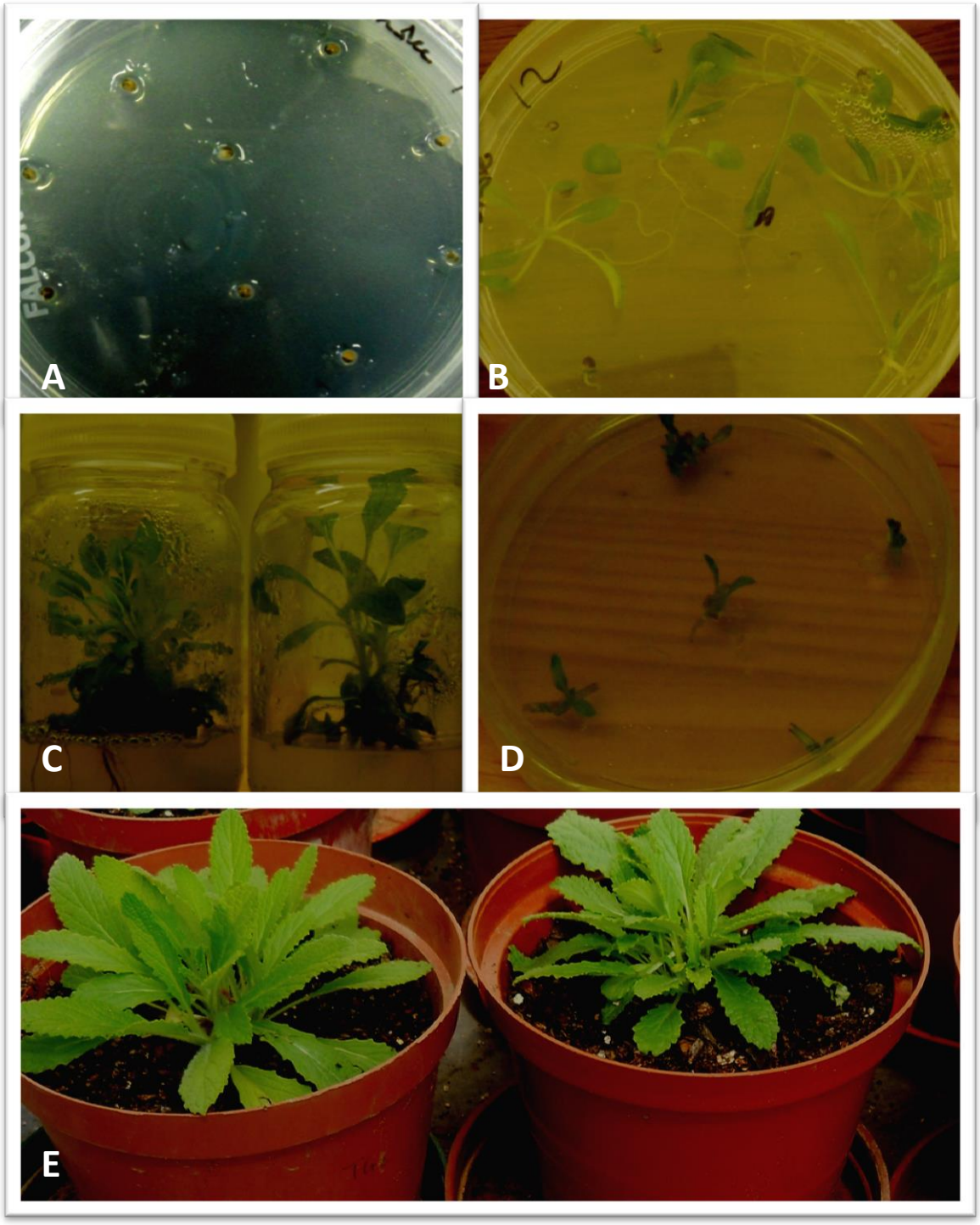

Figure 4.3 A: Seeds set out for germination in tissue culture, B: Seedlings from germinated seeds, C: Culture induction in culture bottles, D: Shoot induction on leaves and nodal sections and E: Acclimatised tissue culture plants in the glasshouse.

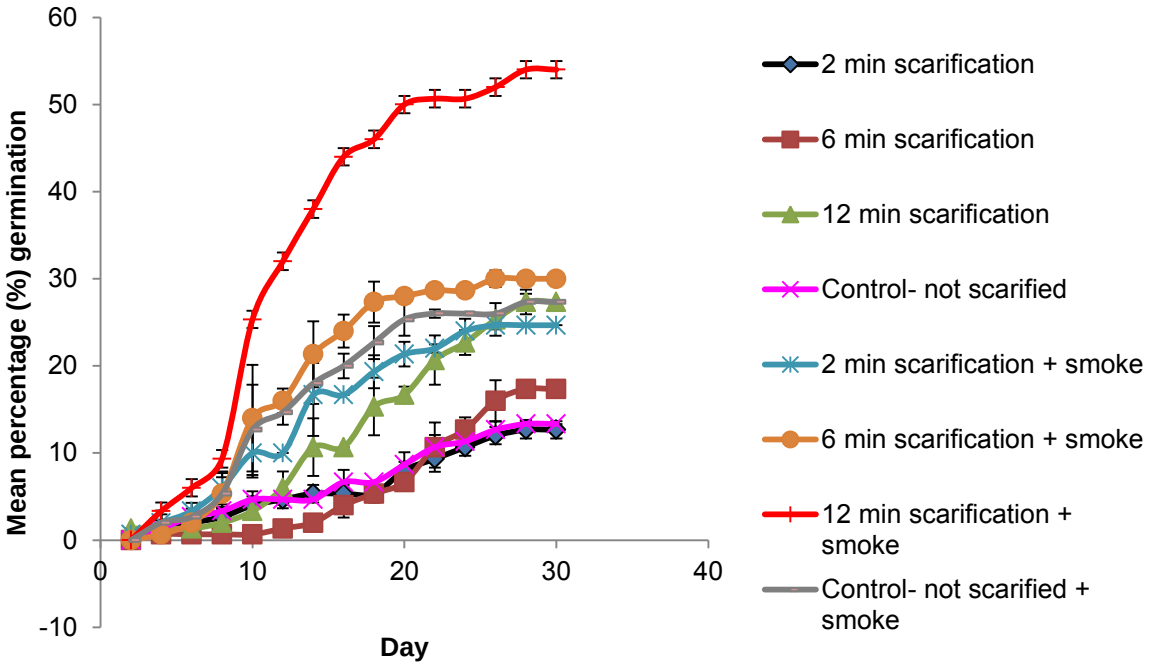

Figure 4.4 Germination rate of *S. repens* seeds after 30 days under light conditions. Seeds were scarified in sulfuric acid for 2 min, 6 min and 12 min with a control that was not scarified, and plated onto 1/10 MS media with and without smoke. Bars represent standard deviation.

Table 4.2 Statistical analysis data of germination of *S. repens* seeds germinated over a 30 days period under different treatments.

Treatment	Mean % germination
Control- not scarified	10.00 a
2 min scarification	12.67 ab
6 min scarification	17.33 abc
12 min scarification	20.00 abcd
2 min scarification + smoke	24.67 bcd
Control- not scarified + smoke	27.33 cd
6 min scarification + smoke	30.00 d
12 min scarification + smoke	54.00 e

Values that share letters are not statistically different at the 95% confidence level. The data was compared using a one-way ANOVA and Fisher LSD test.

A fast and efficient germination and culture induction tissue culture system was successfully developed for *S. repens* (Figure 4.3). Seeds plated out on media containing smoke gave the highest germination rate (Figure 4.4) and final percentage germination (Table 4.2). A significant difference was observed in seeds

that were scarified for 12 min in sulfuric acid and then plated on medium containing smoke. The treatment resulted in a higher rate (Figure 4.4) and higher level of germination (Table 4.2) under light conditions compared with other treatments. *Salvia repens* seeds germinated better when scarified and placed in media containing smoke, showing that their dormancy is effected by both physical (seed coat) and chemical (germination inhibitors) dormancy.

4.3.2 Shoot multiplication and culture induction

Table 4.3 Micropropagation of *S. repens* nodal explants on MS medium supplemented with different combinations of auxins and cytokinins

Treatment	µM	Mean shoot number	Mean shoot length (mm)	Mean root number	Mean root length (mm)	Mean massof regenerating tissue (g)
MS	control	1.64abc	18.02f	1.84bc	33.70d	0.24a
BA	4.4	3.20h	5.38cd	0.48a	4.37ab	0.29a
	8.9	2.60g	6.16bc	0a	0a	0.19a
IAA	2.9	1.48abc	13.39e	1.80b	22.96c	0.16a
	11.4	1.40bc	15.34ef	1.88bc	22.60c	0.22a
BA:IAA	4.4:2.9	2.00a	8.67b	0a	0a	0.19a
	8.9:11.4	2.00a	7.10bc	0.20a	1.41a	0.21a
NAA	2.7	1.74ac	8.12b	2.44c	8.88b	192.85b
	10.7	1.13bf	3.17ad	0.32a	0.88a	0.15a
BA:NAA	4.4:2.7	0.84ef	2.06a	0.12a	2.73a	0.22a
	8.9:10.7	0.50de	1.24a	0a	0a	0.19a
BA:2,4D	8.9:6.8	0.12d	0.13a	0a	0a	0.03a

Values within a column that share letters are not statistically different at the95% confidence level. The data was compared using a one-way ANOVA and Fisher LSD test.

Salvia repens leaves were also set out for shoot and root induction using the same treatments as for nodal explants. The leaf explants did not result in any regeneration after the same time as for nodal explants and were thus discarded for this experiment.

Nodal explants placed on MS media are the best treatment for micropropagation of *S. repens* (Table 4.3). Even though nodal explants placed in BA produced more shoots (with 4.4 µM BA being better than the 8.9 µM BA treatment at 3.2 and 2.6 respectively) than MS at 1.64, the shoots were shorter than those produced by explants on MS (Table 4.3). Explants on BA also produced fewer and shorter roots

compared to explants on MS. For rooting, 2.7 µM NAA produced the most number of roots compared to other treatments. However, the roots were shorter than the ones produced on MS media at 8.88 mm for 2.7 µM NAA and 33.7 mm for MS.The two IAA concentrations gave similar results for shoot and root production. Nodal explants plated on full strength MS media resulted in shoots that were comparable to the IAA treatments in all parameters. Full strength MS media also resulted in longer roots compared with other treatments,with the number of roots produced being comparable to that obtained following treatment with IAA. Longer shoots and roots make it easy to cut off the shoots, and also buds at the shoot tips, for further culture induction. This makes full strength MS medium, without hormonal supplementation, favourable for shoot multiplication and culture induction for *S. repens* nodal explants.

Table 4.4 Callus production from explants of *S. repens* on MS medium supplemented with different combinations of auxins and cytokinins

Treatment	µM	Mean % callus (leaves)	Mean % callus (nodes)
MS	Control	0d	0c
BA	4.4 8.9	16de 48ade	52abde 56abe
IAA	2.9 11.4	0d 40ade	4cd 16cde
BA:IAA	4.4:2.9 8.9:11.4	64abc 64abc	40abcde 40abcde
NAA	2.7 10.7	84bc 76abc	88bf 36acde
BA:NAA	4.4:2.7 8.9:10.7	96c 64abc	40abcde 80abf
BA:2,4D	8.9:6.8	48ade	100f

Values within a column that share letters are not statistically different at the 95% confidence level.The data was compared using repeated measure ANOVA and Fisher LSD test.

Salvia repens explants on full strength MS medium did not produce any callus. For callus production (Table 4.4), the BA: 2,4D combination was optimal as 100% of the nodal explants produced callus as compared to 48% for leaves on same medium. Leaves of *S. repens* placed on BA:NAA at ratios of 4.4:2.7 produced more callus than nodal explants placed on same medium, at 96% and 40% respectively.

These results are not very different from results observed for other *Salvia* species. Makunga and van Staden (2008) and Musarurwa et al. (2010), working on *S.*

africana-lutea and *S. stenophylla* respectively, showed that NAA is the best rooting hormone. For *S. africana-lutea* (Makunga and van Staden, 2008), BA at 4.4 μM did not give rise to more shoots as was the case in this experiment and for Mederos-Molina (2004) working on *S. canariensis* L. and Gostin (2008) working on *S. officinalis.* However, the combination of BA:IAA, which was the second best treatment after full strength MS media in this experiment, was the best treatment for Makunga and van Staden (2008) and Musarurwa et al. (2010), albeit at different ratios, for shoot multiplication.

Salvia repens in vitro plantlets acclimatised readily in the glasshouse, with 100% survival after two weeks. As the plantlets acclimatised readily, they did not have to be treated with any hormones to help with rooting, as was required for *S. africana-lutea* (Makunga and van Staden, 2008) and *S. stenophylla* (Musarurwa et al., 2010).

