CARDIFF UNIVERSITY

PhD Thesis

Topic: Towards Optimisation of L-DOPA synthesis in *Mucuna pruriens.*

By

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(Cardiff School of Biosciences)

This thesis is submitted to Cardiff University in partial fulfilment of the requirements for the degree of **DOCTOR OF PHILOSOPHY (BIOSCIENCES).**

AUGUST 2014.

APPENDIX I.

DECLARATION.

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

DEDICATION.

I dedicate this work to my parents, my academic mentors and all those who have supported me to complete my PhD program.

ABSTRACT.

This study examines the potential for increasing natural L-DOPA drug biosynthesis in *Mucuna pruriens* by silencing or "knocking down" expression of putative DOPA/tyrosine decarboxylase (Mp-ty/ddc) *in situ*. Mp-ty/ddc codes for DOPA/tyrosine decarboxylase (Mp-TY/DDC) which converts L-DOPA to dopamine in plants. The hypothesis of the work was that silencing the Mp-ty/ddc gene would result in accumulation of L-DOPA in the plant tissues. This work involved isolation and characterisation of 1.73 kb putative full-length ORF of Mp-ty/ddc. The gene showed 74% homology with TY/DDC protein alignments of other plants in the same taxa, although no enzyme activity was detected when the gene product was heterologously expressed. In addition, a protocol was developed for *Agrobacterium* mediated transformation of *M. pruriens* so as to be able to manipulate expression of the DOPA genes *in situ*. The cotyledonary nodal and hypocotyl tip explants regenerated shoots on M.S media supplemented with 50 µM BA, 0.5 µM NAA and 50 mg l^{-1} kanamycin selection also the *npt*II transgene was detected by PCR. The *Agrobacteria* strains GV3101 harbouring a pGREEN vector and carrying an Mp-ty/ddc antisense were used for the plant transformation experiments. Further work showed that the Mp-ty/ddc gene copy number was 1, the gene expression was highest in roots and stems, followed by seeds and was very low in leaves. On the other hand, L-DOPA-content in seeds was 17-fold higher relative to leaves and 15 fold relative to stems and roots.

LIST OF ABBREVIATIONS AND ACRONYMS.

Amp: Amperes.

BA: 6-Benzylaminopurine.

BCIP: 5-bromo-4-chloro-3-indolyl phosphate.

Bp: Base pairs of Nucleotides.

ddc: DOPA decarboxylase gene.

DDC: DOPA decarboxylase enzyme

DEPC: Diethylpyrocarbonate.

DNA: Deoxyribonucleic acid.

DOPA genes: Genes in the L-DOPA synthesis and metabolic pathway.

***E. coli*:** *Escherichia coli.*

EDTA: Ethylenediaminetetraacetic acid

eGFP: Enhanced Green Florescent Protein..

ESTs: Expressed sequence tags.

Epi: Epinephrine

HPLC: High performance liquid chromatography

IPTG: Isopropyl-ß-D-1-thiogalactoside.

LB: Luria Bertani medium.

L-DOPA: L-3, 4-dihydroxyphenylalanine.

mM: Millimoles.

mRNA: Messenger ribonucleic acid.

Mp-TY/DDC: Putative DOPA/tyrosine decarboxylase enzyme extracted from *Mucuna pruriens.*

Mp-ty/ddc: Putative DOPA/tyrosine decarboxylase gene isolated from *Mucuna pruriens.*

Mp-TYOH: Putative tyrosine hydroxylase enzyme from *Mucuna pruriens.*

Mp-tyoh: Putative tyrosine hydroxylase gene from *Mucuna pruriens.*

MS: Skoog and Murashige basal nutrient media.

MSD4X2: MSO, NAA, 6-BAP (ref above).

MSO: Skooge and Murashige basal salts x1, Sucrose (3% w/v) (and for solid, Agar (1% w/v)).

NAA: Naphthalene acetic acid.

NBT: Nitro blue tetrazolium.

NCBI: National Council for Biotechnological Information.

nM: Nanomolar

PCR: Polymerase Chain Reaction

PD: Parkinson's disease.

RCF: Relative centrifugal force.

RH: Relative humidity.

RNA: Ribonucleic acid.

RPM: Revolutions per minute.

qRT-PCR: Quantitative Real-time Polymerase Chain Reaction.

SDS: Sodium dodecylsulfate.

SSC: Tri-sodium citrate.

TPL: Tyrosine phenol-lyase.

TY/DDC: DOPA/tyrosine decarboxylase enzyme

ty/ddc: DOPA/tyrosine decarboxylase gene

Tyr: Tyrosine hydroxylase.

X-gal: 5-bromo-4-chloro-3-indolyl1-ß-D-galactopyranoside

ACKNOWLEDGEMENTS.

I extend my sincere gratitude to my academic mentors/Principal investigators; Dr Hillary Rogers and Dr Carsten Muller for their intellectual generosity, excellent supervision and support which has enabled me to complete my PhD study program. In the same vein I extend special to my advisor/mentor Prof James Murray for his distinguished technical advice and support. In addition, I thank Dr Barend de Graaf, Dr Dafydd Jones, staff at Bristol University School of Biological Sciences and colleagues in my PhD study research group at Cardiff University School of Biosciences for the support and advice. I am grateful to the Director Post graduate studies, Prof Hellen White-Cooper, Prof Paul Kemp and the school administration for the enormous support all through my PhD study program. Last but not least, I thank my sponsor The Islamic Development Bank Merit Scholarship office, my parents Dr and Mrs Kirunda Kivejinja, my family and friends for the financial and moral support that has enabled me to pursue and complete my PhD study Program.

CONTENTS

CHAPTER 1: GENERAL INTRODUCTION.

1.1: Introduction.

L- 3, 4-Dihydroxyphenylalanine (L-DOPA, Figure 1.1) is a naturally occurring amino acid produced by animals and has been detected in a few species belong to 44 plant families (Kuklin and Konger, 1995). In plants L-DOPA is produced as an allelochemical to deter competition with other plants and as anti-herbivore (Awang et al., 1997; Nishihara et al., 2005). L-DOPA and is a leading drug used in the treatment of Parkinson's disease (Jaunarajs et al., 2011).

Parkinson's disease is a clinical condition where the brain loses control over movement of motor muscles often leading to body tremors and uncoordinated movement of body limbs (Jaunarajs et al., 2011). The loss of coordination control by the brain is caused by degeneration of dopamine production by the Substantia Nigra (Figure 1.2) in the basal ganglia (midbrain) and as a consequence disables neurotransmission across the synaptic junction between the motor and dopaminergic neurons in the brain (Chan et al., 2010). In the treatment of Parkinson's disease, L-DOPA is administered to the patient intravenously or orally and transferred through the blood circulatory system to the dopamine deficient cells in the brain where it is converted by DOPA decarboxylase to dopamine and used for neurotransmission across synapses with motor neurons (Swiedrych et al., 2004). L-DOPA unlike dopamine is capable of crossing the blood brain barrier to enter the dopamine deficient cells and is thus used as a drug for treatment of Parkinson's disease (Swiedrych et al., 2004; Jaunarajs et al., 2011).

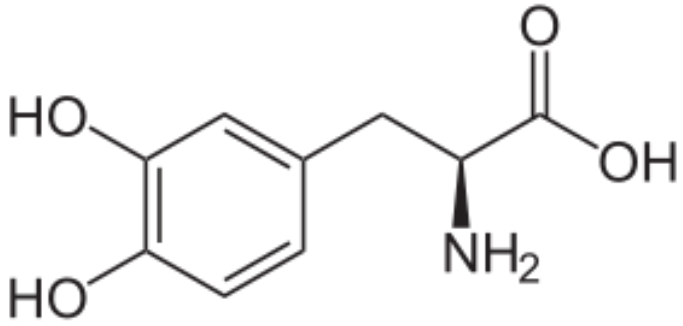


Figure 1.1 chemical structure of L-DOPA. It is formed by addition of a hydroxyl group at position-6 on an L-tyrosine molecule.

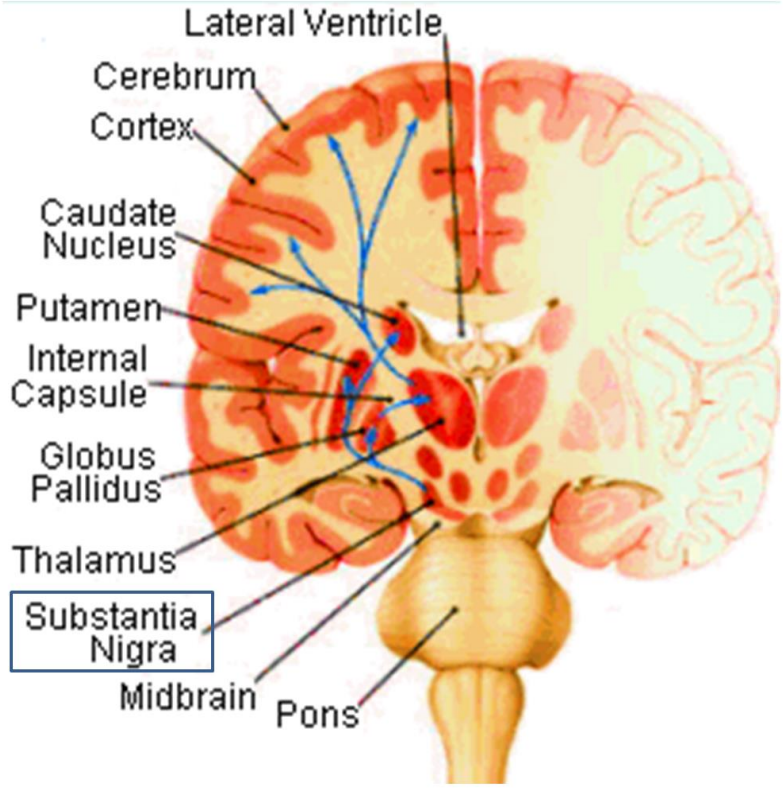


Figure 1.2 Longitudinal section through the human brain. The Substantia Nigra produces dopamine which is then transported to synaptic regions of motor neurons in the cortex region. This diagram was adapted from the website: http://gofree.indigo.ie/~pdpals/pdn1.htm.

Besides treatment of Parkinson's disease, L-DOPA is reported to be effective in the management of DOPA-responsive dystonia and that 750 mg/day of L-DOPA administration caused complete symptomatic relief and improvement in the patients (Rajput et al., 1994). Figure 1.1 shows the chemical structure of L-DOPA.

1.2: Sources of L-DOPA.

1.2.1: Industrial synthesis of L-DOPA.

Commercial L-DOPA is largely produced by chemical synthesis as described in the method by Knowles et al., (1977). Other sources produce smaller quantities of the L-DOPA possibly due to low enzymatic protein production levels than the target of 10 mg l^{-1} required for sustainable commercial scale biosynthesis to meet the desired profit margins (Xu et al., 2011). The initial steps for large scale chemical synthesis of L-DOPA using the method described by Knowles et al., (1977) involve a reaction between precursors; 3-alkoxy-4-hydroxybenzaldehyde (derived from natural vanillin) and acetylglycine in the presence of sodium acetate to form 2-methyl-4-(3'-alkoxy-4'-acetoxybenzal)-5-oxazolone (Figure 1.3).

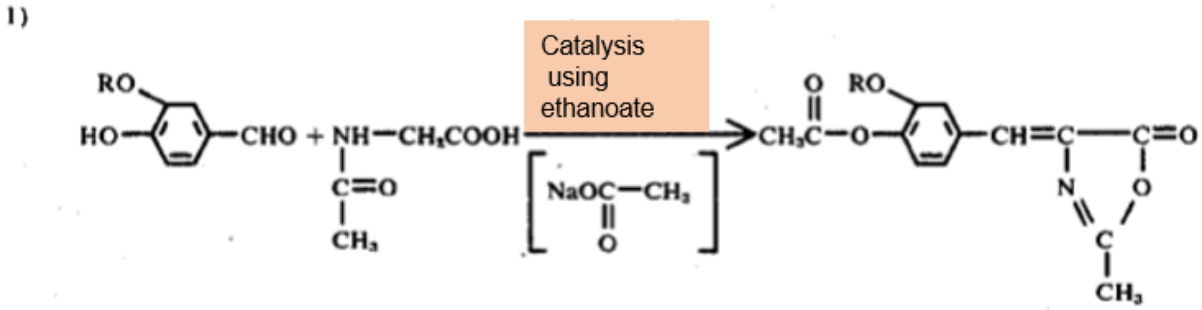


Figure 1.3 Synthesis of 2-methyl-4-(3'-alkoxy-4'-acetoxybenzal)-5-oxazolone by catalytic condensation of precursors 3-alkoxy-4-hydroxybenzaldehyde (derived from natural vanillin) and acetylglycine. R denotes an alkyl group of 1 - 3 carbon atoms (Diagram adapted from http://www.google.com/patents/US4005127 with slight modification).

The resulting 2-methyl-4-(3'-alkoxy-4'-acetoxybenzal)-5-oxazolone is then subjected to mild hydrolysis (Figure 1.4) to obtain α-acetamido-4-hydroxy-3-alkoxy-cinnamic acid acetate. The acetyl group in the 4-position on the benzal group facilitates recovery of the L-enantiomer hence should not be lost during hydrolysis (Knowles et al., 1977).

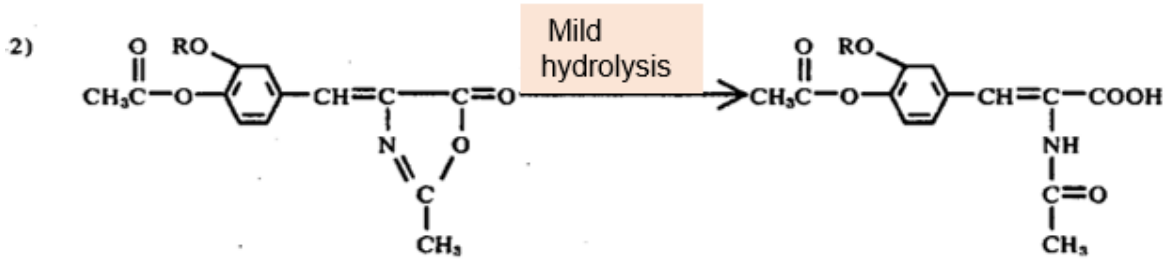


Figure 1.4 Synthesis of intermediate product α-acetamido-4-hydroxy-3-alkoxy-cinnamic acid acetate by hydrolysis. R denotes an alkyl group of 1 - 3 carbon atoms. (Diagram adapted from http://www.google.com/patents/US4005127 with slight modification).

α-acetamido-4-hydroxy-3-alkoxy-cinnamic acid acetate is then converted to L-N-acetyl-3-(4-hydroxy-3-alkoxyphenyl)-alanine acetate (major product) and the D enantiomer (minor product) by catalytic asymmetric hydrogenation (Figure 1.5).

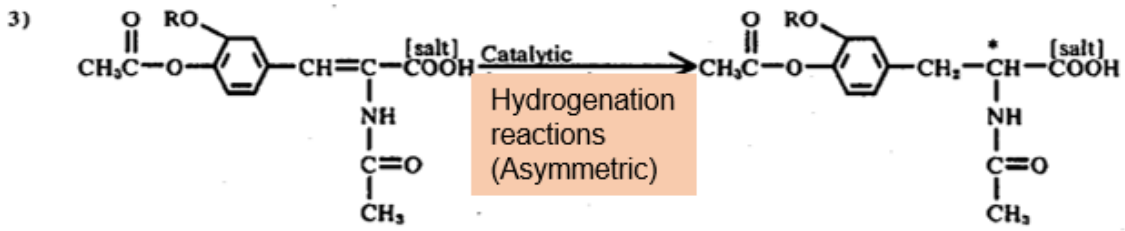


Figure 1.5 Synthesis of N-acetyl-3-(4-hydroxy-3-alkoxyphenyl)-alanine acetate by catalytic hydrogenation of α-acetamido-4-hydroxy-3-alkoxy-cinnamic acid acetate. R denotes an alkyl group of 1 - 3 carbon atoms. (Diagram adapted from http://www.google.com/patents/US4005127 with slight modification).

The L enantiomer of N-actetyl-3-(4-hydroxy-3-alkoxyphenyl)-alanine acetate is purified from the racemic mixture by crystallisation (Figure1.6). Using this method the purified product contained 98% L and 2% D enantiomer (Knowles et al., 1977).

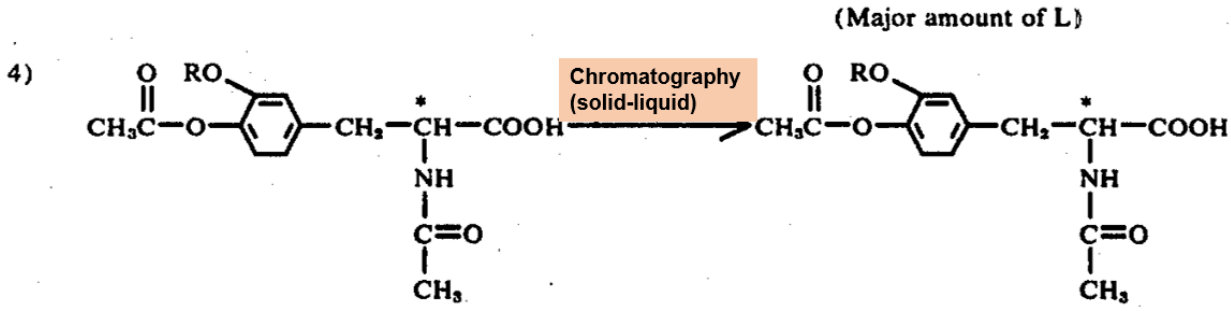


Figure 1.6 Purification of L enantiomorph of N-actetyl-3-(4-hydroxy-3-alkoxyphenyl)-alanine acetate from the mixture containing the D enantiomorph by crystallisation. R denotes an alkyl group of 1 - 3 carbon atoms. (Diagram adapted from http://www.google.com/patents/US4005127 with slight modification).

The L enantiomer is hydrolysed to remove acetyl groups to form L-3-alkoxy-tyrosine or by strong hydrolysis of the L enantiomorph wherein the alkyl group at position-3 on the phenyl ring is also removed resulting in formation of L-DOPA (Figure 1.6).

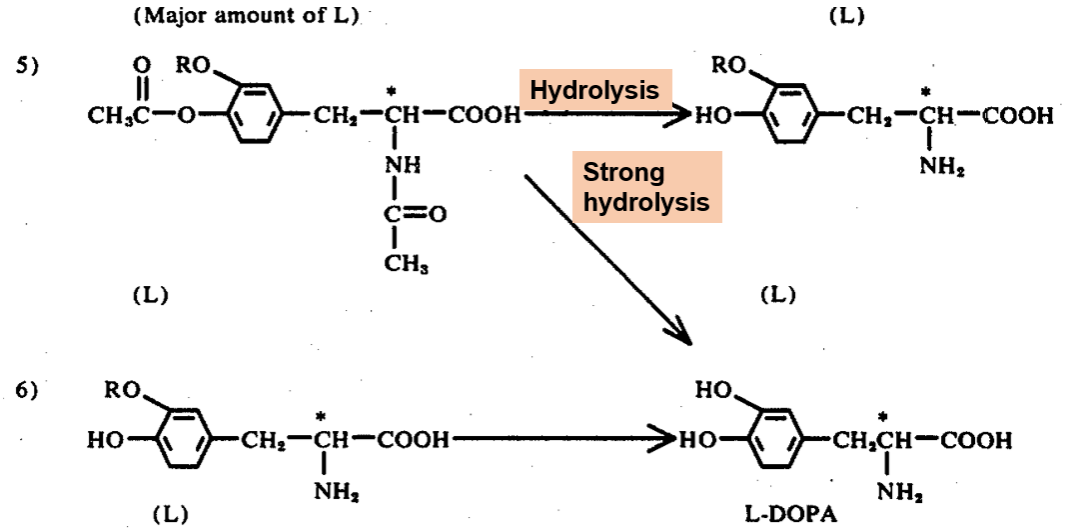


Figure 1.6 Hydrolysis of the N-actetyl-3-(4-hydroxy-3-alkoxyphenyl)-alanine acetate-L to L-3-alkoxy-tyrosine and L-DOPA. (Diagram adapted from http://www.google.com/patents/US4005127 with slight modification).

1.2.2: Natural sources of L-DOPA.

The natural sources of L-DOPA are diverse (See Table 1.1) (Facchini and De Luca, 1994; Patil et al., 2013). L-DOPA is postulated to be produced and in low concentration by about 1000 plant species belonging to the 135 taxonomic families which produce benzylisoquinoline alkaloids (Facchini, 2001). In plants, L-DOPA is converted to dopamine, itself a distant precursor for morphine and benzylisoquinoline alkaloids (Facchini, 2001). In animals on the other hand L-DOPA and dopamine are precursors for the catecholamines; norepinephrine and epinephrine which control the sympathetic and parasympathetic homeostatic responses (Cheng et al., 1996).

Natural sources produce pure L-DOPA or other pharmaceutical products but often in smaller quantities than required for commercial extraction. In light of the above, natural products provide chemical models and formulae which are then used for large scale chemical synthesis (Farnsworth, 1985; Xu et al., 2011).

However, total elimination of minor impurities from synthetic products is almost unattainable and chemically synthesised L-DOPA is reported not to exceed 98% purity and is contaminated with D-DOPA which could not be removed by downstream processing as discussed in Section 1.2.2 below. These minor impurities are postulated to account for some of the side effects experienced by patients in the long term (Daughton and Ruhoy, 2013; Schuster, et al., 2005). In light of the above, efforts have been made to improve the quantity of L-DOPA and other products produced by natural sources. The approaches include; heterologous expression of product biosynthesis pathway genes by using bacteria or yeast cells

and manipulation of the metabolic pathway genes *in situ* to create transgenics which produce higher L-DOPA levels among other strategies as further discussed in Section 1.5 below.

Table 1.0 shows the quantity of L-DOPA produced by different natural sources (This table was adapted with modification from Patil et al., 2013).

Natural sources	**Yield of L-DOPA**
Enzymatic synthesis	
E. intermedia cells (polyacrylamide gels)	5.4 $g \cdot l^{-1}$
E. intermedia cells (carrageenan gel)	7.8 $g \cdot l^{-1}$
Mushroom tyrosinase (Nylon 66)	0.143 $g.l^{-1}$
Chitosan flakes	
Non optimized batch reaction	44.86 mg·l−1·h−1
Optimized batch reaction	54 $mg \cdot l-1 \cdot h^{-1}$
Mushroom tyrosinase (zeolite)	36 $mg \cdot l-1 \cdot h^{-1}$
Cu-alginate	4.5 $mg \cdot l-1 \cdot h^{-1}$
Fungi	
Aspergillus oryzae (mutant)	1.28 mg/ml
Aspergillus oryzae	1.28 mg/ml
Aspergillus oryzae UV7 (double mutant)	1.28 mg ml
Aspergillus oryzae	1.86 mg/ml
Aspergillus oryzae ME2 (Illite)	1.686 mg/ml
Aspergillus oryzae ME2 (Celite)	0.428 mg/ml
Aspergillus oryzae IIB-6	1.34 mg/ml
Acremonium retilum	0.89 mg/ml
Aspergillus niger	0.365 mg/ml
Yeast	
Yarrowia lipolytica NRRL-143	2.96 mg/ml
Egyptian halophilic black yeast	66 ug/ml

Bacteria	
Vibrio tyrosinaticus	4 mg/ml
Pseudomonas melanogenum	8 mg/ml
E. coli W(ATCC 11105) (p-hydroxyphenyl acetate 3-hydroxylase)	48 mM in reaction mixture
Bacillus sp. JPJ	0.497 mg/ml
Recombinant *Erwinia herbicola* cells strain AJ2985	15 g/l/h
Brevundimonas sp. SGJ	3.81 mg/ml
Brevundimonas sp. SGJ	3.361 mg/ml
Plants	
Mucuna atterrima	4.5%
Vicia faba (Fava bean)	3.4 mg/g Dry weight
Stizolobium hassjoo	2 g/l
Mucuna pruriens	3.54% Dry weight
Mucuna pruriens var utilis (velvet bean)	6.36% Weight by weight
Portulaca grandiflora	48.8 mg/l/h
Mucuna monosperma	5.48% Dry weight

In the table above, the range for L-DOPA production by enzymatic synthesis was reported to be 0.14 to 7.8 g l^{-1} while that for batch reactions was 44 to 54 mg l^{-1} h^{-1}. On the other hand, L-DOPA production by fungi ranged from 0.36 to 1.67 mg l^{-1}, while that in plants ranged from 3.5 to 5.4% of total dry weight (DW). The maximum L-DOPA production by from bacterial cell cultures was 48 mM per reaction mixture.

Among the natural sources of L-DOPA, *Mucuna pruriens* was selected for this work towards optimization of L-DOPA biosynthesis for commercial extraction because it is one of the few plant species which produce fairly high quantities of L-DOPA (5.4% DW) and 6.8% weight by weight (Wichers et al., 1994).

1.2.3: A brief account of *Mucuna pruriens* – a potential bio-factory for L-DOPA.

M. pruriens is a diploid (2n= 22), herbaceous tropical plant originally from Asia (India and China) (Duke, 1981; Adepoju and Odubena, 2009). The common English names of *Mucuna* are cowitch, cowhage and velvet bean. According to the Integrated Taxonomic Information System (ITIS, serial number 26797), *Mucuna pruriens* is classified under kingdom- Plantae, Super division-Spermatophyta (seed plant), division-Magnoliophyta (flowering plant), class-Magnoliopsida (dicot), order-Fabales, family-Fabaceae (pea family), sub-family-Faboideae, tribe-Phaseoleae, genus-*Mucuna* and species-*Mucuna pruriens* L. The plants were reported to grow on moist soils, flower in short-day photoperiods under natural conditions with vernalisation as a pre-requisite for flowering (Duke, 1981). The process is stimulated by cooler night temperatures below 21˚C (Hartkamp et al., 2002). The vine cultivar grows up to 15 metres long and flowers after 5 months while the non-vine cultivars have a life-cycle ranging from 100 to 300 days (Duke, 1981). Seed supply laboratories market different cultivars of *M. pruriens* under brand names which describe the seed's physical and growth properties such as the "90-day", "Bush echo", "Bishop black", "Tropical", "Early maturing" and the "vine" cultivars. The "90 day" non-vine cultivar of *M. pruriens* (Figure 1.7) adapted better to growth in the greenhouse and was thus selected for detailed study in this work.

The major chemical components of economic interest produced by *M. pruriens* include proteins-27%, L-DOPA, hallucinogenic tryptamines, phenols and tannins (Ravindran and Ravindran, 1988; Awang et al., 1997). Manyam et al. (1995) reported the common uses of *Mucuna* as a food with large protein reserves, an anti-Parkinson's drug, an aphrodisiac, a mental alertness enhancer, an anti-diabetic, and

a beverage used to make “South American Nescafe” (Ravindran and Ravindran, 1988), while Burkill et al. (1966) reported that *M. pruriens* produces fairly high quantities of L-DOPA, a leading anti-Parkinson’s drug. However, although *M. pruriens* is edible, over consumption of the bean causes development of a confused state of mind, a side effect associated with L-DOPA. Once ingested, L-DOPA undergoes extensive decarboxylation reactions in the gastro-intestinal tract and liver before entering the systemic circulation and the consequent effect of the above reactions is a feeling of dizziness (Garzon-Aburbeh et al., 1985; Awang et al., 1997).

Figure 1.7 shows seed pods *Mucuna pruriens* (“90 day” cultivar) grown in the greenhouse.

1.2.4: Biosynthesis and biochemistry of L-DOPA in *M. pruriens.*

L-tyrosine, is the starting point in the synthesis of L-DOPA (Chattopadhay et al., 1994). In plants, L-tyrosine is produced via prephenate, an intermediate in the shikimic acid pathway (Figure 1.8), while in mammals, L-tyrosine is synthesized from phenylalanine (phe) by the action of the monooxygenase phenylalanine hydroxylase (Swiedrych et al., 2004). In plants, L-tyrosine is converted to L-DOPA by tyrosine hydroxylase and in turn L-DOPA is converted to dopamine by DOPA/tyrosine decarboxylase (Facchini, 2001) (Figure 1.9).

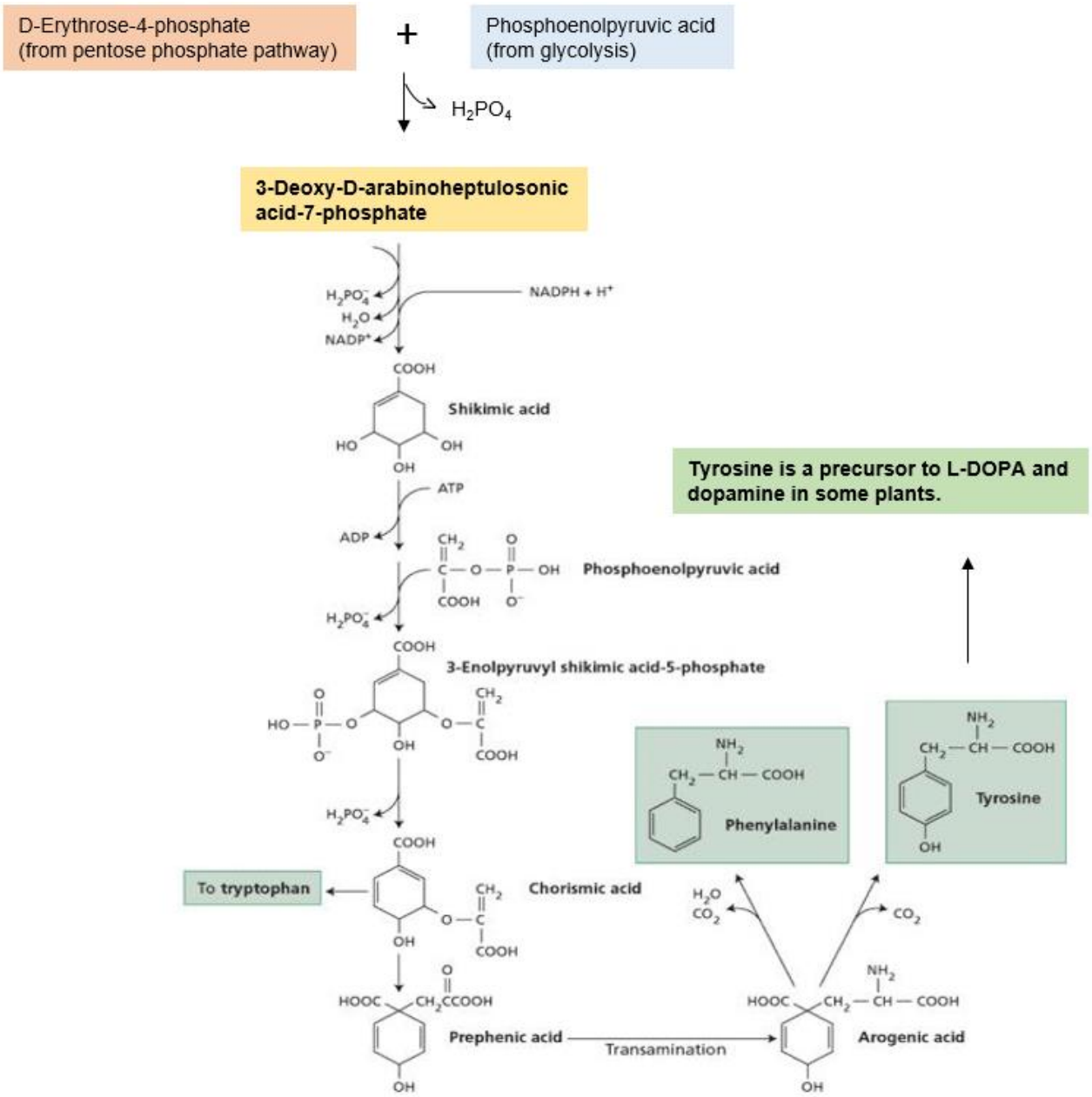


Figure 1.8 Schematic diagrams for the biosynthesis of L-tyrosine in the Shikimic acid pathway in plants. L-tyrosine is a precursor for biosynthesis of L-DOPA. The diagram was adapted with modification from Anderson, (2001).

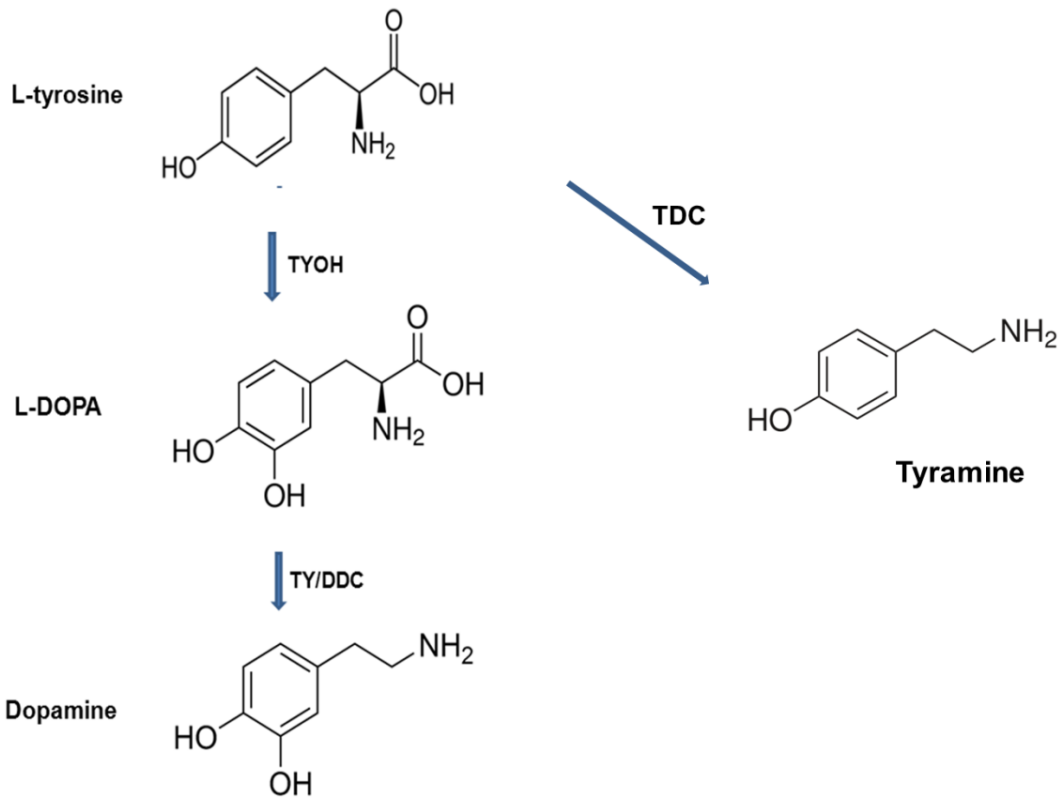


Figure 1.9 Schematic diagram for the L-DOPA biosynthesis from L-tyrosine. L-tyrosine is bioconverted to L-DOPA by tyrosine hydroxylase. L-DOPA is further bioconverted to dopamine by enzymatic action of DOPA decarboxylase in *M. pruriens* and a few plant species.

Figure 1.10 illustrates that both tyramine and dopamine are intermediate precursors for the synthesis of several alkaloids in plants (Facchini, 2001; Taiz and Zeiger, 2006).

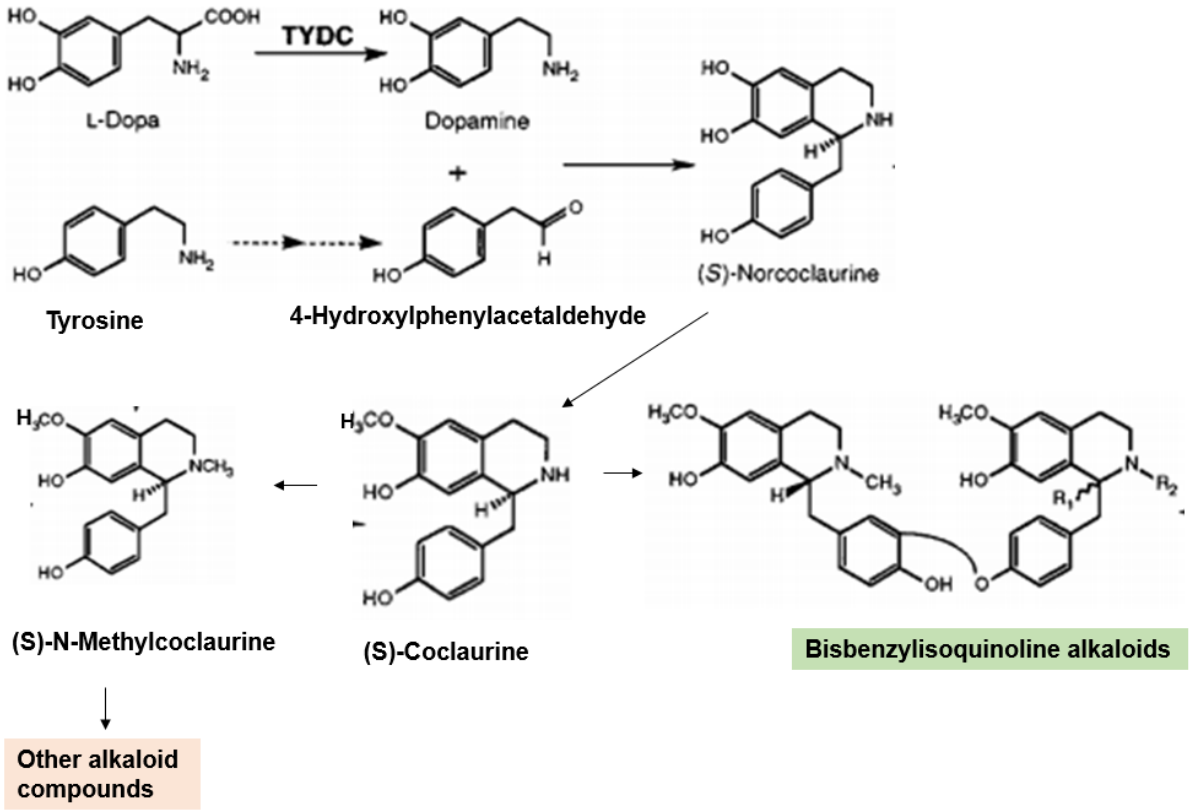


Figure 1.10 Conversion of L-tyrosine to tyramine by action of tyrosine decarboxylase. L-DOPA and tyramine are intermediate precursors for the synthesis of bisbenzylisoquinoline alkaloids which are of high pharmacological value. The diagram was adapted with modifications from Facchini, (2001).

In some plants such as *Musa* species and *Monostroma fuscum,* tyramine can be hydroxylated to dopamine (Facchini and De Luca, 1995; Facchini, 2001) (Figure 1.11).

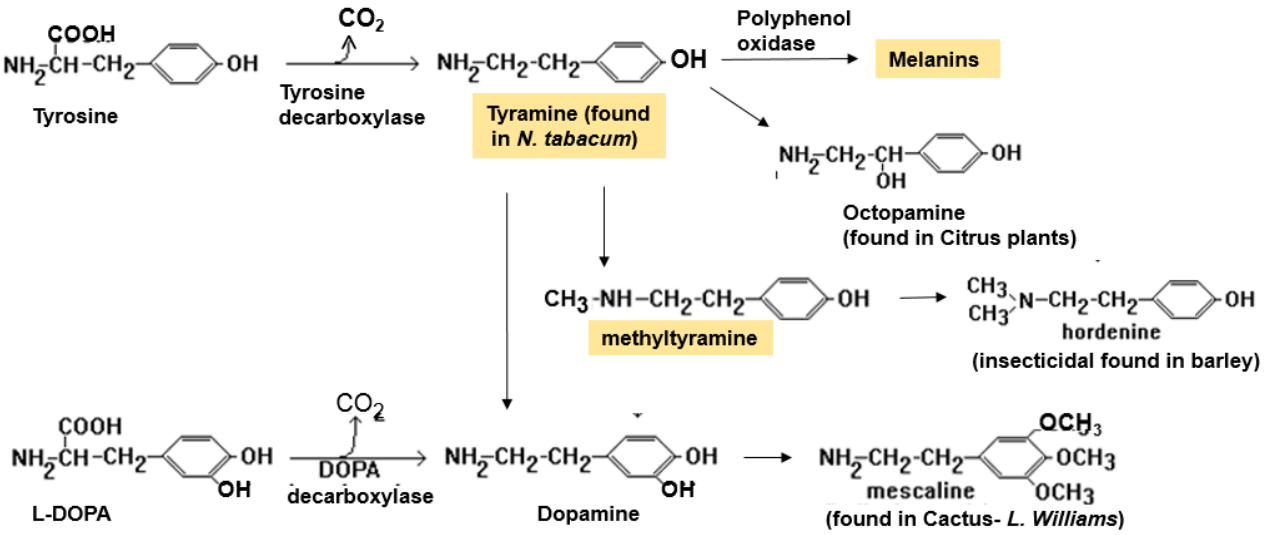


Figure 1.11 Biosynthesis of tyramine from L-tyrosine in plants, the alternate metabolic pathway to conversion of L-tyrosine to L-DOPA.

1.3: Molecular pharming and Advances in Applied Biotechnology.

Molecular pharming refers to the generation transgenic plants which are genetically engineered to maximise production of pharmaceutical and industrial proteins (Obembe et al., 2011). Hacker et al., (2009) reported that about 100 human therapeutic proteins are on the market and work is on-going to develop over 370 more. Molecular approaches which show prospect for high-yield production of L-DOPA or natural product pharmaceuticals both *in situ* or heterologously *in vitro* could include; enhancement of gene transcription and translation efficiency by using optimised constitutive or inducible promoters, engineering enhancers, activators or repressors. Xu et al., (2011) reported that a hybrid promoter comprised of CaM35S

elements and a mannopine synthase promoter of *Agrobacterium* Ti plasmid increased GUS expression by 3 - 5 folds higher when compared to the double enhanced CaMV35S promoter. In another study to enhance promoter efficiency, Lee et al., (2006) demonstrated that foreign protein expression in plant cells increased by 30 folds when an oxidative stress-inducible peroxidase (SWAP2) promoter was used instead the constitutive CaMV35S. In addition, the use of 5' leader sequences from tobacco etch virus or Alfalfa mosaic virus among others as translation elements could enable efficient translation at the 3'end sequence of the transgene (Xu et al., 2011). Other molecular approaches for enhancing yield of natural biopharmaceutical product both *in vitro* and *in vivo*, include strategies for reducing post-translation degradation by for instance co-expressing of protease inhibitors with recombinant proteins (Komarnytsky et al., 2006).

Besides molecular approaches, the yields of natural L-DOPA and other natural pharmaceutical products could be enhanced by using *M. pruriens* cell cultures approaches such as; optimisation of culture medium by supplementing with hormones, precursors or protein stabilising agents such as polyvinyl pyrrolidone (PVP). Other strategies include; immobilisation of exponential growth stage cells a on a porous matrix or alginate which protect the cells from hydrodynamic shear (Bodeutsh et al., 2001). On the other hand, cells cultures used for heterologous expression of foreign proteins could also potentially produce harmful effects to the cells (Joo et al., 2006). The a fore mentioned potential harmful effects could however be avoided by; using a two-phase aqueous culture system such as polyethylene glycol (PEG) and dextran in which cultured cells get immobilised in the PEG phase along with substrates and nutrients while the generated recombinant proteins collect in the dextrin culture phase (Cabral, 2007). Alternatively protein

binding resins binds to the recombinant protein and protects it from proteolysis. James et al., (2002) reported an 8-fold increase in the production mouse HCmAb protein upon supplementing the culture medium of *N. tabacum* cells with protein G resin.

However, there is a limit to which biological systems could be manipulated to enhance production of natural products such L-DOPA or therapeutical proteins besides environmental and related policy matters. In light of the above, using non biological approaches such as improving the engineering designs of bioreactors to achieve commercial scale production of natural products or therapeutic proteins. Optimising bioreactors to enhance sterile culture environment, improved aeration and reduction of shear stress on cells could lead to more efficient natural product synthesis (Paul and Ma, 2011; Xu et al., 2011). A stirred tank bioreactor could for instance improve aeration required especially by rapidly growing bacterial cells during heterologous expression experiments (Sambrook et al., 1989). Disposable plastic or polyvinyl bioreactors could be used to further reduce risks of contamination especially when human pathogens are used in the experiments. On the other hand advanced bioreactors such as fed-batch cultures, perfusion culture, continuous and semi-continuous culture bioreactors allow either continuous nutrient enrichment or replacement with fresh media at intervals. As a consequence of constant nutrient supply and replacement of old cells with new, the cells production potential is maintained at the exponential stage production which could result in increased yield of the natural product (Paul and Ma, 2011; Xu et al., 2011). Besides enhancing production of natural products or recombinant proteins, equally important is devising an efficient method for recovery and purification of natural products such as L-DOPA or recombinant proteins from cultured cells (Sambrook et al., 1989). In both bacterial

and plant cell systems, the target recombinant proteins are either secreted into the medium or are retained inside the cells (Sambrook et al., 1989; Xu, et al., 2011). Proteins secreted into the media are often get diluted, become unstable and tend to require quick purification whereas proteins retained in the cytosol of cells tend to be pure, stable and in high concentration (Ma and Paul, 2011; Xu et al., 2011).

Molecular pharming has consistently shown great potential to emerge as source for commercial production pure natural products such as L-DOPA and therapeutical proteins by using plant and bacterial cells as bio-factories. The fact there is a growing number of patients who are allergic to some synthetic medical drugs and while many drugs have been recalled from the market (Ma and Paul, 2011). The natural product industry on the other hand is increasingly being accepted world-wide as an alternative source for natural pharmaceutical products.

We still depend upon biological sources for a number of secondary metabolites including pharmaceuticals (Pezzuto, 1995), over 80% of the approximately 30,000 known natural products are of plant origin (Balandrin and Klocke, 1988; Fowler and Scragg, 1988; Phillipson, 1990). In 1985, of the 3,500 new chemical structures identified, 2,600 came from higher plants. In addition 75% of the World population rely on plants for traditional medicine and 25% of the pharmaceuticals are based on plant-derived chemicals (Farnsworth, 1985; Payne et al., 1991). The chemistry of *Mucuna pruriens* and for most other plants needs to be characterised so as to explore the potential new chemical models for novel drugs (Cox and Balick, 1994).

1.4: Aim and objectives of the PhD work.

The aim of my PhD was to develop a protocol for genetic transformation of *Mucuna pruriens.* Further aim was to determine the effect on L-DOPA biosynthesis in different tissues of *M. pruriens* by manipulating expression of the putative DOPA/tyrosine decarboxylase gene (Mp-ty/ddc). To achieve this, the Mp-ty/ddc gene was isolated from *M. pruriens*. The protein encoded by Mp-ty/ddc was then characterised to determine whether its enzyme properties were similar to those for DOPA/tyrosine decarboxylase which is responsible for conversion of L-DOPA to dopamine. My hypothesis was that Mp-ty/ddc codes for the DOPA/tyrosine decarboxylase enzyme (TY/DDC) which is responsible for conversion of L-DOPA to dopamine in plants. In light of the above, down regulation or “knock out” of the Mp-ty/ddc gene disables encoding for TY/DDC and as a consequence could potentially lead to accumulation of L-DOPA in tissues of *M. pruriens*.

CHAPTER 2: GENERAL MATERIALS AND METHODS.

2.1: Plant material growth conditions and harvesting of material.

2.1.1: Plant material.

Preliminary studies were made on five cultivars of *Mucuna pruriens* namely; cv. bush echo, cv.tropical, cv.early maturing, cv.90 day and cv.vining. The “90 day” cultivar was then selected for use in this work because it showed better growth and flowering rates. *M. pruriens* seeds were procured from Echo seed bank (USA). Besides *M. pruriens*, some preliminary studies were conducted on *N. tabacum* (TN90) provided by the University greenhouse.

2.1.2: Growth conditions.

M. pruriens is a tropical plant and hence required greenhouse conditions to be grown in the U.K. Seeds were grown in the Cardiff University greenhouse on fine structure peat compost with sand at pH 7, relative humidity of 95, vents at 23˚C, temperature range of 18 - 25 ˚C and at an average light regime of 16 h photoperiod until seed production stage. The vine cultivar of *M. pruriens* flowered after 6 months and produced seed pods after 6.5 - 7 months. On the other hand, the “90 day”, “early maturing”, “tropical” and “bush echo” cultivars of *M. pruriens* took 100 - 120 days to

flower and produced seed pods after 4.5 - 5 months. Among the *M. pruriens* cultivars, the “90 day” cultivar had shortest life-cycle of 4.5 months and also had a higher growth rate hence it was selected as the study cultivar for my PhD research. *N. tabacum* plants were grown under similar greenhouse conditions as *M. pruriens*.

2.1.3: Harvesting and storage of plant material.

Samples of seeds, leaves, stem and roots were collected from *M. pruriens* plants at 14, 28 and 35 day old seed pod stages. Each tissue sample was collected from 30 plants, wrapped in aluminium foil and immediately frozen in liquid nitrogen and transferred to -80 ˚C until used. Before use, each sample of tissue was separately homogenised under liquid nitrogen to fine powder, using a mortar and pestle. Homogenisation of tissues from 30 plants minimised variations among individual plants from being overrepresented in the samples used in this work.

2.2: Molecular Biology procedures.

2.2.1: Genomic DNA extraction.

2.2.1.1: Large scale extraction.

Genomic DNA was extracted from the five cultivars of *M. pruriens* by following the method described by Dellaporta et al. (1983). Leaf tissue (2 g) was ground to fine powder under liquid nitrogen and then mixed with 15 ml of extraction buffer (500 mM

NaCl, 100 mM Tris HCl - pH 8.0 at 25 ˚C, 50 mM EDTA – pH 8.0 and 10 mM β-mercaptoethanol) in a 50 ml centrifuge tube. The tissue was allowed to thaw and then vortexed well before 1 ml of 10% (w/v) SDS was added. The mixture was incubated at 65 ˚C for 10 minutes. Potassium acetate (5 M; 5 µl) was then added and the mixture was incubated on ice for 30 minutes. The mixture was then centrifuged at 3,600 xg using a centrifuge (Beckman Coulter Avanti J-E, USA). The supernatant was filtered through a miracloth (Merck4Bisociences) into a fresh tube containing 10 ml of isopropanol and mixed well. The nucleic acids were precipitated by incubating the mixture at -20 ˚C for 40 minutes. The nucleic acid was then pelleted by centrifugation at 3,600 X g using a centrifuge (Beckman Coulter Avanti J-E, USA) for 30 minutes at 4 ˚C. The supernatant was discarded and the pellet was drained for 5 minutes on a tissue. This was followed by re-suspending the pellet in 700 µl of 5 X TE (50 mM Tris HCl, 10 mM EDTA, pH 8.0) and transferred to a new 1.5 ml tube. RNAse A (20 µl of 10 mg/ml) was added and then allowed to incubate at 37˚C for one hour. Sodium acetate (3 M; 75 µl) was added by flicking and then the mixture was centrifuged for 15 minutes at 8,000 X g using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany). The supernatant was transferred to a fresh tube followed by addition of 500 µl isopropanol to precipitate the DNA. After 5 minutes of incubation at room temperature, the precipitated DNA was pelleted by centrifugation at 8,000 X g for 10 minutes using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany). The DNA pellet was rinsed with 500 µl of 70% ethanol and then centrifuged at 8,000 X g for 3 minutes using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany). The supernatant was discarded and the DNA pellet was air dried. The DNA pellet was re-suspended in sterile distilled water (35 µl).

2.2.1.2: Small scale crude genomic DNA quick extraction protocol for PCR amplification.

The small scale crude DNA was extracted by following the method described in the Plant genomic DNA extraction kit protocol (Sigma). A disc was obtained from a leaf by using the cap and flange of a 0.5 ml PCR microfuge tube. Extraction solution (E) (40 µl) was added to the tube with the leaf disc. Using a micro pestle, the leaf disk was submerged and stabbed in the tube until the buffer turned green. The tube containing the leaf extract was incubated at 95 ˚C for 10 minutes using a PTC-100 thermocycler (MJ Research Inc., Waltham, USA). Dilution buffer (40 µl of D) was then added and the tube was flicked (but not vortexed) to break the tissue. This was then followed by centrifugation at 8,000 X g for 1 minute in a microcentrifuge. 1-2 µl of the supernatant solution was used for analysis by PCR.

2.2.2: RNA extraction.

2.2.2.1: Methods of RNA extraction from leaves, stems and roots of *M. pruriens.*

Different methods and protocols were found to be suited for RNA extraction from the stems, seeds, leaves and roots of *M. pruriens* owing to the substantial differences in their chemical constituents and properties. *M. pruriens* leaf RNA was extracted by following the method described in the RNeasy plant mini kit protocol (Qiagen, Germany).

For RNA extraction from seeds, stems and roots of *M. pruriens*, the tissues were softened by overnight lyophilisation at -60 ˚C using a lab scale lyophiliser (Liaoning,

China) prior to homogenisation in liquid nitrogen using an RNAse free mortar and pestle. Stem and root RNA was extracted by using 0.1% DEPC treated extraction solutions and buffers as follows: Tissue from younger stem parts or roots (200 mg) were ground in liquid nitrogen to a fine powder using a mortar and pestle and then transferred to a 1.5 ml microcentrifuge tube. RNA extraction buffer (650 µl) containing 8 M guanidine-hydrochloride, 20 mM methylethylsulfonate (MES) at pH 7, 20 mM EDTA and 50 mM ß-mercaptoethanol (added just before use, in a chemical fume hood) were added to the tissue powder and the tissue was homogenised by vortexing for 2 minutes. The homogenate was incubated on ice for 5 minutes and centrifuged at 8,000 X g for 10 minutes, at 4 ˚C. To the supernatant, an equal volume of a mixture of phenol (pH 4.3), chloroform and isoamylalcohol in a ratio of 25:24:1 respectively was mixed well under a chemical fume hood, and centrifuged at 8,000 X g for 10 minutes, at 4 ˚C. The resultant supernatant was then collected and to it, 0.2 volume of 1 M acetic acid, and 0.7 volume absolute ethanol were added. The mixture was thoroughly agitated and then incubated for 1 hour at -70 ˚C before centrifugation at 8,000 X g for 10 minutes, at 4 ˚C. The precipitate was washed twice by resuspending in 70% ethanol followed by centrifugation at 8,000 X g for 10 minutes at 4 ˚C to obtain an RNA pellet which was then resuspended in RNAse free water (30 µl).

2.2.2.2: Extraction of RNA from *M. pruriens* seeds.

A different RNA extraction method described by Ding et al. (2007) was used to extract RNA successfully from the protein rich seeds of *M. pruriens.* This RNA extraction method was also used to extract good quality RNA from leaves, stems and roots of *M. pruriens.* The RNA extraction procedure was as follows:

M. pruriens seed or plant tissues (0.5 g) were frozen in liquid nitrogen and ground into a fine powder that was immediately mixed with 400 µl of extraction buffer (5 M NaCl and 100 mM Tris-HCl [pH 8.0]), 100 µl of 2-mercaptoethanol), and 1 ml of Tris-saturated phenol/chloroform (1:1; pH 8.0) in 2-ml polyethylene tubes (Anachem, UK). After vortexing and incubating at RT for 1 minute, the mixture was centrifuged at 8000 X g for 1 minute using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany). The aqueous phase was transferred to a new 2-ml tube. Then 25 µl of 10% polyvinylpyrrolidone (PVP, Sigma P-5288) solution, 25 µl of 5% *N*-lauroyl-sarcosine (Sigma L-5125), 300 µl of 3 M sodium acetate (pH 4.8), and 1 ml of water-saturated phenol/chloroform (1:1) were added and vortexed several times. The mixture was then centrifuged for 5 minutes at 8,000 X g using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany) and the aqueous phase was transferred to a 1.5 ml tube (Anachem, UK), followed by the addition of 200 µl ethanol and 100 µl silica suspension (1 g/ml) (Sigma-S5631) to absorb the RNA. The mixture was vortexed and incubated for 1 minute at room temperature and then centrifuged at 8,000 X g for 15 seconds using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany) to precipitate the silica particles. The pellet was washed with 70% ethanol twice to completely remove the detergent and salts from the silica particles. The pellet was collected by centrifugation at 8,000 X g for 15 seconds using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany) and

dried by draining the tube in a laminar flow hood to remove residual ethanol. The RNA was eluted by resuspending the silica pellet in 35 µl of sterile water, incubating at room temperature for 2 minutes and was then centrifuged at 8,000 X g for 5 minutes. The supernatant (RNA) was transferred to a new tube without disturbing the silica pellet.

The silica suspension (1 g/ml) used in the RNA extraction protocol above was prepared as follows: 6 g silica (Sigma S-5631) was resuspended in 50 ml of 0.1% DEPC treated sterile water, vortexed and allowed to settle for 24 hours. The supernatant containing very fine particles was aspirated and discarded. This process guaranteed that the silica particles used in the purification step were of the correct size (1 - 5 µm) that could be completely recovered by centrifugation. The pellet was then resuspended in 6 ml of 0.1 M HCl, aliquoted and stored in darkness at 4 ˚C.

2.2.3: DNAse treatment.

In order to degrade any genomic DNA “contamination” in the RNA samples, a DNAse treatment / digestion reaction was set up as follows: 10 µg RNA, 10 µl of 10X DNAse 1 buffer (New England Biolabs, Hertfordshire, UK), 2 units of DNAse 1 (New England Biolabs, Hertfordshire, UK) and sterile distilled water up to a total volume of 100 µl. The samples were incubated at 37 ˚C for 30 minutes. Following the incubation, 1 µl of 0.5 M EDTA (Sigma, USA) was added to terminate the reaction. Finally the samples were incubated at 75 ˚C for 10 minutes to inactivate the DNAse 1.

2.2.4: cDNA synthesis.

cDNA was synthesised as follows: 2 µg RNA and Oligo(dT)$_{15}$ (500 µg µl^{-1}) (Promega, Madison, WI, USA) were made to a total volume of 20 µl with sterile distilled water. The samples were incubated at 70 ˚C for 10 minutes and then cooled at 4 ˚C for 10 minutes. The samples were mixed with 6 µl of 10 X M-MLV RT buffer (Promega, Madison, WI, USA), 2 µl of 0.1 M Dithiothreitol (DTT) and 1 µl of 10 mM dNTPs (Promega, Madison, WI, USA), and then incubated at 42 ˚C for 2 minutes. Finally, 1 µl of M-MLV reverse transcriptase (Promega, Madison, WI, USA) was added and the reaction incubated at 42 ˚C for 50 minutes followed by inactivation at 70 ˚C for 15 minutes.

2.2.5: Designing primers.

2.2.5.1: Gene specific primers.

DNA oligo primers based on the partially sequenced Mp-ty/ddc gene sequence portion on the NCBI data bank were designed using the primer 3 software program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). To enhance primer-PCR template annealing efficiency during PCR reactions, primers were further screened using the Oligo analyzer program:
(http://eu.idtdna.com/analyzer/applications/oligoanalyzer/). Primer sequences which formed dimers or hairpin-like structures during screening analysis were discarded or modified. Desalted DNA oligos of scale 0.025 µmol, were synthesised at Sigma (USA).

2.2.5.2: Degenerate primers.

To isolate L-DOPA and dopamine biosynthesis and metabolic pathway genes of *M. pruriens* whose sequence information was not available on the NCBI data bank (www.ncbi.nlm.nih.gov), degenerate primers were designed based on sequence homology. The protein sequences coding for similar genes to the above but in other plant species which are taxonomically related to *M. pruriens* were aligned using the genedoc software program (http://www.nrbsc.org/gfx/genedoc/). Degenerate primers were designed from the conserved protein sequence regions by reverse translation to nucleotide sequence, allowing a degeneracy of 2 - 3 bases at different positions. Desalted DNA oligos of scale 0.025 µmol, were synthesised at Sigma (USA).

2.2.6: Polymerase Chain Reaction (PCR).

PCR amplifications were carried out in a 25 µl volume by following the procedure described in the GoTaq DNA Polymerase kit protocol (Promega, USA). The PCR contained: 5 µl of 5 X GoTaq reaction buffer, 0.5 µl of dNTP mix (10 mM each), upstream and downstream primer (each 0.25 µM), 1.25 units of GoTaq polymerase, 10 ng DNA and the volume was made up to 25 µl using sterile distilled water. A negative control reaction was set up as described above but using sterile distilled water as a template for PCR amplification. This helps to verify and ensure that the PCR reagents are not contaminated. General thermal cycling was performed using a PTC-100 thermocycler (MJ Research Inc., Waltham, USA) and the amplification

was conducted following the thermal profile: Initial denaturation at 94 ˚C for 2 minutes; 34 - 40 cycles (94 ˚C, 30 sec; primer annealing, Tm (˚C), 30 sec; 72 ˚C, 1 min kb^{-1}) and a final extension at 72 ˚C for 10 minutes.

2.2.7: Agarose gel electrophoresis.

For a 1% agarose gel, 1 g of agarose (Bioline, UK), was mixed into a flask with 100 ml of 0.5 X TBE (0.45 M Tris base, 0.44 M Boric acid, 20 ml of 0.5 M EDTA-pH 8.0 made up to 1 litre using sterile water) (Bochringer Mannheim GmbH, Germany). The solution was heated in a microwave oven until the agarose was completely dissolved. Afterwards, the solution was allowed to cool at room temperature until it reached 50 – 55 ˚C and then 5 µl of ethidium bromide (10 mg/ml) per 100 ml of agarose solution was added and gently mixed. The solution was poured into a gel tray and an appropriate number of combs were inserted. The gel was allowed to set for 15 - 30 minutes at room temperature and then placed in an electrophoresis chamber (Power Pac Basic, Bio-Rad, Singapore) covered with 0.5 X TBE.

DNA or RNA samples usually in a final volume of 10 µl that were analysed by gel electrophoresis were mixed with 2 µl of 6 X loading buffer (50 mM Tris HCl pH 7.6, 60% glycerol and bromophenol blue) and then loaded in the wells of the gel. Electrophoresis was carried out at 100 V for 30 minutes or until an optimum separation of the bands was observed. DNA ladder 1 kb (Invitrogen, USA) 250 – 500 ng was used as size marker for most of the electrophoresis experimental sets.

2.2.8: Purification of PCR products using a QIAquick PCR Purification Kit.

PCR products and other enzymatic reactions were purified to remove enzymes and other reagents as follows: 5 volumes of buffer PB (Qiagen, Germany) were added to 1 volume of the PCR sample and mixed. The mix was placed in a QIAquick spin column with a 2 ml collection tube and centrifuged at room temperature for 1 minute at 8,000 X g using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany). The flow-through was discarded and in order to wash the samples, 0.75 ml buffer PE (Qiagen, Germany) was added to the QIAquick columns and then centrifuged at 8,000 X g for 1 minute. The flow-through was discarded again and the samples were centrifuged for an additional 1 minute at 8,000 X g to remove residual wash buffer. The DNA was eluted by placing the QIAquick columns into a sterile 1.5 ml microcentrifuge tube and 35 µl of sterile water was added to the column. Columns were centrifuged for 1 minute at 8,000 X g and the eluate containing the purified DNA stored at 4 ˚C or -20 ˚C until required.

2.2.9: Extraction of a DNA band from agarose gels using a QIAquick Gel Extraction Kit.

Bands of interest were excised from agarose gels using a clean scalpel. 3 volumes of buffer QG (Qiagen, Germany) was added to 1 volume of gel (100 mg ~ 100 µl) and the samples were incubated at 50 ˚C for 10 minutes or until the gel was completely dissolved. One volume of isopropanol was added and the samples were

placed into QIAquick spin columns with a 2 ml collection tube, and then centrifuged at room temperature for 1 minute at 8,000 X g using a microcentrifuge (Heraeus Instruments Biofuge 13, Germany). The flow-through was discarded and to wash, 0.75 ml of buffer PE (Qiagen, Germany) were added to the QIAquick (Qiagen, Germany) column followed by centrifugation for 1 minute at 8,000 X g. The flow-through was discarded and the columns were centrifuged for an additional 1 minute at 8,000 X g to remove residual wash buffer. Finally, the QIAquick columns were placed into a sterile 1.5 ml microcentrifuge tube and DNA was eluted in 35 µl of sterile water by centrifuging the column for 1 minute at 8,000 X g.

2.2.10: Preparation of pZERO-2-T for cloning PCR products.

pZERO-2-T (Invitrogen, USA) cloning vector was prepared as follows: 10 µg of pZERO-2 (Figure 2.1) was digested using 4 units of *Eco*RV (New BioLabs England, Hertfordshire UK) at 37 ˚C for 3 hours. The *Eco*RV digestion product was column purified as described in Section 2.2.8 with the exception that in the final step, the DNA was diluted with 76.5 µl of sterile water instead of 35 µl. In order to introduce a terminal Thymine (T) base at the 5' and 3' ends of the *Eco*RV digested pZERO-2 vector DNA that enables T-A cloning, Taq polymerase PCR was performed. The PCR reagents were as follows: 76.5 µl of the column purified pZERO-2-T DNA, 1 X Go Taq buffer (Promega, USA), 2 mM dTTP (Sigma, USA), 1.5 mM $MgCl_2$ and 1 unit Go Taq polymerase (Promega, USA). Thermal cycling was performed using a PTC-100 thermocycler (MJ Research Inc., Waltham, USA) and the addition of an

overhang-T was conducted following the thermal profile: 94 ˚C for 2 minutes, 94 ˚C for 30 seconds and 72 ˚C for 2 hours. The pZERO-2 plasmid with overhang-T (pZERO-2-T) was column purified as described in section 2.2.8 and the concentration was determined measuring the UV absorbance at 260 nm using a Nanodrop spectrophotometer (LabTech International, USA). The final plasmid DNA concentration was set at 50 ng per microliter of which usually 2 µl was used in the cloning of PCR products.

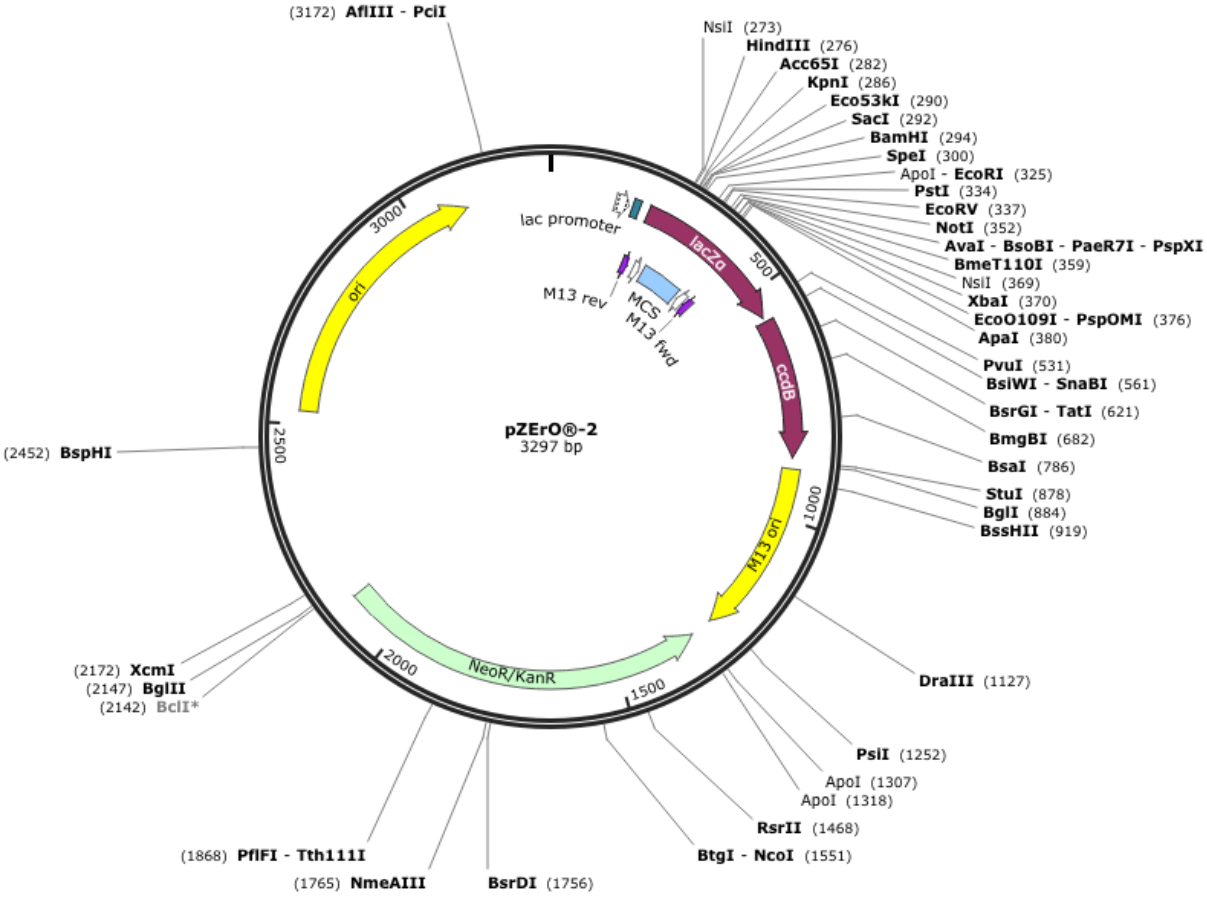


Figure 2.1 Diagram of a pZERO-2 plasmid.

The pZERO-2 cloning vector contains the *ccd*B gene fused to the C-terminus of *Lac*Zα. Insertion of a DNA fragment disrupts the expression of the *lac*Zα – *ccd*B gene fusion, permitting growth of only positive recombinants. Cells without recombinant vector are killed. The *ccd*A and *ccd*B genes are found in the control of cell death (*ccd*) locus on the F plasmid and encode for *Ccd*A and *Ccd*B proteins respectively. *Ccd*B poisons the gyrase-DNA intermediate bond during DNA replication and repair leading to DNA destruction and cell (Van Melderen, 2002).

2.2.11: Dephosphorylation of DNA 5'-termini.

Digested DNA has a 5' phosphate group which enables ligation. Dephosphorylation of digested DNA using a phosphatase such as the Calf Intestinal Alkaline Phosphatase (CIAP) removes the 5' phosphate thereby preventing self-ligation especially of cloning vectors without insert. The reaction was set up following the procedures described in the Fermentas CIAP protocol. Digested DNA (1-20 pmoles) was added to 5 µl of 10 X of CIAP buffer in a 1.5 ml tube. Then 1 unit of CIAP was added to the tube and the volume was adjusted to 50 µl using sterile water. The reagents were gently mixed by inverting the tube a 4 - 6 times before incubating at 37 ˚C for 30 minutes in a Heratherm Incubator (Thermo Scientific, Germany). The reaction was stopped by heating the reaction to 85 ˚C in a hot water bath for 15 min or by extracting DNA using phenol/chloroform followed by isopropanol precipitation as described in Section 2.2.17.

2.2.12: DNA ligation in pZERO-2-T or pGEM-T easy vector.

PCR products were T-A ligated into pZERO-2-T or pGEM-T vector (Promega, Madison, WI, USA) in a ratio of 3:1 respectively for cloning using bacterial cells. The ligation reaction was set up as follows: 100 ng pZERO-2-T (Invitrogen, USA) or 25 ng pGEM-T easy vector (Promega, Madison, WI, USA), 1 µl of 10 X rapid ligation buffer (Promega, Madison, WI, USA) and 1 unit of T4 ligase (Promega, Madison, WI, USA). The reaction was mixed and the volume made up to 10 µl with double distilled water before incubation at 4 ˚C overnight.

2.2.13: Preparation of bacterial competent cells.

Competent cells were prepared for general cloning (*E.coli*DH5α) (Invitrogen, UK), protein expression (*E.coli* (DE3) BL21b) (Novagen, USA) and Tuner 2 (EMD Millipore, USA) as follows: Lysogeny broth (LB medium: 10 g l^{-1} NaCl; 10 g l^{-1} tryptone and 5 g l^{-1} yeast extract, pH 7.0) supplemented with appropriate selection was inoculated with a single bacterial colony. The bacterial culture was incubated overnight at 37˚C with shaking at 225 rpm on a rotor, Incubator Shaker-model G25, (Brunswick Scientific Co Inc, USA). LB medium (50 ml) supplemented with appropriate selection was inoculated with 5 ml of overnight culture in a 250 ml Pyrex flask. The bacterial culture was incubated at 37 ˚C with shaking at 225 rpm on a rotor shaker until $O.D_{600}$ of 0.6 was obtained, measured using a UV/VIS Spectrophotometer, Sp8-400. The culture was incubated on ice for 30 minutes and was then harvested by centrifugation at 3,000 X g for 3 minutes at 4 ˚C in a

Beckman Coulter Avanti J-E centrifuge fitted with a JA-20 rotor, (USA). The supernatant was discarded and the bacterial pellet was resuspended in 1 ml of ice cold 20 mM $CaCl_2$. Aliquots of 100 µl cell suspension were frozen in liquid nitrogen and ultimately transferred to -80 ˚C freezer until used for gene cloning as described in Section 2.2.13.

2.2.14: Transformation of competent cells.

Competent bacterial cells were prepared as described in Section 2.2.12. Competent cells (50 µl) were thawed on ice and then mixed with 10 ng (~2 µl) of the plasmid vector or DNA ligation reaction in 1.5 ml microcentrifuge tubes. The tubes were kept on ice for 20 minutes, then exposed to 42 ˚C for 45 seconds in a water bath and transferred back to ice for 2 minutes. Liquid LB medium (450 µl) without antibiotics was added and the mixture incubated at 37 ˚C for 30 minutes (for Ampicillin resistant strains) or 45 minutes (for kanamycin resistant bacterial cells) shaking at 200 rpm. The cells (100 µl) were plated onto solid LBA medium (LB; 10 g l^{-1} agar, pH 7.0) supplemented with appropriate antibiotics. A positive control experiment was performed by transformation of competent cells (50 µl) using an intact plasmid DNA and were plated under the same conditions as above. The plates were incubated at 37 ˚C overnight and then transferred to 4 ˚C.

2.2.15: Colony PCR.