4.3.3 Phytochemical analysis

4.3.3.1 TLC

Methanol and acetone extracted more metabolites than the other solvents (more evident in the GC-MS results (Figure 4.6)) with 16 bands each followed by dichloromethane and chloroform with 12 bands each for plants in tissue culture (Figure 4.5). For glasshouse plants methanol has 13 bands, acetone has 12 bands, dichloromethane has 10 bands and chloroform has 8 bands. Methanol and acetone were therefore used as the solvents of choice to extract metabolites for biological activity studies.

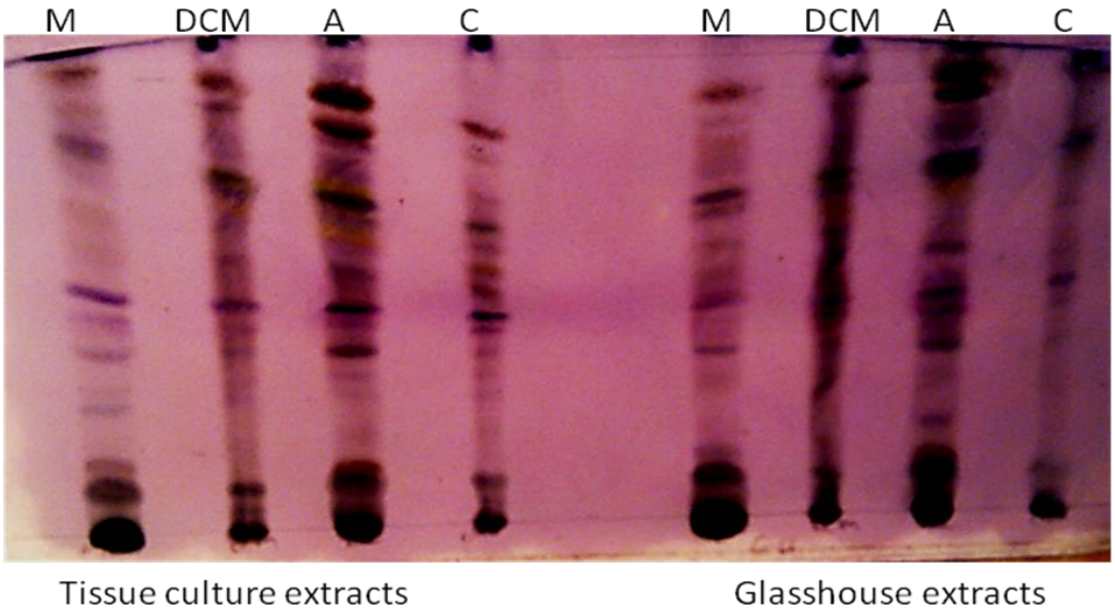

Figure 4.5 A TLC plate showing metabolites separated from different extracts of *S. repens* grown in tissue culture and the glasshouse. M-methanol, DCM-dichloromethane, A-acetone and C-chloroform

4.3.3.2 GC-MS analysis

In total, 37 volatile metabolites were identified from GC-MS analysis (Figure 4.6). Of these, 34 were identified in the glasshouse-acclimatised plantlets and 26 were identified from plantlets in tissue culture.

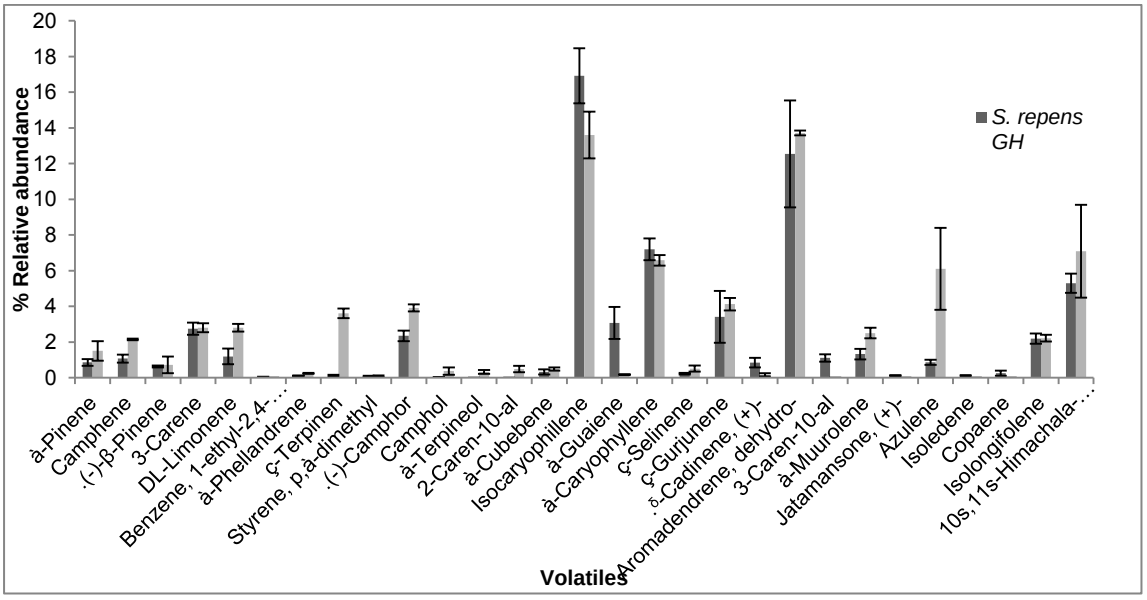

Figure 4.6 Essential oils (volatiles) of *S. repens* identified using Masslynx following head space GC-MS. TC-plants in tissue culture. GH- plants in the glass house. Bars represent standard error

Terpenes found in high relative abundance in both glasshouse and tissue culture plants were isocaryophellene (17.98% TC, 15.39% GH), 10s,11s-himachala-3(12),4-diene (10.63% TC, 7.95% GH), α-caryophellene (6.8% TC, 6.67% GH), ç-gujunene (4.36% TC, 3.77% GH), camphor (3.84% TC, 2.04% GH), 3-carene (3.08% TC, 3.14% GH) and D-limonene (2.66% TC, 1.98% GH). There were also some major discrepancies in metabolite content between tissue-cultured and greenhouse grown plants. The differences were observed in aromadendrene (0.11% TC, 14.22% GH), α-guaine (0.52% TC, 9.19% GH), azulene (2.58% GH), α-muurolene (2.50% TC, 0.085% GH), δ-cadinene (0.29% TC, 2.55% GH), camphene (2.16% TC, 1% GH), α-pinene (2.08% TC, 0.8% GH) and β-pinene (1.24% TC , 0.6% GH).

In other studies pertaining to essential oil contents of *S. repens*, Mayekiso et al. (2008) reported 16.85% β-pinene, considerably more than the 1.24% measured in this study and the 3% that Kamatou et al. (2005) reported. The same goes for α-pinene and limonene, which were recorded at 2.08% and 2.66% respectively, whereas Kamatou et al. (2005) reported 6.6% α-pinene and 9.8% limonene. However, there were some essential oils that the previous studies by Kamatou et al. (2005) reported at low relative abundance but which were measured at higher relative abundances in this study. These were aromadendrene, ç-gujunene, δ-cadinene, and 3-carene. Some metabolites were not detected at all in previous studies but were detected in this study. The metabolites found in this study at high relative abundances and not previously reported in *S.repens* essential oil studies are isocaryophellene,10s,11s-himachala-3(12),4-diene, α-guaine, α-cryophellene and azulene.

The essential oil composition of plants of the same species can vary due to locality (water and climate), as well as genetic and phenotypic variation (Viljoen et al., 2006). It is thus not surprising that in this experiment there are differences in the essential oil composition and content between plants in tissue culture and in the glass house (Figure 4.6), as they were exposed to very different environments. The essential oils that were found at high relative abundance as already mentioned were found at high concentrations in *in vitro* plants as compared to *ex vitro* plants. The essential oils that were found in high concentrations in tissue culture plants include isocaryophellene, α-caryophellene (which were both reported for the first time in this study), camphor,

3-carene and d-limonene. In general even though there are differences, plants propagated in tissue culture did not lose the medicinal value as they contained the essential oils that have been reported before from *S. repens* plants in the wild. The *in vitro* cultured plants are even advantageous as they contain medicinally important essential oils like iso-caryophellene and α-caryophellene which have not been reported in *S. repens* plant extracts from the wild. The essential oil content of tissue cultured plants make *in vitro* farming of *S.repens* to be a commercially viable strategy. The reported essential oils also differ with the essential oils reported by Mayekiso et al. (2008), Kamatou et al. (2005) and Viljoen et al. (2006), who all worked with *Salvia* plants from their natural habitat, but in different locations as discussed above.

Most of the terpenic compounds identified were sesquiterpenes compared to monoterpenes, but the monoterpenes identified were present at high concentrations. Terpenoids are known for their uses as flavourings in food, fragrances in cosmetics such as perfumes, and as medicine (Kamatou et al., 2008). Caryophellene displays anti-inflammatory, antibiotic and anti-oxidant activities, and also acts as a local anaesthetic (Legault and Pichette, 2007). Iso-caryophellene and α-Caryophellene have anti-cancer properties (Legault and Pichette, 2007) and α-Caryophellene also has anti-inflammatory properties (Fernandes et al., 2007). Monoterpenes such as camphor, pinene and 3-Carene are used as flavourings and fragrances (Weitman and Major, 2010) and d-limonene have medicinal properties, such as anti-cancer activities (Weitman and Major, 2010).

Non-volatile metabolites were also identified and quantified. A total of 94 metabolites were identified (Table 4.5), including an array of amino acids, sugars, lipids/fats and most importantly for medicinal plants, secondary metabolites.

Table 4.5 Non-volatile metabolites tentatively identified from the GC-MS data of *S. repens* extracts using AMDIS. Metabolites were measured in % relative abundance/g FW

Metabolite/solvent	Acetone		Methanol		Dichloromethane		Chloroform		
Name	**GH**	**TC**	**GH**	**TC**	**GH**	**TC**	**GH**	**TC**	**Description**
Alanine	-	-	0.62	0.50	-	-	-	-	Alpha amino acid
Alanine, 3-cyano-	-	-	-	0.11	-	-	-	-	Alpha amino acid
alpha-D-Galactopyranosyl-	-	-	22.21	-	-	-	-	-	
Asparagine [-H2O]	-	-	-	1.34	-	-	-	-	Amino acid
Aspartic acid	-	-	-	0.40	-	-	-	-	Alpha amino acid
Arabinonic acid	0.08	-	-	-	-	-	-	-	
Benzoic acid,	0.26	0.79	-	0.28	0.09	0.23	-	-	
beta-D-Allose	-	9.36	-	-	-	-	-	-	
beta-Galactopyranosyl-1,3-arab	-	-	0.06	-	-	-	-	-	Saponin,secondary metabolite
Butane, 1,2,4-trihydroxy-	-	1.37	-	-	-	-	-	-	
Caffeic acid 8	31.98	29.99	9.15	9.20	-	-	-	-	Phenolic, secondary metabolite
Campesterol	2.76	4.44	1.50	0.81	0.83	1.10	-	0.06	Phytostero,secondary metabolite
Citric acid	-	-	0.03	-	-	-	-	-	
Erythronic acid	3.85	-	6.27	7.43	-	-	-	-	
Farnesol	-	-	-	-	6.76	-	-	1.02	Sesquiterpene, secondary metabolite
Ferulic acid, cis-	3.99	0.10	1.90	-	0.08	-	-	-	Secondary metabolite
Ferulic acid, trans-	0.14	0.30	-	-	0.03	-	-	-	Secondary metabolite
Fumaric acid	0.69	0.85	1.09	1.08	-	0.03	0.05	0.04	TCA cycle
Galactinol	9.99	-	428.88	100.51	0.80	0.81	1.48	0.50	
Galactonic acid	-	-	0.86	-	-	-	-	-	
Galactopyranoside, 1-O-methyl-	-	55.06	2.21	18.59	-	-	-	-	
Galactose	2.19	-	5.33	29.75	-	1.26	0.16	1.01	Sugar monosaccharide
Gentiobiose	-	4.05	-	-	-	-	-	-	Glucose-glucose disaccharide