Single colonies of bacteria generated in Section 2.2.14 above were inoculated as separate individual spots on LBA medium supplemented with the appropriate selection (50 µg ml^{-1} of ampicillin or kanamycin) on a 90 X 16.2 mm Petri dish. This Petri dish (referred to as the "master clone plate") was incubated at 37 ˚C overnight (12 - 16 hours) in a Heratherm Incubator (Thermo Scientific). PCR was used to screen individual colonies to confirm the presence of cloned DNA fragments into the plasmid. Colony PCR was set up as described in Section 2.2.6 but with the exception that the template for PCR amplification was a single colony picked from a "master clone plate" using a 200 µl pipette tip (Gilson, UK). The M13 forward (5-GTAAAACGACGGGCCAGT-3') and reverse (5'-AACAGCTATGACCATG-3') primers which flank the transgene on the plasmid used for transformation were used for the colony PCR. A few colonies were further screened for successful ligation of the transgenes in the plasmid by PCR using a combination of an upstream transgene specific primer with a downstream plasmid M13 primer. PCR amplification products were analysed by agarose gel electrophoresis as described in Section 2.2.7. The single colonies on the "master clone plate" which produced the correct size PCR product were selected for recovery of the cloned plasmid as described in Section 2.2.14.

2.2.14: Plasmid DNA purification using QIAprep Spin Miniprep Kit.

A QIAprep Spin Miniprep Kit protocol (Qiagen, Germany) was used for purification of plasmid DNA. A single colony was used to inoculate 5 ml of liquid LB medium with the appropriate antibiotic selection. The bacterial culture was incubated at 37 ˚C overnight with shaking at 225 rpm on a rotor, Incubator shaker-model G25 (New Brunswick Scientific Co. Inc, USA). The bacterial cells were collected by centrifugation at 3,000 X g at room temperature (RT) for 5 minutes using a centrifuge (Beckman Coulter Avanti J-E, USA). The bacterial pellet was resuspended in 250 µl Buffer P1 (Qiagen, Germany) and transferred to microcentrifuge tube. Buffer P2 (250 µl) (Qiagen, Germany) was added and mixed by inverting the tube 4 - 6 times. Then 350 µl of Buffer N3 was added and the tubes mixed by inverting 4 - 6 times followed by a centrifugation step (8,000 X g for 10 minutes) at room temperature using the microcentrifuge. The supernatant was transferred to a QIAprep spin column (Qiagen, Germany) followed by centrifugation for 1 minute (8000 X g) at room temperature using a microcentrifuge and the flow through was discarded. The QIAprep spin column was then washed using 0.75 ml Buffer PE (Qiagen, Germany) and this was followed by centrifugation at 8,000 X g for 1 minute. The flow through was discarded as before followed by centrifugation at 8,000 X g at room temperature for an additional 1 minute to remove residual wash buffer. To elute plasmid DNA from the QIAprep spin column this was placed in a sterile 1.5 ml microcentrifuge tube. Sterile water 35 µl was added to the centre of the column and was let to stand for 1 minute at room temperature before centrifugation for 1 minute. The DNA concentration was measured using a Nanodrop, ND-1000 Spectrophotometer, (LabTech International, UK).

2.2.15: Purification of Nucleic Acids using Phenol-Chloroform.

Purification of nucleic acids using the phenol-chloroform method was conducted as described by Sambrook et al., (1989) and Dale and Von Schantz, (2003). The nucleic acid was transferred to a polypropylene 2ml tube (Anachem, UK) and an equal volume of phenol: chloroform (1:1), pH 8 was added. The contents were mixed until an emulsion formed. This was followed by centrifugation at 8,000 X g for 3 minutes at room temperature using a microcentrifuge (Heraeus Instruments Biofuge 13, Germany). The aqueous phase was transferred to a fresh tube. All the phenol-chloroform extraction steps were repeated until no protein was visible at the interface of the organic and aqueous phases. An equal volume of chloroform was then added and the contents were mixed until an emulsion formed as before and this was followed by centrifugation at 8,000 X g for 3 minutes. The aqueous phase was transferred to a fresh tube. The nucleic acid was then recovered by precipitation using isopropanol as described in Section 2.2.16 or by ethanol precipitation as an alternative method described by Sambrook et al., (1989).

2.2.16: Purification and concentration of nucleic acids by Isopropanol precipitation.

Nucleic acids which were partitioned into the protein free aqueous phase during phenol-chloroform extraction as described in Section 2.2.15 contained salts and other non-protein impurities. To obtain purified nucleic acids and with higher concentration, isopropanol precipitation was carried out as described by Sambrook

et al., (1989) and Dale and Schantz, (2003). To the nucleic acid extract obtained in Section 2.2.17 in the aqueous phase, 0.1 volume of sodium acetate (3 M; pH 4.8) (Sigma, USA) was added by flicking. This was then followed by centrifugation at 8,000 X g at room temperature for 10 minutes using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany). The supernatant was transferred to a fresh tube and 0.7 volume of isopropanol was added and mixed well. The reaction mix was incubated at room temperature for 5 minutes before centrifugation at 8,000 X g for 10 minutes to pellet the DNA using a microcentrifuge. The DNA pellet was rinsed thoroughly with 100 - 200 µl of 70 % ethanol. The pellet was then collected by centrifugation at 8,000 X g for 3 minutes. Finally the DNA pellet was resuspended in 20 - 35 µl of sterile water.

2.2.17: Restriction enzyme digestion of plasmid DNA.

Digestion reactions were set up as follows: 1 unit of restriction enzyme(s), 2.5 µl of 10 X Buffer (NEB) and 400 ng of plasmid DNA were made up to 25 µl using sterile distilled water in a 1.5 ml tube and were incubated at 37 ˚C for 2 h.

2.2.18: Analysis of sequence data.

Plasmids were sequenced at an external company (Eurofins MWG Sequencing, Germany). Sequence results were analysed using Vector screen (used to delete plasmid vector sequence from that of the transgene clone) and Blast (used to draw

protein alignments). Both Vector screen and Blast software programs are available on the NCBI webpage (www.ncbi.nih.gov/BLAST/). Further analysis of sequences was performed using the generunner (http://www.generunner.net/) and Bioedit software programs. For high precision analysis very long sequences (>1 kb) of multiple clones, a combination of the 4.8 Sequencher (http://sequencher.software.informer.com/4.8/ and Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) software programs were used. Sequence alignments were drawn using the genedoc software (programhttp://www.nrbsc.org/gfx/genedoc/).

2.2.19: SDS-PAGE.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used for protein analysis (Laemmli). For protein separation, using a BIORAD Mini-PROTEAN Tetra Cell unit, 10% SDS-PAGE gels were prepared by mixing 6.6 ml of 30% acrylamide/bis-acrylamide (Melford Laboratories), 5.2 ml 1 X separation buffer (1.5 M Tris-HCl, pH 8.8; 10% SDS), 8.0 ml sterile water, 200 µl of freshly prepared 10% ammonium persulfate (APS), 8 µl N,N,N,N'-tetramethyl-ethylenediamine (TEMED) (Sigma) in a total of 20 ml. The gel mix was cast between two glass plates (Bio-Rad) and let to solidify for about 15 minutes. Stacking gels containing 1.66 ml 30% acrylamide/bis-acrylamide, 1.36 ml 1 X stacking buffer (1.0 M Tris-HCl, pH 6.8; 10% SDS), 6.8 ml sterile water, 0.1 ml 10% APS, 10 µl TEMED were cast on top of the separation gel and let to set for about 5 minutes. At the same time, protein samples were mixed with appropriate amount of 2 X protein loading buffer (0.125 M

Tris-HCl, pH 6.8; 4% SDS, 20% glycerol, 0.5 M DDT, 0.004% Bromophenol blue) and boiled for 5 minutes, then allowed to cool for 3 minutes before loading on the SDS-PAGE gel. During SDS-PAGE gel electrophoresis, the protein samples were size separated in 1 X SDS-PAGE gel electrophoresis buffer (0.025 M Tris-HCl; 0.2 M Glycine; 1% SDS) at 120 V for about 2 hours in a Bio-Rad gel system. Finally, the protein bands were visualised by staining the gel with Coomassie Brilliant Blue staining solution: 0.25% Coomassie Brilliant Blue R-250 (Sigma); 20% methanol, 10% glacial acetic acid and sterile water for about 1 hour. The excess Coomassie stain was then destained from the SDS-PAGE gel using destaining solution (10% acetic acid; 40% methanol). The size of protein bands was estimated from the protein size marker loaded alongside the samples in a separate well (Bio-Rad).

CHAPTER 3: L-DOPA PROFILE IN *MUCUNA PRURIENS.*

3.1: INTRODUCTION.

M. pruriens is a herbaceous plant in the family Leguminosae and is reported to produce L-DOPA, dopamine and tyramine naturally (Duke, 1981; Pras, et al., 1993) as well as a number of other secondary metabolites. Besides L-DOPA, *M. pruriens* seeds are reported to contain other alkaloids such as Mucunine, tryptamine, prurienine and prurieninine (Awang et al., 1997). Bell and Nulu (1971) also showed that *M. pruriens* contains 1-methy-3-carboxy-6, 7-hydroxy-1, 2, 3, 4-tetrahydroisoquinoline and L-3-carboxy-6,7-dihydroxy-1, 2, 3, 4-tetrahydroisoquinoline in addition to L-DOPA. Only a few plant species have been reported to produce L-DOPA. These include some species of *Mucuna*, *Vicia faba*, *Papaver somniferum*, *Baptisia* and *Lupinus* (Huizing, et al., 1985). However levels of L-DOPA are modulated by the presence of enzymes that catabolise L-DOPA to dopamine. There are four pathways for L-DOPA metabolism in living systems and the principal pathway is decarboxylation to produce dopamine by enzymatic action of aromatic dopa decarboxylase. The second but less prominent metabolic pathway is the methylation of L-DOPA to 3-O-methyldopa by catechol-O-methyltrasferase. Other pathways are transamination and oxidation (Muzzi, et al., 2008). In addition, Bell and Nulu (1971) and Saito et al., (1982) reported that besides conversion to melanine by polymerisation, L-DOPA and dopamine could serve as early stage precursors for the synthesis of tetrahyroisoquinolines in *M. pruriens* and other plants.

L-DOPA has been detected in several organs of the plant. Reports differ on the level of L-DOPA in *M. pruriens* seeds: Siddhuraju and Becker (2001) reported 4.96% of L-DOPA with no information on L-DOPA-content in other tissues of the plant provided, while Bell and Janzen (1971) reported 5.9 - 6.4% (excluding seed coat which represented 15-20% of seed weight). Wichers, et al., (1993) demonstrated that L-DOPA-content in leaves, stems and roots of *M. pruriens* plants drastically decreased within 3 weeks after germination (See Table 3.1).

Table 3.1 shows L-DOPA content in tissues of germinating *M. pruriens*

M. pruriens tissue	L-DOPA on Day-1 (% of DW)	L-DOPA on Day-21 (% of DW)	L-DOPA on Day-30 (% of DW)
Leaf	9.8	1.0	0.5
Stem	5.0	2.2	1.8
Root	9.8	1.8	1.0

On the other hand, dopamine was detected in the leaves but not in the stem and roots of germinating *M. pruriens* plants. The dopamine-content of the leaves of *M. pruriens* was shown to drastically increase to a maximum concentration during the first 3 weeks after germination before a sharp decline and a consequent stabilisation at a significantly higher concentration than for L-DOPA (Wichers et al., 1993) (See Table 3.2).

Table 3.2 shows dopamine content in tissues of *M. pruriens*.

M. pruriens tissue	Dopamine on Day-1 (% of DW)	Dopamine on Day-21 (% of DW)	Dopamine on Day-30 (% of DW)
Leaf	1.8	7.7	4.0

In contrast, L-DOPA-content in cell suspension cultures of *M. pruriens* was 16 - 80 fold higher than dopamine (Wichers et al., 1993) suggesting that this might be a promising route to producing higher concentrations of this compound. It was also reported that addition of 2,4-Dichlorophenoxyacetic acid (2,4-D) to cell suspension cultures of *M. pruriens* induced accumulation of dopamine but caused a decrease in L-DOPA levels in addition to inhibition of biomass production (Wichers et al., 1985). In order to discern whether the effect of 2,4-D on cell biomass and L-DOPA production was not due to salt stress but rather a hormonal response, sodium chloride salt was added to *M. pruriens* cell suspension culture. Consequently, Wichers et al., (1993) reported that addition of sodium chloride at concentrations higher than 0.25 mol l^{-1} to *M. pruriens* cell suspension cultures, caused a very significant increase in the L-DOPA levels besides plasmolysing the cells. The maximum increase in L-DOPA levels in the *M. pruriens* cell suspension cultures was 74% and was caused by addition of sodium chlororide at concentration of 0.5 mol l^{-1}. Notably, no dopamine could be detected in the plasmolysed cell cultures (Wichers, et al., (1993). The above observation suggested that the lowering of L-DOPA concentration and biomass production in cell cultures was not due to salt stress. In an earlier study, Brain (1976) reported high L-DOPA production (15 mg l^{-1}) by *M. pruriens* cotyledonary cell culture grown on M.S suspension medium supplemented

with 2.5 mg l^{-1} 2,4-D and 10% coconut milk. In a later study, Chattopadhyay et al., (1994) reported enhancement of L-DOPA production by over 2.5% of dry weight and a 14-fold increase in growth rate in two-stage *M. pruriens* cell suspension cultures grown in constant light. The first-stage was reported to significantly enhance cell biomass production and it involved growing cells in M.S supplemented with 2% sucrose (M.S.I) but without calcium chloride. On the other hand the second-stage was reported to enhance L-DOPA production and it involved growing cells harvested from the first-stage culture in M.S.I medium supplemented with 42.5 mg l^{-1} KH_2PO4 and 4% sucrose (Chattopadhyay et al., 1994). The occurrence of L-DOPA and dopamine in tissues of *M. pruriens* and other plants will be the result of either of the following two biosynthetic pathways: 1.) the primary metabolite, tyrosine a product from the shikimic acid pathway could be ortho-hydroxylated into L-DOPA by tyrosine hydroxylase. L-DOPA was then converted to dopamine by DOPA decarboxylase; 2.) tyrosine could be decarboxylated into tyramine by tyrosine decarboxylase and tyramine ortho-hydroxylated into dopamine by phenoloxidase (Wichers, et al., 1984; Facchini, 2001). However, whereas tyrosine was detected in extracts from leaves and in cell cultures of *M. pruriens*, tyramine was not, suggesting that the first pathway may be the main biosynthetic pathway for L-DOPA in *M. pruriens* (Wichers, et al., 1984; Facchini, 2001).

Chemical analysis of L-DOPA in plants was performed using a non-specific calorimetric test in early reports (Maggi and Cometti, 1972; Szent-Kiralyi, 1979). L-DOPA was extracted from *M. pruriens* seeds with hot water, purified by column chromatography and quantified using UV spectrophotometry (Daxenbichler et al., 1972) or using an amino acid analyser (Bell and Janzen, 1971; Prakash and Tewari, 1999). A non-aqueous titration for determination of L-DOPA is used in the British

Pharmacopoeia (B.P) and the US Pharmacopoeia (U.S.P), which also describes a UV assay (Reynolds, 1989). These earlier methods were however laborious and specific only for L-DOPA detection (Modi et al., 2008). Reverse-phase high performance liquid chromatography (RP-HPLC) provided a comprehensive separation and detection of L-DOPA, L-3-carboxy 6,7-dihydroxy-1,2,3,4-tetrahydro-isoquinoline and 1-methyl-3-carboxy-6,7-dihydroxy-1,2,3,4-tetrahydro-isoquinoline in *M. pruriens* seeds var *utilis* (cultivar from Tamil Nadu, India) (Siddhuraju and Becker, 2001).

However, different methods for extraction of L-DOPA vary significantly in efficiency and hence affect reported L-DOPA levels in the plant extract samples ranging from 50% as determined by thin layer chromatography after extraction with water and sulphurdioxide as an antioxidant to 0.98% following re-crystallisation (Table 3.3) (Misra and Wagner, 2007).

Table 3.3 Percentage recovery of L-DOPA by re-crystallisation from seed extracts of *M. pruriens* (Misra and Wagner, 2007).

Extraction solvent	L-DOPA yield (% crystallised)
Ethanol/Water (1:1) ascorbic acid as antioxidant	1.78
Water, sulphurdioxide as anti-oxidant	0.98
Chloroform (pH 6.6)	4.00

Misra and Wagner (2007) demonstrated that the efficiency of extraction of L-DOPA and isoquinoline alkaloids from *M. pruriens* seeds was dependant on the type of alcohol. Methanol was determined to be the most efficient alcohol used in crude extraction of L-DOPA (Table 3.4).

Table 3.4 shows the efficiency of different alcohols used in the crude extraction of a mixture of L-DOPA and isoquinoline alkaloids from *M. pruriens* seeds (Misra and Wagner, 2007).

Extraction solvent	**Yield of L-DOPA and alkaloidal components (%)**	**TLC identification using reference compounds**
n-Butanol	7.6	Amino acids, L-DOPA (minor product).
Ethanol	6.7	Amino acids, L-DOPA, isoquinoline alkaloids.
Methanol	9.7	Amino acids, L-DOPA (major product).

Siddhuraju and Becker (2001) demonstrated an efficient method for extraction of L-DOPA from *M. pruriens* seeds by using hydrochloric acid (0.1 M) as the extraction solvent. Pure L-DOPA was then separated from the tetrahyroisoquinoline alkaloids in the extract by HPLC followed by UV detection. The concentration of pure L-DOPA extracted using the method described by Siddhuraju and Becker (2001) from *M. pruriens* seeds was 4.96% of dry weight. The second highest concentration of pure L-DOPA reported to be extracted from *M. pruriens* seeds was 4% of dry weight and was achieved by using chloroform (pH 6.6) as the extraction solvent (Table 3.3) (Misra and Wagner, 2007). It was observed that the use of different alcohols as extraction solvents generated crude L-DOPA hence it was not possible to discern the actual dry weight of pure L-DOPA from the data (Table 3.4). In light of the above, the method described by Siddhuraju and Becker (2001) for L-DOPA extraction and quantification of L-DOPA using seeds of *M. pruriens* seems to be among the most efficient. The method was thus used to determine more accurately the L-DOPA-content in different tissues of *M. pruriens* in this study.

3.1.1: Aims, objectives and approaches used in Chapter 3.

The aims of this chapter was to determine the L-DOPA-content in seeds, leaves, stems and roots of *M. pruriens* "90 day" cultivar.

3.2: MATERIALS AND METHODS.

3.2.1: Plant material.

Seeds of three *Mucuna pruriens* cultivars: "90 day", "early maturing" and "tropical" were purchased from Echo seed bank (USA) and were stored at 4 ˚C.

M. pruriens seeds were grown in the greenhouse as described in Chapter 2 Section 2.1.2. Seeds, leaves, stem and roots were collected from 30 plants at 14, 28 and 35 day old seed pod stages and were stored at -80 ˚C for future use.

3.2.2: Extraction of L-DOPA from *M. pruriens.*

L-DOPA and dopamine were extracted from seeds, leaves, stems and roots of *M. pruriens* by following the method described by Siddhuraju and Becker, (2001) with modifications as described below.

Samples of leaf, stem, root and seeds were collected from 30 plants. Leaf, stem and root samples were ground to a fine powder under liquid nitrogen using mortar and pestle. Seeds were first ground with an electric hand blender (Bosch MSM6300GB Hand Blender, 600W) for 30 seconds before manual grinding with mortar and pestle as described above. Powdered samples were stored at – 80 ˚C until used for L-DOPA/dopamine extraction and analysis.

The dry weight of *M. pruriens* leaf, stem, root and seeds was determined by drying 100 mg fresh weight of powdered samples at 105 ˚C in an oven to a constant weight.

The fresh weight equivalent of 70, 140 and 250 mg dry weight of plant tissue samples were used initially for extraction of L-DOPA and analysis.

Powdered samples were placed in glass tubes and to each; 10 ml of 0.1 N formic acid was added. Samples were stirred with a magnetic stirred for 10 minutes at room temperature (22 ˚C), homogenized in an ice bath with an Ultra-turrax T25 homogenizer (20500 min^{-1}) for 30 seconds and subsequently stirred with a magnetic stirrer for 2 h at room temperature. The supernatant was collected by centrifugation (3,000 X g, 15 min). The pellet was re-extracted twice with 0.1 N formic as described above, and supernatants of the three extractions were pooled and filtered through miracloth (Merck4Biosciences). The extracts were stored at 2 - 5 ˚C and were analysed within 8 h of preparation.

3.3.3: HPLC analysis of *M. pruriens* extracts.

3.3.3.1: Preparation of standards.

Stock solution (5 mM) of L-DOPA was prepared by dissolving 9.9 mg L-DOPA (Sigma HPLC grade/98% Pure) in 10 ml of 0.1 N formic acid. Similarly the stock solutions of dopamine (5 mM) and L-tyrosine (5 mM) ware prepared by dissolving 9.5 mg dopamine (Sigma, HPLC grade/98% Pure) and 9.7 mg L-tyrosine (Sigma, HPLC grade/98% Pure) respectively in 10 ml of 0.1 N formic acid. The stock solutions were kept in darkness at 4 ˚C for 2 days. Standard solutions for calibration were prepared from stocks at 1 mmol/l, 0.2 mmol/l, 0.04 mmol/l and 0.008 mmol/l level by dilution with 0.1 N formic acid.

3.3.3.2: Measurement of L-DOPA and dopamine-content by HPLC.

Analyses were carried out on a Thermo Separation HPLC system consisting of a Quaternary gradient pump (P4000, Thermo Finnigan) with photo-diode array detection (6000LP, Thermo) and autosampler (A-7200 Model). 10 µl of standard or sample were resolved over a 250 X 4.6 mm I.D column Nucleosil C18 (Machery-Nagel) with guard column at room temperature with a flow rate of 1.2 ml/min in a binary gradient system of A = water (975.5 mL): methanol (19.5 mL): phosphoric acid (1 mL), pH 2, B = 70% methanol and the following time program: 100% (A) and 0% (B) up to 12 min, next 5 min solvent (B) increase from 0 to 100% with 100 to 0% decrease of solvent A, increase A to 100% and decrease B to 0% in the next 5 min, and then the column is washed with solvent A alone in the next 15 min to adjust the column to the starting conditions (A 100% and B 0.00%). Isocratic elution was performed at room temperature (22 ˚C). The data generated were integrated for a wavelength of 280 nm using Excalibur (Thermo Scientific, Germany). However, the preliminary range finder trial experiments to determine the minimum detection level for L-DOPA were performed using RP-HPLC (Waters, Model-2006, UK).

3.4: RESULTS.

3.4.1: Preliminary range finder trial.

L-DOPA eluted after 3.4 min with an absorption maximum at 280 nm and its detection limit was 0.32 mg/l (= 3.2 ng/injection). The results showed that L-DOPA could not be detected in extracts of 28 day old leaf, stem and root *M. pruriens* tissues of less than 100 mg dry weight. L-DOPA was however detected in extracts from *M. pruriens* seeds of dry weight 70 and 250 mg (Figure 3.1).

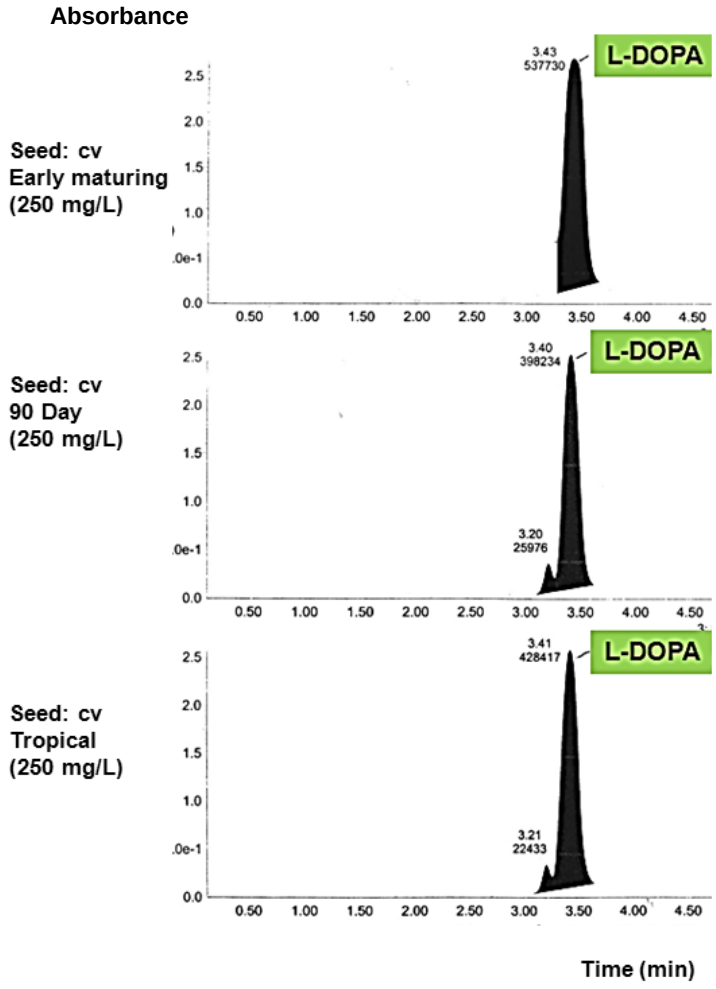


Figure 3.1 Chromatograms of *M. pruriens* seed extracts for the three cultivars under study obtained at 3.4 minute elution time and absorption spectra of 280 nm using a Reverse phase-HPLC (Waters, UK).

Since L-DOPA could not be detected in extracts from 100 mg dry weight of leaf, stem and root, 250 mg dry weight was used for further analyses. In this experiment a further analysis was carried out using a Thermo Separation HPLC system (See Section 3.3.3.2) to determine the purity of the L-DOPA previously extracted from the seed tissue (Figure 3.2 b). Pure L-DOPA (Sigma) eluted after 9.7 min with an absorption maximum at 280 nm (Figure 3.2a).

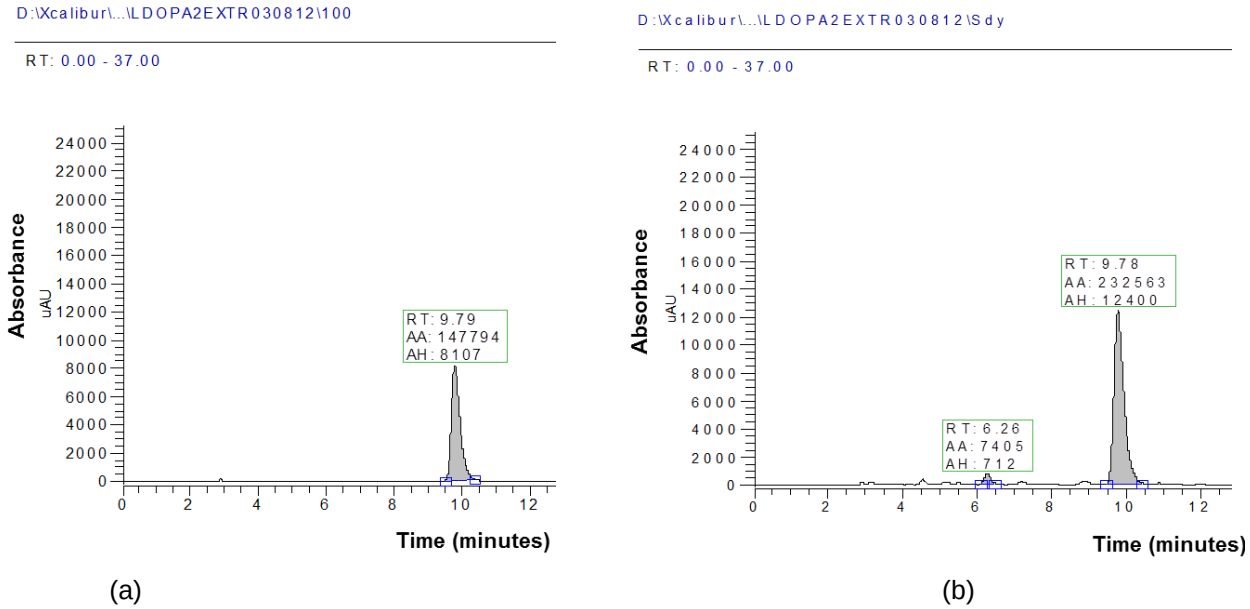
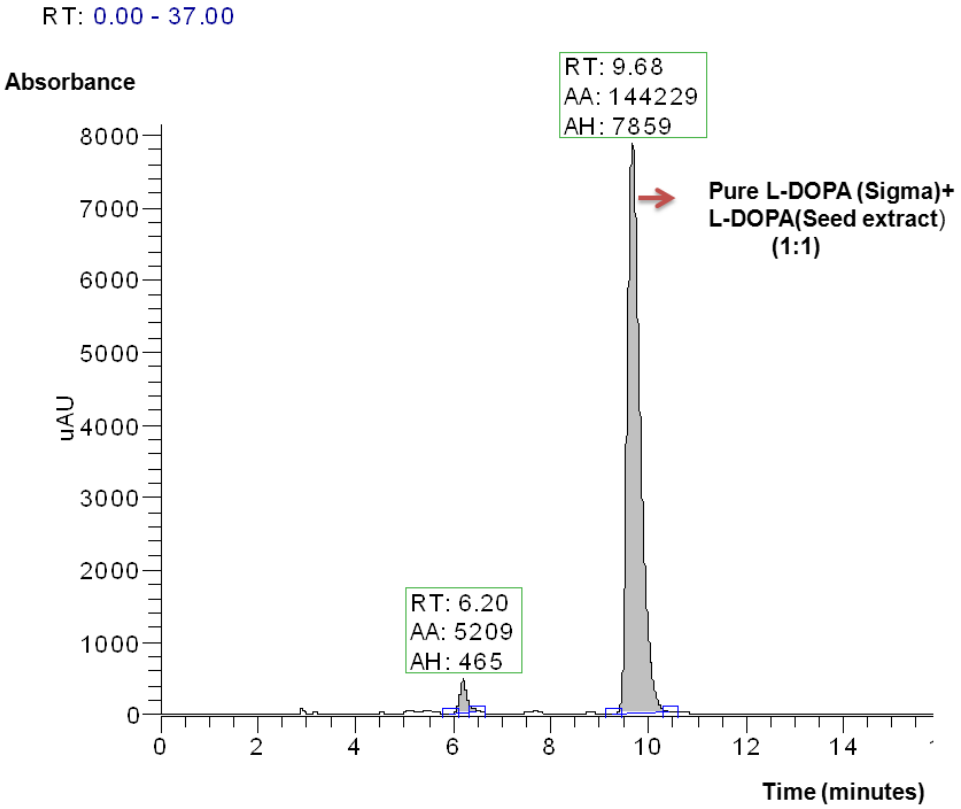


Figure 3.2 Chromatogram for (a) pure L-DOPA (Sigma, 100 µg/ml) and (b) extract from *M. pruriens* seeds had an absorption spectrum of 280 nm and an elution time of 9.7 minutes upon analysis using a Thermo Separation RP-HPLC system.



(c)

Figure 3.2 (c) A single chromatogram with absorption spectra of 280 nm and an elution time of 9.7 minutes which is characteristic for pure L-DOPA was obtained by analysis of a mixture of pure L-DOPA (sigma) and extracts from *M. pruriens* seeds using a Thermo Separation Reverse phase-HPLC system (USA).

3.4.2: L-DOPA concentrations.

L-DOPA-content in different parts of *M. pruriens* at 28 day old (seed pod) stage was higher than at the 14 day old stage (See Table 3.1).

Table 3.1 L-DOPA concentration in tissues of *M. pruriens* at 14 and 28 d post flowering.

M. pruriens **tissue**	**L-DOPA-content (µg g^{-1})**	
	14 d	**28 d**
Seed	282.0	581.0
Leaf	30.5	33.3
Stem	31.1	37.2
Root	26.7	37.5

In each case, triplicate samples were run on the RP-HPLC and obtained the same value. The L-DOPA-content in seeds obtained from 28 day old seed pods was 580.7 µg g^{-1} and about two-fold higher than that in seeds from 14 day old (282.2 µg g^{-1}) (Table 3.1). L-DOPA-content in seeds from 28 day old seed pods (580.7µg g^{-1}) was about 17-fold higher than in leaves at 28 day stage (33.3 µg g^{-1}), about 15-fold higher than in stems and root tissues at the 28 day stage (Table 3.1).

3.5: DISCUSSION.

L-DOPA is produced by *M. pruriens* and a few other plant species during secondary metabolism (Facchini, 2001; Awang et a., 1997). Siddhuraju and Becker (2001) reported L-DOPA-content of 4.96 g g^{-1} in *M. pruriens* seeds of a cultivar obtained from Tamil Nadu, India. The L-DOPA profile analysis here for *M. pruriens* (90 day cultivar) shows the L-DOPA-content in seed tissues (0.58 mg g^{-1}) was 17-fold more than that in leaves and 15-fold more than that in stem and root tissues (Table 3.1). However, Siddhuraju and Becker, (2001) reported a much higher L-DOPA-content (49.6 mg g^{-1}) in seeds of *M. pruriens* cultivar grown under a natural environmental conditions in Tamil Nadu, India. The finding suggests very significant differences in L-DOPA-content among cultivars of *M. pruriens.* Similarly, the finding also suggests *M. pruriens* plants grown in their natural environmental conditions had significantly higher L-DOPA-content compared to those grown in a greenhouse. Despite the relatively high L-DOPA-content in *M. pruriens* seeds, they are not a reliable source for constant supply of L-DOPA because most *M. pruriens* cultivars and especially the "wild type" took 3-5 months to flower, before producing seeds. On the other hand leaves grow and accumulate biomass within 3 weeks and as thus would be the preferred tissue to target for continous large scale extraction of L-DOPA. The challenge however is that the L-DOPA-content in leaves is very low (~33.3 µg g^{-1}) as shown in Table 3.1, hence this would be a serious limitation for large scale extraction of L-DOPA. A possible remedy could be extraction of L-DOPA from leaf cell suspension cultures instead leaf tissues. Wichers et al., (1993) reported that leaf extracts contained more dopamine than L-DOPA, but in continuously grown *M. pruriens* cell suspension cultures, the L-DOPA-content was 16 - 80 fold higher than

dopamine, which could be further increased up to 74% by subjecting the cell suspension culture to higher salt stress which plasmolysed the cells (Wichers et al., 1993). These findings also seem to suggest that L-DOPA is stored in cell vacuoles.

The L-DOPA-content in tissues of *M. pruriens* plants at 28 day old seed pod stage was higher than at 14 day seed pod stage (Table 3.1). L-DOPA is an alkaloid produced by a few plants such as *M. pruriens* as a secondary metabolite used in plant defense mechanism as an allelochemical (Nishihara et al., 2005). The increase of L-DOPA content in leaf, stem and root of *M. pruriens* between the 14 and 28 day old seed pod stage is relatively small when compared to that of seeds (Table 3.1). Wichers et al., (1993) demonstrated that L-DOPA-content in cells of *M. pruriens* increased with an increase in biomass and this relatively small increase in L-DOPA-content in leaf, stem and root tissues is likely to reflect such a response. On the other hand the L-DOPA-content in seeds obtained from 28 day old pods was two-fold higher than for the 14 day old seed pods of the same plants and 15 - 17 times higher than in other tissues. In contrast, the reported L-DOPA-content in leaves, stems and roots of germinating *M. pruriens* seedlings was reported to decrease drastically within 3 weeks, however, starting from intially extremely high concentrations (9.8%D.W) (Wichers, et al., 1993). The high L-DOPA concentrations would deter herbivory of the tender young leaves due its hallucinogenic properties (Facchini, 2001; Taiz and Zeiger, 2006).

CHAPTER 4: ISOLATION, CHARACTERISATION AND EXPRESSION OF A *MUCUNA PRURIENS* DOPA DECARBOXYLASE LIKE-GENE (*Mp-ddc*)

4.1: INTRODUCTION.

M. pruriens is one of the few plant species which produces L-DOPA (Bell and Janzen, 1971; Brain, 1976). To better understand this process with the longer term aim of manipulating it, one aim of my work was to isolate the genes which encode enzymes in the biosynthesis and metabolism of L-DOPA in *M. pruriens*. I focussed on two genes: tyrosine hydroxylase (Mp-tyoh) and DOPA/tyrosine decarboxylase (Mp-ty/ddc) (Figure 4.1). These encode enzymes responsible for the synthesis of L-DOPA from L-tyrosine and its conversion to dopamine respectively (Fitzpatrick, 1999; Facchini et al., 2000). In this chapter describes the experiments undertaken to try to clone these genes from *M. pruriens*.

In animals, tyrosine hydroxylase (TYOH) is the first rate limiting enzyme in the biosynthesis of catecholamines (CA), derived from L-DOPA (Hillas and Fitzpatrick, 1996) and is likely the first and rate limiting step also in plants (Facchini and De Luca 1995; Kuklin and Conger, 1995) (Figure 4.1). TYOH is an iron-containing, biopterin-dependant amino acid hydroxylase which catalyses the hydroxylation of L-tyrosine produced via prephenate in the shikimic acid pathway to L-DOPA as shown in Figure 4.1. TYOH, phenylalanine hydroxylase and tryptophan hydroxylase belong to a family of catalysts that use tetrahydrobiopterin (BH_4) and oxygen to hydroxylate tyrosine to L-DOPA, phenylalanine to tyrosine and tryptophan to 5-

hydroxytryptophan (5-HTP) respectively (Fitzpatrick, 1999). A monophenol monooxygenase with TYOH activity was isolated from *M. pruriens* suspension cultures (Wichers at el., 1985) but to date no gene sequences are available from this species.