Metabolite/solvent	Acetone		Methanol		Dichloromethane		Chloroform		
Name	**GH**	**TC**	**GH**	**TC**	**GH**	**TC**	**GH**	**TC**	**Description**
Glucoheptonic acid-1,4-lactone	2.03	-	1.11	-	-	-	-	-	
Glucopyranose [-H20]	1.06	2.54	0.88	1.80	0.08	-	0.05	0.11	
Glucopyranose, D-	1.84	0.64	-	-	-	0.70	-	0.39	
Glucose, 1,6-anhydro, beta-	53.53	92.63	-	-	-	-	-	-	
Glucose, 1,6-anhydro, beta-	0.48	0.06	-	6.54	-	-	-	-	
Glutamic acid	-	-	-	0.09	-	-	-	-	Amino acid
Glutamine [-H2O]	-	-	-	-	-	-	-	0.02	Amino acid
Glyceric acid	1.20	0.50	23.08	1.24	-	-	-	-	
Glycerol	3.25	1.65	5.04	13.02	1.19	4.67	0.15	-	Lipid,glycerine
Glycerol-3-phosphate	4.36	2.49	6.08	4.97	-	-	-	-	
Glycerophosphoglycerol	-	-	0.15	-	-	-	-	-	
Glyoxylic acid	0.07	-	-	-	-	-	-	-	
Gulonic acid	-	-	-	0.15	-	-	-	-	
Homoserine	-	-	-	trace	-	-	-	-	Alpha amino acid
Hydroquinone	-	-	-	-	0.11	-	-	-	Aromatic, type of phenol
Inositol, myo-	7.89	0.95	85.41	62.68	0.06	0.68	0.34	0.67	
Inositol-2-phosphate, myo-	-	-	1.15	0.75	-	-	-	-	
Isoleucine	-	-	0.02	0.76	-	-	-	-	Alpha amino acid
Kestose, 6-	0.74	1.48	0.57	149.38	-	-	-	-	Sugarkestose
Kestose, 1-	-	-	-	202.57	-	-	-	-	Sugarkestose
Lactic acid	0.10	-	2.43	-	-	-	-	-	
Laminaribiose M	-	-	0.40	-	-	-	-	-	Disaccharide
Leucine	-	-	-	0.76	-	-	-	-	Amino acid
Leucine, cyclo-	0.18	1.56	-	0.05	-	-	-	0.05	Nonproteogenic amino acid
Limonene, (+/-)-	-	trace	-	-	-	-	-	-	Terpene, secondary metabolite
Lyxonic acid	-	-	-	0.63	-	-	-	-	

Metabolite/solvent	Acetone		Methanol		Dichloromethane		Chloroform		
Name	**GH**	**TC**	**GH**	**TC**	**GH**	**TC**	**GH**	**TC**	**Description**
Lyxose	0.87	-	0.55	-	-	-	-	-	Sugar monosaccharide
Maleic acid	0.69	0.85	-	-	-	-	-	-	TCA cycle
Malic acid	1.50	-	23.66	4.53	-	-	-	-	Calvin cycle, citric acid cycle malate
Maltotriitol	0.40	-	-	-	-	-	-	-	
Mannitol	0.93	2.64	3.25	8.51	-	-	-	-	
Mannosamine, N-acetyl-	-	0.08	-	-	-	-	-	-	Rare monosaccharide
Menthol, L-(-)-	1.13	1.55	0.62	-	-	0.24	-	-	Secondary metabolite
Norleucine	-	-	-	0.76	-	-	-	-	Amino acid
Norvaline, DL-	-	0.06	0.41	2.23	-	-	-	-	Amino acid
Oleanolic acid	1.37	1.53	0.20	0.07	1.26	9.23	0.04	-	Triterpenoid,secondary metabolite
Phenol	0.04	0.04	0.04	-	-	-	0.13	0.08	
Phosphoric acid	9.73	10.89	9.56	25.87	0.90	7.35	5.42	5.69	Mineral,inorganic acid
Phylloquinone	-	0.07	-	-	-	-	-	-	Polycyclic aromatic ketone, vitamin K1
Phytol	1.13	1.55	0.62	0.36	0.17	0.24	-	-	Diterpene alcohol for synthesis of Vitamin e and K
Proline	-	-	-	0.09	-	-	-	-	Alpha amino acid
Psicose	-	-	-	0.89	-	-	-	-	Sugar monosaccharide
Pyroglutamic acid	0.18	1.56	10.03	380.92	-	-	-	0.05	Uncommon amino acid
Pyruvic acid	-	-	0.02	-	-	-	-	-	Citric acid cycle
Raffinose	2.46	0.06	146.41	52.80	-	-	-	-	Sugar trisaccharide
Ribulose-5-phosphate	-	-	0.17	-	-	-	-	-	Pentose phosphate pathway and calvin cycle intermediate
Rosmarinic acid	63.97	90.10	0.52	1.50	-	-	-	-	Polyphenol secondary metabolite
Sarcosine	-	-	0.62	0.50	-	-	-	-	Amino acid
Serine	-	-	0.23	5.26	-	-	-	-	Amino acid
Shikimic acid	1.08	-	1.12	-	-	-	-	-	Hydroaromatic intermediate for plant phenolics
Sitosterol, beta-	55.21	118.95	30.46	22.75	31.61	39.76	4.67	14.26	Phytosterol, secondary metabolite
Sorbitol	-	-	-	7.35	-	-	-	-	Sugar alcohol

Metabolite/solvent	Acetone		Methanol		Dichloromethane		Chloroform		
Name	**GH**	**TC**	**GH**	**TC**	**GH**	**TC**	**GH**	**TC**	**Description**
Sorbose BP	2.54	1.65	4.62	162.79	-	0.91	0.18	1.37	Ketose sugar, monosaccharide
Sphingosine	0.03	4.15	-	1.49	-	-	-	-	Cell membrane lipid
Stigmasterol	0.66	7.71	0.56	2.05	0.24	1.62	-	0.19	Phytosterol, secondary metabolite
Succinic semialdehyde	0.20	-	-	-	-	-	-	-	
Tagatose	-	4.03	-	-	-	-	-	-	Sugar monosaccharide
Tartaric acid	0.51	-	0.52	-	-	-	-	-	Organic acid secondary metabolite
Taxifolin	0.06	0.06	-	0.08	-	-	-	-	Flavononol secondary metabolite
Threonic acid	3.85	0.24	6.27	7.43	-	-	-	-	Secondary metabolite
Threonic acid-1,4-lactone	0.83	-	-	-	-	-	-	-	
Threonine	-	-	0.21	2.97	-	-	-	-	
Tocopherol, alpha-	0.43	1.74	-	0.04	-	-	-	-	Vitamin E
Tryptophan, 5-hydroxy-	-	0.04	-	-	-	0.04	-	-	Amino acid
Uracil	-	-	-	0.10	-	-	-	-	Nucleotide
Uracil, dihydro-	0.32	3.12	0.92	19.01	0.42	0.83	0.68	21 227.85	
Uridine	-	-	-	399.31	-	-	-	-	
Ursolic acid	8.12	8.21	4.23	1.44	27.77	17.88	1.53	-	Secondary metabolite
Valine	-	0.06	0.41	2.23	-	-	-	-	Alpha amino acid
Xylobiose, D- B	-	4.40	-	2.97	-	-	-	-	Disaccharide
Xylose	-	2.61	-	-	-	-	-	1.54	Sugar monosaccharide

The different solvents used for extraction resulted in different metabolite yields; methanol extracted the most metabolites, followed by acetone, dichloromethane and then chloroform. Acetone and methanol extracted the most secondary metabolites in total and are the recommended solvents for extraction of metabolites for this plant.

The medicinal properties of the identified secondary metabolites agreed with the traditional medicinal uses of this plant, as reported by Jäger and van Staden, (2000). These include treatment of sores in the body, stomach ache and diarrhoea (anti-inflammatory and anti-oxidant activities). The plant extracts contained caffeic acid, ferulic acid (a caffeic acid monomer) and rosmarinic acid (caffeic acid dimer) which are known to give these plants their anti-bacterial, anti-oxidant, anti-inflammatory and anti-cancer properties (Kamatou et al., 2008). Other secondary metabolites which are worthy to noteare the triterpenoids ursolic and oleanolic acids, as they display anti-inflammatory, anti-tumor, anti-microbial, anti-hyperlipidemic and hepatoprotection activities (Liu, 1995). Phytosterols which have been reported to inhibit cholesterol absorption, campesterol, β-sitosterol and stigmasterol (Nair et al., 1984) were also identified. Metabolites which are used in the cosmetics industry, such as farnesol (Derengowski et al., 2009), menthol (Corvalán et al., 2009) and phytol (Lasseur et al., 2006), were also identified.

4.3.3.3 Biological activity assays

Mayekiso et al. (2008) tested the solvent extracts of *S. repens* for anti-bacterial activities and Kamatou et al. (2005; 2006; 2008) tested the biological activities, such as anti-inflammatory, anti-oxidant, anti-bacterial, anti-cancer and anti-mycobacterium activities, of the solvent and essential oil extracts of *S. repens*. As far as can be ascertained by the authors, this report represents the first assessment of the anti-fungal activities of *S. repens* extracts.

Table 4.6 Minimum Inhibitory Concentration (mg/ml) values of anti-microbial activity of *S. repens*extracts against Gram + and Gram – bacterial pathogens.

Extract (50 mg/ml)	***E. coli*** **(Gram -)**	***B. subtilis*** **(Gram +)**	***S. aureas*** **(Gram +)**	***K. pneumoneae*** **(Gram -)**
Methanol TC	3.125	3.125	3.125	3.125
Acetone TC	6.25	6.25	6.25	6.25
Methanol GH	1.56	3.125	0.8	1.56
Acetone GH	1.56	0.8	1.6	1.56

Streptomycin inhibited bacterial growth at MIC of 0.16 mg/ml. MIC values are averages of three replicates per concentration. Plant extracts with MIC values less than 1 mg/ml are highlighted in red.

Even though *S. repens* extracts inhibited bacterial growth, the minimum inhibitory values are higher than the recommended 1 mg/ml (Van Vuuren 2008) for most and thus cannot serve as leads for new drugs development. However, the minimum inhibitory concentration value for the methanol glasshouse-grown plant extracts was 0.8 mg/ml against *S. aureas* and acetone glasshouse-grown plant extracts also had an MIC value of 0.8 mg/ml against *B. subtilis* (Table 4.6). The latter extracts can thus be studied further for use against the respective bacterium.

Table 4.7 MIC values of anti-fungal activity of *S. repens* extracts against three *Fusarium* species

Extract (10mg/ml)	***F. verticillioides*** **MRC826**				***F. proliferatum*** **MRC6908**				***F. subglutinans*** **MRC0115**			
	48h	72h	96h	120h	48h	72h	96h	120h	48h	72h	96h	120h
Methanol TC	0.32	0.32	0.625	1.25	0.315	0.6	1.25	1.25	0.08	0.4	0.625	1.04
Methanol GH	0.067	0.21	0.32	0.32	0.08	0.11	0.3	0.32	0.1	0.3	0.32	0.32
Acetone TC	0.32	0.32	0.625	1.04	0.07	0.3	1.25	2.0	0.16	0.32	0.5	1.0
Acetone GH	2.5	2.5	2.5	2.5	0.625	2.5	2.5	2.5	0.16	1.25	2.5	2.5

Control inhibitory MIC: 0.03 mg/ml Amphotericin. MIC values are averages of three replicates per concentration. Plant extracts with MIC values less than 1mg/ml after 4 and 5 days are highlighted in red.

The plant extracts displayed very high antifungal activities against the three *Fusarium* spp. used (Table 4.7) over a period of 5 days. The methanol extracts from glasshouse-grown plants and acetone extracts from tissue culture-derived plantlets were particularly active. *Fusarium* produces mycotoxins, leading to agricultural losses (Thembo et al., 2010). Currently, artificial fungicides, which pose danger to the environment as they are mostly not biodegradable and to which pathogens have become resistant (Wilson et al., 1997), are used to control these pathogens. These fungicides are not accessible or affordable to most rural African subsistence farmers (Thembo et al., 2010). There is thus a need for an affordable, accessible and environmental friendly control measure. Therefore further studies on *S. repens* extracts are worthwhile for the fungicide commercial industry.

4.4 Conclusion

A highly regenerative tissue culture protocol was developed for *S. repens* as a commercialisation and conservation strategy to serve the phyto-pharmaceutical industry. The plant displayed high fungal inhibition activity, especially the methanol extracts from the tissue cultured material, indicating the ability for plants to continue producing secondary metabolites. Further work is therefore worthwhile in this plant, especially developing biotechnological strategies to identify and then increase the production of the active compound/compounds.

CHAPTER 5: Over-expression of *Arabidopsis* MYB transcription factors in *Salvia stenophylla*

5.1 Introduction

Plants produce secondary metabolites which are not needed by the plant at all times, but which rather help plants to defend themselves against diseases or stressful conditions. The same products can serve as nutrients, flavourants, colorants, fragrances and medicines to people (Wu and Chappel, 2008). Interest has grown world-wide in the use of plant metabolites in medicines, more so as the products are natural. Nearly two-thirds of the drugs approved since 1981 have been derived from such plant metabolites.