DOPA/tyrosine decarboxylase (TY/DDC) is responsible for decarboxylation of L-DOPA to dopamine and the decarboxylation of L-tyrosine to tyramine (Figure 4.1) (Facchini et al., 2001). In plants and other organisms, TY/DDC enzymes are dependent on coenzyme pyridoxal 5'-phosphate (PLP) (Facchini and De Luca, 1994). DOPA/tyrosine decarboxylase genes have been isolated from a number of plant species (Facchini et al., 2001).

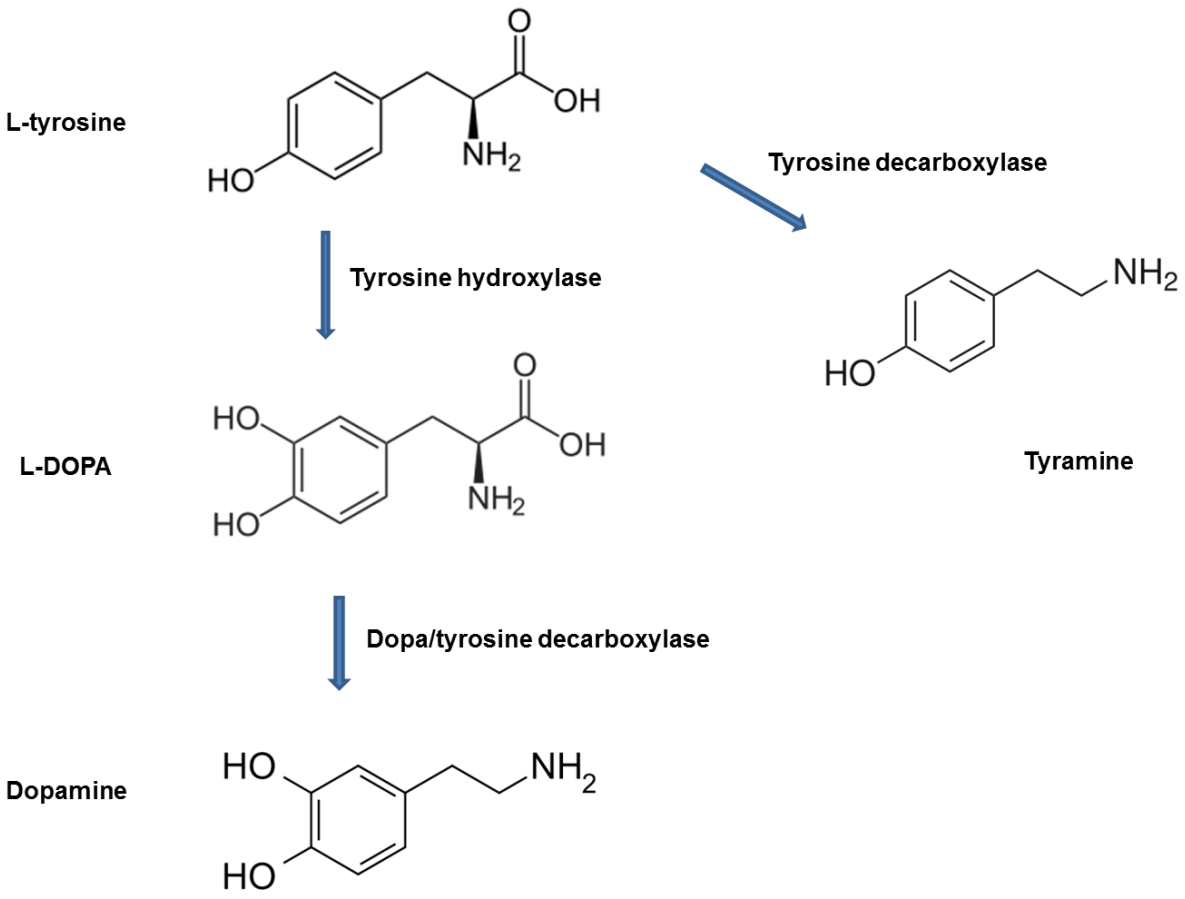


Figure 4.1 Schematic diagram showing enzymes responsible for bioconversion of L-tyrosine to L-DOPA or tyramine pathways in plants.

The enzymatic action of tyrosine decarboxylase (TYDC) on L-tyrosine to produce tyramine is a more conventional pathway especially in plants which do not produce L-DOPA (Guillet and De Luca, 2005). Tyramine is a precursor for the synthesis of important isoquinoline alkaloids and a wide range of plant defense system compounds (Facchini, 2001). The chemistry of isoquinoline alkaloids is widely appreciated but little is known about the genes and the enzymology regulating the isoquinoline biosynthesis in plants (Facchini, 2001).

The most studied and characterised plant DOPA/tyrosine decarboxylase (TY/DDC) is that for *Papaver somniferum* (opium poppy), encoded by a gene family (Ps-ty/ddc) of 10 to 14 members (Facchini and De Luca, 1994). The Ps-ty/ddc gene family is divided into two subsets (Ps-ty/ddc1 and Ps-ty/ddc2) based on sequence homology among members of the gene family and they encode proteins of predicted molar mass of 56,983 and 59,323 Daltons (Da) respectively (Facchini and De Luca, 1994). In each of the subsets (ty/ddc1 or ty/ddc2) the genes exhibit greater than 90% identity but share less than 75% identity between subsets. A genomic southern analysis revealed that the Ps-ty/ddc gene family of *P.somniferum* comprised of 6-8 and 4-6 genes in the Ps-ty/ddc1 and Ps-ty/ddc2 subsets respectively (Facchini and De Luca, 1994). *In situ* RNA hybridisation using Ps-ty/ddc1 and Ps-ty/ddc2 probes was used to demonstrate the differential and organ-specific expression of Ps-ty/ddc genes in mature plants. The Ps-ty/ddc1-like genes were highly expressed in roots although trace expression levels were detected in stems. On the other hand, Ps-ty/ddc2-like genes were substantially more expressed in stems than in roots. *In situ* RNA hybridisation revealed the Ps-ty/ddc gene expression is confined to the metaphloem and protoxylem in the vascular bundles. This is consistent with the hypothetical developmental origin of alkaloid rich Latificers (internal secretory cells) which grow adjacent to vascular tissues (Facchini and De Luca, 1995). In addition, Ps-ty/ddc2-like transcripts were detected in the calyx a day before anthesis and in carpels a day after anthesis. However, carpels showed low levels of Ps-ty/ddc gene expression despite having abundant alkaloid-rich latex suggesting that carpels/capsules of *P. somniferum* are storage sites for the alkaloids while the actual biosynthesis occurs in the stems and roots (Facchini and De Luca, 1995). The Ps-ty/ddc differential expression and the correspondent accumulation of different

alkaloids in specific plant tissues suggest a coordinated regulation of specific biosynthetic genes under the influence of developmental programs.

4.1.1: Aims, objectives and approaches used in Chapter 4.

The primary aim of this chapter was to isolate genes related to the rate-limiting enzyme steps in the biosynthesis and metabolism of L-DOPA in *M. pruriens*. This was approached by searching publicly available databases for already annotated genes or sequences showing homology to genes already identified in other plants. Another approach attempted was to design degenerate PCR primers from sequences available in data bases for other plants and use them to amplify gene fragments from *M. pruriens* cDNA. The further aims were to obtain full length cDNA sequences using 3' and 5' RACE, to study the copy number of any identified genes by Southern blotting and analyse their expression across different *M. pruriens* tissues.

4.2: METHODS AND MATERIALS.

4.2.1: Plant Materials.

M. pruriens seeds were procured from Echo seed bank (USA) and grown in the under greenhouse conditions as described in Chapter 2 Section 2.1.2. Samples of *M. pruriens* tissues were harvested at 14 and 28 day stages after flowering as described in Chapter 2 Section 2.1.3.

4.2.2: Extraction of nucleic acids and cDNA synthesis

DNA and RNA were extracted from plant tissues by using the methods described in Chapter 2 Sections; 2.2.1 and 2.2.2.2 respectively. cDNA synthesis was performed as described in Chapter 2 Section 2.2.4.

4.2.3: Rapid amplification of cDNA ends.

4.2.3.1: 3' RACE and Primer design.

The principle of 3' RACE is to use the characteristic poly-A nucleotide sequence at the 3' terminal of a eukaryotic gene to design a 3' adapter primer using a DNA oligo d(T) primer to which an adapter sequence is added. This is then used for cDNA synthesis. The oligo d(T) used for 3' RACE cDNA synthesis here was designed by adding 34 bp of G-C rich "adapter" sequence to the 5' end of the primer sequence: $dT_{3RACE\text{-}GC}$ 5'-GCGAGCACAAGAATTAATACGACTCACTATAGG**T$_{(20)}$**TVN-3'. The 3'

adapter sequence was used to design two 3' RACE "nested" reverse primers; R1 (3RACE): and R2 (3RACE): for nested PCR amplification of the un-sequenced 3' end of the target genes (Table 4.1). 3' RACE "nested" forward primers F1**(3RACE)** and F2**(3RACE)** were designed as described in Section 2.2.5.1, from the partial Mp-ty/ddc gene (EF101921.1). Figure 4.2 shows the position of the primers which were used in 3' RACE PCR.

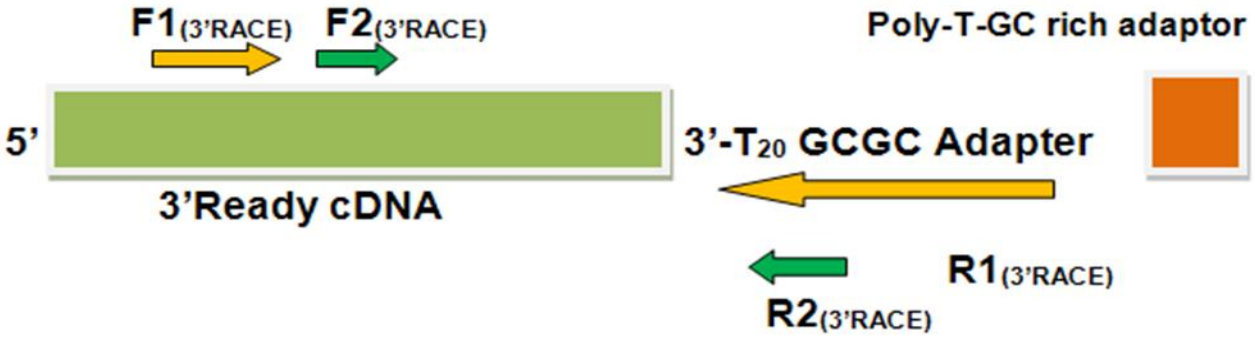


Figure 4.2 Position of the 3' RACE primers on the Mp-ty/ddc cDNA.

Table 4.1 Primers used in 3' RACE PCR on the Mp-ty/ddc gene.

No.	3' RACE Primers (10 nm; 1µl)	Sequence
1	3' RACE adapter primer; $dT_{3RACE-GC}$	5'GCGAGCACAAGAATTAATACGACTCACTA TAGG**T**$_{(20)}$TVN-3'
2	F1 **(3RACE):**	5'-GCCACAGAGCTCGAAGCCTT-3'
3	R1 (3RACE):	5'GCGAGCACAGAATTAATACGACT 3'
4	F2 **(3RACE):**	5'-CTCTGGTGAGGGTGGGGTGCTTTTGGG-3'
5	R2 (3RACE):	5'- GAATTAATACGACTCACTATAG-3'

4.2.3.2: 3’ RACE cDNA synthesis and PCR amplification.

Total RNA (2 µg) was extracted from 4 week (“young”) and 8 week old (“mature”) healthy leaf tissues of *M. pruriens* plants using the method described in Chapter 2 Section 2.2.2.2. The “young” and “mature” stages were chosen because some genes related to secondary metabolism are switched on or off depending on the developmental stage of a plant. Total RNA was used as a template for cDNA synthesis by following the method described in the 3’ RACE manual (Ambion, USA) as follows; 2 µg of leaf or seed RNA was then mixed with 1 µl of 3’ adapter primer (10 nM) and incubated at 70 ˚C for 2 min and then cooled on ice for 5 min. The following components were then added; 5 X AMV reverse transcriptase (RT) buffer (5 µl), dNTPs (10 mM; 2.5 µl) (Sigma, USA), 1 µl of Rnasin Ribonuclease inhibitor (Ambion, USA), AMV RT (0.5 units) (Promega, USA) and the total volume was made up to 25 µl with RNAse free water and mixed well. The reaction mixture was incubated at 58 ˚C for one hour.

The (3’ RACE ready) leaf cDNA (1 µl) was used as a template for the 3’ RACE PCR was performed using primers $F1_{(3'RACE)}$ and $R1_{(3'RACE)}$ described above (Fig 4.2). The PCR product from this reaction (15 ng) was used as a template for a 3’ RACE “nested” PCR reaction using the inner primers $F2_{(3'RACE)}$ and $R2_{(3'RACE)}$ (Figure 4.2). Both PCRs were carried out as follows; 94 ˚C for 2 min, 34 cycles (94 ˚C for 30 sec, 55 ˚C for 30 sec, 72 ˚C for 2 min) and 72 ˚C for 10 min (extension time).

4.2.3.3: 5’ RACE and Primer design.

4.2.3.3.1: Principles of 5’ RACE.

5’ RACE is a PCR based technique used to isolate a full length cDNA from a gene whose 5’ terminal sequence data is unknown. 5’ RACE was performed using a BD SMART^{TM} RACE cDNA Amplification kit manual (Clontech, UK). BD SMART technology (Zhu et al., 2001) facilitates the amplification of full length cDNAs by the joint action of the BD SMART II ^{TM} A Oligonucleotide and the BD PowerScript Reverse Transcriptase (RT). BD PowerScript RT is a variant of MMLV RT that, upon reaching the d(C)-rich cDNA tail uses it as an extended template for RT. BD PowerScript RT switches templates from the mRNA molecule to the BD SMART oligo, generating a complete cDNA of the original RNA with the additional BD SMART sequence at the end (Figure 4.3). 5’-RACE-Ready cDNA was synthesized as described for the 3’ RACE using 12 µM of the provided 5’ RACE CDS primer (5’-CDS;): 5’-T_{25}VN-3’ (V = A,C,G, or T; V = A,G, or C) and 1 µg total RNA using also the BD SMART II Oligonucleotide 5’-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3’, provided by the kit which anneals to the 5’ C extension and provides the template at the 5’ end of the cDNA for subsequent PCR. The tubes were incubated at 70 ˚C for 2 minutes followed by cooling on ice for 2 minutes. The tubes were then briefly spun to collect the contents at the bottom before the following were added to each reaction: 5 X First-strand buffer (2 µl), DTT (20 mM; 1 µl), dNTP mix (10 Mm; 1 µl), BD PowerScript Reverse Transcriptase (1 µl). The contents of the tubes were mixed by gentle pipetting before being collected to the bottom by a brief spin as above. The tubes were then incubated at 42 ˚C for 1.5 hours in a hot-lid PTC-100 thermal cycler (MJ Research Inc., Waltham, USA).

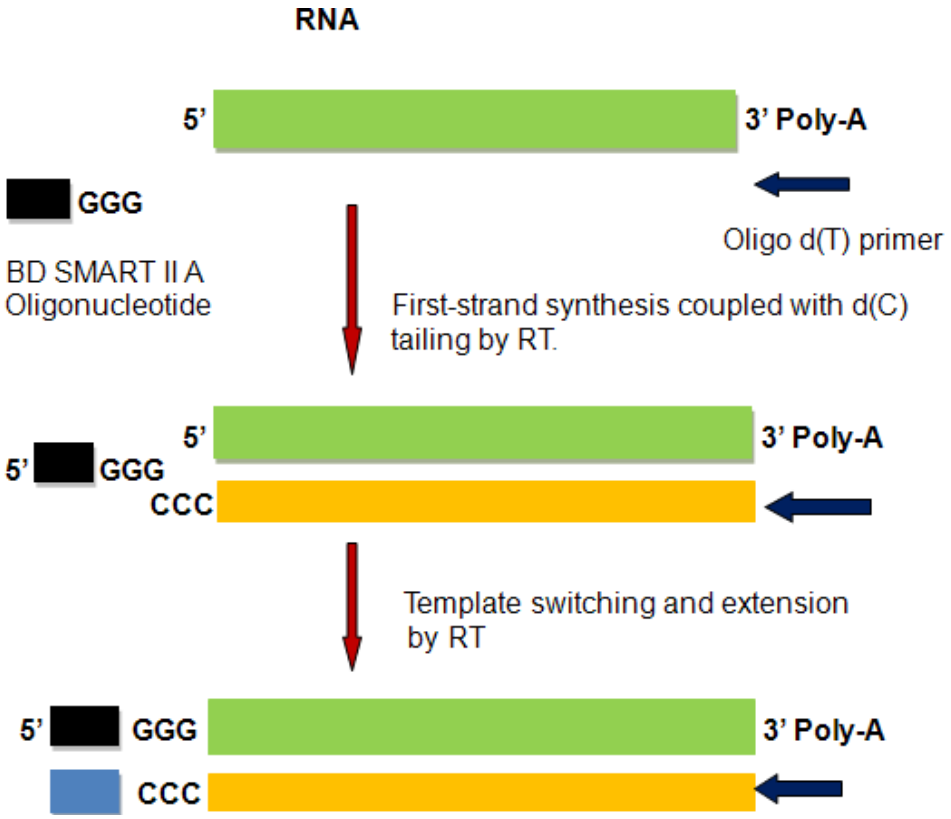


Figure 4.3 Mechanism of BD SMART cDNA syntheses. First-strand synthesis is primed using a modified oligo d(T) primer. After reverse transcriptase reaches the 5' end of the mRNA template, it adds several d(C) residues. The BD SMART II A Oligo nucleotide anneals to the cDNA and serves as an extended template for BD PowerScript RT.

The first-strand reaction product was diluted with 100 µl of Tricine-EDTA buffer before the reaction was stopped by denaturing the enzyme at 72 ˚C for 7 minutes. The synthesised 5' RACE-Ready cDNA samples were then stored at -20 ˚C for three months.

4.2.3.4: 5’ RACE and PCR amplification.

PCR-grade water (34.5 µl), 10 X BD Advantage 2 PCR buffer, 50 X BD Advantage 2 Polymerase mix (1 unit), dNTP mix (10 mM; 1 µl) and were mixed by vortexing and the reaction tubes briefly spun in a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany). The following components were then added; 5’-RACE-Ready cDNA (2.5 µl), UPM (10 X; 5 µl), Mp-ty/ddc-R1$_{(5'RACE)}$ reverse primer (10 mM; 1 µl) (Table 4.1). Two negative control 5’ RACE experiments were set up as described above but lacking at least one primer; either the Mp-ty/ddc-R1$_{(5'RACE)}$ reverse primer (10 mM; 1 µl) or the UPM (10 X; 5 µl), in each case sterile water added to make a total volume of 50 µl.

Touchdown 5’ RACE PCR was performed using a PTC-100 thermal cycler (MJ Research Inc., Waltham, USA) as follows: 5 cycles (94 ˚C, 30 seconds; 72 ˚C, 3 minutes), 5 cycles (94 ˚C, 30 seconds; 70 ˚C, 30 seconds; 72 ˚C, 3 minutes), and 25 cycles (94 ˚C, 30 seconds; 68 ˚C, 30 seconds; 72 ˚C, 3 minutes), for primers whose melting temperature (Tm) was greater than 70 ˚C. For 5’ RACE gene specific primers whose Tm was 65 - 70 ˚C, the thermal cycler program ran was: 25 cycles (94 ˚C, 30 seconds; 68 ˚C; 30 seconds; 72 ˚C, 3 minutes). In cases where the primary PCR reaction failed to give a distinct band of interest or produced a smear, a “nested” PCR reaction was performed using the NUP and the nested Mp-ty/ddc-R2$_{RACE}$ primers (Table 4.1) to re-amplify from 2.5 ul of the primary 5’ RACE PCR product, using the thermal cycler conditions: 25 cycles (94 ˚C, 30 seconds; 68 ˚C, 30 seconds and 72 ˚C; 3 minutes). Other reaction components were as above.

The 5' RACE PCR products were analysed by UV agarose gel electrophoresis as described in Section 2.2.7. The DNA band of interest was purified from the agarose gel as described in Section 2.2.9 and was ligated in a pZERO-2-T plasmid (Invitrogen, USA) or pGEM-T vector (Promega, USA) as described in Section 2.2.11. The transgenic plasmid was transformed and cloned using *E.coli*DH5α as described in Section 2.2.12. The bacterial cells carrying the transgene clone of interest were screened by colony PCR and a single transgenic bacterial colony inoculated on LB medium as described in Sections 2.2.13 and 2.2.14 respectively. The transgenic plasmid was recovered from the bacterial culture as described in Section 2.2.14. The purified transgenic plasmid was further screened by double digestion using restriction enzymes flanking either sides of the cloned 5' RACE PCR product as described in Section 2.2.14. The size of the released 5' RACE PCR fragment was confirmed by UV agarose gel electrophoresis as described in Section 2.2.7 and consequently a plasmid sample (80 ng μl^{-1}) was sequenced at Eurofins MWG Sequencing (Germany). The 5' RACE sequence result was analysed using the generunner software program before it was analysed using Blast against the NCBI data bank (www.ncbi.nih.gov/BLAST/) to draw protein alignments.

4.2.4: Southern analysis.

4.2.4.1: Principle of Southern blot analysis.

Southern analysis is used for determining the copy number of a specific sequence in DNA samples (Sambrook et al., 1989). It involves restriction digestion of a DNA sample, separation of the DNA fragments through an agarose gel by electrophoresis,

and transfer of the separated DNA fragments from the gel to a nylon membrane by capillary action. The DNA is fixed to the nylon membrane and used to perform hybridisation assays with sequences of known genes as probes. The DNA probe used in this study was 1.5 kb ORF of Mp-ty/ddc, radioactively labelled with P-^{32}. The copy number of the Mp-ty/ddc gene in the genome was determined by comparing the number of hybridisation signals observed to the expected number of gene fragments from the known 1.727 kb sequence for a given restriction enzyme. The copy number of a gene was deduced to be one, for a signal to fragment ratio of 1:1, and two for the 2:1 ratio.

4.2.4.2: Restriction digestion and Gel electrophoresis.

Genomic DNA used for the Southern analysis was extracted from leaves of *M. pruriens* as described in Section 2.2.1. The sequenced 1.727 bp of Mp-ty/ddc gene restriction map was used to select the appropriate restriction enzymes used for genomic DNA digestion and gene copy number analysis. The Mp-ty/ddc gene has one *Eco*RV restriction site at position 1.5 kb (3' end of the Mp-ty/ddc ORF) which upon digestion and radiolabelled probing, was predicted to produce a single hybridisation signal for a single copy of a gene. *Hind*III on the other hand has two restriction sites at positions; 487 and 520 bp, hence upon digestion and probing should produce two visible hybridisation signals and a small invisible signal corresponding to the DNA fragments carrying the 487, 520 and the 33 bp Mp-ty/ddc gene portions respectively (See Figure 4.3).

M. pruriens genomic DNA was digested using *Hind*III and *Eco*RV enzymes as shown in Figure 4.4. In addition *Eco*RI and *Xho*I which have no restriction sites in the known Mp-ty/ddc gene sequence were also used to digest genomic DNA for Southern analysis. The DNA digestion reactions were set up in 1.5 ml Eppendorf tubes as follows: DNA (20 µg), using 10X NEB buffer 4 (15 µl) for *Eco*RI-HF (10 units); 10 X NEB buffer 3 (15 µl), 10 X BSA (15 µl) for *Eco*RV (10 units); 10 X NEB buffer 4 (15 µl), for *Hind*III (10 units) and 10 X Tango buffer 3 (15 µl) (Thermo Scientific), 10 X BSA (15 µl) for *Xho*I (10 units). The total volume was made up to 150 µl with sterile water. The reactions were gently mixed before incubation at 37 ˚C (Heratherm Incubator, Thermo Scientific) overnight for 14 - 16 hours. The digested DNA was extracted by phenol-chloroform DNA purification followed by isopropanol precipitation as described in Sections 2.2.17 and 2.2.18 respectively.

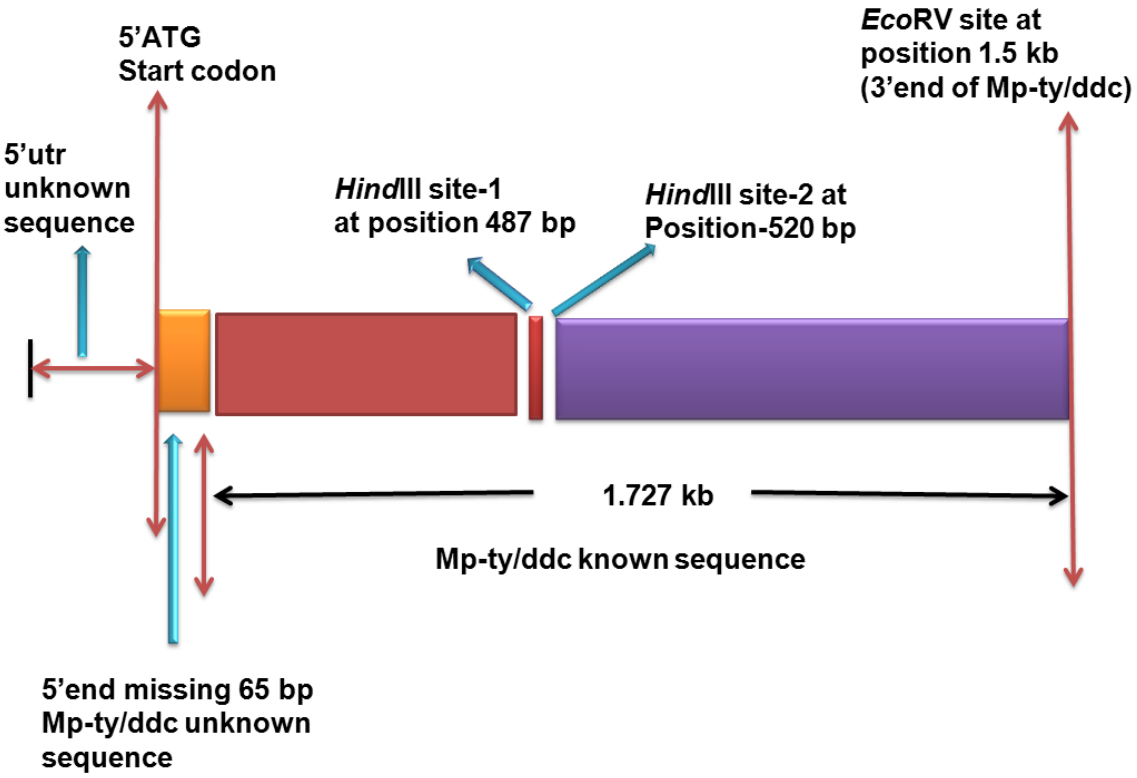


Figure 4.4 shows 1.727 kb Mp-ty/ddc restriction map shows that *Hind*III cleaves the gene at restriction sites 487 and 520 bp which results in release of three Mp-ty/ddc fragments whereas *Eco*RV cleaves the DNA at position 1.5 kb and releases a single Mp-ty/ddc fragment.

A positive control for the Southern blot analysis was generated by linearisation by *Eco*RI digestion of a pET21b-Mp-ty/ddc plasmid harbouring 1.5 kb Mp-ty/ddc full length ORF transgene. The plasmid DNA was linearised as follows; pET21b-Mp-ty/ddc DNA (0.6 ng; 5.5 µl), NEB buffer 4 (10 X; 3 µl), *Eco*RI (1 unit) and the volume was made up to 30 µl using sterile water. The reaction was incubated for 3 hours at 37 ˚C, after which the plasmid was column purified as described in Chapter 2 Section 2.2.8. The linearised plasmid was diluted to a concentration to 5 pg/µl, equivalent to a single copy Mp-ty/ddc gene expression (Sambrook et al., 1989). About 100 pg of the diluted linearised plasmid DNA was used a positive control on the Southern analysis agarose gel electrophoresis as described below.

A 0.8% agarose gel stained with ethidium bromide (0.5 µg ml^{-1}) was submerged in 2000 ml 1X TAE [40 ml of 50x TAE: Tris base (242 g); acetic acid (100%, 57.1 ml); EDTA (0.5 M, 100 ml)], in a gel electrophoresis tank (Power Pac Basic, Bio-Rad, Singapore). The digested DNA fragment samples and the positive control were loaded in separate wells and were electrophoresed at 40 volts, 35 mAmps for 16 hours. Separated DNA fragments were analysed by UV imaging using a genedoc machine (Syngene genedoc, Japan) as described in Section 2.2.7. The separated DNA fragments were then denatured *in situ* as follows; the agarose gel was submerged in depurination solution (0.125 M HCl) and agitated gently for 10 - 20 minutes. This was followed by submerging the gel in denaturation buffer [1.5 M NaCl (87.66 g); 0.5 M NaOH (20 g); volume made to 1 litre with sterile water], and incubated for 30 minutes with gentle agitation. This alkaline denaturation enhances binding of the negatively charged thymine residues of DNA to the positively charged amino groups of the hybond nylon membrane (Amersham, UK). Finally the gel was then submerged in neutralisation buffer [1.5 M NaCl (87.66 g); 0.5 M Tris (60.5 g); volume made up to 1 litre with sterile water], and was incubated for 30 minutes with gentle agitation.

4.2.4.3: Southern blot

The *in situ* denatured DNA fragment smear was transferred to a hybond nylon membrane (+) (Amersham, UK) by capillary blotting based on the principle of capillarity. The nucleic acid transfer buffer [10 X SSC: Tri-sodium citrate (44.115 g); NaCl (82.82 g); sterile water added to 1 litre; pH 7.5], was drawn by capillary action

from the region of high water potential (plastic tray), to that of low water potential (paper towels) as shown in Figure 4.4 below. During this process, DNA was transferred from the gel to the membrane; DNA was bound to the membrane by ion exchange interactions between the negative charge of the DNA and the positive charge of the membrane.

A plastic tray was half filled with nucleic acid transfer buffer (10 X SSC). Then a support stand was covered with 3 pieces of Whatman paper (3 MM) wick with no air bubbles trapped in between them was assembled in 10 X SSC buffer as shown in Figure 4.4. The treated gel was placed upside down on the wick platform removing any air bubbles. The gel sides were then surrounded with cling film to prevent the buffer from being absorbed directly into the paper towels. A hybond nylon membrane of the same size as the gel was pre-soaked in 10 X SSC buffer and then placed on top of the gel without trapping any air bubbles. Three sheets of Whatman 3 MM paper cut to the size of the gel were saturated in 10 X SSC buffer and then placed on top the membrane. Trapped air bubbles were removed by rolling a glass rod over the sheets of Whatman paper. Finally, a stack of absorbent towels were placed on top of the Whatman paper sheets to enhance the capillary pressure and upward movement of transfer buffer through the gel. Pressure was applied evenly to the gel by placing a weight of 0.5 kg on top of the absorbent towels.

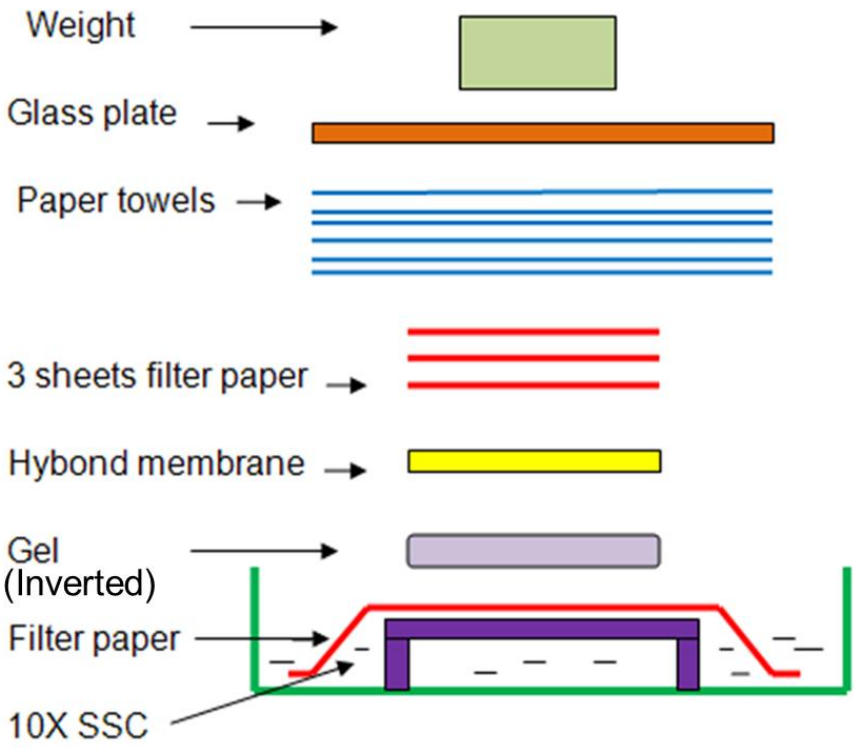


Figure 4.5 Diagrammatic representation of the capillary blotting apparatus used to transfer the DNA from the agarose gel to a hybond nylon membrane (Southern blot). The Filter paper dipped in 10 X SSC buffer served as a wick while the paper towels drew the solution upwards through the gel and in the process DNA was transferred on to the hybond nylon membrane.

DNA was then immobilised on the membrane by UV-crosslinking (100 mJ/cm^2) using a UV-cross linker (302 nm, 230 V; China). The membrane was wrapped in aluminium foil and stored at -80 ˚C until used.

4.2.4.4: Pre-hybridisation and Probe generation.

The nylon membrane was pre-hybridised by rinsing in Church buffer: Phosphate buffer (pH 7.2) [Na_2HPO_4 (1 M; 171 ml), NaH_2PO_4 (1 M; 79 ml)]; SDS (10%, 700 ml); EDTA (0.5 M; pH 8) as described below. The membrane was submerged (DNA side-up) in Church buffer (50 ml) and was incubated at 65 ˚C with gentle shaking on an orbital shaker (Helix 150, Labstrong, USA) for 6 hours. Pre-hybridisation served to block the membrane surface and target DNA from non-specific binding with the probe. After pre-hybridisation, the immobilised DNA fragments on the membrane blot were probed using a radioactive labelled Mp-ty/ddc PCR product. The probe was approx. 1.5 kb Mp-ty/ddc full length ORF, previously cloned in the 3.3 kb pET21b expression plasmid. The 1.5 kb probe was thus recovered from the *Eco*RI and *Xho*I cloning sites on the pET21b plasmid by restriction digestion using the respective enzymes as follows; pET21b-Mp-ty/ddc plasmid (2 µg), NEB buffer 4 (10X; 5 µl), *Eco*RI (3 units), *Xho*I (3 units), BSA (10 X) and the volume was made up to 50 µl using sterile water. The reaction was incubated at 37 ˚C for 4 hours after which the DNA fragments were separated by ethidium bromide stained agarose gel electrophoresis as described in Chapter 2 Section 2.2.7. The 1.5 kb Mp-ty/ddc gene fragment was gel purified as described in Chapter 2 Section 2.2.9. The gene fragment was then diluted to a probe concentration of 100 ng/ul.

The 1.5 kb Mp-ty/ddc gene probe was radioactively labelled by primer extension based on the procedure described in the Affymetrix primer-It II kit (Affymetrix, USA) as follows; DNA (100 ng; 5 µl) and Mp-tyddc primer mix: 5’-CTAGACCCTGAAGAGTTCAGAAGACAAGG-3’ and 5’-

CCACGTCCTCGTACGGTTAAGAGACAA-3' (10 µM each; 2 µl) were denatured in a 1.5 ml Eppendorf tube at 95 ˚C for 2 minutes, and then allowed to cool for 1 minute. The following reagents were then added to the reaction tube; -CTP mix (5 X; 2 µl) (Sigma, USA), α^{32}P dCTP (10 µCi/0.37 MBq; 1 µl) (Sigma, USA), Exo (-) Klenow fragment of DNA polymerase I (2.5 U; 0.5 µl) (Affymetrix, USA). The reagents were mixed gently by pipetting before incubation at 37 ˚C for 10 minutes. During this step, new Mp-ty/ddc DNA strands were synthesised on a denatured DNA template and in the process the radioactive α^{32}PdCTP nucleotides were incorporated in the DNA sequence. The radioactively labelled DNA (probe) generated was diluted to 100 µl by adding Tris-EDTA buffer (pH 8). Unincorporated nucleotides were then removed by passing through an autoclaved Sephadex G50 spin column (1 g/15 ml TE) (Sigma, USA) as shown in Figure 4.6. The DNA bound α^{32}PdCTP was collected by centrifugation (Centaur 2, UK) at 3,000 X g for 3 minutes while the small molecules of unbound α^{32}PdCTP where retained in the column. Perspex glass (5 mm thick) was used to shield the parts of my body exposed to radiation emitted during radioactive labelling of the probe, hybridisation and membrane washing. A Geiger Müller counter (Series 900 mini-monitor) was used to detect any radioactive spillages.