5.1.1*Salvia stenophylla*

Salvia stenophylla is a medicinal plant belonging to the family Lamiaceae. It is indigenous to South Africa, Lesotho and Botswana, where it is used in traditional medicine to treat a variety of medical conditions. The secondary metabolites that the plant produces have been investigated by Kamatou et al. (2005; 2006; 2008). The species contains high concentrations (28.9-41%) of epi-α-bisabolol in relative abundance to other metabolites (Viljoen et al., 2006), which has anti-inflammatory bioactivities (Jäger and Van Staden, 2000; Kamatou et al., 2008), as compared with 17% relative abundance in chamomile (*Matricaria chamomilla*), which is usually the preferred species for isolation of this terpenoid. Epi-α-bisabolol is used in aromatherapy and cosmetics industries, making it economically important. Musarurwa et al. (2012) reported an increase in this commercially important terpenoid when *S. stenophylla* was cultured in media with an increased supply of macronutrients.

Salvia stenophylla wild populations may become endangered as whole plants are harvested by the local people for commercial and informal trade sectors (Kamatou et al., 2008). Biotechnology offers an alternative way in which plants can be preserved and also metabolite production enhanced. *Salvia* plants have been exposed to *Agrobacterium*-mediated transformation to enhance the production of important

secondary metabolites (Yan and Wang, 2007). The plants have been subjected to hairy roots regeneration using the wild *Agrobacterium rhizogenes rol* genes. In our laboratory, hairy roots were produced using wildtype *A. rhizogenes* strains in *S. africana-lutea* (Ramogola et al., 2009) and on *S. runcinata* (Figlan, 2012) but not for *S. stenophylla* which is also cultured in our laboratory. The same is true for *Salvia* species across the world. Examples include work by Yan and Wang (2007) where susceptibility of *Salvia miltiorrhiza* to transformation by wildtype *A. Tumefaciens* containing the pCambia 2301 expression vector was investigated. Marchev et al. (2011) compared the common transformation of leaf disks using *A. rhizogenes* compared to the two-phase temporary immersion system for genetic transfer in *Salvia tomentosa*. Khawar et al. (2003) showed susceptibility to transformation of the two species *S. sclareae* and *S. pratense* by *A. tumefaciens*. These examples were mainly looking at susceptibility of *Salvia* plants to transformation by wild type *Agrobacterium* species which have not been transformed by addition of any foreign genes. *Salvia stenophylla* has not yet been exposed to these trials of transformation susceptibility by *A. rhizogenes* and thus need to be investigated.

Agrobacterium tumefaciens gives rise to new plantlets. Explants transformed with *A. tumefaciens* can start off by producing callus. Plants can be regenerated from the produced callus and be analysed for metabolites to see the effect of the gene of interest in the metabolite composition of the plant. *Agrobacterium rhizogenes* on the other hand results in production of hairy roots which, unlike normal roots, grow plagiotropically (loss of gravity response). They have a high degree of lateral branching which results in the production of several meristems (Srivastava and Srivastava, 2007). Hairy root production is always an indication and proof of transformation in plants (Chilton et al., 1982). Growth rate of hairy roots is high compared to normal roots and they are biochemically and genetically stable (Srivastava and Srivastava, 2007) and are thus ideal for studies on metabolite accumulation.

Some *Salvia* plants have been transformed to over-express particular genes or effect changes in different pathways, either to produce targeted secondary metabolites or to make the plants more stress tolerant. Musarurwa (2012) transformed *S. stenophylla* using recombinant *A. tumefaciens* to enhance the production of

secondary metabolites, especially essential oils. Han et al. (2007) successfully transformed *S. miltiorrhiza* using *A. tumefaciens* with the *TaLEA1* (*Late Embryogenesis Abundant1*) gene from wheat, making the plant more salt and drought tolerant.

5.1.2*Arabidopsis thaliana* MYB transcription factors

Arabidopsis thaliana MYB proteins are members of a super-family of transcription factors (Yanhui et al., 2006) that have been reported to be multifunctional. The MYB transcription factors are involved in a wide range of processes such as the regulation of meristem activity and dorsi-ventral polarity, cellular specification and morphogenesis, intracellular signalling and, most significantly for this study, in the control of secondary metabolite pathways such as the phenylpropanoid pathway (Martin and Paz-Ares, 1997; Jin and Martin, 1999).

Transcription factors AtMYB3, AtMYB6, AtMYB7, AtMYB8, AtMYB13, AtMYB14, AtMYB15 and AtMYB32 were used in this study. These transcription factors belong to two different families of the R2R3 MYB transcription factors, subfamily II and subfamily IV. Subfamily II (AtMYB13, AtMYB14 and AtMYB15) have been reported to enhance the phenylpropanoid pathway (Marin et al., 2011), whereby when over-expressed they produced more metabolites from this pathway. A study by Chen et al. (2006) reported that *AtMYB15* was over-expressed when *A. thaliana* was wounded and resulted in up-regulation of the shikimate pathway. Members of subfamily IV (AtMYB3, AtMYB6, AtMYB7, AtMYB8 and AtMYB32), on the other hand, have been reported to repress the phenypropanoid pathway. Secondary metabolites which are of medicinal value in *S. stenophylla* are produced via the phenylpropanoid pathway. Transcription factors from both subfamilies should therefore have interesting effects on the accumulation of secondary metabolites in S*. stenophylla*.

The aims and objectives of this study were therefore: i) to transform *S. stenophylla* explants with two commonly-used *A. rhizogenes* strains in order to determine which is more efficient for hairy root production; ii) to clone *AtMYB* transcription factors into S*. stenophylla* through *Agrobacterium*-mediated transformation in order to attempt to increase the production of secondary metabolites; and iii) to characterise functions of

the *AtMYB*s by determining their effects on the metabolite composition of plants by comparing non-transgenic and transgenic plant cultures.

5.2 Materials and Methods

5.2.1 Plant material and culture

Plantlets of *S. stenophylla* were maintained in continuous culture, using a protocol from Musarurwa et al. (2010). Nodal sections of approximately 1cm with two axillary buds were used as explants, placing each axillary bud in direct contact with the medium. Explants were cultured on MS salts (4.43 g/l, Highveld Biological) supplemented with 30 g/l sucrose, 0.1 g/l myo-inositol (Fluka Analytical) and 10 g/l agar-agar powder (Biolab) at a pH 5.7-5.8. They were grown at 25°C under a 16 h light/8 h dark photo-period, with light provided by cool, white fluorescent tubes (50 µmolphotons.m^{-2}.s^{-1}, 58W Osram L fluorescent tubes). The plants were sub-cultured onto new media every 4 weeks. All media used for this study were autoclaved at 121°C for 15 min at 103 kPa and then poured upon cooling into bottles of ~12 cm x 4.5 cm dimensions.

5.2.2 Amplification and isolation of *E. coli* plasmids containing transcription factors

The isolation of the Gateway®-compatible plasmids containing the various transcription factors was performed as described in section 3.2.2.1.

5.2.2.1 Construction of pCambia2200-AtMYB Gateway® destination vectors

The expression vector pCAMBIA2200 (Figure 5.3B) containing the Cauliflower Mosaic Virus 35S (CaMV35S) promoter and neomycin phosphotransferase (*npt*II) plant selectable marker gene (kanamycin resistance), was used to transform leaf explants of *Salvia* plants. The vector confers chloramphenicol resistance in bacteria and kanamycin resistance in plants. The pBinAR multiple cloning site containing the CaMV35S promoter and 3-OCS terminator, cleaved with *Eco*RI and *Hin*dIII, was ligated into the *Eco*RI and *Hin*dIII sites of the pCAMBIA2200 vector (highlighted red in Figure 5.3C) by Dr James Lloyd (IPB). The pCAMBIA2200-BinAR vector was converted into a Gateway® destination vector by ligating the GRFCA into the *Sma*I

site, according to the manufacturer's protocol (Invitrogen, 2008), to generate pCAMBIA2200-GRFCA (Figure 5.3D). This procedure was kindly carried out by Mr Marnus Smith (IPB).

5.2.2.2 Mobilisation of transcription factors into the plant expression vectors

This was performed as described in section 3.2.2.3, except that the LB agar medium was supplemented with 34 µg/ml chloramphenicol for selection.

5.2.2.3 Verification of gene insertion in plant transformation vectors

This was performed as described in section 3.2.2.4.

5.2.2.4 Restriction Digests

This was conducted as described in section 3.2.2.5. Expected fragment sizes following restriction analysis are presented in Table 5.1.

Table 5.1 Expected band sizes from single and double restriction digests of pCAMBIA-2200 containing the *AtMYB* genes *AtMYB6* and *AtMYB14*

Vector	*Eco*RI	*Hind*III	*Bam*HI	*Kpn*I+*Xba*I	*Kpn*I+*Bam*HI
pCambia2200-*AtMYB6*	9878bp	9256bp 622bp	9203bp 675bp	8877bp 1001bp	8884bp 675bp 319bp
pCambia2200-*AtMYB14*	9730bp	8918bp 812bp	9730bp	8877bp 853bp	8884bp 846bp

5.2.2.5 PCR reaction

Amplification via PCR was performed as described in section 3.2.2.6. Details of PCR primers are given in Table 3.2.

5.2.3 Mobilising the pCambia2200-AtMYB constructs into *Agrobacterium* and transformation of *Salvia*

5.2.3.1 Tri-parental mating

Agrobacterium tumefaciens EHA105 and *Agrobacterium rhizogenes* LBA9402 were transformed via a tri-parental mating protocol adapted from Armatige et al. (1988) and Walkerpeach and Velten (1994). Untransformed *Agrobacterium* strains were

grown at 28°C for 2 d in the dark on YEP (10 g/l peptone, 10 g/l yeast extract powder, 5 g/l NaCl and 12 g/l bacteriological agar) solid plates, containing appropriate antibiotics. For *A. tumefaciens* EHA105, 50 µg/ml rifampicin and 25 µg/ml streptomycin were used, whilst only rifampicin was used for *A. rhizogenes* LBA9402. The *E. coli* cultures containing the pCAMBIA2200-AtMYB plasmids were grown on solid LB medium containing 34 µg/ml chloramphenicol for selection. The *E. coli* strain containing the helper plasmid pRK2013 was also grown on LB plates with 50 µg/ml kanamycin for selection. Single colonies were picked from each plate and inoculated into 5 ml YEP liquid culture for *Agrobacterium* strains and liquid LB for *E. coli* strains, and grown at the respective incubation temperatures without antibiotics. The liquid cultures were then mixed on one plate containing solid YEP medium without antibiotics by spreading 50 µl of each strain onto the plate before incubating overnight in the dark at 28°C. Next, 5 ml of sterile 10 mm $MgCl_2$ was added to each plate which was then incubated for 30 min at room temperature with gentle shaking. Serial dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were made from the liquid culture from these plates and 100 µl of each were plated onto YEP solid medium containing rifampicin and chloramphenicol, with the addition of streptomycin for *A. tumefaciens* EHA105, for selection and the plates were incubated for 2 d at 28°C in the dark. Plasmid DNA was isolated from single colonies and PCR used to verify transformation and PCR conditions were as described in Table 3.2.

5.2.3.2 *Agrobacterium*-mediated transformation and selection of transformants

Agrobacterium-mediated transformation was performed as described by Nigro et al. (2008). Firstly wildtype strains A4T and LBA9402 of *A. rhizogenes* were used to transform 100 (1x1.5cm) leaf explants of *S. stenophylla* in order to determine which one was more efficient. After that the more efficient strain was used for *Agrobacterium*-mediated transformation after it was successfully transformed with pCambia-AtMybs vector as described in Chapter 3 via tri-parental mating. The same transformation protocol was used to transform explants of *S. stenophylla* with wildtype *A. rhizogenes* and transgenic *Agrobacterium* strains. The *Agrobacterium* strains were each grown in 25 ml liquid YEP media containing appropriate antibiotics and 250 µM acetosyringone to a saturated culture of OD_{600}=1 -2, in the dark at 28°C. Then cells were harvested by centrifuging 13 ml of the liquid culture at 7000 *xg*

at 25°C for 15 min. The supernatant was discarded and the pellet re-suspended in liquid MS media and centrifuged for 10 min after which the supernatant was discarded and the cells re-suspended in 10 ml liquid YEP medium. Then 100 *Salvia* nodal explants (1x1.5 cm) leaf explants for each wildtype *A. rhizogenes* and *Agrobacterium-AtMYB* were incubated in the bacterial suspension for 1 h at room temperature (25°C). After incubation, the explants were removed and blotted dry on sterile tissue paper and placed in the dark for 3 d on MS media containing 250 µM acetosyringone. The explants were then removed and washed with half-strength MS medium containing 250 µg/ml cefotaxime to wash off the bacteria and then placed on solid half-strength MS selection medium containing 50 µg/ml kanamycin (not included for wildtype *A. rhizogenes* strains) and 250 µg/ml cefotaxime for shoot induction or the same media with 2 mg/l 2,4-D for callus formation. The explants were monitored every 3 days for organogenesis.