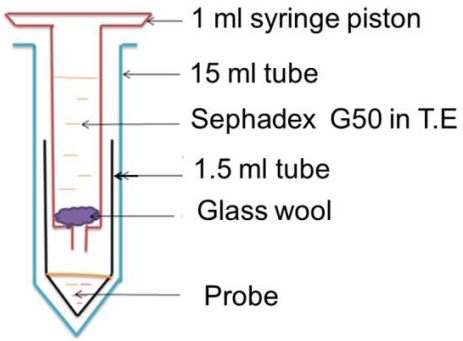


Figure 4.6 Sephadex G50 spin column used to purify the Mp-ty/dcc radioactive labelled probe.

The specificity the probe to bind only with target DNA was enhanced by adding Salmon sperm (2.5 mg/ml; 250 µl) in Church buffer (10 ml).

4.2.4.5: Hybridisation.

The pre-hybridisation buffer was replaced with 20 ml of fresh Church buffer at 65 ˚C. The membrane was then submerged in the buffer (DNA side-up) in a glass tray and the probe was then added. The tray was sealed and incubated at 65 ˚C overnight for 12 - 14 hours, with gentle shaking.

4.2.4.6: Washing and development.

After hybridisation, the membrane was washed in 2 X Tri-sodium citrate (SSC)/ Sodium dodecylsulfate (SDS) (0.1%) low stringency buffer at 60 ˚C for 20 minutes on a gently shaking rotor. This wash eliminates background and unbound probe. The buffer was carefully discarded in a sink, flowing with running water. The low stringency wash was repeated with fresh buffer at 65 ˚C for 15 minutes with gentle shaking and the buffer was discarded as described in the previous step. The membrane was then washed with 0.1 X SSC/SDS (0.1%) high stringency buffer at 65 ˚C for 10 minutes with gentle shaking before discarding the buffer as described above. The high stringency wash eliminates non-specific binding of the probe to

DNA and membrane surface. The membrane was then wrapped in saran wrap and exposed to X-ray film (Kodak, USA) in an X-ray cassette (AX-II, China) with intensifying screens at -80 ˚C overnight. The X-ray cassettes were removed from the -80 ˚C freezer and left to warm at room temperature then developed using developing fluid (Kodak HC-110 developer, Kodak, USA) for 5 minutes with gentle agitation. After rinsing in water it was fixed (Kodak, USA) for 5 minutes with gentle agitation, then rinsed in water and dried.

4.2.5: Real-time PCR (qRT-PCR).

Real-time PCR (qRT-PCR) is a standard PCR but with the advantage of detecting and quantifying the copies of DNA or cDNA amplicon during each PCR cycle by using DNA fluorescence as described in Section 4.2.5.1 below. qRT-PCR results show less variability than standard PCR due to the sensitive fluorescent chemistry and elimination of post-PCR detection procedures (Wong and Medrano, 2005; Kubista et al., 2006). Real-time PCR (qRT-PCR) was performed to determine the Mp-ty/ddc expression profile across the different tissues relative to endogenous gene expression in “wild type” *M. pruriens*.

4.2.5.1: Principle of Real- time PCR (qRT-PCR).

The intensity of the fluorescence emitted during qRT-PCR correlates to the amount of DNA product formed. Fluorescence exponentially increases as the DNA template is amplified until the fluorescence saturates the detector of the real-time PCR machine. The SYBR-based chemistries use fluorophores or fluorescent dyes that can intercalate and bind within double-stranded DNA. The fluorescent signal of intercalated dye is several orders of magnitude higher than that of unbound dye. SYBR Green is most common fluorophore used in qRT-PCR and was choice my study (Figure 4.7).

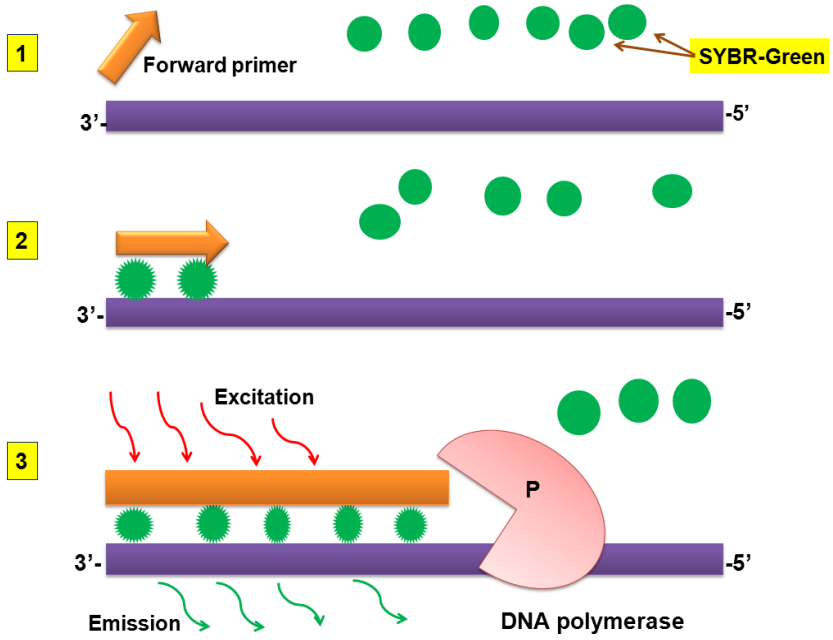


Figure 4.7 SYBR-Green fluorescence-based Real-time PCR. (1) cDNA or a single strand of denatured DNA and free low fluorescent SYBR-Green molecules in the reaction mix exhibit low fluorescence. (2) Primers anneal to the single stranded DNA template. SYBR-Green molecules bind between the primer and the DNA. This enhances SYBR-Green fluorescence upon excitation by light. (3) DNA polymerase elongates the template and more SYBR-Green molecules bind to the product formed resulting in exponential increase in the fluorescence level.

4.2.5.2: qRT-PCR Primer Design.

4.2.5.2.1: Designing Degenerate primers for endogenous genes in *M. pruriens.*

Since there was very limited *M. pruriens* gene sequence information on NCBI and other public data bases, degenerate primers for endogenous genes; Elongation factor-1 (EF1 alpha), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Ubiquitin and 18S ribosomal RNA (18S rRNA) were designed from conserved proteins/nucleotide sequence regions for similar genes in species of the family Phaseoleae to which *M. pruriens* belongs as described in Chapter 2 (Section 2.2.5.2).

4.2.5.2.2: Designing Gene-specific qRT-PCR primers.

Gene-specific primers were then designed to PCR-amplify 120 - 200 bp from a sequence region of approximately 600 bp region close to 3' end of Mp-ty/ddc and for *M. pruriens* endogenous genes; EF1 alpha, GAPDH, Ubiquitin and 18S rRNA as described in Chapter 2 Section 2.2.5.1. This was because sequences closer to the 3'end of a gene tend be unique for a given gene (Sambrook et al., 1989; Higuchi et al, 1992; Wong and Medrano, 2005). The most endogenously expressed gene of the above was used as the reference control in qRT-PCR.

Table 4.3 Primers used for qRT-PCR analysis for the Mp-ty/ddc and 18S rRNA.

No.	qRT-PCR Primers	Sequence
1	Forward primer, $F_{(18S)}$: (10 mM; 1.75 µl)	5'-TGA CGG AGA ATT AGG GTT CG-3'
2	Reverse primer, $R_{(18S)}$: (10 mM; 1.75 µl)	5'-CCT CCA ATG GAT CCT CGT TA-3'
3	Forward Primer, $F_{(MpqRT)}$: (10 mM; 1.75 µl)	5'-TTA GGA TTG CAC CAT CAG CT-3'
4	Reverse primer, $R_{(MpqRT)}$: (10 mM; 1.75 µl)	5'-CTC CTT CAA CCT CAC CAT GA-3'

4.2.5.3: Normalisation of endogenous gene expression.

RNA was extracted as described in Section 2.2.2.2, purified from DNA contamination by DNAse treatment (Section 2.2.3) and the concentration RNA (1 µl) was determined by measuring spectrophometric absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK) by following instructions in the instrument's user manual. Good quality RNA had a 260/280 ratio of approximately 2. RNA concentration for all samples was made uniform by adding appropriate volumes of sterile water calculated using the formulae: Stock concentration X Stock volume = Dilution concentration X Dilution volume (Sambrook et al., 1989). RNA (2 µg) was used to synthesise cDNA for each sample as described in Section 2.2.4. qRT-PCR was performed on endogenously expressed

genes; EF1 alpha, Ubiquitin, GAPDH and 18S rRNA as described in Section 4.2.6.4 below. The number of cycles required for the copies of DNA synthesised by qRT-PCR to exceed the background threshold (Cycle threshold or Ct) is directly proportional to concentration of cDNA or DNA template. Of these, the 18S rRNA gene which had minimum discrepancies in Ct values for all seed, leaf, stem and root cDNA samples was used as the endogenous control. By using the formula; 1 Ct value = 2 X RNA concentration (Livak and Schmittgen, 2001), the Ct values for the 18S rRNA gene expression in all cDNA samples was normalised to a common value in the range of 15 - 20 by adjusting the cDNAs with appropriate amount of sterile water. qRT-PCR was then performed on triplicate samples of normalised cDNA samples. The normalisation steps and subsequent qRT-PCR amplification were repeated until all samples and their triplicates had the same Ct value (+/- 0.5). The normalised cDNA samples were then used as qRT-PCR templates for gene expression analysis of the Mp-ty/ddc gene as described in Section 4.2.6.4 below.

4.2.5.4: Real-time PCR Amplification.

Real-time PCR amplification was performed using thermocycler DNA Engine 2 Opticon (MJ Research Inc, USA). Normalised cDNA samples described in Section 4.2.6.3 above were used as templates for qRT-PCR amplification. The qRT-PCR reaction mixture for was as set up as follows in triplicate samples; 2 X SYBR-Green Mix (Thermo Scientific) (12.5 µl), 10 mM; 1.75 µl of each primer, normalised cDNA (5 µl) and sterile water (4 µl). qRT-PCR amplification was performed using the

following program; 95 ˚C; 15 min, 49 cycles (95 ˚C; 15 sec, 55 ˚C; 30 sec, 72 ˚C; 15 sec, Plate Read), Melting Curve (55 ˚C – 98 ˚C), read every 0.5 ˚C, hold (1 sec).

4.2.5.5: Analysis of Real-time PCR results.

Results for gene amplification by qRT-PCR were analysed using the Opticon 3 software program of the real-time PCR machine, DNA Engine 2 Opticon (MJ Research Inc, USA). The threshold for background fluorescence was manually fixed at 0.012 which corresponded to the start point of the log phase on the qRT-PCR gene amplification curve. Purity of the qRT-PCR product was determined by melt profile analysis. A pure qRT-PCR product was denoted by; a single and sharp amplicon melting-curve peak obtained at a common melting temperature for all qRT-PCR amplified samples. The melt profile for qRT-PCR amplicons of endogenously expressed 18S rRNA gene of *M. pruriens* and the Mp-ty/ddc gene of interest, have different melting temperatures. The 18S rRNA and Mp-ty/ddc gene expression in seed, leaf, stem and root cDNA samples was determined from their corresponding Ct values. The Ct values were determined by using the Opticon 3 software program for the thermocycler, DNA Engine 2 Opticon (MJ Research Inc, USA). The triplicate Ct values for each sample were then imported into the Microsoft Excel program. The Mp-ty/ddc gene expresion, normalised to that of endogenously expressed 18S rRNA control as described in Section 4.2.5.3 above, was analysed based on Livak and Schmittgen (2001) derived equation below.

$$\textbf{Mp-ty/ddc expression} = 2^{-\Delta\Delta Ct},$$

Where; $\mathbf{\Delta\Delta Ct = (Ct_{(Mp\text{-}ty/ddc)} - Ct_{(18S\ rRNA)})_{Time\ X} - (Ct_{(Mp\text{-}ty/ddc)} - Ct_{(18S\ rRNA)})_{Time\ 0}}$. Time X is any time point and Time 0 represents the 1 X expression of Mp-ty/ddc gene normalised to 18S rRNA. The mean Ct values for both Mp-ty/ddc and the 18S rRNA were determined at time zero and were used in the gene expression equation above. The fold change in the Mp-ty/ddc gene, normalised to 18S rRNA and relative to the expression at time zero, was calculated for each sample the gene expression equation above. The mean, standard deviation and standard error were then determined from the triplicate samples at each time point. Using this analysis, the value of the mean fold change was expected to be very close to one ($2^0 = 1$).

4.3: RESULTS.

4.3.1: Isolation of a portion of a putative *M. pruriens* DOPA/tyrosine decarboxylase gene DC1.

A 493 bp sequence was found in the NCBI databank (EF101921.1) which was annotated as "*Mucuna pruriens* partial DOPA/tyrosine decarboxylase DC1". To verify this sequence, primers were designed from it (5'-CTCTGGTGAGGGTGGTGG-3' and 5'-GTAAGCTGCATCTACGTGGA-3') as described in Section 2.2.5.1. High molecular weight genomic DNA was extracted from leaf tissues of five cultivars of *M. pruriens:* "90 day", "Tropical", "Bush echo", "Vine", "Early maturing" following the method of Dellaporta et al. (1983) (Figure 4.8 a) as described in Section 2.2.1. (Plant material is described in Section 2.1). The aim was to find out if the Mp-ty/ddc gene was present in the genome of different cultivars of *M. pruriens* and if there were clues to different gene family members of Mp-ty/ddc that could be deduced from the different cultivars. The above primers were then used to isolate 406 bp of the Mp-ty/ddc gene from genomic DNA of *M. pruriens* by PCR as described in Chapter 2 Section 2.2.6. The PCR product was analysed by agarose gel electrophoresis and UV imaging as described in Chapter 2 Section 2.2.7. The DNA was then purified from the gel as described in Section 2.2.9 and ligated into pGEM-T vector (Promega, USA) as described in Chapter 2 Section 2.2.11. The ligation was transformed into *E.coli*DH5α bacterial cells as described in Chapter 2 Section 2.2.13 and the putatively transformed white colonies that regenerated on selection media were screened by colony PCR (as described in Chapter 2 Section 2.2.14). Plasmid DNA was extracted from the transformed bacterial cells as described in Chapter 2 Section

2.2.15 followed by restriction digestion using *Eco*RI and this led to the release the cloned 0.4 kb Mp-ty/ddc fragment (Figure 4.8 b). The 0.4 kb Mp-ty/ddc gene portion was isolated from five cultivars of *M. pruriens* as described in Chapter 2 Section 2.2.6 and sequenced as described in Chapter 2 Section 2.2.15. The aim was to find out if the Mp-ty/ddc gene was present in the genome of different cultivars of *M. pruriens* and if there were clues to different gene family members of Mp-ty/ddc that could be deduced from the different cultivars.

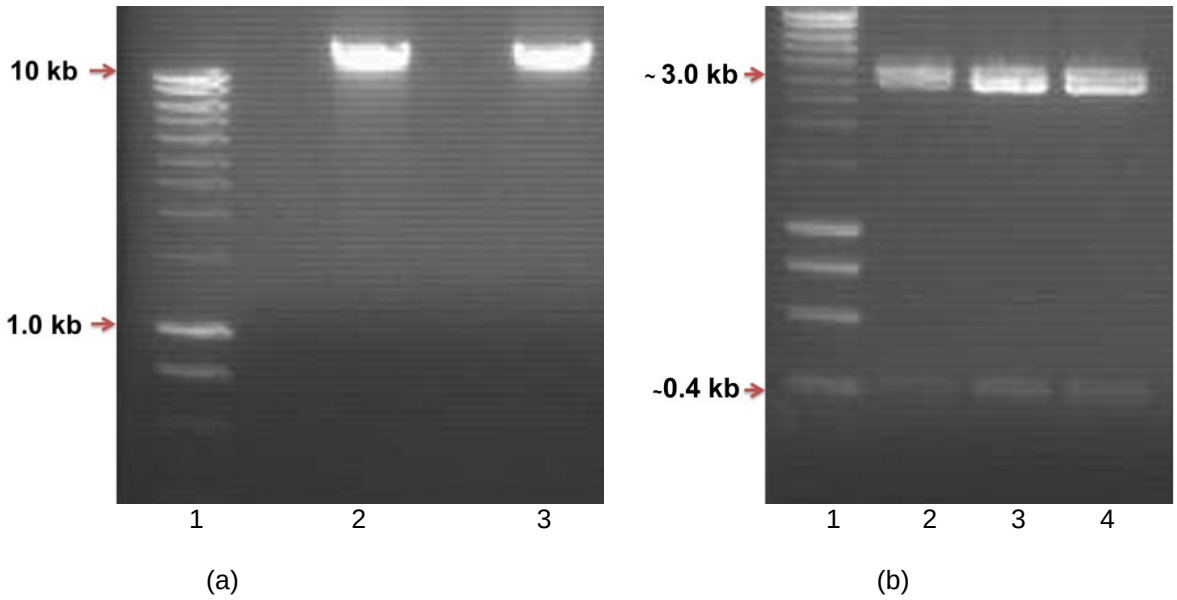
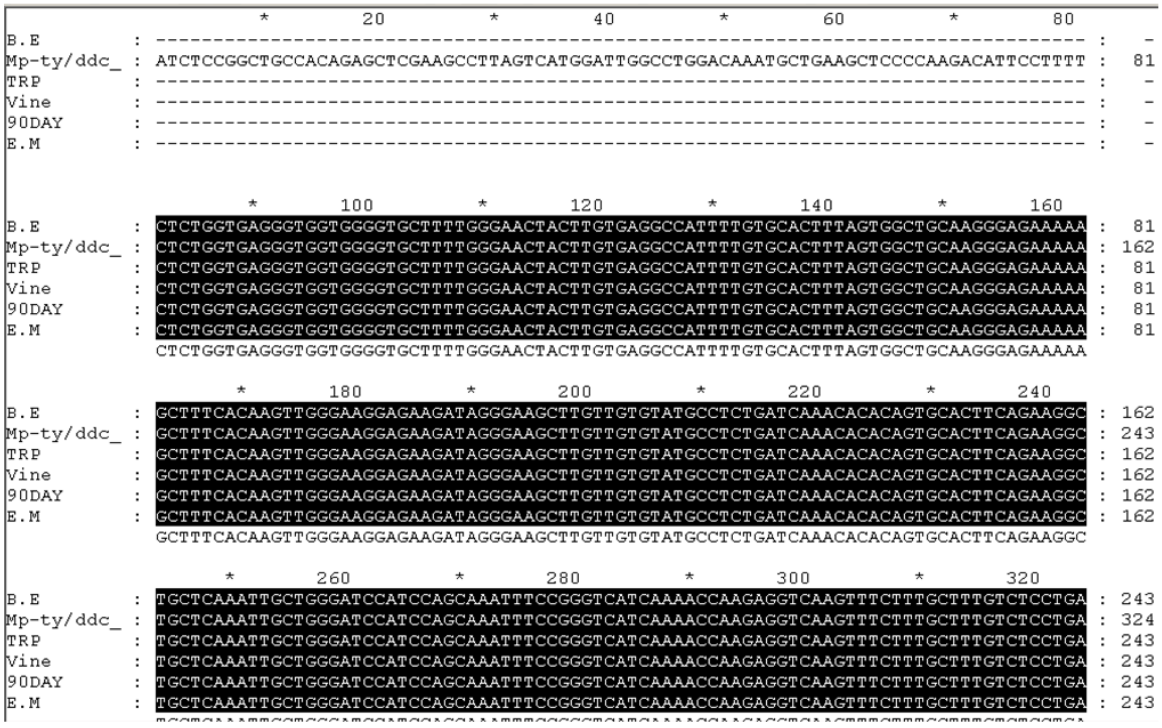


Figure 4.8 is (a) Agarose gel electrophoresis of gDNA (5 µg) extracted from the "90 day" (lane 1) and vining cultivars (lane 2) of *M. pruriens.* (b) Gel electrophoresis of cloned 0.4 kb Mp-ty/ddc PCR products recovered from the pGEMT plasmid vector by *Eco*RI digestion (lanes 2-4).

4.3.2: Sequence results for the cloned 0.4 kb Mp-ty/ddc genomic PCR product.

Sequencing yielded an identical 406 bp for the five cultivars of *M. pruriens* (Figure 4.9). This led to the decision of selecting only one cultivar (the "90 day") for gene isolation. The "90 day" cultivar had shown better germination and flowering rates in the greenhouse.



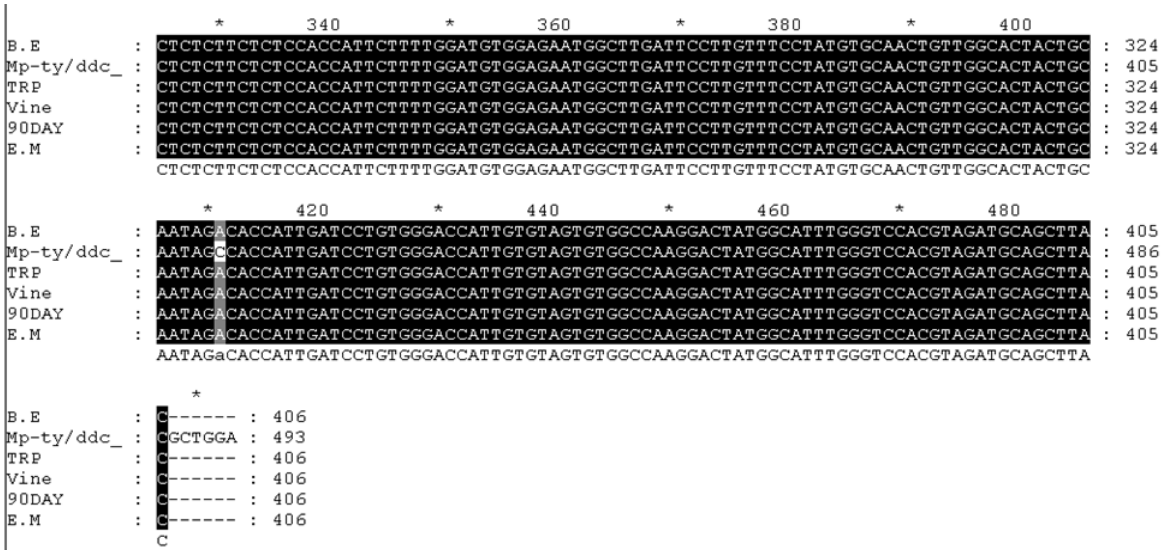
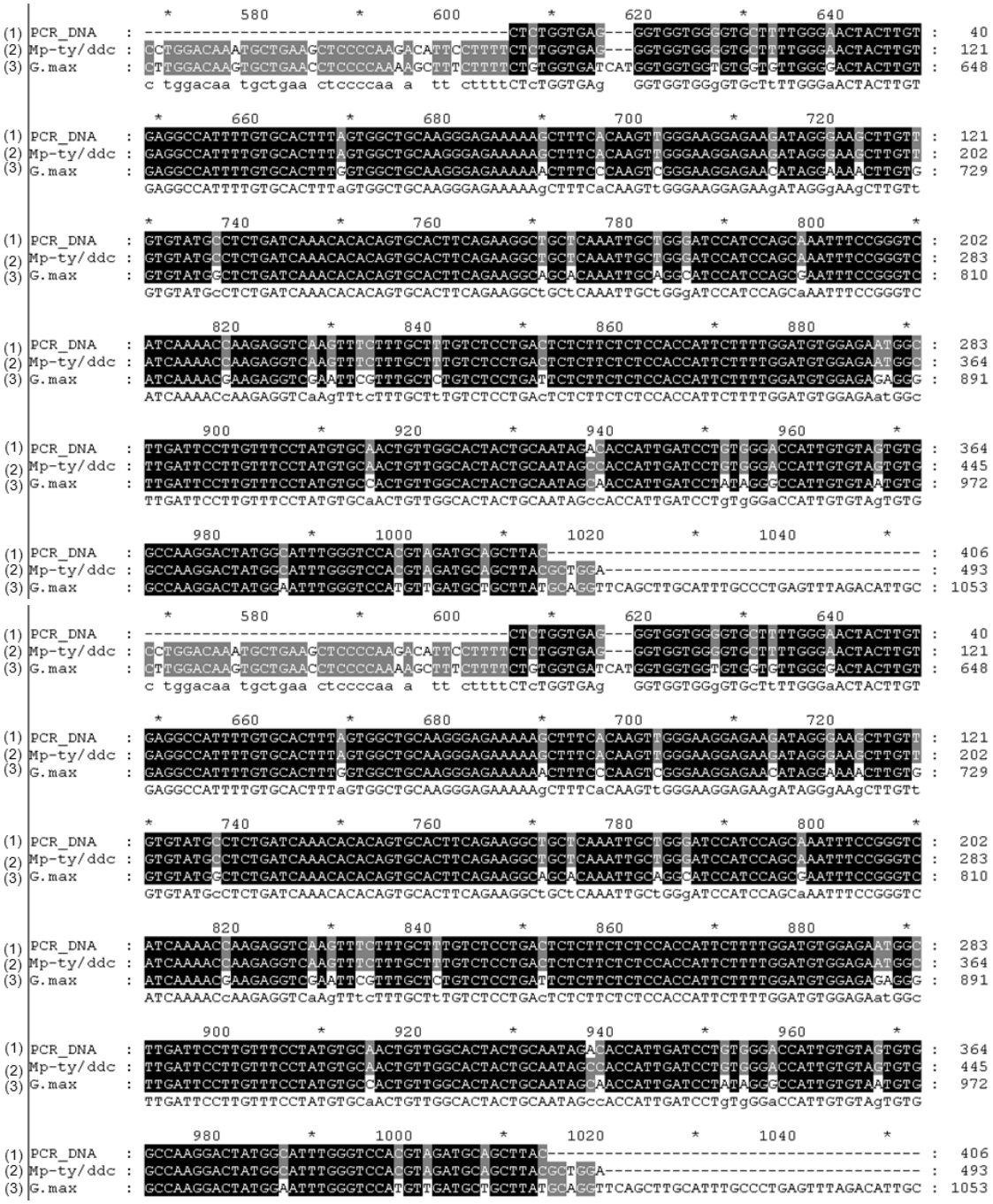


Figure 4.9 Alignment of the 406 bp genomic PCR sequence result to the Mp-ty/ddc (EF101921.1) on the NCBI data bank. The 406 bp genomic PCR sequence result for the “Bush Echo” (B.E), “Tropical” (TRP), “Vine”, “90 day” and “Early maturing” (E.M) cultivars of *M. pruriens* were identical and drew 100% complete alignment in the overlap region (82-406 bp) with the Mp-ty/ddc sequence (EF101921.1) on the NCBI data bank**.**

The 406 bp sequence result showed 100% homology with the Mp-ty/ddc partial sequence (EF101921.1) on the NCBI data bank. When the 406 bp sequence was Blast analysed on the NCBI data bank, very significant alignments were drawn with the DOPA/tyrosine decarboxylase gene (Figure 4.10 a) and protein (Figure 4.10 b) sequences for *Glycine max* and other species in family Phaseoleae of *M. pruriens.*

(a)



(b)

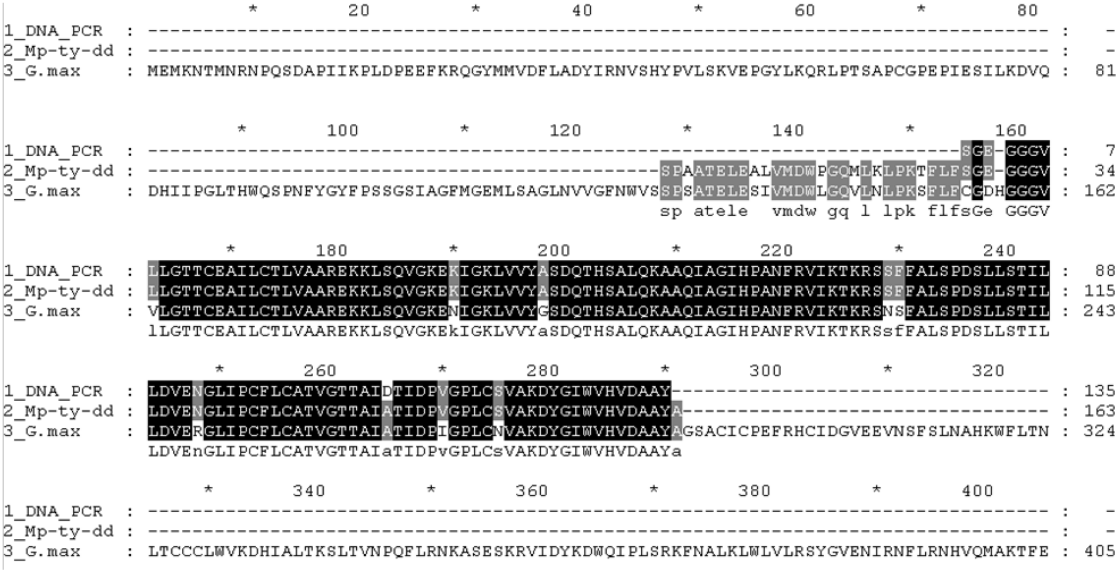


Figure 4.10 Alignments of (a) DNA sequences (a) and amino acid sequences of the DOPA/tyrosine decarboxylase for; (1) the 406 bp Mp-ty/ddc sequenced DNA fragment (2) the database Mp-ty/ddc accession in the overlap region (616 - 1014 bp) (EF101921.1) and (3) *Glyicine max* (XM_003529857

The 406 bp sequence obtained was a portion of the Mp-ty/ddc gene but the rest of the sequence was unknown. In order to obtain the complete open reading frame, 3' and 5'RACE PCR approaches were used on leaf and seed cDNA of *M. pruriens* as described in Sections; 4.3.3 and 4.3.4 respectively below. Besides RACE, degenerate primers were designed from conserved sequence regions for the TY/DDC protein/genes as described in Chapter 2 Section 2.2.5.2 and were used to isolate more Mp-ty/ddc sequence (See Section 4.3.4).

4.3.3: Obtaining the full 3' end of the Mp-ty/ddc mRNA using 3' RACE.

Firstly, there was a need to establish a protocol for RNA extraction from different tissues of *M. pruriens* for use in cDNA synthesis and gene expression analysis. RNA extraction from the proteinaceous seed was recalcitrant to the conventional guanidine-hydrochloride extraction method used in most commercial RNA extraction kits. However, good quality RNA was successfully extracted using the method described in the protocol by Ding et al. (2007) (see Chapter 2 Section 2.2.2.1).

3' RACE was used to obtain the complete 3' end of the Mp-ty/ddc mRNA using 3' RACE- Ready cDNA from derived from leaf tissue RNA (as described in Section 4.2.1.1), as a template. This was PCR amplified using a gene specific primer; $F1_{(3RACE)}$: 5'-CTCTGGTGAGGGTGGTGGGGTGCTTTTGGG-3', 406 bp from the 3' end of the 493 bp of the partial Mp-ty/ddc sequence (EF101921.1) on the NCBI data bank and a supplied primer; $R1_{(3RACE)}$: (3' RACE PCR kit, Ambion USA). The PCR product was re-amplified using "nested" gene specific primer; $F2_{(3RACE)}$: 5'-GAAGGAGAAGATAGGGAAGCTTGT-3', 310 bp from the 3' end of the genomic fragment cloned in Section 4.3.1 and a supplied primer; $R2_{(3RACE)}$ 5'-GAATTAATACGACTCACTATAG-3' (3' RACE PCR kit, Ambion, USA). A PCR product of approximately 1.2 kb was obtained from the 3' RACE reaction (See Figure 4.11 a). To verify this product, primers; 5'-CTCTGGTGAGGGTGGTGGGGTGCTTTTGGG-3' and 5'-GTAAGCTGCATCTACGTGGA-3' previously used to PCR amplify the 406 bp Mp-ty/ddc fragment (Section 4.3.1) were used to detect by PCR the presence of the sequenced 406 bp Mp-ty/ddc gene fragment in the 1.2 kb 3' RACE PCR product (Figure 4.11 b). The 1.2 kb fragment was then cloned into pGEM-T vector

(Promega, USA) and transformed into *E. coli*DH5α as described Sections: 2.2.11 and 2.2.13 respectively. Colony PCR was used to identify colonies transformed with the pGEM-T-Mp-ty/ddc transgene plasmid (as described in Chapter 2 Section 2.2.14). Plasmid DNA was extracted from bacterial cultures which produced positive colony PCR result for the Mp-ty/ddc transgene. Samples of the purified plasmid DNA were further screened for the Mp-ty/ddc transgene by double digestion using *Eco*RI. (Figure 4.11 c). Three independent 3' RACE PCR product clones were each sequenced using the T7 and Sp6 primers designed from the plasmid vector sequence as described in Chapter 2 Section 2.2.5.1. The sequence result for the individual clones were 100% identical in the overlapping region but varied slightly in length with one sequence reaching the poly A tail at the 3' end of the Mp-ty/ddc gene (Figure 4.11).

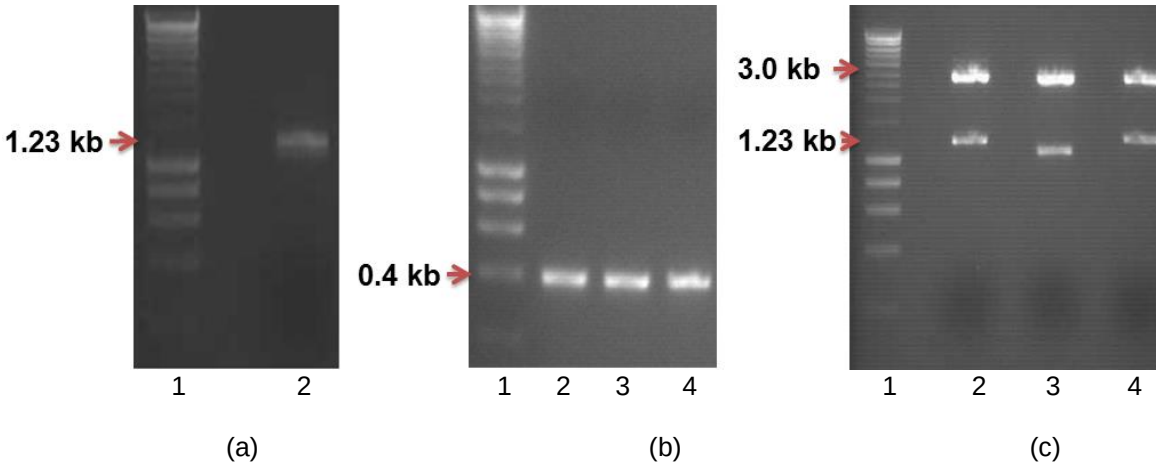
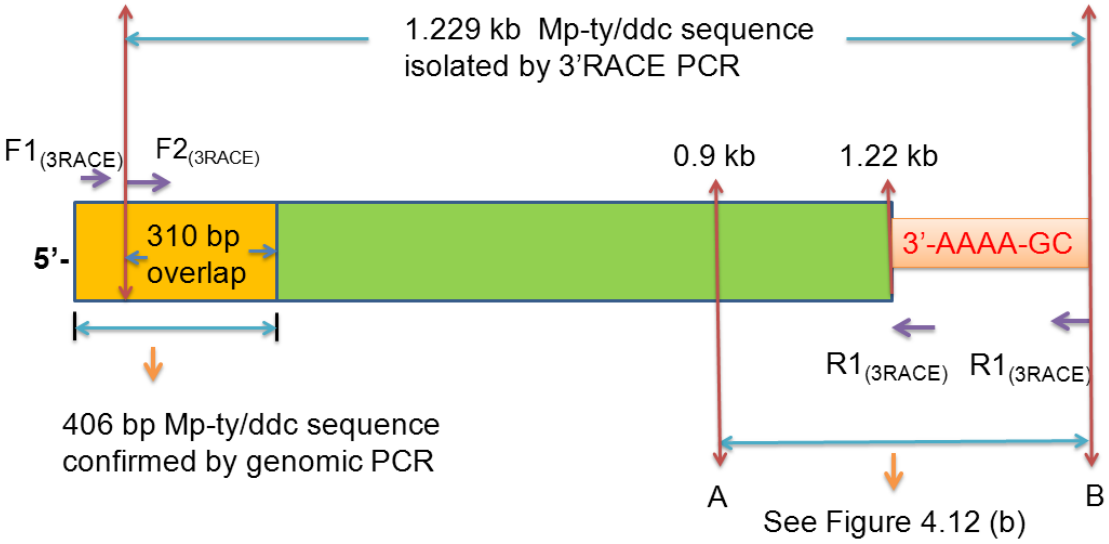


Figure 4.11 Gel electrophoresis of; (a) Approximately 1.2 kb 3' RACE PCR product (lane 2). (b) Approximately 0.4 kb PCR product isolated from the 1.2 kb 3' RACE PCR template using Mp-ty/ddc internal primers designed to PCR amplify 0.4 kb of the known Mp-ty/ddc gene sequence (lanes 2 - 4). (c) pGEM-T plasmids (lane 2 and 4) harbouring 1.2 kb 3' RACE clones were double digested using *Eco*RI to release the clones. The 3 kb plasmid vector and the recovered 1.2 kb clone were separated according to size by gel electrophoesis.

a)



(b)

```
          760         *         780         *         800         *         820         *         840
3RACE1 : CAATGGTACTGAAGCATGCTATAATGGGAAACTGATGGATGATGAGTATAGGGTGAATGAAGTCAATCGTAAATTGCTTGATTC :  840
3RACE2 : CAATGGTACTGAAGCATGCTATAATGGGAAACTGATGGATGATGAGTATAGGGTGAATGAAGTCAATCGTAAATTGCTTGATTC :  840
3RACE3 : CAATGGTACTGAAGCATGCTATAATGGGAAACTGATGGATGATGAGTATAGGGTGAATGAAGTCAATCGTAAATTGCTTGATTC :  840
         CAATGGTACTGAAGCATGCTATAATGGGAAACTGATGGATGATGAGTATAGGGTGAATGAAGTCAATCGTAAATTGCTTGATTC

                  *         860         *         880         *         900         *         920
3RACE1 : AATTAATAGTTCTGGCAATGTATTCATGACTCATGGTGAGGTTGAAGGAGCCTTTGTGATTAGATGTGCTATTGGTGCAACTTT :  924
3RACE2 : AATTAATAGTTCTGGCAATGTATTCATGACTCATGGTGAGGTTGAAGGAGCCTTTGTGATTAGATGTGCTATTGGTGCAACTTT :  924
3RACE3 : AATTAATAGTTCTGGCAATGTATTCATGACTCATGGTGAGGTTGAAGGAGCCTTTGTGATTAGATGTGCTATTGGTGCAACTTT :  924
         AATTAATAGTTCTGGCAATGTATTCATGACTCATGGTGAGGTTGAAGGAGCCTTTGTGATTAGATGTGCTATTGGTGCAACTTT

                *         940         *         960         *         980         *        1000
3RACE1 : AACAGAGGAACACCATGTGATTATGGGCATGGAAGTTGGTGCAGGAGCATGCCAATTCTCTGTTAGGTAACTTCTAAAACAAAT : 1008
3RACE2 : AACAGAGGAACACCATGTGATTATGGGCATGGAAGTTGGTGCAGGAGCATGCCAATTCTCTGTTAGGTAACTTCTAAAACAAAT : 1008
3RACE3 : AACAGAGGAACACCATGTGATTATGGGCATGGAAGTTGGTGCAGGAGCATGCCAATTCTCTGTTAGGTAACTTCTAAAACAAAT : 1008
         AACAGAGGAACACCATGTGATTATGGGCATGGAAGTTGGTGCAGGAGCATGCCAATTCTCTGTTAGGTAACTTCTAAAACAAAT

          *      1020         *      1040         *      1060         *      1080         *
3RACE1 : TGTACTTTCATTTAGTTATCCGGTTGTTGATATCAAAATTATTCAGGCAGACTTGGAAAAGTCATCGATGATAGAGAACCAACA : 1092
3RACE2 : TGTACTTTCATTTAGTTATCCGGTTGTTGATATCAAAATTAT---------------------------------------- : 1050
3RACE3 : TGTACTTTCATTTAGTTATCCGGTTGTTGATATCAAAATTATTCAGGCAGACTTGGAAAAGTCATCGATGATAGAGAACCAA-- : 1090
         TGTACTTTCATTTAGTTATCCGGTTGTTGATATCAAAATTATTcaggcagacttggaaaagtcatcgatgatagagaaccaa

             1100         *      1120         *      1140         *      1160         *
3RACE1 : TCGATATTGCTATCAGGTCGGTGATGACAGTTGGAAATTACTAGAAGTTATTCCTAATTAAAATATTTATCTATAAATACCAAT : 1176
3RACE2 : ---------------------------------------------------------------------------------- :    -
3RACE3 : ---------------------------------------------------------------------------------- :    -

         1180         *      1200         *      1220
3RACE1 : TTCTACAATGTAATAAAGTTGGAACTTTGTCATTAAAAAAAAAAAAAAAAAAA : 1229
3RACE2 : ------------------------------------------------------ :    -
3RACE3 : ------------------------------------------------------ :    -
```

Figure 4.12 (a) A diagramatic representation of the 1.229 kb Mp-ty/ddc sequence obtained by 3' RACE. (b) Alignment of the 3' RACE sequences isolated from three independent clones. The 3' RACE forward primers; $F1_{(3RACE)}$ and $F2_{(3RACE)}$ designs were based on the 406 bp sequence result for the Mp-ty/ddc isolated by genomic PCR from *M. pruriens* (Section 4.3.1). The 3' RACE product was isolated from leaf cDNA using primers; $F1_{(3RACE)}$ and $R1_{(3RACE)}$ followed by "nested" PCR using primers; $F2_{(3RACE)}$ and $R2_{(3RACE)}$. This generated a 310 bp overlap with the sequence result for the 406 bp genomic PCR product (Section 4.3.1). The sequence result for the 3' RACE independent clones varied slightly in length at the 3'end (region A-B).

The 406 bp Mp-ty/ddc sequence isolated by genomic PCR (See Section 4.3.1) was extended to 1.229 kb by 3' RACE PCR (See Figure 4.12 b). The forward primer for 3'RACE, $F2_{(3RACE)}$, was designed approximately 107 bp from 5'end of the 406 bp genomic PCR sequence result. When the longest 3' RACE sequence was aligned with the original 406 bp sequence cloned from genomic DNA, an overall of 100% homology was seen in the 310 bp overlap region (Figure 4.13).

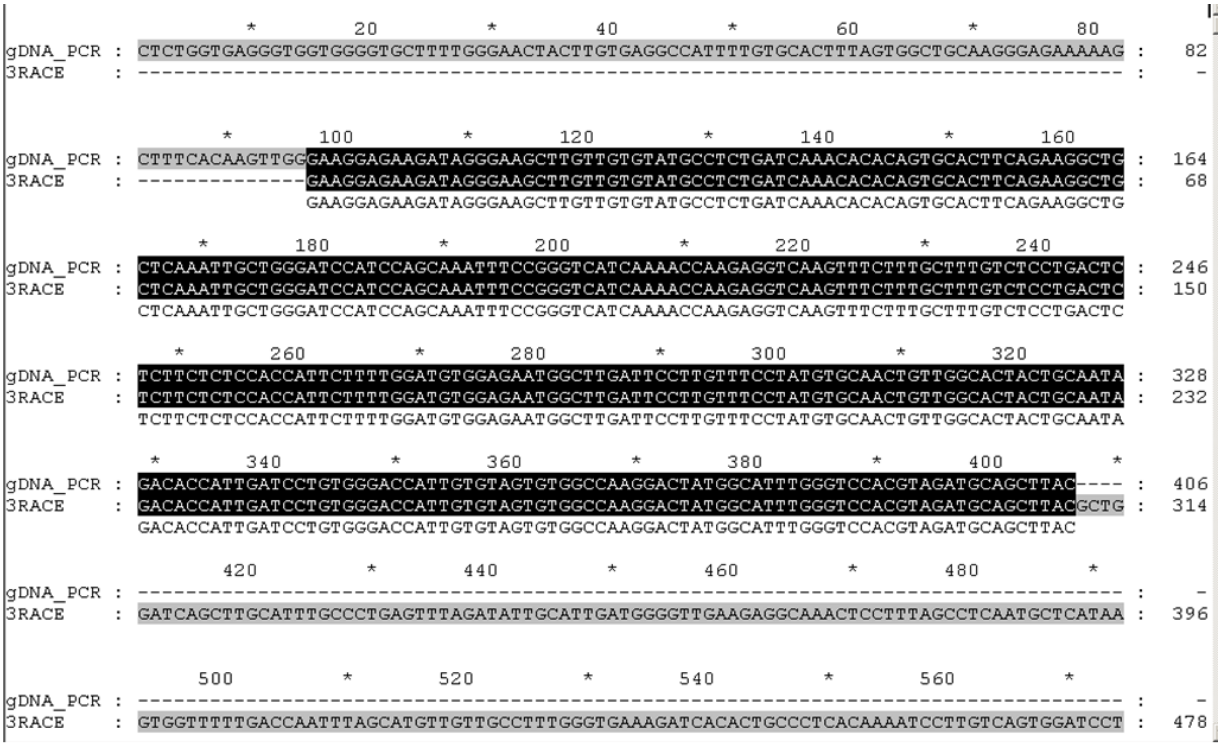


Figure 4.13 Alignment of the 406 bp Mp-ty/ddc sequence (gDNA_PCR); obtained by genomic PCR approach, to the 3' RACE sequence result. 310 bp at the 5' end of 3' RACE sequence result showed 100% homology with the gDNA_PCR sequence result in the overlap region (100 - 410) for the two sequences. The sequence overlap confirmed the 406 bp Mp-ty/ddc sequence was enxtended to up to 3' end poly A tail by 3' RACE.

Analysis of the 3' RACE sequence result by Blast on the NCBI data bank drew very significant alignments with DOPA/decarboxylase TY/DDC sequences of the family Leguminosae of *M. pruriens* (Figure 4.14). *Glycine max*, *Cicer arietinum* showed the highest homology with the 1.229 kb 3' RACE Mp-ty/ddc sequence result. The overlap region for the three sequences showed approximately 79% of the ty/ddc gene was conserved (Figure 4.14). The ty/ddc sequence for *Glycine max* was approximately 30 bp longer at the 3' end. The 3' UTR for the Mp-ty/ddc 3' RACE sequence was largely unconserved but extended up to the 3' terminal end of the gene, characterised by poly- adenylation (Figure 4.14).

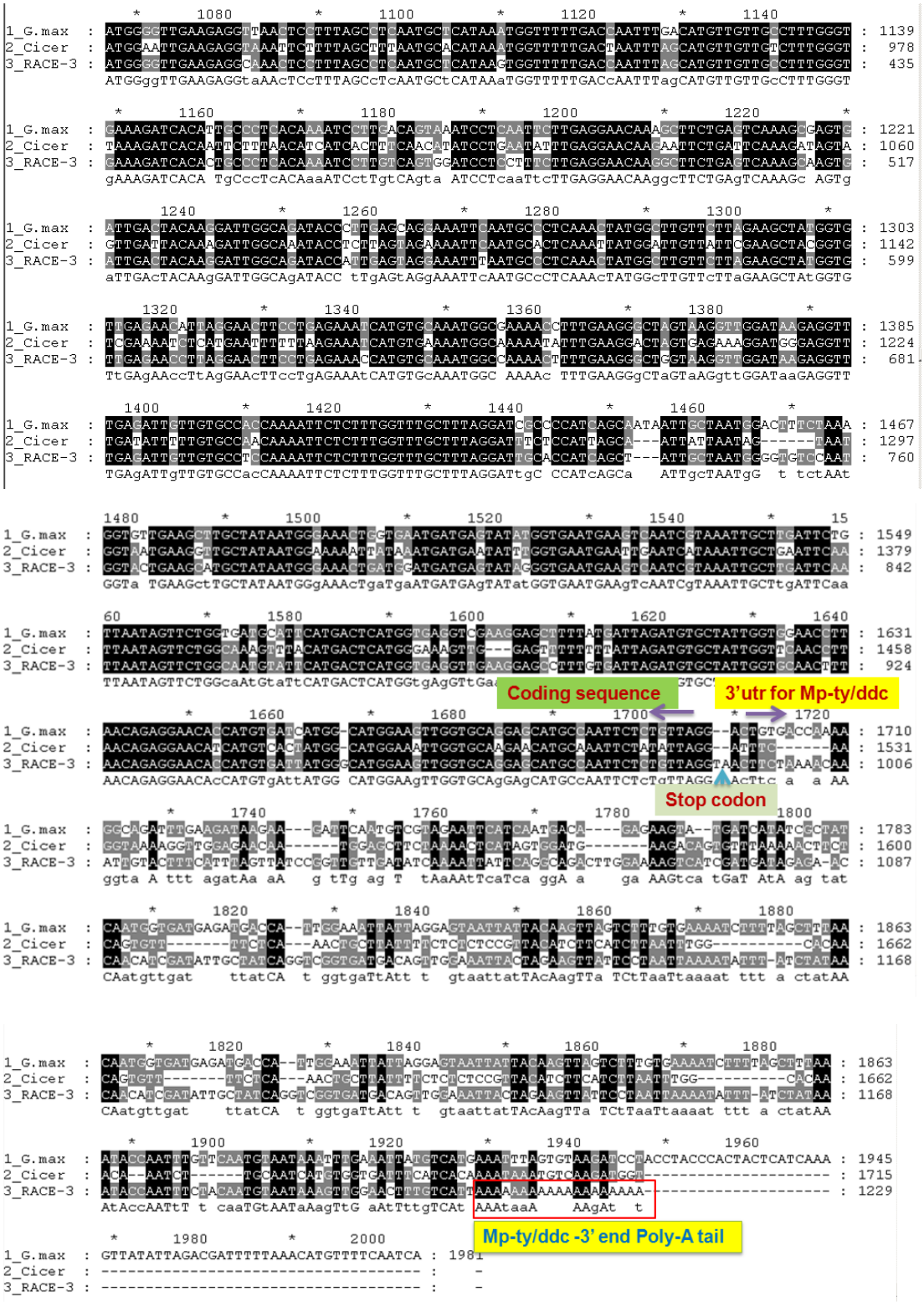


Figure 4.14 ty/ddc alignments for (1) *Glycine max* (XM_003529857.1) and (2) *Cicer arietinum* (XM_004510674.1) to (3) the 1.229 kb Mp-ty/ddc 3'RACE sequence result. The overlap region (715 - 1710 bp) shows 79% of the gene sequence is conserved. The Mp-ty/ddc sequence was extended by 3' RACE up to the poly-A tail at the 3' terminal end of the gene. The alignments were drawn using the genedoc software program.

Similarly, the TY/DDC amino acid alignments for *Glycine max* (XP_003529905.1) and Cicer *arietinum* (XP_004510731.1) to the 3' RACE sequence showed approximately 83% homology in the overlap region (190 - 530) (Figure 4.15).

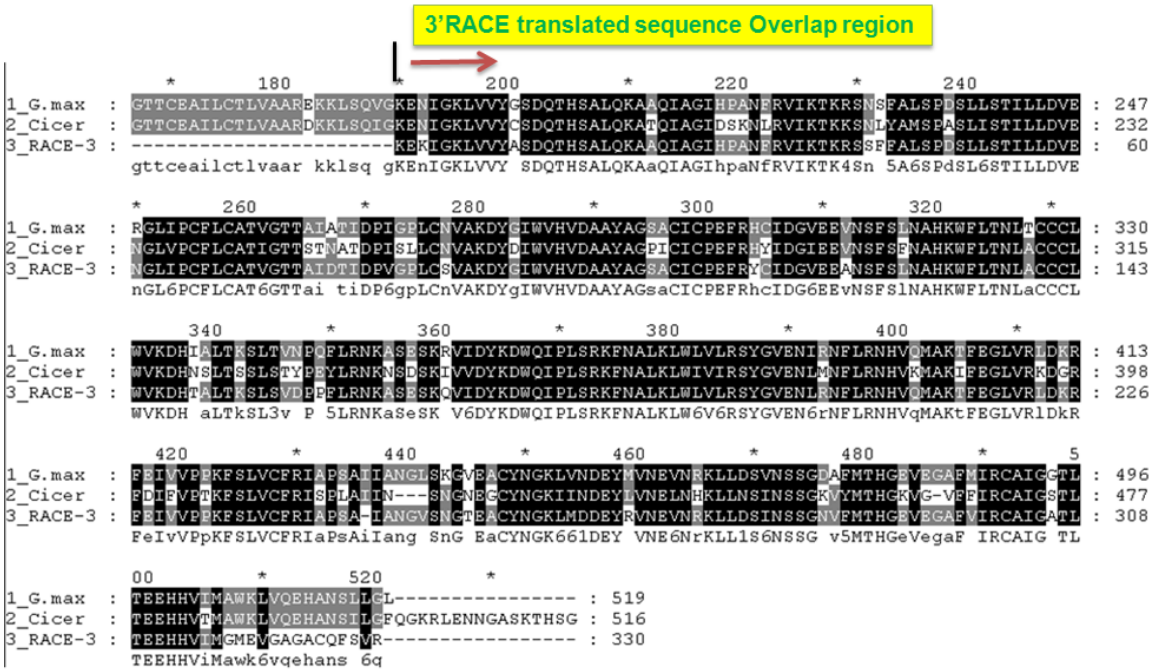


Figure 4.15 ty/ddc alignments for (1) *Glycine max* (XP_003529905.1) and (1) *Cicer arietinum* (XP_004510731.1) to (3) the 1.229 kb Mp-ty/ddc 3' RACE sequence result. The overlap region (190 - 530) shows approximately 83% of the gene sequence is conserved. The alignments were drawn using the genedoc software program.

4.3.4: Towards obtaining the full 5' end of the Mp-ty/ddc mRNA using 5'RACE.

5' RACE was used to isolate sequences of Mp-ty/ddc 5' to the 406bp fragment, using seed cDNA (See Section 4.2.2.1 for methods). A 0.5 kb DNA fragment (See Figure 4.16 a) was isolated using primers; Mp-ty/ddc-R1$_{(5'RACE)}$, 378 bp from the 5' end of

the 406 bp sequence cloned above (See Section 4.3.1) and a UPM as described in Section 4.2.4.3 above (Figure 4.16 a). The 5' RACE product was cloned in pGEMT plasmid (Promega, USA) using *E.coli*DH5α as described in Chapter 2 Section 2.2.13 and colonies screened by PCR (Figure 4.16 b). The plasmid containing the insert was isolated and sequenced as described in Chapter 2 Section 2.2.15.

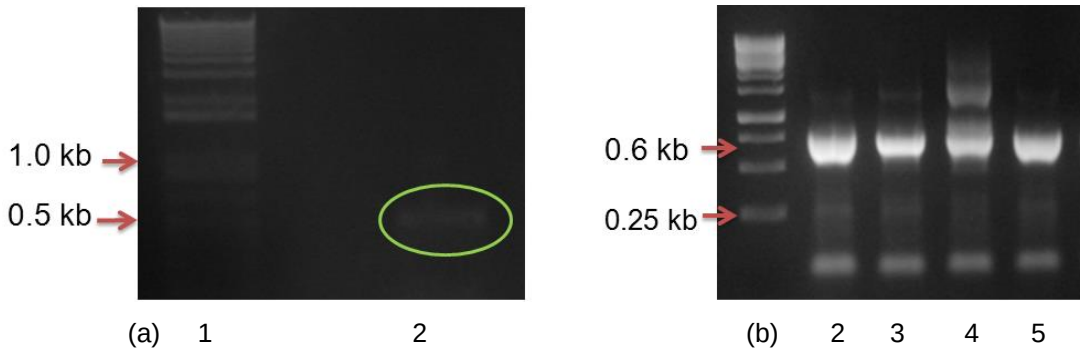
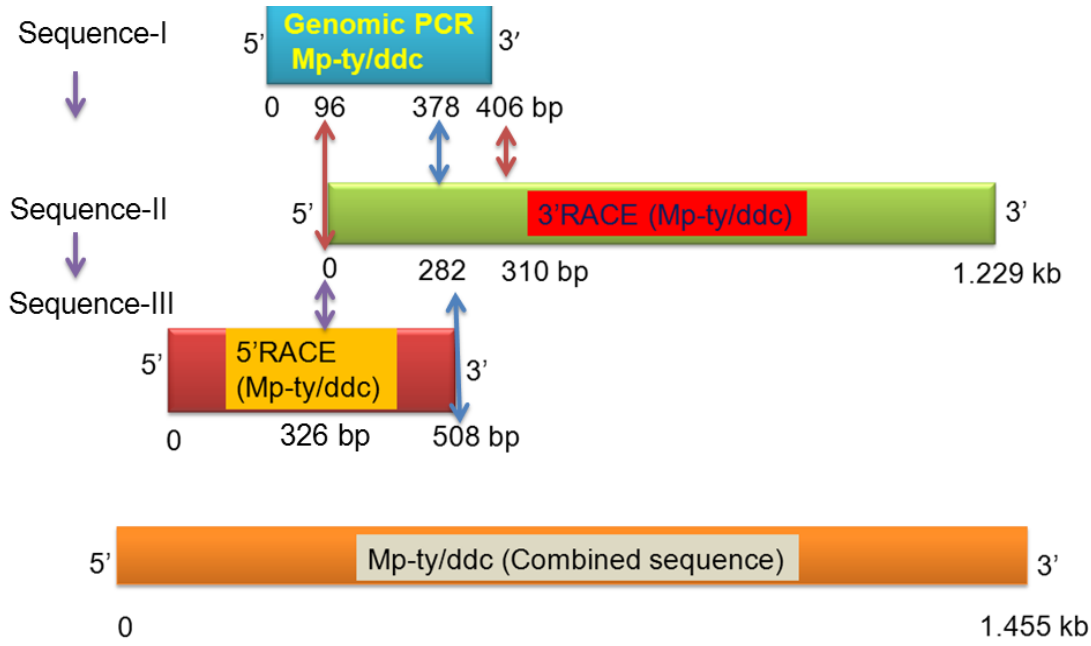


Figure 4.16 (a) 0.5 kb DNA band (lane 2) was isolated from *M. pruriens* seed cDNA by 5' RACE PCR. Lane 1 is a DNA marker (Bioline, UK). (b) Approximately 0.6 kb colony PCR products (lanes 2 - 5) were amplified using vector plasmid primers T7 and Sp6 primers, from single bacterial colonies transformed with a pGEM-T plasmid harbouring a cloned 0.5 kb 5' RACE product. Colony PCR product is 0.6 kb because the additional 0.1 kb sequence was PCR amplified from the vector plasmid using the T7 and Sp6 primers which flanked the 5' RACE clone.

The 5' RACE sequence of 508 bp obtained showed 100% homology in the overlap region (227 - 508 bp) with earlier sequences obtained by 3' RACE and the genomic PCR approach (Figure 4.17 a). Besides the overlap region, the 5' RACE sequence extended that for 3' RACE by 326 bp towards the 5' end of the Mp-ty/ddc (Figure 4.17 b) and the combined sequence obtained by 3' RACE and genomic PCR was extended by approximately 227 bp (region 0 - 227 bp) for the Mp-ty/ddc gene (Figure 4.17 b). The 100% homology in the overlapping region strongly indicating that the new sequence was indeed part of the same gene.

(a)



(b)

```
                *       100         *       120         *       140         *       160
PCR_DNA  : ----------------------------------------------CTCTGGTGAGGGTGGTGGGGTGCTTTTGGGAACTAC  :   36
3RACE    : ------------------------------------------------------------------------------  :    -
5RACE    : ATGGATTGGCCTGGACAAATGCTGAAGCTCCCCAAAACATTCCTTTTCTCTGGTGAGGGTGGTGGGGTGCTTTTGGGAACTAC  :  166
                                                          ctctggtgagggtggtggggtgcttttgggaactac

              *       180         *       200         *       220         *       240
PCR_DNA  : TTGTGAGGCCATTTTGTGCACTTTAGTGGCTGCAAGGGAGAAAAAGCTTTCACAAGTTGGGAAGGAGAAGATAGGGAAGCTTG  :  119
3RACE    : ------------------------------------------------------------GAAGGAGAAGATAGGGAAGCTTG  :   23
5RACE    : TTGTGAGGCCATTTTGTGCACTTTAGTGGCTGCAAGGGAGAAAAAGCTTTCACAAGTTGGGAAGGAGAAGATAGGGAAGCTTG  :  249
           ttgtgaggccattttgtgcactttagtggctgcaagggagaaaaagctttcacaagttggGAAGGAGAAGATAGGGAAGCTTG

           *       260         *       280         *       300         *       320         *
PCR_DNA  : TTGTGTATGCCTCTGATCAAACACACAGTGCACTTCAGAAGGCTGCTCAAATTGCTGGGATCCATCCAGCAAATTTCCGGGTC  :  202
3RACE    : TTGTGTATGCCTCTGATCAAACACACAGTGCACTTCAGAAGGCTGCTCAAATTGCTGGGATCCATCCAGCAAATTTCCGGGTC  :  106
5RACE    : TTGTGTATGCCTCTGATCAAACACACAGTGCACTTCAGAAGGCTGCTCAAATTGCTGGGATCCATCCAGCAAATTTCCGGGTC  :  332
           TTGTGTATGCCTCTGATCAAACACACAGTGCACTTCAGAAGGCTGCTCAAATTGCTGGGATCCATCCAGCAAATTTCCGGGTC

                340         *       360         *       380         *       400         *
PCR_DNA  : ATCAAAACCAAGAGGTCAAGTTTCTTTGCTTTGTCTCCTGACTCTCTTCTCTCCACCATTCTTTTGGATGTGGAGAATGGCTT  :  285
3RACE    : ATCAAAACCAAGAGGTCAAGTTTCTTTGCTTTGTCTCCTGACTCTCTTCTCTCCACCATTCTTTTGGATGTGGAGAATGGCTT  :  189
5RACE    : ATCAAAACCAAGAGGTCAAGTTTCTTTGCTTTGTCTCCTGACTCTCTTCTCTCCACCATTCTTTTGGATGTGGAGAATGGCTT  :  415
           ATCAAAACCAAGAGGTCAAGTTTCTTTGCTTTGTCTCCTGACTCTCTTCTCTCCACCATTCTTTTGGATGTGGAGAATGGCTT

             420         *       440         *       460         *       480         *       5
PCR_DNA  : GATTCCTTGTTTCCTATGTGCAACTGTTGGCACTACTGCAATAGACACCATTGATCCTGTGGGACCATTGTGTAGTGTGGCCA  :  368
3RACE    : GATTCCTTGTTTCCTATGTGCAACTGTTGGCACTACTGCAATAGACACCATTGATCCTGTGGGACCATTGTGTAGTGTGGCCA  :  272
5RACE    : GATTCCTTGTTTCCTATGTGCAACTGTTGGCACTACTGCAATAGACACCATTGATCCTGTGGGACCATTGTGTAGTGTGGCCA  :  498
           GATTCCTTGTTTCCTATGTGCAACTGTTGGCACTACTGCAATAGACACCATTGATCCTGTGGGACCATTGTGTAGTGTGGCCA

           00          *       520         *       540         *       560         *       580
PCR_DNA  : AGGACTATGGCATTTGGGTCCACGTAGATGCAGCTTAC----------------------------------------------  :  406
3RACE    : AGGACTATGGCATTTGGGTCCACGTAGATGCAGCTTACGCTGGATCAGCTTGCATTTGCCCTGAGTTTAGATATTGCATTGAT  :  355
5RACE    : AGGACTATGG------------------------------------------------------------------------  :  508
```

Figure 4.17 (a) Schematic diagram showing the overlap regions for Mp-ty/ddc gene portions isolated by different methods (b) Alignments of Mp-ty/ddc sequence portions obtained by genomic PCR approach ((Section 4.3.1) and 3' RACE (Section 4.3.2) to the 508 bp 5' RACE. The overlap region (227 - 508 bp) for the three sequence portions showed 100% homology.

Analysis of the 5’ RACE sequence using Blast against the NCBI data bank drew very significant gene and protein alignments for DOPA/tyrosine decarboxylase of species in family Leguminosae of *M. pruriens* as well as for species in other plant taxa. However some of the gene sequences that drew very significant alignments to the Mp-ty/ddc were longer by approximately 0.3 kb at the 5’ end. This indicated that the isolated Mp-ty/ddc sequence might be partial. However, additional sequence data obtained from over 80 further 5’ RACE experiments including using varied approaches and modifications, did not reveal alignment with any known DOPA decarboxylase gene sequence in the NCBI data bank. The approaches used to optimise the 5’ RACE experiments included; performing replica experiments as described in Section 4.2.4.3 above, and using different gene-specific primers. In addition the integrity of the 5’ RACE-Ready cDNA quality by extraction of fresh high quality RNA coupled with cDNA synthesis after every 3 - 5 trial 5’ RACE experiments. In addition, touchdown PCR was used as described in Section 4.2.3.4 to improve specificity for PCR amplification. Further approaches tried included: changing the enzyme used for cDNA synthesis; from GeneScript Reverse Transcriptase (Clontech, UK) to SuperScript Reverse Transcriptase (Takara, Japan) and lowering the Tm for 5’ RACE from 68 - 70 ˚C (optimal range) to 65 67 ˚C suited for amplification of difficult templates (BD SMART RACE protocol, Clontech, UK) (See Appendix IV).

4.3.5: Isolation of a further 5’ fragment of the Mp-ty/ddc gene based on degenerate PCR primers.

Since obtaining the 5’ end of the Mp-ty/ddc gene by 5’ RACE proved intractable, an alternative strategy was sought. Degenerate primers (described in Chapter 2

Section 2.2.5.2) were designed based on nucleotide sequences for *Glycine max, Papaver somniferum* and other species in family Leguminosae of *M. pruriens* (Figure 4.18).

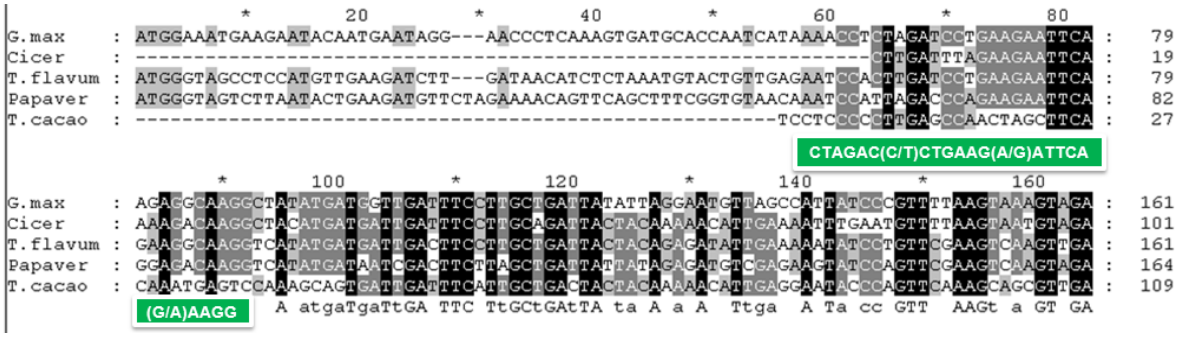


Figure 4.18 Mp-ty/ddc forward degenerate primer was designed from the conserved sequence (region 64-95 bp) of DOPA/tyrosine decarboxylase (ty/ddc) gene. The alignments were drawn from the ty/ddc 5'end sequence for *Glycine max* (356522541), *Cicer arietinum* (502157020), *Thalictrum flavum* (AF314150.1), *Papaver somniferum* (AF025434.1) and *Theobroma cacao* (XM_007030181.1) using the genedoc software program.

An approximately 0.7 kb Mp-ty/ddc PCR product was isolated from *M. pruriens* seed cDNA, (Figure 4.19 a) using a forward degenerate primer; $R_{(DEG)}$: (5'-CTAGAC(C/T)CTGAAG(A/G)ATTCA(G/A)AAGG-3') for the conserved sequence approximately 60 bp from to the 5'end of fully sequenced ty/ddc (Figure 4.20) and an Mp-ty/ddc gene specific reverse primer (GSP); $R_{(X\text{-}DG)}$: (5'-CTATTGCAGTAGTGCCAACAGTTGC-3'), 102 bp from the 3'end of the 406 bp gene fragment isolated by genomic PCR (See Section 4.3.2). The PCR was performed at an annealing temperature of 58 ˚C and the PCR product was cloned as described in Chapter 2, Section 2.2.13. A 0.5 kb Mp-ty/dc transgene clone (Figure 4.19 b) was amplified by colony PCR as described in section 2.2.14 using Mp-ty/ddc

gene specific primers; $F_{(X\text{-}DG)}$: (5'-GTGAGATGCTAAGCACTGGATTC-3'), 10 bp from the 5' end of the 5' RACE sequence and $R_{(X\text{-}DG)}$ primer described above. The plasmids extracted from the positive colony PCR cultures were further screened by PCR using plasmid primer; T7: (5′-TAATACGACTCACTATAGGG-3′) and Mp-ty/ddc primer; $R_{(X\text{-}DEG)}$ at annealation temperature of 49 ˚C. The approximately 0.7 kb cloned gene was PCR amplified from the transgene plasmids (Figure 4.19 c) and sequenced.

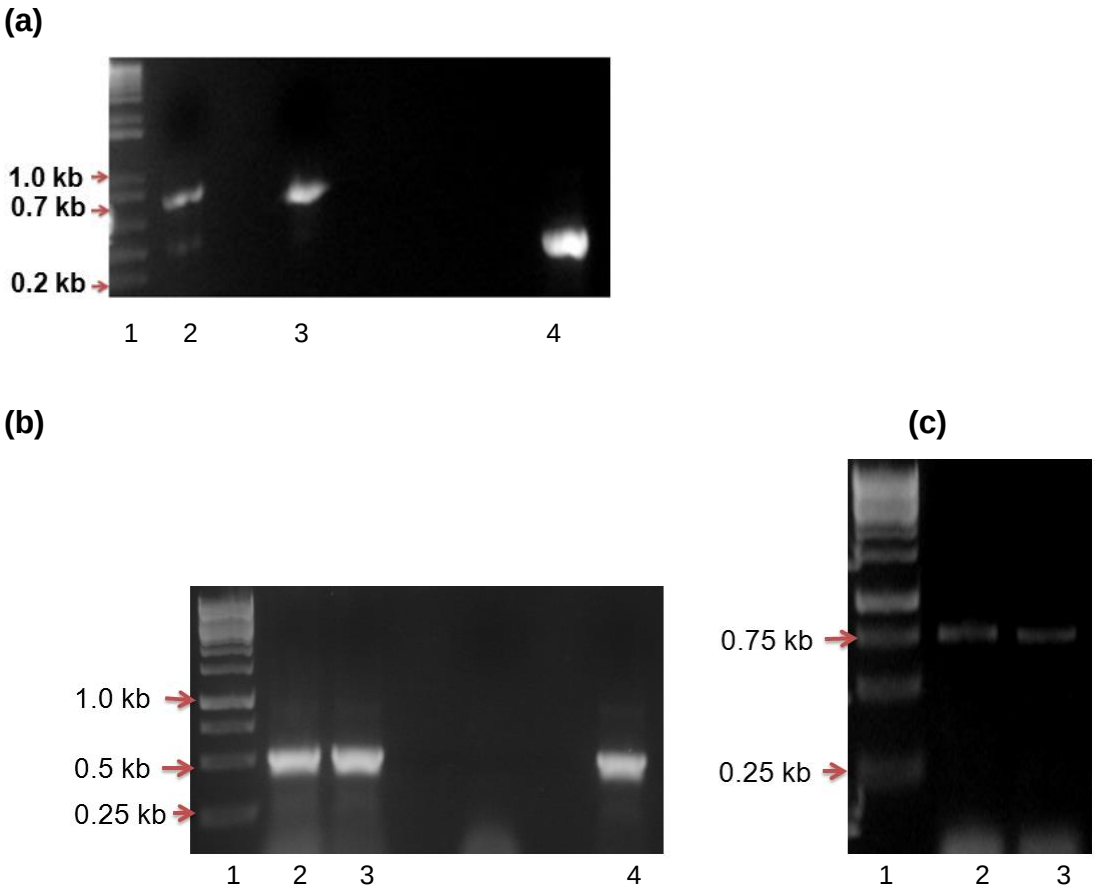
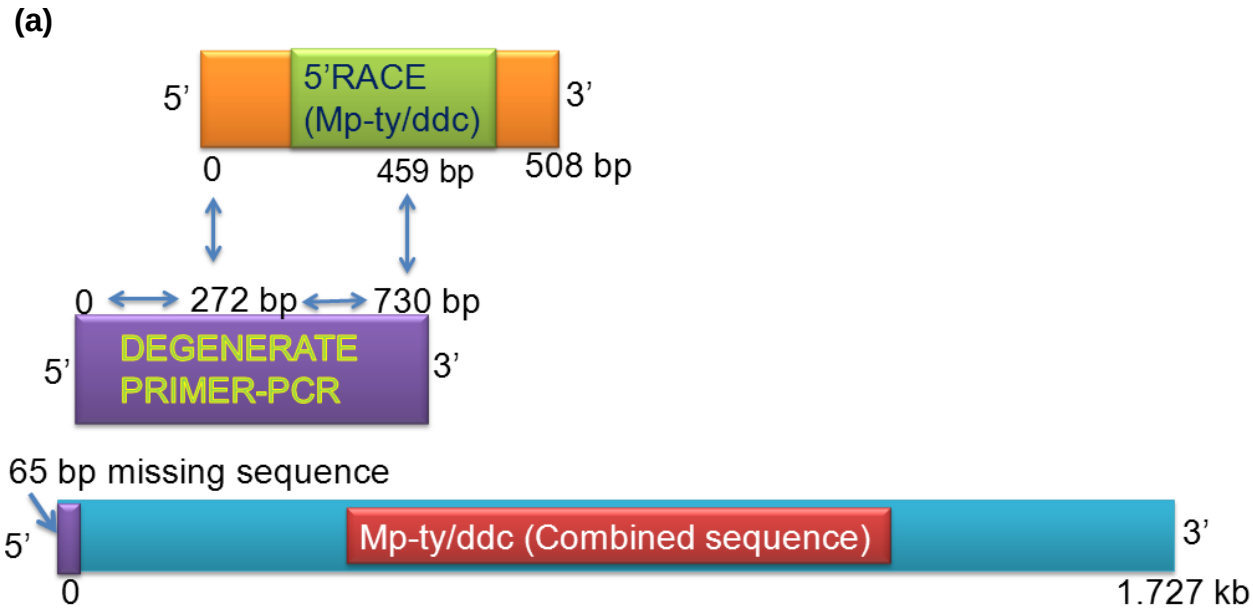


Figure 4.19 (a) A 0.7 kb Mp-ty/ddc PCR product isolated from seed cDNA (lane 2) and genomic DNA (lane 3) of *M. pruriens* using a degenerate primer; $F_{(DEG)}$) and GSP; $R_{(DEG)}$. A positive control of 0.5 kb (lane 4) PCR amplified from gDNA using Mp-ty/ddc primers $F_{(X\text{-}DG)}$ and $R_{(X\text{-}DG)}$. (b) A 0.5 kb colony PCR products (lanes 2-3) isolated using primers; $F_{(X\text{-}DG)}$ and $R_{(X\text{-}DG)}$. A 0.5 kb PCR product was isolated from gDNA (Positive control) and none from sterile distilled water (negative control). Lane 1 is a DNA marker (Fermentas).