The transformation experiments were repeated twice and the transformation (number of transgenics) data were collected daily after the first hairy root for *A. rhizogenes* strains or fresh calli for *A. tumefaciens* made an appearance and was collected for a period of 65 days. Data were analysed using Statistica version 11 software. One-way ANOVA was used for analysis and the data subjected to FISHER LSD and the significant differences recorded at 95% confidence levels. The *S. stenophylla* explants transformed with wildtype *A. rhizogenes* strains were used as controls for the *A. rhizogenes* strains containing *AtMYB* transcription factors.

5.2.4 Molecular analysis: Verifying transformation

5.2.4.1 Isolation of genomic DNA

DNA was extracted as described in 3.2.3.2. The DNA concentrations were then determined and the samples stored at -20°C until analysis.

5.2.4.2 PCR on genomic DNA

The isolated genomic DNA was analysed to check for transgenesis. Integration for the *rol* genes was determined using the PromegaGoTaq® PCR kit as described in section 3.2.3.3. Primers for *rolA*, *rolB* and *rolC* (Table 6.1) were purchased from IDT (USA).

Table 5.2 Primer sequences, annealing temperatures and expected band sizes for *rol* genes in *A. rhizogenes*

Primer	Sequence	Annealing temp(°C)	Amplicon Size (kb)
rol A	5'- CAG AAT GGA ATT AGC CGG ACT A –3'forward 5'- CGT ATT AAT CCC GTA GGT TTG TTT -3' reverse	54	0.3
rol B	5'- ATG GAT CCC AAA TTG CTA TTC CTT CCA GA-3'forward 5'- TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC -3'reverse	54	0.4
rol C	5'- CAT TAG CCG ATT GCA AAC TTG -3'forward 5'- ATG GCT GAA GAC GAC CTG -3'reverse	54	0.6

5.2.5 Phytochemical analysis: Plant extracts and GC-MS analysis

Metabolites were extracted from wildtype *S. stenophylla* and from transgenic *S. stenophylla* twice for 40 min in the sonicator using a 1:1 mixture of methanol: chloroform. The samples were filtered through Whatman® no.1 filter paper into a previously weighed beaker and the solvents dried off. Samples were then re-suspended in fresh solvent to a total concentration of 50 mg/ml and used for GC-MS analysis, using the Glassop et al. (2007) GC-MS protocol as outlined in section 3.2.6.3. The GC-MS analysis was performed only once as there was insufficient hairy root material to repeat the test.

5.3 Results and Discussion

5.3.1 Restriction analysis

The vector constructs containing the transcription factors were all cleaved at the expected sites for both the single and double restriction digests (Figure 5.1). This suggested successful cloning of the transcription factors into the plant expression vector pCAMBIA2200-GRFCA.

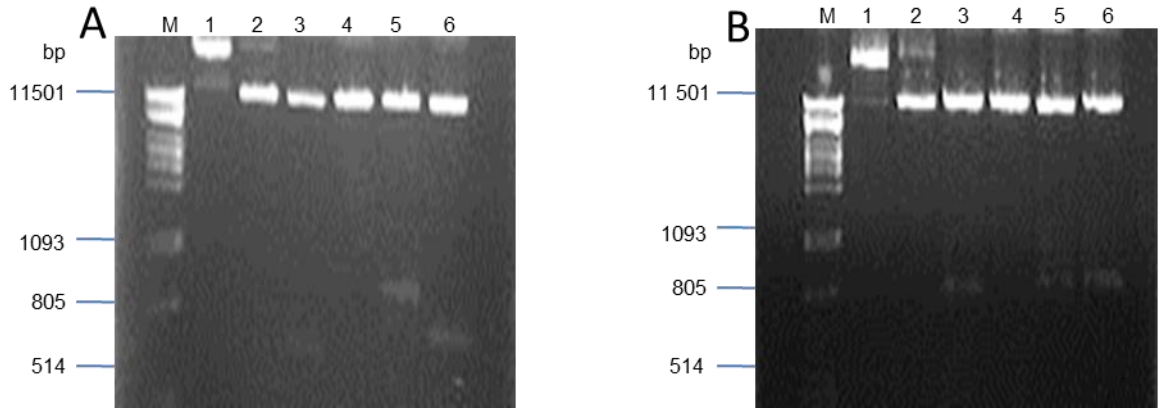

Figure 5.1 1% agarose gel image showing different bands obtained for pCAMBIA2200-AtMYB6(A) and pCAMBIA2200-AtMYB14 (B). (M-λ*Pst*I molecular weight marker,1-intact isolated plasmid DNA, 2-*Eco*RI, 3-*Hin*dIII, 4-*Bam*HI,5-*Kpn*I+*Xba*I and 6-*Kpn*I+*Bam*HI)

5.3.2 PCR analysis

All eight *Arabidopsis thaliana* MYB transcription factors used in the study were successfully cloned into the plant expression vector. Gene specific primers were used to verify this using PCR and all generated fragments of the expected sizes (Figure 5.2).

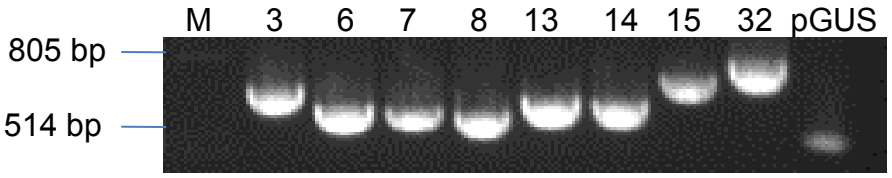

Figure 5.2 pCambia2200-AtMYB constructs ready for transformation. The numbers represent the *AtMYB* transcription factors and the expected band sizes are as shown in Table 3.2

As confirmed by the restriction digests (Figure 5.1) and PCR (Figure 5.2), the *AtMYB* transcription factors were ligated into the plant expression vectors successfully as represented in Figure 5.3.

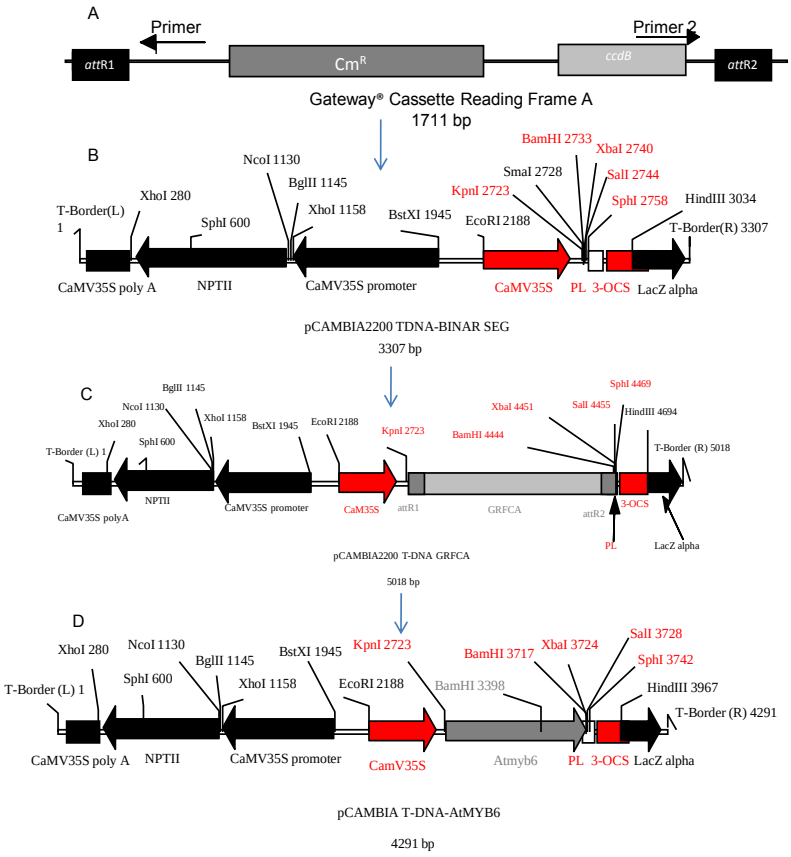

Figure 5.3 Vector constructs The Gateway® reading frame cassette frame A - GRFCA (A) which was ligated into the expression vectors pCAMBIA2200 expression vector (B) showing the section from BINAR highlighted in red, pCAMBIA2200-GRFCA (C) with the GRFCA ligated into the *Sma*I site of the expression vector and (D) pCAMBIA2200-AtMYBwith *AtMYB6* as an example.

5.3.3 Comparing transformation efficiency of two *Agrobacterium rhizogenes* strains

Agrobacterium rhizogenes agropine synthesizing strains LBA9402 (a hyper-virulent strain) and A4T occur naturally in the wild. They are amongst the most widely used strains for transformation as they possess the strongest induction ability (Hu and Du, 2006), which is why they were used in the study. The two strains were used in order to determine which one is optimal for *S. stenophylla* transformation, to enable transformation of the species with the *AtMYB* genes.

The Ri plasmid found in *A. rhizogenes* carries oncogenes that lead to neoplastic outgrowths when expressed in plants. The expression of the oncogenes leads to the organised growth of adventitious roots that are hairy (Nilson and Olsom, 1997), referred to as hairy roots disease. A study by White et al. (1985) showed that only insertions in four of the potential 18 loci on the T-DNA of the Ri plasmid affected the

morphology of the hairy roots that were produced. These loci are what is now known as the *rol* genes (root locus genes) and have been named *rolA*, *rolB*,*rolC* and *rolD* (Slighton et al., 1986). Hairy roots generated from mutants of the *rolA* gene grow straight out from the wound, and do not have a curly appearance as is typical. Root locusB (*rolB*) mutants were totally avirulent. Reduced growth of hairy roots was observed when insertions were made in the *rolC* locus and mutations in the *rolD* locus resulted in an increased production of callus and weakened root growth (Nilson and Olsom, 1997). Spena et al. (1987) showed that a combined action of *rolA*+*rolB*+*rolC* results in high production of hairy roots. It was verified that *rolB* is vital as it can act on its own to produce hairy roots and *rolA* can do the same but to a lesser extent than *rolB* (Spena et al., 1987).

The leaf explants of *S. stenophylla* were successfully transformed with the two *A. rhizogenes* strains (Figure 5.4C-D and Figure 5.5). Chilton et al. (1982) stated that production of hairy roots is always proof of transformation, unlike the production of callus following transformation by *A. tumefaciens* (Figure 5.4A), which, without selection, could consist of both transformed and untransformed cells (Nilson and Olsom, 1997). The genomic DNA of the hairy roots was, however, isolated and PCR carried out using gene-specific primers for *rolA*, *rolB* and *rolC* to confirm transformation (Fig. 5.5).

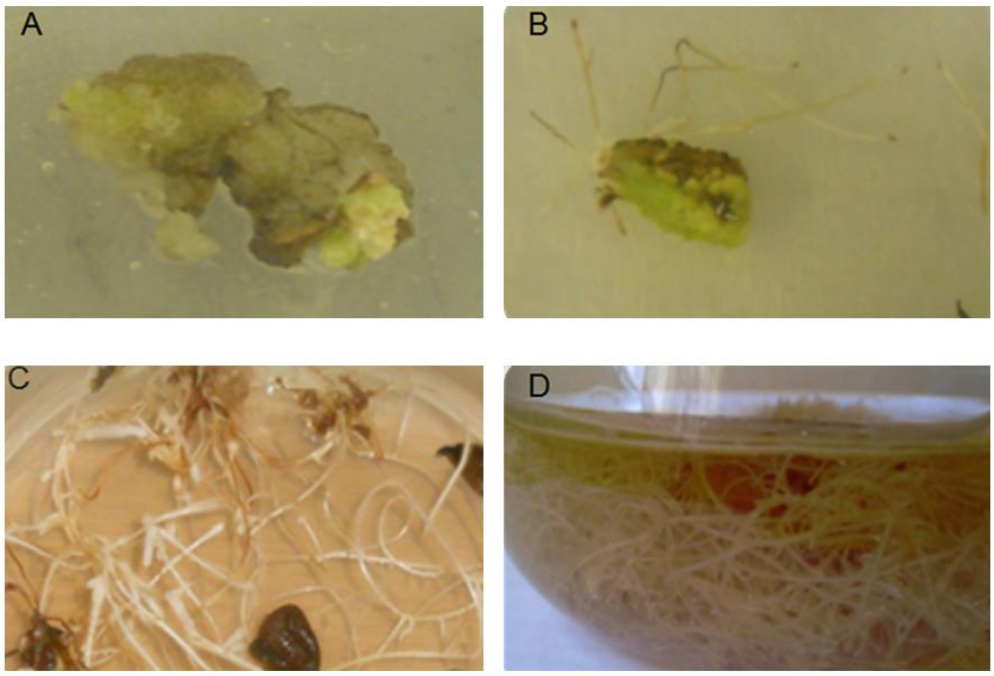

Figure 5.4 A: Putative transformant of *S. stenophylla* producing callus on selection media after transformation using *A. tumefaciens* EHA105 containing pCambia-AtMYB6. B: Hairy roots of *S. stenophylla* in selection media after transformation using *A. rhizogenes* LBA9402 containing pCambia-AtMYB6. C: Hairy roots of *S. stenophylla* after transformation using wildtype LBA9402 *A. rhizogenes* strain. D: Transgenic hairy roots of *S. stenophylla* in suspension culture.