A 0.73 kb sequence was obtained by the PCR approach using degenerate primers. The overlap region (272 - 720 bp) showed 100% homology with the Mp-ty/ddc sequence obtained earlier by 5' RACE (See Section 4.3.3) (Figure 4.20). The 5' RACE sequence was thus extended at the 5' end by 272 bp of Mp-ty/ddc again strongly indicating that the extended sequence derived from the same gene.



(b)

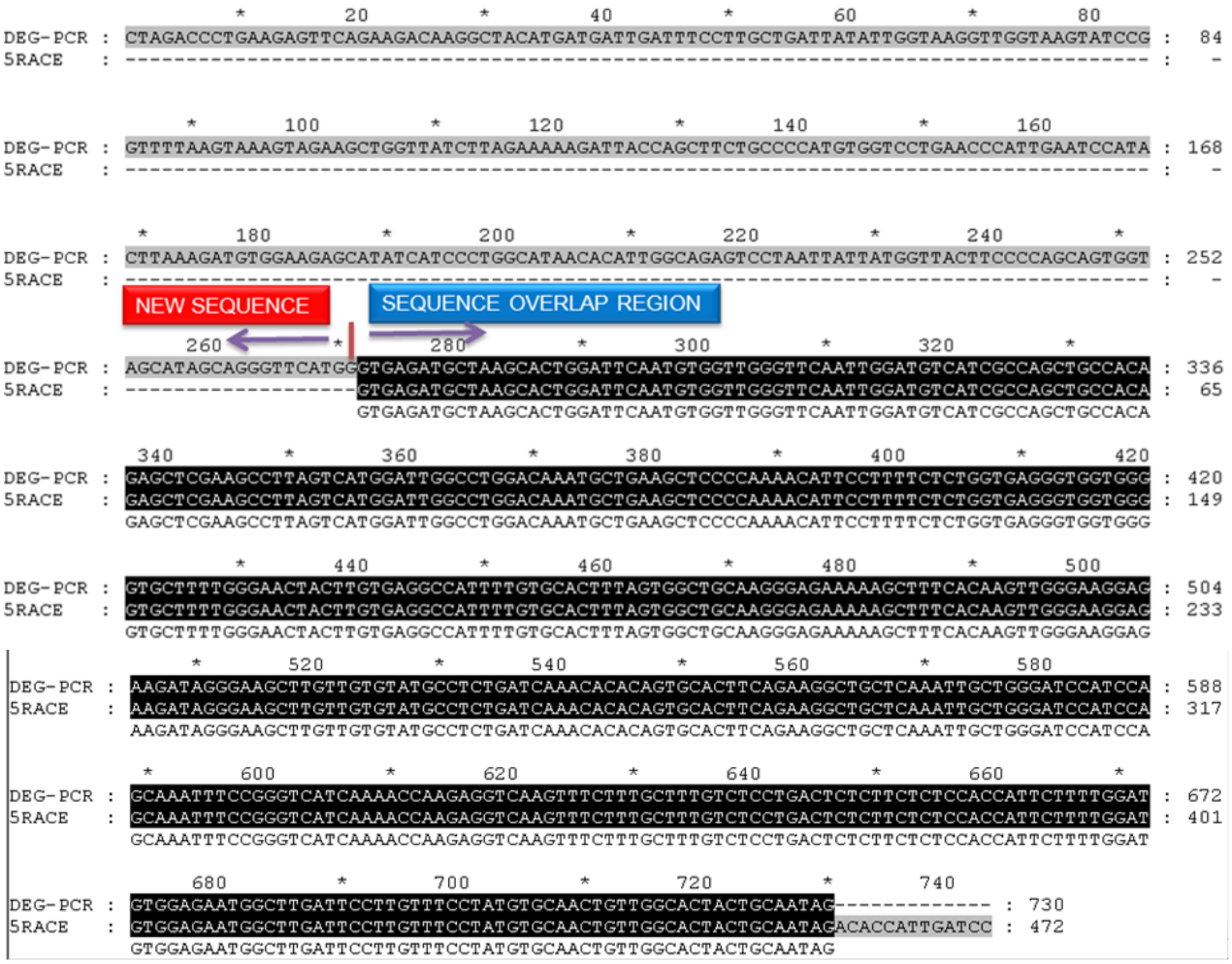
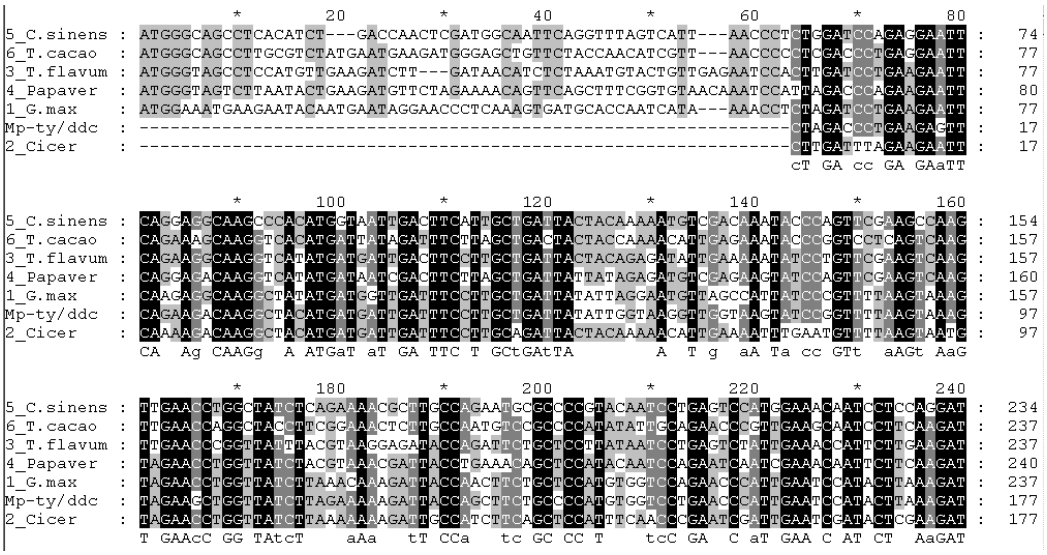


Figure 4.20 (a) Schematic diagrams of the overlap regions for Mp-ty/ddc gene portions isolated by 5' RACE and degenerate PCR. (b) Alignment of the 5' RACE sequence to that obtained by degenerate primer PCR (DEG-PCR). The overlap region (273 - 730 bp) showed 100% homology. The Mp-ty/ddc sequence obtained earlier by 5' RACE was extended by 272 nucleotides at the 5' end.

The sequence towards the 5' end of Mp-ty/ddc sequence obtained was analysed using Blast against the NCBI data bank. This drew very significant alignments of DOPA/tyrosine decarboxylase for taxonomically closely related species to *M. pruriens* (Figure 4.21).

(a)



(b)

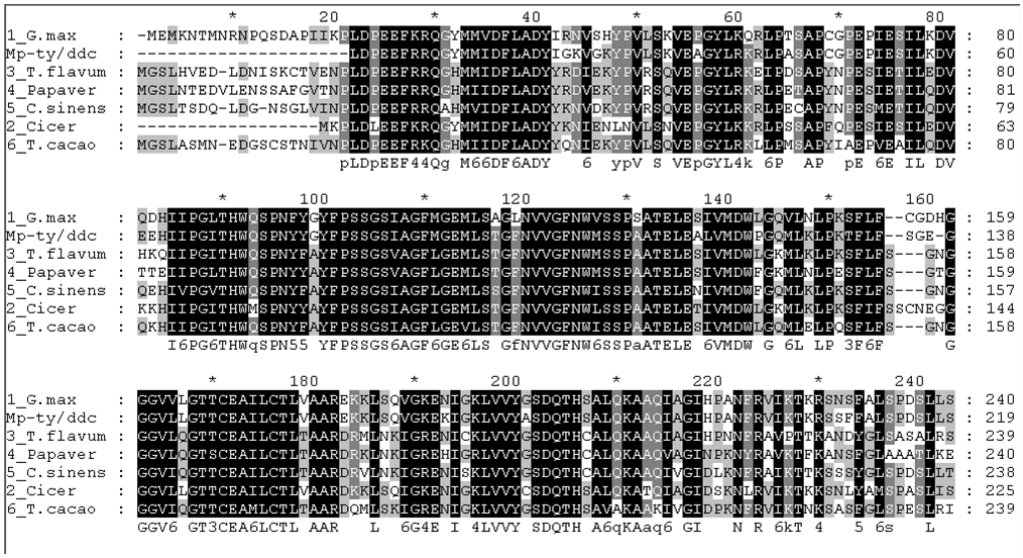


Figure 4.21 Shows 5’ end DOPA/tyrosine decarboxylase alignments for; (1) *Glycine max* (XM_003529857.1), (2) *Cicer arietinum* (XM_004510674.1), (4) *Papaver somniferum* (AF025434.1), (5) *C. sinensis* (XP_006473252), (6) *Thalictrum cacao* (AF314150.1) to the Mp-ty/ddc sequence obtained; (a) nucleotide (b) amino acid sequences. The alignments were assembled using the genedoc software program.

The Mp-ty/ddc sequence obtained however appeared to be 65 bp (20 amino acids) shorter at the 5’ end, than the longest plant DOPA/tyrosine decarboxylase sequences on the NCBI data bank. The putative missing 65 bp and 20 amino acids at 5’end of ty/ddc were highly divergent (Figure 4.21).

4.3.6: Analysis of the Mp-ty/ddc assembled sequence.

The combined sequence for Mp-ty/ddc was obtained by alignment of the overlapping sequence portions obtained as described in Sections; 4.3.1, 4.3.2, 4.3.3 and 4.3.4. A continuous sequence of 1.727 kb was obtained upon assembling the overlapping Mp-ty/ddc sequence portions obtained as shown in the Figure 4.22 below. Analysis of the sequence revealed an open reading frame (ORF) of 496 amino acids and with a protein size of 54.6 kd (Figure 4.22).

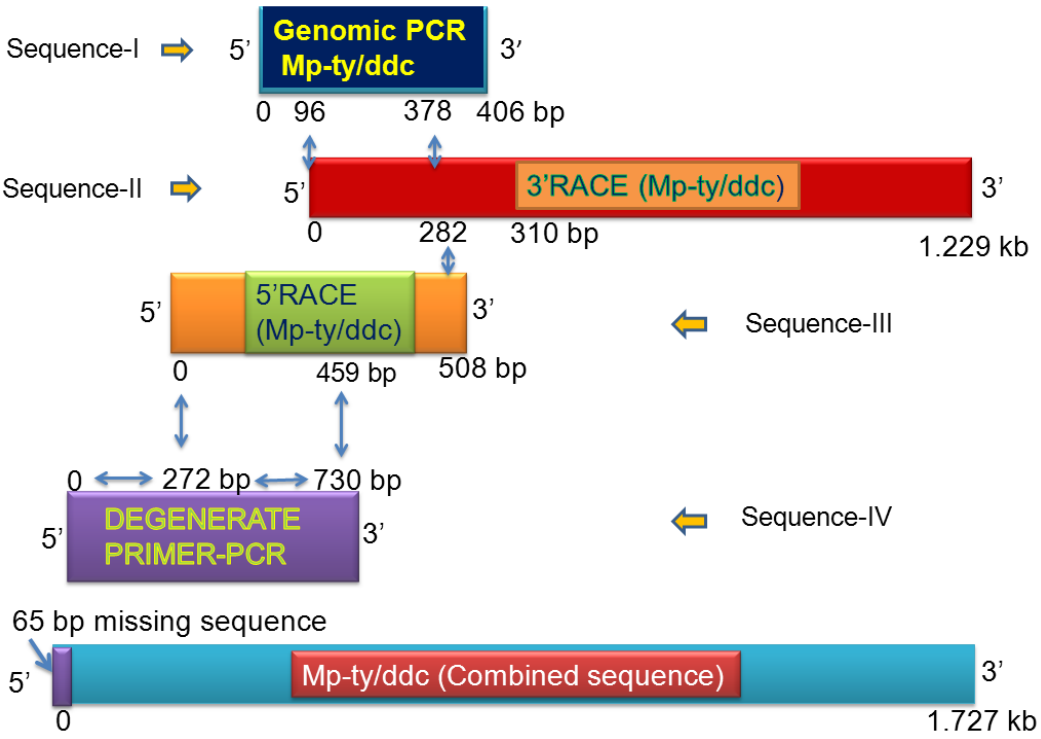


Figure 4.22 a Schematic diagram showing the overlap regions for Mp-ty/ddc gene portions isolated by different methods. The region (96 - 378 bp) of the genomic PCR sequence overlapped with region (0 - 310 bp) of the 3' RACE sequence. Region (0 - 282 bp) of the 3' RACE sequence overlapped with region (326 - 508 bp) of the 5' RACE sequence. The degenerate primer PCR sequence region (272 - 730 bp) overlapped with region (0 – 459 bp). The overall Mp-ty/ddc sequence was 1.727 kb.

```
tctagaccctgaagagttcagaagacaaggctacatgatgattgatttccttgctgattat
  L  D  P  E  E  F  R  R  Q  G  Y  M  M  I  D  F  L  A  D  Y
attggtaaggttggtaagtatccggttttaagtaaagtagaagctggttatcttagaaaa
 I  G  K  V  G  K  Y  P  V  L  S  K  V  E  A  G  Y  L  R  K
agattaccagcttctgccccatgtggtcctgaacccattgaatccatacttaaagatgtg
 R  L  P  A  S  A  P  C  G  P  E  P  I  E  S  I  L  K  D  V
gaagagcatatcatccctggcataacacattggcagagtcctaattattatggttacttc
 E  E  H  I  I  P  G  I  T  H  W  Q  S  P  N  Y  Y  G  Y  F
cccagcagtggtagcatagcagggttcatgggtgagatgctaagcactggattcaatgtg
 P  S  S  G  S  I  A  G  F  M  G  E  M  L  S  T  G  F  N  V
gttgggttcaattggatgtcatcgccagctgccacagagctcgaagccttagtcatggat
 V  G  F  N  W  M  S  S  P  A  A  T  E  L  E  A  L  V  M  D
tggcctggacaaatgctgaagctccccaaaacattccttttctctggtgagggtggtggg
 W  P  G  Q  M  L  K  L  P  K  T  F  L  F  S  G  E  G  G  G
gtgcttttgggaactacttgtgaggccattttgtgcactttagtggctgcaagggagaaa
 V  L  L  G  T  T  C  E  A  I  L  C  T  L  V  A  A  R  E  K
aagctttcacaagttgggaaggagaagatagggaagcttgttgtgtatgcctctgatcaa
 K  L  S  Q  V  G  K  E  K  I  G  K  L  V  V  Y  A  S  D  Q
acacacagtgcacttcagaaggctgctcaaattgctgggatccatccagcaaatttccgg
 T  H  S  A  L  Q  K  A  A  Q  I  A  G  I  H  P  A  N  F  R
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 V  I  K  T  K  R  S  S  F  F  A  L  S  P  D  S  L  L  S  T
attcttttggatgtggagaatggcttgattccttgtttcctatgtgcaactgttggcact
 I  L  L  D  V  E  N  G  L  I  P  C  F  L  C  A  T  V  G  T
actgcaatagacaccattgatcctgtgggaccattgtgtagtgtggccaaggactatggc
 T  A  I  D  T  I  D  P  V  G  P  L  C  S  V  A  K  D  Y  G
atttgggtccacgtagatgcagcttacgctggatcagcttgcatttgccctgagtttaga
 I  W  V  H  V  D  A  A  Y  A  G  S  A  C  I  C  P  E  F  R
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 Y  K  D  W  Q  I  P  L  S  R  K  F  N  A  L  K  L  W  L  V
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 F  S  L  V  C  F  R  I  A  P  S  A  I  A  N  G  V  S  N  G
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 T  E  A  C  Y  N  G  K  L  M  D  D  E  Y  R  V  N  E  V  N
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 R  K  L  L  D  S  I  N  S  S  G  N  V  F  M  T  H  G  E  V
gaaggagcctttgtgattagatgtgctattggtgcaactttaacagaggaacaccatgtg
 E  G  A  F  V  I  R  C  A  I  G  A  T  L  T  E  E  H  H  V
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 I  M  G  M  E  V  G  A  G  A  C  Q  F  S  V  R  -  L  L  K
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 Q  I  V  L  S  F  S  Y  P  V  V  D  I  K  I  I  Q  A  D  L
gaaaagtcatcgatgatagagaaccaacatcgatattgctatcaggtcggtgatgacagt
 E  K  S  S  M  I  E  N  Q  H  R  Y  C  Y  Q  V  G  D  D  S
tggaaattactagaagttattcctaattaaaatatttatctataaataccaatttctaca
 W  K  L  L  E  V  I  P  N  -  N  I  Y  L  -  I  P  I  S  T
atgtaataaagttggaactttgtcattaaaaaaaaaaaaaaaaaaa
```

Figure 4.22 b The putative full-length Mp-ty/ddc gene and the encoded 496 amino acid sequence for the Mp-TY/DDC. The open reading frame (ORF) is 1.489 kb (approximated to 1.5 kb). The first in-frame ATG and methionine as well as the in frame stop codon are shaded in red. The active site loop is underlined.

To confirm that he assembled sequences derived from a single mRNA, primers were designed to amplify the whole coding region which was then cloned and sequenced. Primers; $F_{(5'DC)}$: 5'-CTAGACCCTGAAGAGTTCAGAAGACAAGG-3' and $R_{(3'DC)}$: 5'-CCTAACAGAGAATTGGCATGCTCCTGCAC-3' designed from the 5' and end of the Mp-ty/ddc ORF (as described in Section 2.2.5.1) were used at an annealing temperature of 55˚C, to isolate approximately 1.5 kb Mp-ty/ddc ORF sequence from seed cDNA by PCR (as described in section 2.2.6). The PCR product was cloned and sequenced (as described in sections 2.2.11, 2.2.13 and 2.2.15). The sequence of 1.5 kb obtained was identical to the assembled ORF shown in Figure 4.20 and hence validated the assembled sequence obtained. When the 1.5 kb ORF sequence was analysed using Blast against the NCBI data bank, DOPA/tyrosine decarboxylase protein sequences (TY/DDC) for *Glycine max* and *Cicer arietinum* of the family Leguminosae of *M. pruriens* drew very significant alignments. In addition, Plant DOPA/tyrosine decarboxylase genes for species of varied taxa such as *P. somniferum*, also drew significant alignments (Figure 4.23). The Mp-TY/DDC amino acid sequence showed 74% homology with the TY/DDC of *Glycine max* and *Cicer arietinum* (overlap region 20 - 506; Figure 4.23 a). *Glycine max* and *Cicer arietinum* are taxonomically related to *M. pruriens* in the family Leguminosae (Duke, 1981). However, TY/DDC alignments with plant species belonging to a cross-section of taxonomically diverse families other than the Leguminosae family of *M. pruriens*, showed approximately 48% homology with the Mp-TY/DDC sequence (See Figure 4.23 b).

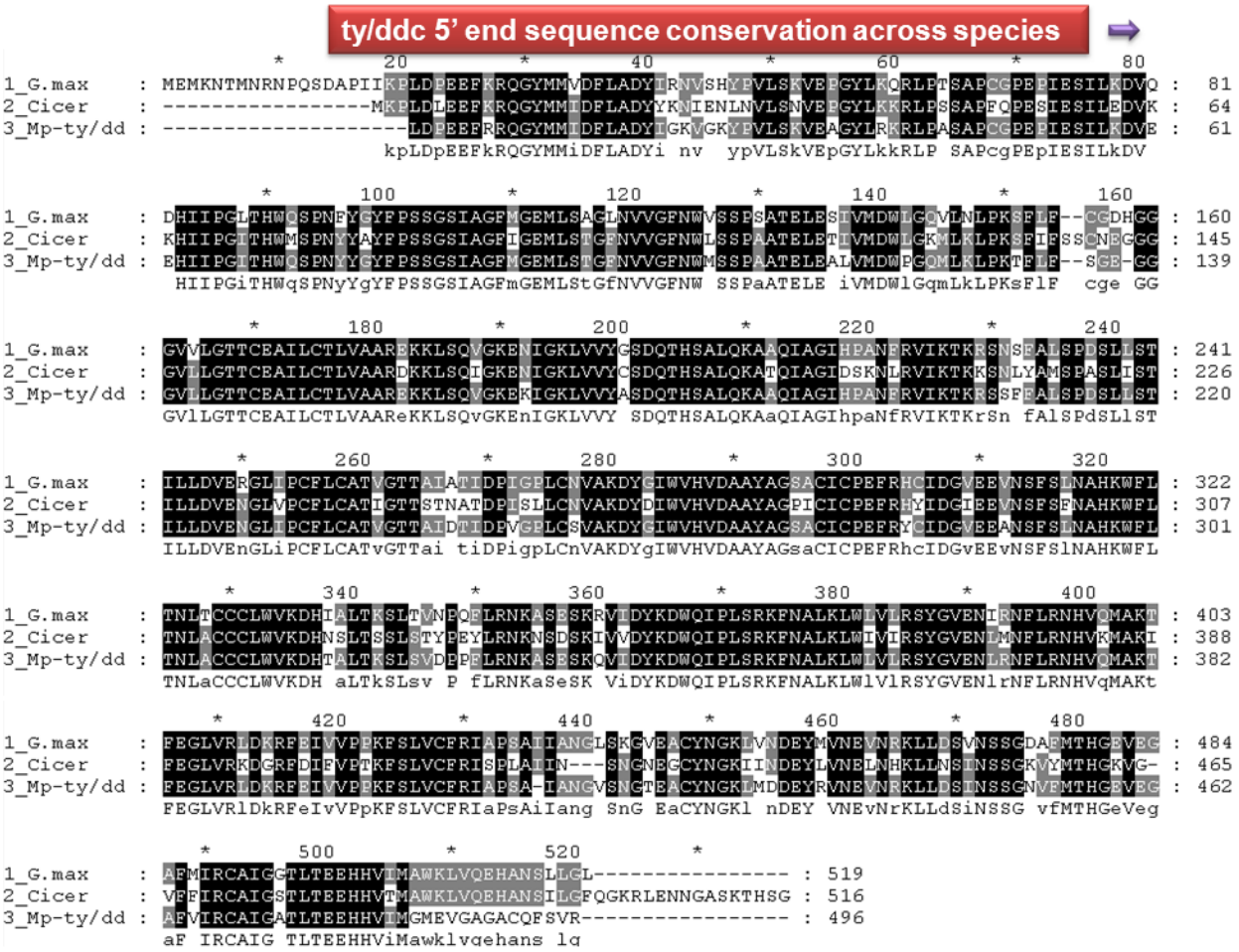


Figure 4.23 a DOPA/tyrosine decarboxylase amino acid alignments for; (1) *Glycine max* (XM_003529857.1) and (2) *Cicer arietinum* (XP_004510731.1) to the 1.5 kb *orf* sequence of Mp-ty/ddc. The alignments were drawn when the Mp-ty/ddc sequence was Blast analysed against the NCBI data bank. The alignments were assembled using the genedoc software program. The overlap region (20 - 506 bp) showed 74% sequence homology.

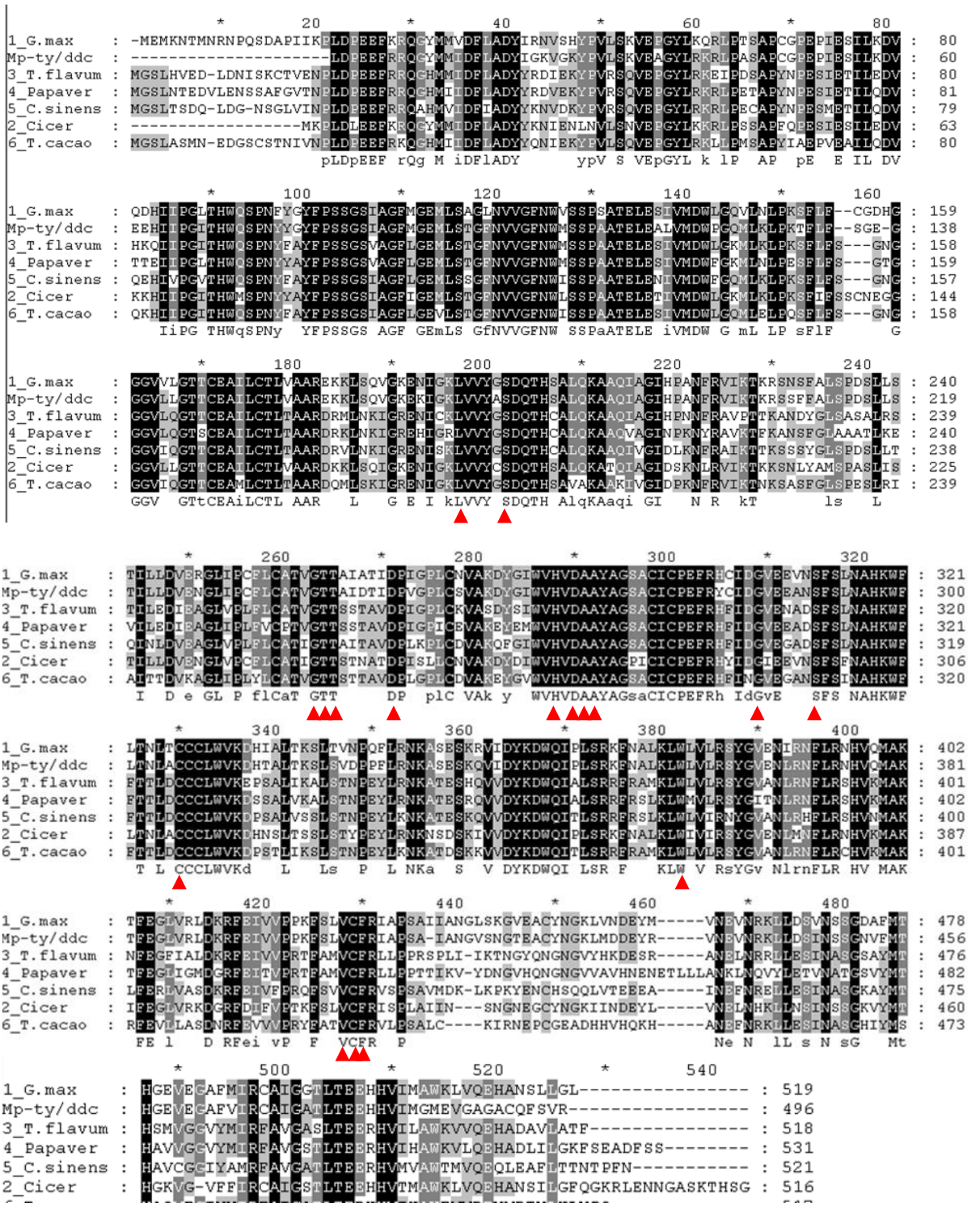


Figure 4.23 b TY/DDC amino acid alignments for; (1) *Glycine max* (XM_003529857.1), (2) *Cicer arietinum* (XP_004510731.1), (3) *Thalictrum cacao* (AAG60665.1), (4) *Papaver somniferum* (AAC61843.1) to the 1.5 kb ORF sequence of Mp-ty/ddc. The alignments were drawn when the Mp-ty/ddc sequence was Blast analysed on the NCBI data bank. The alignments were assembled using the genedoc software program. Red arrows indicate conserved amino acids in all pyridoxal phosphate-dependent decarboxylases, blue arrow indicates the putative pyridoxal binding site.

All the amino acids previously identified as conserved in pyridoxal phosphate-dependent decarboxylases such as L-histidine decarboxylase (Sandmeier, 1994) as well as L-glutamate decarboxylase (Baum et al*.,* 1993) and are also conserved in the *P. somniferum* TY/DDC (Facchini and De Luca, 1994) are conserved in the Mp-TY/DDC amino acid sequence (shown on Figure 4.23). These include the putative pyridoxal binding site.

4.3.7: Southern analysis and Mp-ty/dc gene copy number *in M. pruriens* genome.

Southern analysis was performed as described in Section 4.2.3 to determine the Mp-ty/ddc gene copy number in the genome of *M. pruriens.* The Mp-ty/ddc restriction map reveals the *Eco*RI and *Xho*I restriction sites among others to be are absent in the Mp-ty/ddc gene while three restriction sites for *Hind*III and one for *Eco*RV were present (Figure 4.24). These restriction sites were selected for Southern analysis because they tend to be randomly distribited through out the genome of many plants (Sambrook et al., 1989) producing fragments of approximately 2 - 7 kb on average depending on the species (http://tools.neb.com/~vincze/gnsites/). The gene copy number was deduced from the ratio of restriction fragments to hybridisation sginals, were a ratio of one implied a single copy. Four genomic DNA samples were each digested with one of these four restriction enzymes as described in Section 4.2.5.2 and ethidium bromide staining following gel electrophoresis (Figure 4.24) showed good digestion of the DNA.

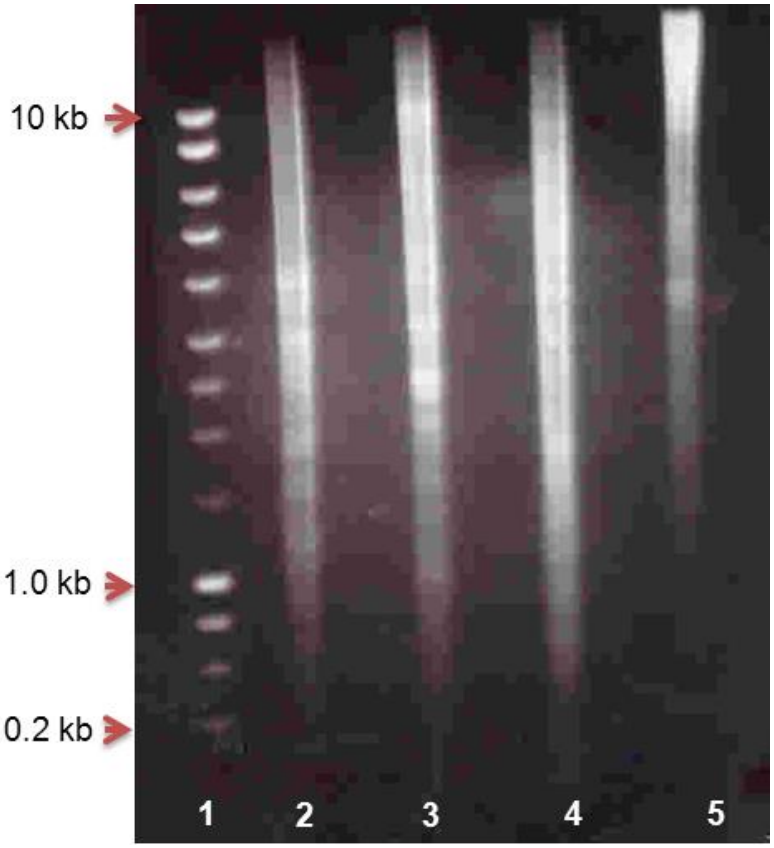


Figure 4.24 Gel electrophoresis of *M. pruriens* gDNA (20 µg) restriction digested by *Eco*RI (lane 2); *Eco*RV (lane 3); *Hind*III (lane 4) and *Xho*I (lane 5). Lane 1 was labelled using a DNA size marker (NEB).

The digested DNA was transferred to a membrane (See Section 4.2.3.3) and hybridised with a radiolabelled 1.5 kb Mp-ty/ddc probe as described in Section 4.2.5.4. Hybridisation signals of 10 kb and 13 kb were produced for *Eco*RI and *Xho*I digested genomic DNA respectively. A 4.0 kb hybrisation signal was obtained for *Eco*RV digested DNA, while a 1.0 kb and a 6.0 kb hybridisation signals were produced for the *Hind*III digested genomic DNA (Figure 4.25). A pET2b plasmid harbouring an Mp-ty/ddc gene clone was used as the positive control (5) and produced a hybridisation signal of 8 kb.

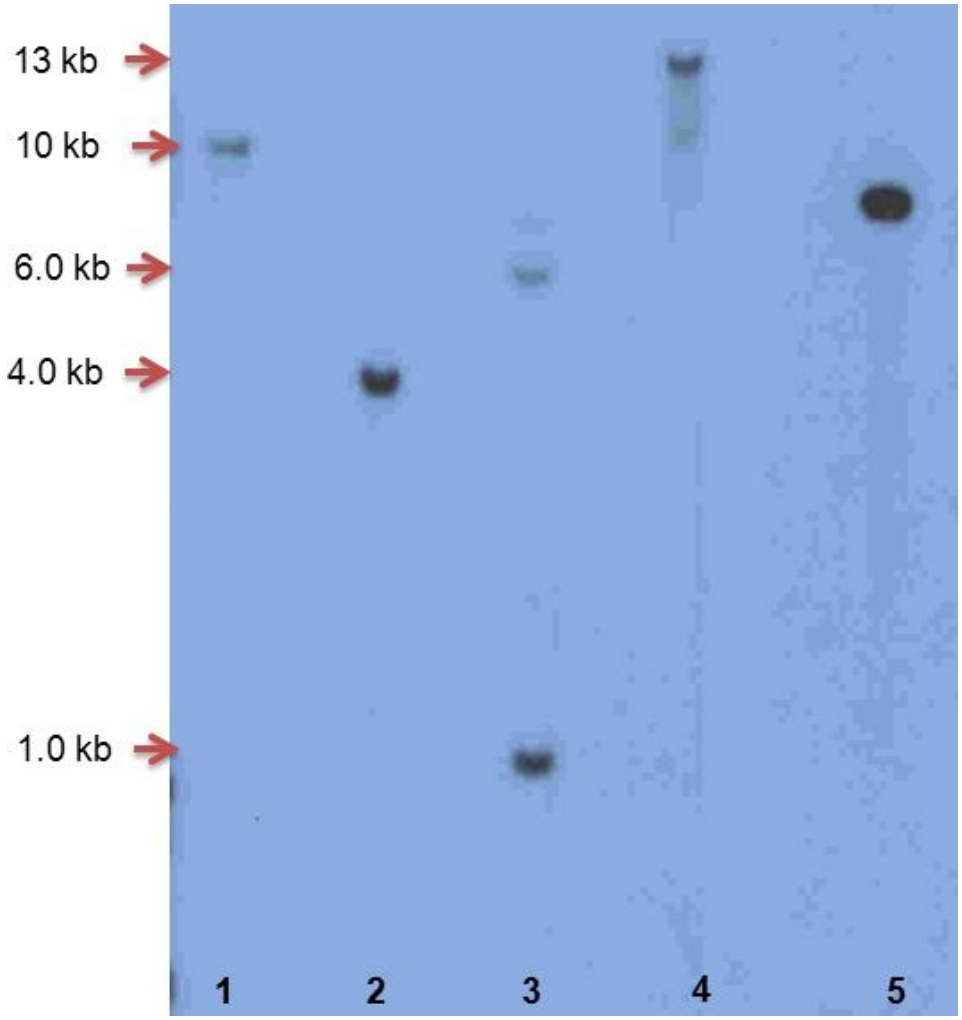


Figure 4.25 (a) Autoradiograph showing radiolabelled Mp-ty/ddc probe hybridisation on the Southern blot of *M. pruriens* gDNA. Hybridisation signals were; (lane 1) *Eco*RI digested DNA, (lane 2) *Eco*RV digested DNA, (lane 3) *Hind*III digested DNA and (lane 4*) Xho*I digested DNA and (lane 5) pET2b-Mp-ty/ddc plasmid.