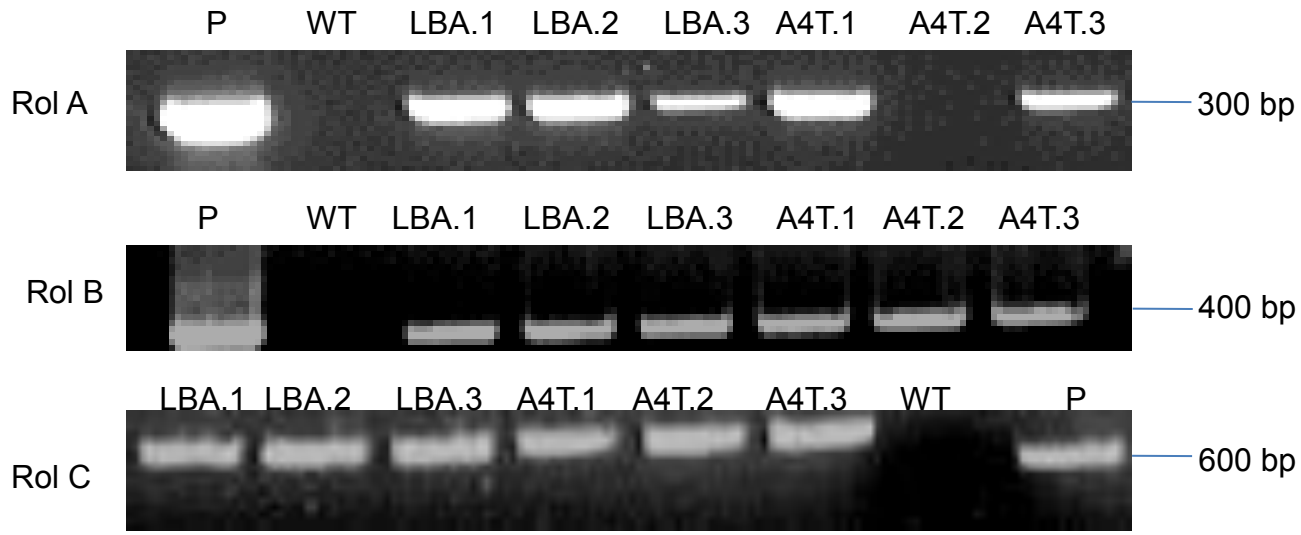

Figure 5.5 The *rol* genes were confirmed in genomic DNA in hairy roots of wildtype *A. rhizogenes* strains A4T and LBA9402in *S. stenophylla*. P: Positive control, pure plasmid extracted from *A. rhizogenes*. WT: Wildtype roots of *S. stenophylla*. LBA.1: First line of hairy roots transformed with *A. rhizogenes* LBA9402, LBA.2: Second line of hairy roots transformed with *A. rhizogenes* LBA9402. LBA.3: Third line of hairy roots transformed with *A. rhizogenes* LBA9402, A4T.

5.3.4 Data analysis of hairy roots from the two wildtype *A. rhizogenes* strains

Of the two *A. rhizogenes* strains tested for their efficacy in transforming *S. stenophylla*, *A. rhizogenes* LBA9402 produced the largest number of hairy roots. This strain resulted in the production of a total of 328 hairy roots, with an average of 41 hairy roots per explant. This was almost 4 times the number produced by the *A. rhizogenes* A4T strain (Table 5.3). Therefore *A. rhizogenes* LBA9402 was chosen as the favoured strain to be used for the transformation of *S. stenophylla* after it was transformed with the plant expression vector pCambia-AtMYB.

Table 5.3 Total number of hairy roots produced by *Salvia stenophylla* explants following transformation by *A. rhizogenes* strains LBA9402 and A4T.

Wildtype strain	Total No. of hairy roots	Mean No. of hairy roots
LBA9402	328	41a ± 10.03
A4T	89	11.125b ± 6.67

Different letters in the same column indicate values that are significantly different at the 95% confidence levels. p=0.02

The production of hairy roots by the two wildtype strains in *S. stenophylla* were significantly different at the 95% confidence level.

5.3.5 Confirming presence of *AtMYB* Transcription Factors in p-Cambia plant expression factor and confirming the presence of pCambia-MYB in *Agrobacterium* to be used for transformation

The transcription factors were successfully incorporated into the plant expression vectors (Figure 5.2) as confirmed using colony PCR. The plant expression vectors were then mobilised into the two *Agrobacterium* strains, *A. tumefaciens* strain EHA105 (Figure 5.6A) and *A. rhizogenes* strain LBA9402 (Figure 5.6B) successfully.

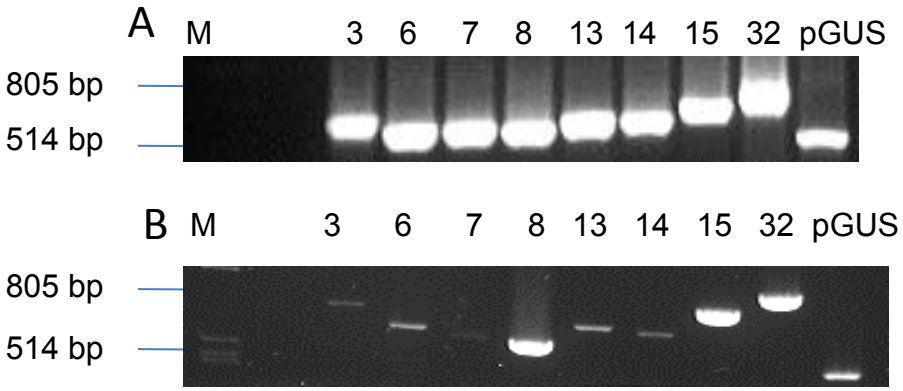

Figure 5.6 A: Colony PCR on the two *Agrobacterium* strains used following transformation with *AtMYB* transcription factors, *A. tumefaciens* EHA105 (A) and *A. rhizogenes* LBA9402 (B) showing all transcription factors successfully moved into the *Agrobacterium.* M:λ*Pst*I marker; numbers represent amplification using gene specific primer pairs for the corresponding *AtMYB* transcription factor, pGUS-positive GUS control as provided in the Gateway® kit.

5.3.5.1 Selection and confirmation of transgenic callus and hairy roots of *S. stenophylla* transformed with pCambia-*AtMYB6* and pCambia-*AtMYB13*

Salvia stenophylla transformed with *A. tumefaciens* containing pCambia-*AtMYB6* resulted in proliferation of callus (Figure 5.4A), for other transcription factors the explants died off on the selection media. For *S. stenophylla* transformed with *A. rhizogenes* only explants transformed with the plant expression vectors harbouring *AtMYB6* and *AtMYB13* resulted in hairy roots proliferation. The other six transcription factors did not result in hairy root production but died off on selection media.

It took 9 months of monthly transformation trials to finally get hairy roots formation in plant discs transformed with *Agrobacterium* which harboured the AtMYB transcription factors 6 and 13, no hairy roots could be generated from the remaining six constructs. As the eight transcription factors used in this study are grouped into two subfamilies and subfamilies of MYB transcription factors have been reported to be functionally redundant (Sanz 2010), it was decided to continue with the two transcription factors which had resulted in hairy roots proliferation in order to save time. *AtMYB6* represented the R2R3MYB transcription factor subfamily IV which represses the phenylpropanoid pathway and *AtMYB13* represented the R2R3MYB

transcription factors subfamilyII, members of which which have been reported to enhance the phenylpropanoid pathway.

The callus and hairy roots produced by the putatively transgenic *S. stenophylla* explants were analysed to verify transformation using PCR (Figure 5.7) and gene expression (Figure 5.8) using RT-PCR (*AtMYB13* gene expression gel not shown). Unfortunately, for callus produced by explants transformed by *A. tumefaciens*, PCR could not show incorporation of the transgenes in all the tested callus from the explants. As a result, the callus was not used for plantlet regeneration and no further work was done with *A. tumefaciens*. Hairy roots from explants transformed using *A. rhizogenes*, on the other hand, were found to be stably transformed and expressed as confirmed by the amplification of the *AtMYB* genes (Figure 5.7 A and B and Figure 5.8) and the three *rol* genes (Figure 5.9 *rol A*, *rolB* and *rolC*) from genomic DNA by PCR.

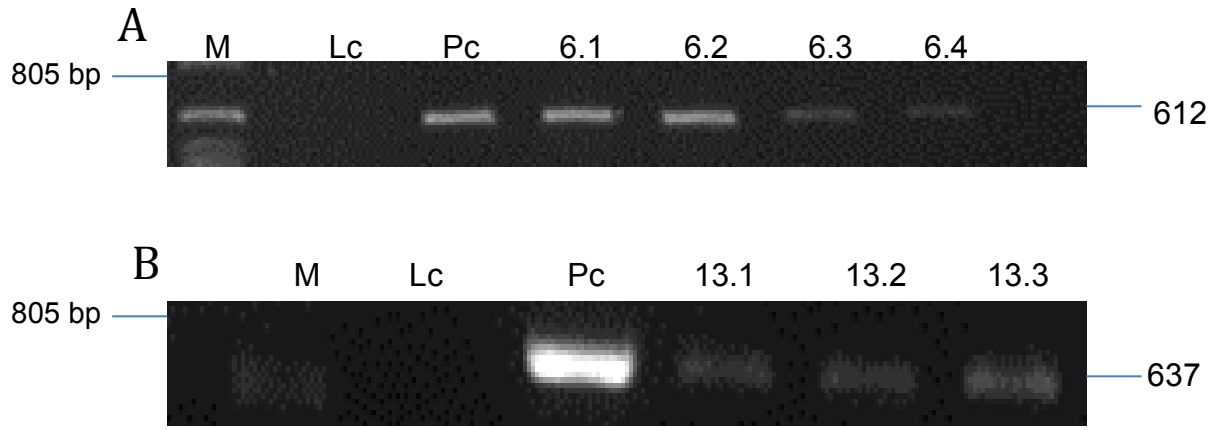

Figure 5.7 *AtMYB6* (A) and *AtMYB13* (B) were successfully incorporated into the genomic DNA of *S. stenophylla.* M: λ*Pst*I marker; Lc: *A. rhizogenes* LBA9402 plasmid without the *AtMYBs* used as negative control, Pc: pCambia plant expression vector containing respective *AtMYB* for the gel used as positive control, the numbers represent the respective *AtMYB* transformation lines which have successfully incorporated the AtMYBs.

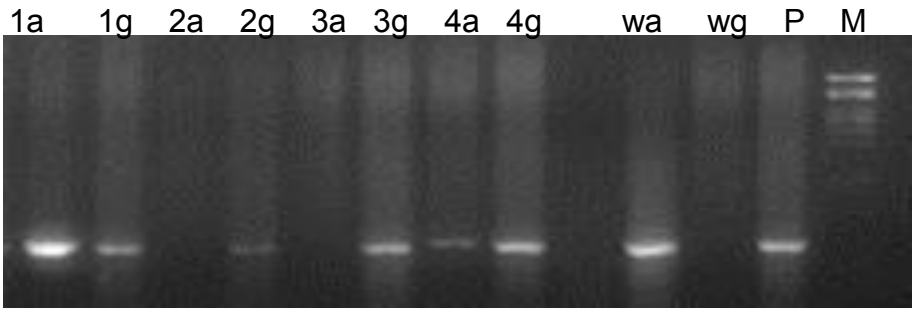

Figure 5.8 *AtMYB6* expressed in cDNA of *S. stenophylla* hairy roots with actin as the housekeeping gene. The numbers represent the clone with a and g representing where actin and gene were loaded respectively. M-λ*Pst*I, P- plasmid as positive control and w-wildtype.

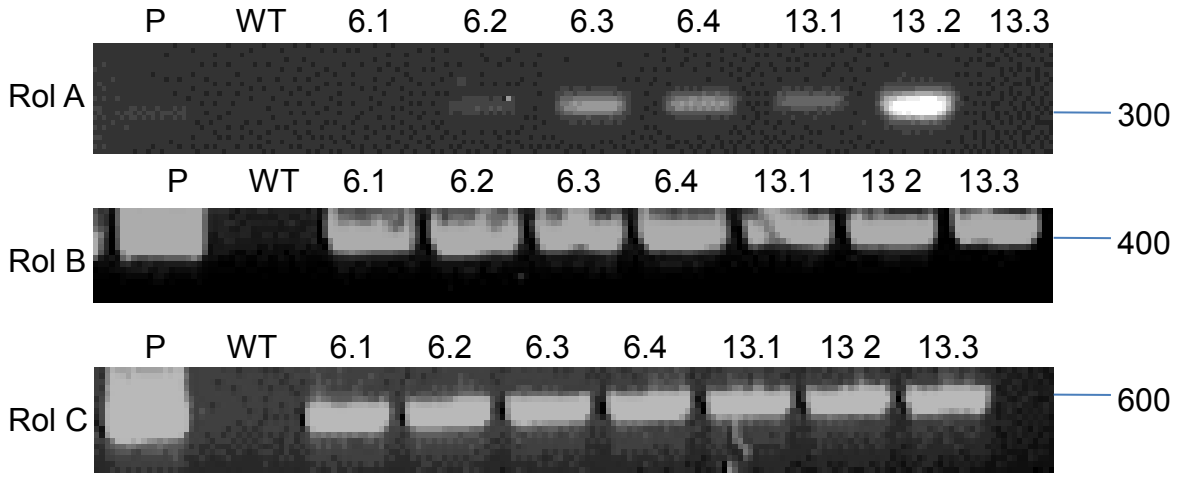

Figure 5.9 The Ri plasmid *rol* genes were effectively transferred into *S. stenophylla*. The *rol* genes were confirmed in genomic DNA in hairy roots of *S. stenophylla* hairy roots transformed with A. *rhizogenes* LBA9402 harbouring *AtMYB*s. P: Positive control plasmid extracted from *A. rhizogenes*. WT: Wildtype roots of *S. stenophylla*. The numbers represent the respective AtMYB transformation lines which have been successfully incorporated into genomic DNA of *S. stenophylla* hairy roots.

The production of hairy roots by the wildtype and transgenic *A. rhizogenes* strains in *S. stenophylla* were not significantly different (Table 5.4.)

Table 5.4 Total number of hairy roots produced by wildtype strain of *A.rhizogenes* LBA9402 and transformed *A. rhizogenes* LBA9402-AtMYB6 and LBA9402-AtMYB13 leaf explants of *S. stenophylla*.

***A. rhizogenes* strain**	**Total number of hairy roots**	**Mean number of hairy roots**
LBA9402-WT	328	29.8a ± 9.1
LBA9402-AtMYB6	248	22.5a ± 9.03
LBA9402-AtMYB13	167	15.1a ± 6.63

The mean values were not statistically different at 95% confidence levels. p=0.6

5.3.6 Phytochemical analysis

Hairy root cultures are able to synthesize root-derived secondary metabolites over successive generations without losing genetic or biosynthetic stability (Giri and Nasaru, 2000). This property can be utilized by genetic manipulations to increase biosynthetic capacity secondary metabolites (Giri and Nasaru, 2000). Hairy roots that have been transformed by genes related to the production of secondary metabolites, provide a promising alternative for the accumulation of secondary metabolites as hairy roots grow at a significantly faster rate than non-transgenic root cultures (Bourgaud et al., 2001).

5.3.6.1 Plant extracts and GC-MS analysis

Most of the metabolites identified in *S. stenophylla* hairy roots for both transgenic and non-transgenic hairy roots (for both wildtype strains) were sugars. Each hairy root produced, even if from the same explant, is regarded as an independent transformation event. Because of this independence, different metabolites were produced by the transgenic lines, even when transformed by the same *Agrobacterium* strain (with or without *AtMYBs*, though there are some similarities.

Table 5.5 Metabolites identified by GC-MS from hairy roots of *S. stenophylla* produced by wildtype strains of *A. rhizogenes* LBA9402 (denoted as LBA) and A4T (denoted as A4T) with metabolites produced by *A. rhizogenes* LBA9402 containing the *AtMYB*s 6 and 13 with numbers representing transgenic lines. RT: retention time. Metabolites were measured in % relative abundance/g FW. The data is an average of two GC-MS analyses.

Metabolites	RT	LBA1	LBA2	LBA3	LBA13-1	LBA13-2	LBA13-3	LBA6-1	LBA6-2	LBA6-3	LBA6-4	A4T1	A4T2	A4T3
Valine	9.77											0.0002		
Benzoic acid	10.61											0.001		
Phosphoric acid	11.11				0.001	0.003	0.01	0.01	0.001	0.01	0.001	0.03	0.004	
Pyroglutamic acid	16.50			0.02	0.03	0.02	0.02	0.02	0.01	0.01	0.01	0.19	0.01	0.02
Leucine, cyclo-	16.51				0.01									
Proline	17.62											0.001		
Lyxose	19.72		0.01		0.003			0.01	0.01	0.01		0.06	0.01	0.01
Erythritol	21.76				0.3									
Glycerol	21.79				2									
Glucose,1,6-anhydro, beta-	21.82				0.72									
Fructose	26.67	0.01			0.05	0.08		0.07	0.22	0.04			0.03	0.01
Psicose	26.68											0.04	0.03	0.02
Sorbose	26.69	0.01	2.62	1.07	0.25	1.14	2.76	0.54	2.67	0.71	0.57	2.62	0.5	0.39
Idose	27.15				0.01							0.62		
Allose	27.16											0.10		
beta-D-Allose	27.40									0.01				
Hexopyranose	27.41											0.004		
Glucopyranose, D-	27.42	0.01	0.01	0.98	0.0001	0.001	0.003	0.01	0.003	0.02	0.01	0.06	0.02	0.003
Galactose	27.55	0.02	1.07	0.14	0.06	0.16	0.19		1.05	1.81	0.28		0.26	0.1
Glucose	27.58	0.016	2.98	1.22	0.5	0.94	1.87	2.37	4.72	3.64	0.48	0.57	0.95	1.2
Mannose	28.22		0.28	0.14	0.02	0.06	0.05	0.25	0.32	0.29	0.12	1.32	0.24	0.1
Galactopyranoside, 1-O-methyl-	29.22				0.02									
Hexadecanoic acid	33.65				0.001					0.002		0.08		

Metabolites	RT	LBA1	LBA2	LBA3	LBA13-1	LBA13-2	LBA13-3	LBA6-1	LBA6-2	LBA6-3	LBA6-4	A4T1	A4T2	A4T3
Galactosamine, N-acetyl-	34.61				0.003							0.01		
Inositol, myo-	34.82		0.03	0.04	0.01	0.05	0.035	0.06		0.01	0.02	0.37	0.01	0.03
Caffeic acid, trans-	37.15									0.002		0.01		
Triethanolamine	40.69				5.68									
Octadecanoic acid	41.48			0.01	0.002		0.003	0.01			0.01	0.20	0.003	0.002
Sucrose	56.89	0.23	5.14	5.95	0.74	0.96	0.92	5.35	1.12	3.02	3.03	14.0	4.82	4.42
Kestose, 6-	56.92		0.39		0.07							0.0003		
Cellobiose, D-	60.13				0.0002									
Galactinol	63.63				0.002	0.002	0.002					0.01		
Campesterol	67.18				8.18									
Stigmasterol	67.46				0.001							0.01		
Sitosterol, beta-	68.15				9.96					0.002			0.01	

Reports from different studies have shown that the most important secondary metabolites in *Salvia* plants are phenolics (Lu and Foo, 2002) as these are the main metabolites responsible for the medicinal properties of these plants. The main phenolics found in *Salvia* are phenolic acids and flavonoids, with phenolic acids being the major constituents (Lu and Foo, 2002). The most important phenolic acid found in *Salvia* is caffeic acid. Caffeic acid plays a central role in the biochemistry of these Lamiaceae plants and occurs either in its true form or, predominantly, in the dimer form as rosmarinic acid (Gerhardt and Schroeter, 1983). It is the building block of a variety of plant metabolites, ranging from the simple monomers to multiple condensation products that give rise to a variety of oligomers (Lu and Foo, 2002) such as lignin. In this study, the two phenolic acids which were identified are caffeic acid and benzoic acid (Table 5.5). Caffeic acid is known for its anti-oxidant, anti-microbial and anti-inflammatory properties (Kamatou et al., 2008), whilst benzoic acid displays anti-microbial properties (Ashraf et al., 2011).

Metabolite analysis of hairy roots of *S. africana–lutea* by Ramogola (2009) transformed using the *A. rhizogenes* LBA9402 wildtype identified caffeic acid and benzoic acid. Figlan (2012) also identified caffeic acid (and its dimer rosmarinic acid) and benzoic acid in hairy roots of *S. runcinata* transformed by the wildtype *A. rhizogenes* strain A4T. In this study, only 1 line of wildtype *A. rhizogenes* A4T hairy roots in *S. stenophyla* produced caffeic acid. Hairy roots from transformation by the *A. rhizogenes* LBA9402 wildtype strain did not contain any caffeic acid or its derivatives. Aerial parts of *S. stenophylla* in tissue culture (Musarurwa, 2012) and from the wild (Kamatou et al., 2006) have also been reported to contain caffeic acid and its derivatives.

Caffeic acid was identified in LBA9402-*AtMYB6* and A4T (Table 5.5). The wildtype A4T contained 5 times more caffeic acid than the LBA9402-*AtMYB6* hairy roots. The *S. stenophyla* hairy roots produced by wildtype *A. rhizogenes* strain LBA9402 and the wildtype *S. stenophylla* normal roots did not contain caffeic acids in this study. *Arabidopsis thaliana MYB6* belongs to the R2R3MYB sub-family IV which also includes *AtMYB3*, *AtMYB4*, *AtMYB7* and *AtMYB32* (Martin et al., 2011). These AtMYBs are negative regulators of general phenylpropanoid metabolism (Jin et al., 2000). The most likely roles for this subgroup of R2R3MYB proteins, as reported by

Martin et al. (2011), is that they all negatively regulate flavonoid biosynthesis, even though they may have different target genes. Over-expression of *AtMYB4* in *Arabidopsis thaliana* in a study done by Jin et al. (2000) resulted in the production of sinapoyl malate being repressed and thus the plants being sensitive to ultra violet light. As *AtMYB4* and *AtMYB6* belong to the same subgroup it was expected that lines over-expressing *AtMYB6* would show reduced or repressed production of some metabolites in the phenylpropanoid pathway as compared to lines over-expressing *AtMYB13*. However, Jin et al. (2000) did report that the over-expression of *AtMYB4* resulted in increased production of the caffeoyl coenzyme A O-methyl-transferse and did not in any way affect the production of caffeic acid O-methyl-transferase, which are both involved in lignin biosynthesis. It is worth noting that, even though they are in the same subgroup, the transcription factors act on different genes in the pathway in different ways, even though in most cases they act as repressors, and this can explain the presence of caffeic acid in the *AtMYB6* transgenic lines whereas it was not detected in the wildtype and in the *AtMYB13* transformants.

Hairy roots of S*. stenophylla* that were transformed with LBA9402-*AtMYB13* did not contain any phenolics (Table 5.5). Studies on *AtMYB13* reported that it is likely a regulator of general phenylpropanoid metabolism, as are *AtMYBB14* and *AtMYB15* (Martin et al., 2011). The absence of phenolics in transformants of *AtMYB13* was not expected as it contradicts the function of its subgroup that they enhance the phenylpropanoid pathway. Dubos et al. (2010) reported that *AtMYB13* is involved in ABA-mediated responses to environmental signals, as is *AtMYB15*. Different growth media have been reported to result in enhanced growth rates and increased metabolite production in *S. miltiorrhiza* hairy roots (Chen et al., 1999). Rosmarinic acid and lithospermic acid were produced in MS-NH_4 (MS-without NH_4NO_3) and 6,7-V (which is a modification of the PRL-4-C medium of Gamborg [1966] by Veliky and Martin [1970]) at higher concentrations than in the clones growing on MS, B5 (Gamborg et al., 1968) and WPM (Woody Plant Media, Lloyd and McCown, 1980). Additionally, secondary metabolites are mainly produced under stressful conditions such as pathogen attack, cold stress, drought stress, etc. In the absence of these stresses, plant cultures might not produce metabolites as there will not be any trigger for their synthesis in the plant. This phenomenon was shown by Wu et al. (2007) where hairy root cultures of *S. miltiorrhiza* produced more tanshinone when co-

cultured with *Bacillus cereus* bacteria. Therefore the low production of secondary metabolites in this study could be due to the conditions in which these hairy roots were cultured, which might not be favourable for production of secondary metabolites.

5.4 Conclusion

Salvia stenophylla was successfully transformed with *A. rhizogenes* LBA9402 and A4T. The *A. rhizogenes* strain LBA9402 was more effective at transforming *S. stenophylla* than A4T at averages of 41 ± 10.03 and 11.125 ± 6.67 respectively and signinificantly so at p value of 0.02. However, if one is interested in the production of more metabolites (especially phenolics) the *A. rhizogenes* A4T strain is recommended. *Arabidopsis thaliana MYB6* and *MYB13* transcription factors were transformed into the genomic DNA of *S. stenophylla* using *A. rhizogenes* LBA9402 strain. The two transcription factors which were over-expressed belong to two subfamilies which work in contrast to each other, as *AtMYB6* is a repressor whereas *AtMYB13* is an enhancer of the phenylpropanoid pathway. It was expected that the lines over-expressing *AtMYB13* would result in increased production of phenolic metabolites as compared to *AtMYB6* lines, but this did not happen. The effect of the MYB transcription factors *AtMYB6* and *AtMYB13* on production of metabolites in *S. stenophylla* hairy roots could not be determined as the different transgenic lines did not give consistent results for the particular transcription factor.

It is proposed that conversion of the hairy roots to generate transgenic plants be carried out in order to better understand the effects of over-expressing the 2 or even all of the eight AtMYB transcription factors on secondary metabolism at a whole plant level. This may be important as the aerial parts of *S. stenophylla* are preferred in traditional medicine and for the extraction of high-value chemicals for various industries.

Chapter 6: General Conclusion

Out of the total of eight *AtMYB* transcription factors used in the study, only five (*AtMYB3*, *AtMYB6*, *AtMYB7*, *AtMYB13* and *AtMYB32*) were successfully verified to be present in the sugarcane callus genome. Plants were regenerated from the transgenic sugarcane callus but they did not survive for more than 2 weeks when acclimatized in the glasshouse. The carbohydrates (glucose, fructose, sucrose and starch) content of the transgenic sugarcane callus, either on solid or in suspension culture media, varied from one clone to another for a given *AtMYB*. However, clones of *AtMYB13* overall resulted in increased sucrose production and all *AtMYB7* transgenic lines resulted in sucrose reduction. Clones of *AtMYB7 and AtMYB13* resulted in more starch production. Other transcription factors gave inconsistent results. The metabolites identified from the GC-MS data were mainly sugars followed by amino acids. The effect of overexpressing the *AtMYB* transcription factors 3,6,7,13 and 32 in overall metabolite production of sugarcane could therefore not be determined due to the inconsistent data.

An easy and efficient micropropagation system was established for *Salvia repens*. The germination protocol, shoot multiplication, culture induction and acclimatisation protocols could all be done in two months, producing material that can be used for metabolite analysis. The metabolites produced varied according to locality being *in vitro* or *ex vitro*. Both the volatile and non-volatile metabolites that were identified and quantified using GC-MS proved the medicinal value of *S. repens*. The plant extracts have metabolites that have anti-bacterial, anti-inflammatory, anti-oxidant and even anti-cancer activities. Most importantly *S. repens* metabolites were extracted and tested against bacteria and fungi. The extracts of *S. repens* were tested against fungi for the first time in this study according to our knowledge. The *S. repens* extracts were shown to be highly active against *Fussarium* species over a long period of time (4-5 d) at MIC values lower than 1 mg/ml as recommended by van Vuuren (2008). The potency of the metabolites even though shown to be more in glasshouse plants was still significant even in tissue cultured plants. This makes the production of *S. repens in vitro* a viable option for commercialization purposes.

Production of hairy roots was established for *S. stenophylla* using *Agrobacteria* strains LBA 9402 and A4T for the first time in this study. *Agrobacterium rhizogenes*

LBA9402 was more efficient at producing hairy roots than the A4T strain. Phytochemically, A4T produced more metabolites as compared to LBA9402 in terms of variety and relative abundance, with the GC-MS method that was used in the study. *Arabidopsis thaliana* MYB transcription factors were cloned into *S. stenophylla* successfully via *Agrobacterium*-mediated transformation. The metabolites that resulted from these transgenics did not really differ that much from the wild type hairy roots. However, it was evident that A4T *Agrobacterium* strain, even though it had the lowest transformation frequency, had greater effects on metabolite production compared to LBA9402 strain. Further work testing the biological activity of the hairy roots for both wildtype and transgenic clones (containing the *AtMYB6* and *AtMYB13*) is worthwhile as this will give an indication as to whether it is viable to produce the hairy roots or not. The hairy roots could be compared to the activity of the aerial parts of the plant both wildtype and transgenic in order to be able to conclude on the effects of *AtMYB6* and *AtMYB13* on metabolite content of *S. stenophylla*.

In general, the biological activity of the *AtMYB* transcription factors which were successfully cloned into the two plants transformed in this study (sugarcane and *S. stenophylla*) have not been established or ascertained compared to previous studies by other groups such as Prof Cathie Martin's group (John Innes Center, United Kingdom). Further work cloning these *AtMYBs* in other plants need to be carried out in order to fully determine and conclude on the effects of these transcription factors in plants, whether in heterologous or homologous systems.

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Appendix 1 Metabolites identified by GC-MS from transgenic sugarcane calli over-expressing *AtMYB3*, *AtMYB6*, *AtMYB7*, *AtMY13* and *AtMYB32* transcription factors grown on solid media. The numbers represent transgenic lines; wt-Wildtype sugarcane callus. Metabolites were measured in % relative abundance/g FW.

Metabolite	WT	3_1	3.3	3_4	3_5	6.1	6_2	6_8	6_9	6_11	6_12	7_1	7_4	7_6	7_8	7_9	13_1	13_2	13_7	13_8	32_4	32_5	32_6	32_7	32_10
Sorbose	0	0.17	0	4.76	0	3.51	0	12.49	0	7.011	0	7.14	16.84	5.58	13.77	3.16	0.41	6.53	22.72	0	0	3.12	0	10.73	7.84
Galactose	0	0.95	0	3.43	4.08	0	1.53	0	0	12.46	6.25	6.03	0	8.31	5.54	4.62	1.46	5.79	0	4.05	0	0	0	10.74	0
Glucose	3.67	1.34	16.50	24.07	3.67	12.96	0	18.8	0	0	9.78	17.78	23.87	19.10	25.70	44.64	39.18	24.74	36.41	0	19.10	28.01	33.31	18.85	33.87
Cellobiose, D-	0	10.55	0	1.47	0.40	0.44	0	0	0	0	0.46	0.53	0	0.24	0	0.14	1.44	0.64	0	0	0	1.29	0	0.366	0
Glucose, 1,6-anhydro, beta-	0	10.55	0	2.41	0	1.47	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Inositol, myo-	2.09	4.54	1.21	0	2.81	1.47	0	1.59	3.93	0	1.15	0.79	0.04	2.05	1.19	2.70		0.25	0	0	0.53	3.91	3.94	3.12	0.30
Sucrose	29.76	17.05	0	34.59	43.16	16.89	33.34	24.83	45.44	30.31	22.21	11.75	0.19	11.06	1.50	3.78	22.83	18.73	0	45.18	32.78	16.41	24.19	5.26	6.38
Fructose	1.64	0	1.18	0	0.12	3.69	0	10.04	0	0	4.81	6.58	5.36	4.84	3.19	4.53	2.42	0	10.18	0	0	0	0.62	5.81	0
Idose	4.30	0	7.22	6.90	0	15.32	0	0	0	0	0	3.32	3.79	10.28	4.96	4.87	0	14.33	1.25	0	0	11.22	0	0	13.83
Mannose	0.49	0	0	2.74	0	1.47	0	0	0	0	1.06	2.61	2.91	0	3.69	0	2.53	2.47	2.97	0	0	2.97	0	2.94	4.59
Valine	0	0	0	0	0	0	0	2.33	0	5.94	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phosphoric acid	0	0	0	0	0	0	0	2.06	0	6.30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glucopyranose, D-	0	0	0	0	0	0	0	3.43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Isoleucine	0	0	0	0	0	0	0	0	0	1.14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Serine	0	0	0	0	0	0	0	0	0	1.19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hexadecanoic acid	0	0	0	0	0	0	0	0	0	6.33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Psicose	0	0	0	0	0	0	0	0	0	0	3.03	1.49	0	0	2.08	0	0	0	0.96	0	0	0	0	0	2.31
Kestose, 1-	0	0	0	0	0	0	0	0	0	0	3.05	0	0	0	0	0	0	1.28	0	4.23	0	0	0	1.25	0
Tagatose	0	0	0	0	0	0	0	0	0	0	0	4.52	2.15	0	0	0	0	0	0	0	0	0	0	2.97	1.80
Sphingosine	0	0	0	0	0	0	0	0	0	0	0	0.59	0	0	0	0	0	0	0	0	0	0	0	0	0
beta-D-Fructofuranosyl-(2,1)-be	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.54	0	0	0	0	0	0	0	0	0
Kestose, 6-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.91	0	0	0	0	0	0	0	0	0
beta-D-Galactopyranoside, 1-is	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.56	0	0	0	0	0	0	0	0
Gallic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01	0	0	0	0	0	0

Appendix 2 Metabolites identified by GC-MS from transgenic sugarcane calli over-expressing *AtMYB3*, *AtMYB6*, *AtMYB7*, *AtMY13* and *AtMYB32* transcription factors grown in liquid media. The numbers represent transgenic lines; wt-Wildtype sugarcane callus. Metabolites were measured in % relative abundance/g FW.

Metabolite	WT	3_1	3_4	3_2	3_3	6_8	6_9	6_11	6_12	7_1	7_6	7_8	7_9	13_1	13_2	13_6	13_8	32_4	32_5	32_6	32_8
Sorbose	15.56	14.38	7.89	12.30	12.12	15.01	0.06	0.02	8.52	22.90	22.06	22.65	15.16	26.97	21.74	11.81	9.15	2.42	11.21	11.77	7.11
Galactose	32.18	24.58	13.86	10.52	9.14	22.21	0.10	0.03	16.38	10.68	8.01	8.31	22.40	8.97	16.60	12.19	9.45	11.13	22.88	14.92	21.06
Glucose	29.72	26.09	14.15	24.49	17.82	24.90	0.10	0.04	14.45	28.37	32.37	32.96	20.74	36.91	40.47	12.20	9.45		8.87	16.75	0
Inositol, myo-	0.10	2.34	2.72	7.50	1.64	8.65	0.01	0.001	9.04	3.24	5.43	4.29	3.18	3.38	0.55	15.38	11.91	1.44	3.31	2.52	1.95
Sucrose	4.21	11.39	45.81	24.24	35.50	11.95	0.17	0.02	36.59	5.49	4.04	6.77	10.48	0	0	0	19.05	72.35	27.24	33.94	34.44
Fructose	0	0	2.16	0	0	0	0	0	0	7.63	6.18	0	0	0	0	0	9.15	2.35	0	0	9.92
Mannose	0	0	0	0	0	0	0	0	0	0	1.57	0	0	0	0	0	0	0	0	0	0
Valine	0.22	0.91	0.64	1.08	0.44	0.19	0.001	0	0	0.09	0.14	0.11	0.11	0.76	0.19	4.39	3.39	0.81	1.43	0.44	0
Phosphoric acid	1.68	1.23	2.65	1.82	2.27	1.14	0.005	0	2.95	0.76	0.76	0.79	0.56	0	0.97	4.90	3.79	2.82	3.23	4.52	0
Isoleucine	0.10	0.43	0.39	0.35	0.22	0	0	0	0.19	0.02	0.06	0.13	0.02	0.33	0	5.11	3.96	0.12	0.58	0.35	0
Serine	0.04	0.21	0.24	0	0.19	0	0	0	0	0	0	0	0	0	0	0	0	0.11	0.38	0.29	0
Hexadecanoic acid	0.22	0	0	0	0.47	0.82	0	0	0	0.87	0	0	1.11	0	0	0	0	0	0.54	0.69	0
Gallic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Norleucine	0	0.73	0.52	0	0.39	0.12	0	0	0	0	0	0	0	0	0	0	3.80	0	1.34	0.58	0
Threonine	0	0.16	0.15	0	0.17	0	0	0	0	0	0	0	0	0	0	0	0	0	0.53	0.30	0
Glycine	0	0	0.08	0	0.10	0	0	0	0.27	0	0	0	0	0	0	0	0	0	0.18	0.23	0
Proline	0	0	0	0	0.06	0	0	0	0	0	0	0	0	0	0	5.16	0	0		0.06	0
Lyxose	0	0	0	0	0.15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Octadecanoic acid	0	0	0	0	0.21	0.36	0	0	0	0.41	0	0	0.38	0	0	0	0	0	0	0	0
Butanoic acid, 4-amino	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0