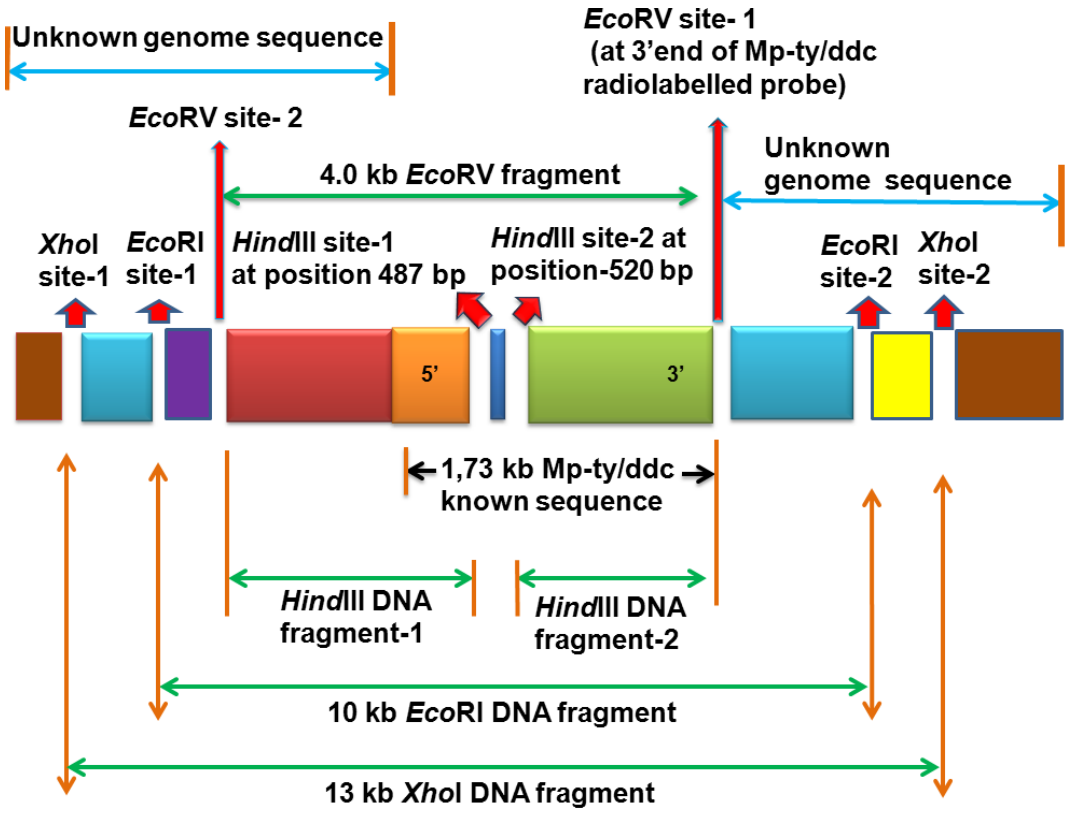


Figure 4.25 (b) Schematic diagram showing the number and size of the genomic Southern DNA fragments and how they relate to the observed hybridisation signals. The ratio of DNA fragments to hybridisation signals was one.

The Southern analysis result showed that the number of expected Mp-ty/ddc fragments produced by each of the four restriction enzymes used were equal to the number of Mp-ty/ddc probe hybridisation signals produced (See Figure 4.26). This indicates there is a single copy of Mp-ty/ddc gene in the genome of *M. pruriens*. This is because gene family members often differ in size and to some extent, sequence homology. This would result in the DNA fragments produced by restriction digestion of different gene family members to vary in size and hence different sized hybridisation signals would be observed. Having established that the Mp-ty/ddc was

likely a single copy gene, the expression profile was determined in tissues of *M. pruriens* by real-time PCR (qRT-PCR). The gene was intronless.

4.3.8: Real-time PCR (qRT-PCR) for Mp-ty/ddc.

4.3.8.1: Determining the endogenous control gene in *M. pruriens.*

The Mp-ty/ddc gene expression in the seeds, leaves, stems and roots of *M. pruriens* was determined by quantitative real-time PCR (qRT-PCR) as described in Section 4.2.6. Given the lack of *M. pruriens* sequences on publicly available data bases it was necessary to first identify an endogenous control gene of *M. pruriens,* for use in real-time PCR. Degenerate primers for, GAPDH, EF1 alpha, Ubiquitin and a gene specific primer for *M. pruriens* 18S rRNA, were designed as described in Chapter 2 Section 2.2.5.2. In order to determine the appropriate endogenous gene, semi-quantitative PCR was performed on seed, leaf, stem and root cDNA for all four genes 18S rRNA, GAPDH, Ubiquitin and EF1 alpha. The semi-quantitative PCR results showed GAPDH and EF1 alpha were suitable as control genes (Appendix-IV) thus the four primer sets were also tested using qRT-PCR. However, the qRT-PCR analysis showed the GAPDH expression was consistently poor in seed cDNA samples whereas EF1 alpha expression was poor in root cDNA samples of *M. pruriens.* On the other hand, the qRT-PCR analysis for the 18S rRNA showed the gene was uniformly expressed. These preliminary experiments also revealed that the cDNA concentration was too low as the 18S rRNA showed relatively high cycle threshold (Ct) values (>25). This was remedied using 3 - 5 fold concentrated cDNA as described in Section 4.2.5.3. Consequently, Ct values for the 18S rRNA gene

(endogenously expressed) were lowered to an optimal range of 18 - 20 (Figure 4.26).

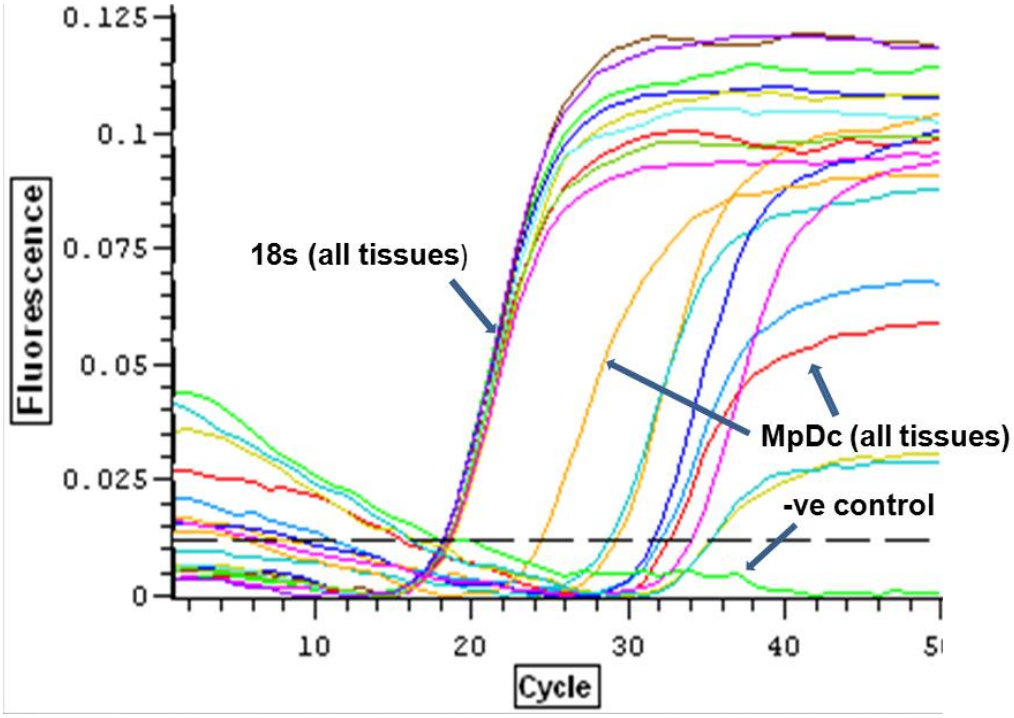


Figure 4.26 qRT-PCR analysis graph showing the expression profile of Mp-ty/ddc (MpDc) in the cDNA of different *M. pruriens* tissues relative to the endogenously expressed 18S rRNA (18s).

4.3.8.2: Mp-ty/ddc expression profile.

The endogenous gene (18S rRNA) expression in seed, leaf, stem and root cDNA of *M. pruriens* was normalised as described in Section 4.2.6.3 to a cycle threshold reference Ct value of 18.82 (Figure 4.27). The Mp-ty/ddc gene was then qRT-PCR amplified from the normalised cDNAs for each *M. pruriens* tissue under study as described in Section 4.2.5.3.

Purity of the qRT-PCR products was determined from their respective melting curves. The melt profile analysis for the qRT-PCR products, using the Opticon 3 software program (MJ Research Inc, USA), revealed a single pure product peak at 79 ˚C and 84 ˚C for the Mp-ty/ddc and 18S rRNA respectively (Figure 4.27).

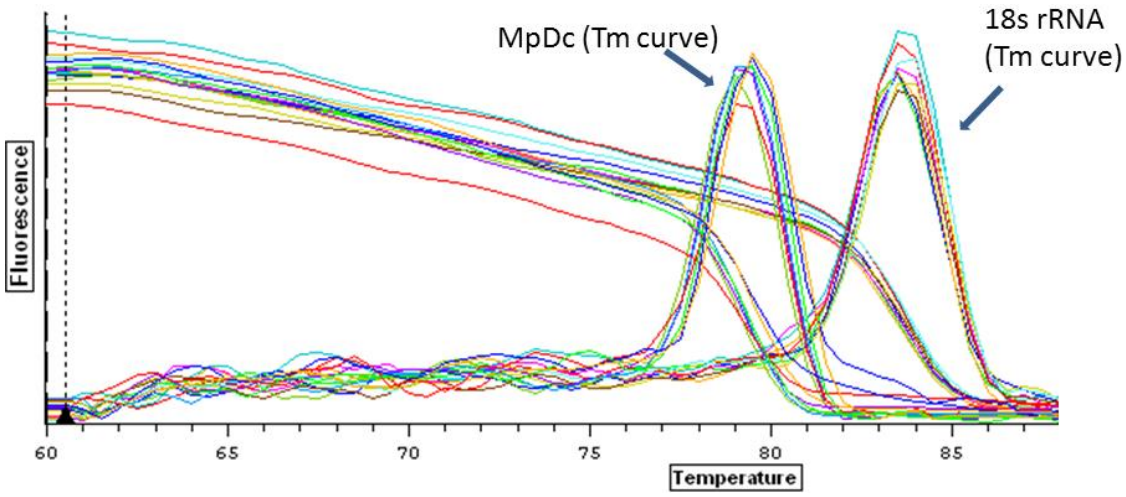


Figure 4.27 Melting curves for the Mp-ty/ddc (MpDc) and 18S rRNA (18s) gene expression profile analysis in different tissues of *M. pruriens.*

Statistical analysis of the mean, standard deviation and standard error in the Ct values of the triplicate qRT-PCR amplicons for seed, leaf, stem and root cDNA was performed, and the Mp-ty/ddc expression for each triplicate set of normalised cDNA of seed, leaf, stem and root relative to endogenous (18S rRNA) expression in *M. pruriens* was calculated using the equation below derived by Livak and Schmittgen (2001) as described in Section 4.2.5.5.

The results showed the Mp-ty/ddc gene expression in *M. pruriens* was highest in the roots (1.66 +/- 0.1 S.E), followed by stems (1.37 +/- 0.3 S.E), then seed (1.0) and least expression was observed in the leaves (0.195 +/- 0.02 S.E) (Figure 4.28). (The one-way analysis of variance (ANOVA) to be performed using the Statistica software program).

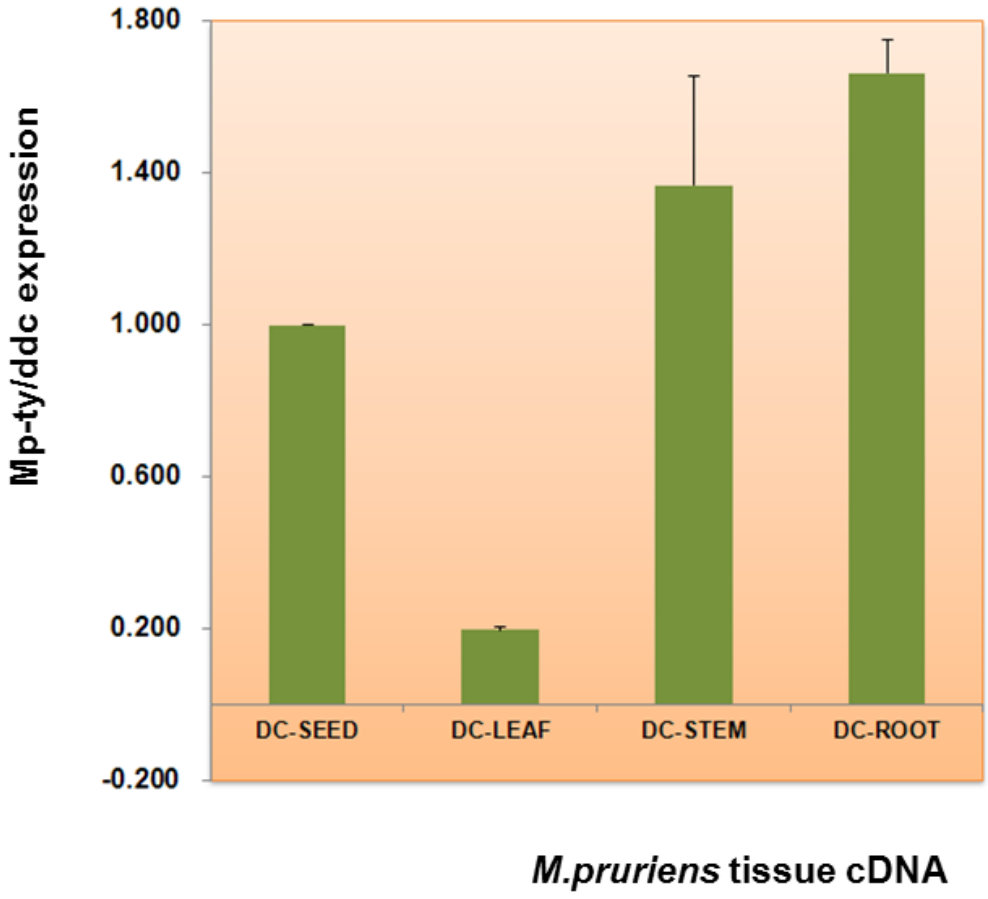


Figure 4.28 Mp-ty/ddc expression profile in seeds, leaf, stem and roots of *M. pruriens*. Mp-ty/ddc expression was normalised relative to endogenous 18S rRNA expression by qRT-PCR (mean ± SE; n=3).

4.4: DISCUSSION.

The primary aim of this chapter was to isolate and characterise genes related to the biosynthesis and metabolism of L-DOPA, with a longer term aim of manipulating it. Initial focus was on two genes; tyrosine hydroxylase (Mp-tyoh) and DOPA/tyrosine decarboxylase (Mp-ty/ddc) which encode for the enzymes responsible for synthesis of L-DOPA from L-tyrosine and dopamine from L-DOPA respectively (Fitzpatrick, 1999; Facchini, 2001).

Attempts to isolate Mp-tyoh genes from *M. pruriens* using degenerate primers based on available sequences from the databases were not successful (Appendix IV). However a short sequence for a putative ty/ddc gene from *M. pruriens* was available (NCBI databank accession number EF101921.1), hence efforts were focussed on obtaining a full-length sequence from this gene and characterising gene copy number and expression in different *M. pruriens* tissues.

Initial PCR amplification using genomic DNA from five different *M. pruriens* cultivars showed that at least in this portion of the gene, sequences were identical, indicating a lack of sequence divergence between cultivars. Although several studies have documented differences between ecotypes and cultivars of *M. pruriens* (http://hal.archives-ouvertes.fr/docs/00/13/79/62/PDF/T304Asongwed.pdf) but very

little is known about sequence differences. The cultivars used here were; '90 day', vine, early maturing, tropical and cultivar and Bush Echo. Clearly more sequence data are required but data here would indicate relatively little divergence between these cultivars of very different growth habits.

Homology of the assembled Mp-ty/ddc gene to decarboxylase genes from related and more distant species is consistent with the putative annotation of this gene as encoding a TY/DDC enzyme. Of particular significance is the conservation of the active site loop and the conserved amino acids in all pyridoxal phosphate-dependent decarboxylases, including the putative pyridoxal binding site (Facchini and de Luca, 1994). However in *Arabidopsis* very similar proteins encode tyrosine decarboxylases, one of which is annotated as an aromatic aldehyde synthase (AT2G20340) and the other as a TYDC (AT4G28680). An important difference between these two genes is a change from a Y to an F in the active loop. The Mp-ty/ddc gene encodes an F at this position (amino acid 267), which might indicate that it is not in fact an active TY/DDC. However the *P. somniferum* gene also encodes an F in this position and has been shown to have TY/DDC activity *in vitro* (Facchini and De Luca, 1994). It is therefore difficult to assign a function to the encoded enzyme based solely on the sequence.

A total of 1,727 bp of Mp-ty/ddc gene sequence was obtained with an open reading frame starting from the first in frame methionine to the first stop codon of 496 amino

acids. However, alignment of the translated Mp-ty/ddc gene sequence to the ty/ddc sequences of other species, revealed that some sequences, and specifically the *P. somniferum* sequence were longer at the 5' end. This suggested the possibility that the isolated Mp-ty/ddc gene might be missing the N terminal amino acids. One of the shortest sequences is that of, the TY/DDC for *Cicer arietinum* (XP: 004510731.1) which is 18 amino acids shorter at the N-terminal than the longest plant TY/DDC alignment (Figure 4.24). However it is three amino acids longer than the translated Mp-ty/ddc sequence and the 11 amino acids upstream of the first in frame methionine of the Mp-ty/ddc sequenced from taxonomically closely related and less related species (Figure 4.23). This strongly suggests that the Mp-ty/ddc gene sequence may be missing between 3 and about 20 amino acids at the N-terminus. Given this probability strenuous efforts were made to isolate further 5' sequences using further degenerate primers and by numerous attempts with 5'RACE. Further attempts were also made to isolate the missing sequences using an inverse PCR approach as described in appendix IV. However, the inverse PCR product DNA was of low concentration but could not be re-PCR amplified us for direct sequencing or cloning (See Appendix IV). Possible explanations for the difficulty could be secondary structure of the RNA that impedes cDNA synthesis, perhaps due to a GC-rich region. Although overall the GC content of Mp-ty/ddc open reading frame is only 44%, if the missing amino acids were the same as those in *C. arietum* (MKP) this

could result in the sequence GCCC just 5' to the sequence isolated in the Mp-ty/ddc gene which could potentially form strong secondary structures with any complementary sequences further upstream. RACE is known to often produce incomplete termini (Pfeffer et al., 1995; Schramm et al., 2000) and although the kits used are designed to circumvent these problems, difficulties may still arise. Inverse PCR is a powerful technique for the isolation of flanking sequences but it too is not without its difficulties (Moreau et al., 2002), again compounded by secondary structure.

Since the TY/DDC genes in *P. somniferum* form a gene family, it was important to establish whether the Mp-ty/ddc was also the member of a gene family or is a single copy gene. Southern blot analysis was therefore performed. The general observation in the Southern analysis was that the number of hybridisation signals was equal to the number expected DNA fragments generated by restriction digestion. This result is expected for genes with a single copy in the genome (Figure 4.23). This was an unexpected result given the multiple genes in *P. somniferum* (Facchini and De Luca, 1994). It is however supported by all the PCR products sequenced for this gene from *M. pruriens* none of which showed any differences in sequence apart from slight differences in the length of the 3' UTR. In particular many enzymes involved in defence have shown linear specific expansion such as

the cytochrome P450 family (Lespinet et al., 2002) indicating there may be substantial divergence in gene family size between lineages and species. Unfortunately there are no data on the C-value database for this species (data.kew.org/c values). The Leguminosae (Fabaceae) cover an extremely wide range of C-values from 0.3 pg to 27 pg. *P. somniferum* has a C-value of 3.8 pg and is thus substantially bigger than the smallest members of the Fabaceae.

Another possibility is that there are related genes in the *M. pruriens* genome but that that lower stringency conditions are required, and thus that they are substantially divergent. This could be further explored by further Southern blot analysis. Transcriptome sequencing, which is now becoming much more accessible would also be a method to reveal whether there are other similar sequences expressed.

The next task was to determine the expression profile of Mp-ty/ddc in different tissues of *M. pruriens*. This was performed using the real-time PCR as described in Chapter 4 Section 4.2.6.8. Mp-ty/ddc expression profile in different tissues of "wild-type" *M. pruriens* was normalised relative to the 18S rRNA endogenous expression. Mp-ty/ddc expression was highest in cDNA for the roots then stems, and seed cDNA whereas the least expression was seen in leaf. The high Mp-ty/ddc expression in mid mature stem tissues and roots of *M. pruriens* is in agreement with the studies by Facchini and De Luca (1995) in *P. somniferum*. They reported that highest

expression of a similar gene (DOPA/tyrosine decarboxylase; Ps-ty/ddc) in *Papaver somniferum* was highest in the phloem and protoxylem of mature stems and roots, while the lowest gene expression was reported in the leaf tissue. The RNA hybridisation studies on a cross section of leaf tissues of *P. somniferum* revealed that the Ps-ty/ddc gene was confined to the midrib region in young leaves but no expression was observed in leaf blade tissues (Facchini and De Luca, 1995). However, in *P. somniferum,* Ps-ty/ddc expression in reproductive tissues (Carpels) was also low and decreased as the seed capsule matured. In contrast Mp-ty/ddc expression was relatively high in maturing/mature *M. pruriens* seeds.

In conclusion a single copy TYDC-like gene was isolated from *M. pruriens* that is most highly expressed in stems, roots and seeds respectively. It appears to show significant homology to DOPA/tyrosine decarboxylases and appears to be almost full length. The next step was to determine whether the isolated sequence had enzymatic activity when expressed *in vitro*.

CHAPTER 5: RECOMBINANT EXPRESSION AND ACTIVITY OF *M. PRURIENS* PUTATIVE DOPA DECARBOXYLASE GENE.

5.1: INTRODUCTION.

DOPA/tyrosine decarboxylase (Mp-TY/DDC) is the putative enzyme responsible for decarboxylation of L-DOPA to dopamine in *M. pruriens* and other L-DOPA producing plants (Facchini, 2001). In animals, bioconversion of L-DOPA to dopamine has been reported to be controlled by DOPA decarboxylase.

M. pruriens is an ecologically endangered plant species and has been reported to be among the few plant species which produce L-DOPA, a drug used to treat Parkinson's disease (Siddhuraju and Becker, 2001). In *M. pruriens*, one of the primary metabolic products of the Shikimic acid pathway; L-tyrosine is converted to L-DOPA by enzymatic action of tyrosine hydroxylase (Mp-TYOH) (Pras et al., 1993; Swiedrych et al., 2004; Nishihara, et al., 2005). Consequently, L-DOPA is converted to dopamine by DOPA/tyrosine decarboxylase (Mp-TY/DDC) (See Figure 1.9). The Mp-TY/DDC enzyme is putatively encoded for by a single copy of Mp-ty/ddc gene based on the results obtained by performing a genomic Southern analysis (See Chapter 4, Section 4.3.6). In contrast, DOPA/tyrosine decarboxylase (Ps-TY/DDC) of *Papaver somniferum* is encoded for by a family of 15 genes (Ps-ty/ddc), which is subdivided into two subsets (ty/ddc1 and ty/ddc2) based on sequence homology (Facchini and De Luca, 1994) (See Chapter 4, Section 4.1). In plants, L-tyrosine has

also been reported to be converted to tyramine by action of tyrosine decarboxylase (TYDC) (Facchini, 2001) (See Figure 1.9). In *M. pruriens, P. somniferum* and a few other L-DOPA producing plants species, tyramine and dopamine are precursors in the early steps of benzylisoquinoline alkaloid biosynthesis (Facchini and Park, 2003). Benzylisoquinoline alkaloids consist of more than 2,500 structurally defined natural products including a wide range of phytoalexins and several pharmacologically important compounds such as analgesics; morphine and codeine (Facchini and Park, 2003).

DOPA/tyrosine decarboxylase, like tyrosine decarboxylase (TYDC) (See Figure 1.9) is an aromatic amino acid decarboxylase (AADC) present in a wide range of species and has substrate specificity for L-DOPA and tyrosine respectively (Facchini, 2001). However, studies on aromatic amino acid decarboxylase (AADC) proteins demonstrated that besides the decarboxylation observed in TY/DDC and TY-DDC, some previously annotated AADC were found to be aromatic acid synthesis (AAS) (Torrens-Spence, et al., 2013). Despite sharing over 50% homology, AAS performs additional physiological roles when compared with the true AAADs. The AASs catalysed decarboxylation-oxidative deamination process of aromatic amino acids to produce aromatic acetaldehydes, CO_2, ammonia and hydrogen peroxide instead of the AAAD-derived arylalkylamines and CO_2 (Torrens-Spence, et al., 2013). Plant AAS enzymes have also been reported to play a role in the synthesis of volatile flower scents and defensive secondary metabolite, phenolic acetaldehyde (Torrens-Spence, et al., 2013).

This chapter focusses on characterisation of the Mp-ty/ddc gene isolated from *M. pruriens* genome. A similar enzyme to Mp-ty/ddc referred to as DOPA

decarboxylase (DDC) has been characterised in a wide range of animal species including humans and showed sequence homology in some conserved domains.

The predicted three-dimensional structure of DDC reveals a tight association of α_2-dimer of two monomers with each composed of three distinct domains (Figure 5.2). The PLP binding site is in the large domain of DDC which contains seven strands of mixed β-sheet at the centre, encircled by eight α-helices in a typical α/β fold. The C-terminal small domain has four antiparallel β-sheet strands with three helices packed facing the large domain. Besides these two characteristic domains for the α-family enzymes, DDC has an N-terminal domain (residues 1 - 85) which comprises of two helices and linked by an extended strand. The helix of one subunit aligns antiparallel to the equivalent helix of another resulting in a flap over-like structure. The N-terminal domain is unlikely to be an autonomous folding unit but stabilises by creating an interface between the two monomers. The active site of DDC is positioned close to the interface of the two monomers although its residues are largely from one monomer (Figure 5.1) (Burkhard et al., 2001).

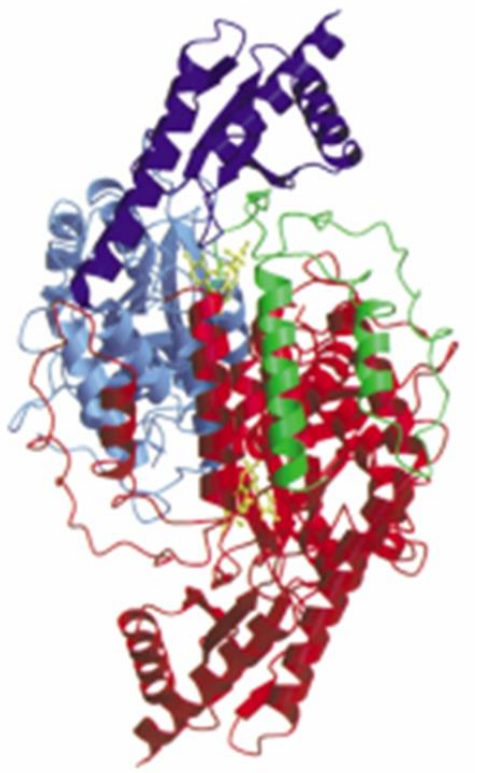


Figure 5.1 The two-fold symmetry axis view for the DDC polypeptide backbone shows the two monomers (red and green) and the large (cyan) and small (blue) domains. The yellow ball and stick represents the cofactors (PLP) and the DDC inhibitor. The picture was extracted from Burkhard et al., 2001).

5.1.1: An overview of the methodology.

The *in vitro* recombinant protein expression plasmid was built by cloning the transgene in the *lac* operon of the pET21b plasmid as described in Chapter 2 Section 2.2.14 and hence its expression required induction by IPTG. IPTG binds to the *lac* repressor detaching it from the *lac* operator and thereby enabling transcription of the genes in the *lac* operon such as the recombinant Mp-ty/ddc and

the gene which codes for β-galactosidase (Sambrook et al., 1989; Dale and Von Schantz, 2003). Recombinant protein expression in bacterial hosts is reported to be temperature dependent (Sambrook, et al., 1989; Francois and Mirna., 2004). Bacterial strains; BL21 (DE3) (Novagen, USA) and Tuner 2 cells (Novagen, USA) harbouring the pET21 protein expression vector which carries a cloned Mp-ty/ddc transgene insert were used as hosts for the *in vitro* recombinant protein expression. The BL21 (DE3) strain was designed to achieve protein overexpression upon induction using appropriate concentration of IPTG (BL21 cells user manual, Novagen, USA). On the other hand, Tuner 2 strain is a mutant of BL21 and was produced by deletion of the *lac* ZY which consequently enabled adjustment of protein expression levels throughout all cells in culture (Tuner 2 cells user manual, Novagen, USA). Mutation of the *lac* permease (*lac* Y) makes the bacterial cell walls or membranes to be uniformly permeable to IPTG. This enhanced a more proportionate response in protein expression with respect to varying IPTG concentrations. By lowering IPTG concentration, low level protein expression which could enhance solubility and activity of previously insoluble target proteins may be achieved (Tuner 2 user manual, Novagen, USA).

5.1.2: Bioassays on L-DOPA.

Plant enzymes have been used as biopharmaceuticals to produce novel drugs that are free from side effects which are largely associated with synthetic chemical products (Kutney, 1993 and Pras et al., 1995). Pras et al. (1995) reports advantages of using plant enzymes in drug synthesis; they have the ability to catalyse the reactions stereospecifically to produce chirally pure products. In addition, plant

enzymes can perform regiospecific modifications that are not easily carried out by chemical synthesis or by microorganisms. Kutney (1993) reports that the World Health Organisation (WHO) has paid special attention to two of the therapeutically important groups of natural plant products namely the podophyllotoxin and the catechols including L-DOPA. Podophyllotoxin and related ligands are used as starting compounds for the synthesis of anti-tumour drugs. There are a few reports on L-DOPA drug synthesis by bioconversion using bacterial cells or plant cell cultures. Wichers et al. (1983) used cells of *M. pruriens* immobilised in calcium alginate to bioconvert L-tyrosine to L-DOPA. There are also reports on biosynthesis of L-DOPA from tyrosine using enzyme tyrosine phenol-lyase (TPL). TPL normally catalyses the degradation of L-tyrosine to pyruvate, phenol and ammonia (Tsuchida et al., 1994). This reaction is reversible and L-DOPA is produced when catechol is substituted for phenol, hence maintaining high concentrations of the substrates (pyruvate, catechol and ammonia), drives the equilibrium towards formation of L-DOPA (Enei and Yamada, 1986; Foor et al., 1993; Tsuchida et al., 1994). In another study, Chattopadhay and Arail (1990) reported using tyrosine hydroxylase of *Aspergillus terreus to* bioconvert L-tyrosine to L-DOPA, although the procedure requires sophisticated control measures to prevent further oxidation of L-DOPA by the enzyme to L-2, 3, 4-trihydroxy phenylalanine. Lee and Xun (1998) reported obtaining relatively high levels of L-DOPA by incubating *E. coli* cells of strain W (ATCC 11105) suspended in Luria-Bertani (LB) medium with 1 mM L-tyrosine for 2 h and the supernatant were analysed by HPLC. The compound was eluted at 3.5 min and had a peak maximum of 280 nm which is a typical of authentic L-DOPA. The *E. coli* strain W has a broad substrate range enzyme p-hydroxyphenylacetate 3-hydroxylase which only recognises a hydroxyl group attached to an aromatic ring

and it catalyses its further hydroxylation, hence it is capable of bioconverting L-tyrosine to L-DOPA and the energy for the process is derived from oxidation of NADH. Glycerol was thus added to the incubation medium to maintain the reducing potential inside the cells so that NADH could be regenerated (Lee and Xun, 1998). On an industrial perspective using plant enzymes in bioconversion to produce pharmaceuticals, Pras et al. (1995) reported that phenoloxidase isolated from cell cultures of *M. pruriens* can be used to produce a new pharmaceutical, the dopaminergic agent 7, 8 dihydroxy N-di-n-propyl 2-aminotetralin (7, 8-$(OH)_2$DPAT) by regiospecific hydroxylation of the synthetically prepared precursor 7-hydroxy N-di-n-propyl 2-aminotetralin (7-OH DPAT).

Plants have the potential to of being a major source for novel enzymes, pharmaceuticals or chemical models for drugs in the coming centuries because the chemistry of the majority of plants is yet to be characterised (Cox and Balick, 1994).

5.1.3: Aims and objectives of Chapter 5.

The aim of this chapter was to express the DOPA/tyrosine decarboxylase fusion protein (Mp-TY/DDC) using bacterial cells. This involved isolation and sequencing the putative full-length Mp-ty/ddc gene and then cloning the gene in a protein expression vector (pET21b) using bacterial cells. Further aims were to establish the optimal conditions for inducing expression of the gene product and to determine its

enzyme properties. This involved establishing the specific substrate(s) requirements for enzyme action and the subsequent product(s) formed.

5.2: MATERIALS AND METHODS.

5.2.1: DOPA/tyrosine decarboxylase expression in *E. coli.*

5.2.1.1: Cloning of Mp-ty/ddc into an expression vector.

To express the recombinant DOPA/tyrosine decarboxylase *in vitro* required; the Mp-ty/ddc which encodes for the recombinant protein to be cloned on a protein expression vector in a bacterial cells as described below. Putative full length ORF of Mp-ty/ddc gene (1.45 kb) was PCR amplified from a pZERO-T plasmid carrying an 1.727 kb Mp-ty/ddc (See Chapter 4 Section 4.3.5) using primers; $F_{(PET\text{-}DC)}$: 5'-TAGAATTCATTGATTTCCTTGCTG-3' and $R_{(PET\text{-}DC)}$: 5'-ATCTCGAGCCTAACAGAGAATTGG-3' at annealation temperature of 58 ˚C. The primers were designed to incorporate flanking 5' *Eco*RI and *Xho*I restriction endonuclease sites, and which enabled directional cloning of PCR products in the pET21b expression vector. The primers were used to PCR amplify the Mp-ty/ddc gene region between the putative 5' end translation start codon at position 37 bp and position 1.45 kb adjacent to the stop codon as described below. The PCR was performed following the method described in the protocol for Phusion high-fidelity DNA polymerase (NEB, UK). The enzyme performs DNA sequence proof-reading during thermocycling and produces blunt ended PCR products. The reaction mix was setup as follows; Phusion high fidelity buffer (5 X; 10 µl), dNTPs (10 mM; 1 µl), $F_{(PET\text{-}DC)}$ (10 µM; 2.5 µl), $R_{(PET\text{-}DC)}$ (10 µM; 2.5 µl), Template DNA (80 ng), Phusion DNA polymerase (1 U), and the total volume was made up to 50 µl using sterile

distilled water. Thermocycling was then performed using a PTC-100 thermocycler (MJ Research Inc., Waltham, USA) and the amplification was conducted following the thermal profile: Initial denaturation at 98 ˚C for 30 sec; 35 cycles (98 ˚C; 10 sec, 60 ˚C; 30 sec, 72 ˚C; 30 sec) and final extension at 72 ˚C for 10 minutes. Simultaneously, a pZERO-2 plasmid (Invitrogen, USA) restriction digested at the *Eco*RV site to enable blunt end ligation of PCR products. The digestion reaction was set up as follows; pZERO plasmid DNA (1 µg), *Eco*RV (2 units) (NEB), NEB buffer 3 (10 X) and the volume was made up to 25 µl using sterile distilled water. The reaction was incubated at 37 ˚C for 2 hours. The plasmid vector was analysed on ethidium stained agarose gel by electrophoresis as described in Chapter 2 Section 2.2.7 and was then purified from the gel as described in Chapter 2 Section 2.2.9. The 1.45 kb Mp-ty/ddc ORF amplified by phusion PCR was then blunt ligated into the prepared *Eco*RV site of pZERO plasmid vector as follows; pZERO-2 (Invitrogen, USA) (100 ng), T4 ligation buffer (Promega, Madison, WI, USA) (10X; 1 µl) and T4 ligase (Promega, Madison, WI, USA) (1 U). The ligation reaction volume was made up to 10 µl, mixed by vortexing and centrifuged for 30 sec at 8,000 X g using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany). The reaction was then incubated at 4 ˚C overnight. The ligation reaction was then transformed in *Ecoli*DH5α and cloned (See Chapter 2 Section 2.2.14). The plasmid was then purified (See Chapter 2 Section 2.2.16) and sequenced as described in Chapter 2 Section 2.2.19. The plasmid sample confirmed to harbour the Mp-ty/ddc clone was then restriction digested using *Eco*RI and *Xho*I enzymes and consequently released the 1.45 kb Mp-ty/ddc transgene. The transgene was then directionally cloned in the *Eco*RI and *Xho*I restriction sites of a prepared pET21b plasmid as described in Chapter 2 Section 2.2.14 (Figure 5.3). The resulting recombinant plasmid DNA

(pET-Mp-ty/ddc) was then transformed in bacterial strains; BL21(DE3) (Novagen, USA) and Tuner 2 (Novagen, US) as described in Chapter 2 Section 2.2.4 for *in vitro* protein expression analysis as described in Section 5.3.1.2 below. BL21 (DE3) strain was designed for protein overexpression and is especially useful for proteins produced at very low concentrations (BL21 user manual, Novagen, USA). On the other hand, Tuner 2 strain is a *lac* ZY deletion mutant of BL21 which enable adjustable levels of protein expression throughout all cells in culture (Tuner 2 user manual, Novagen, USA). The *lac* permease (*lac* Y) mutation allows uniform entry of IPTG into cells in the population. This allows induction with IPTG to occur in a true concentration-dependent fashion that is exceptionally uniform throughout the culture.

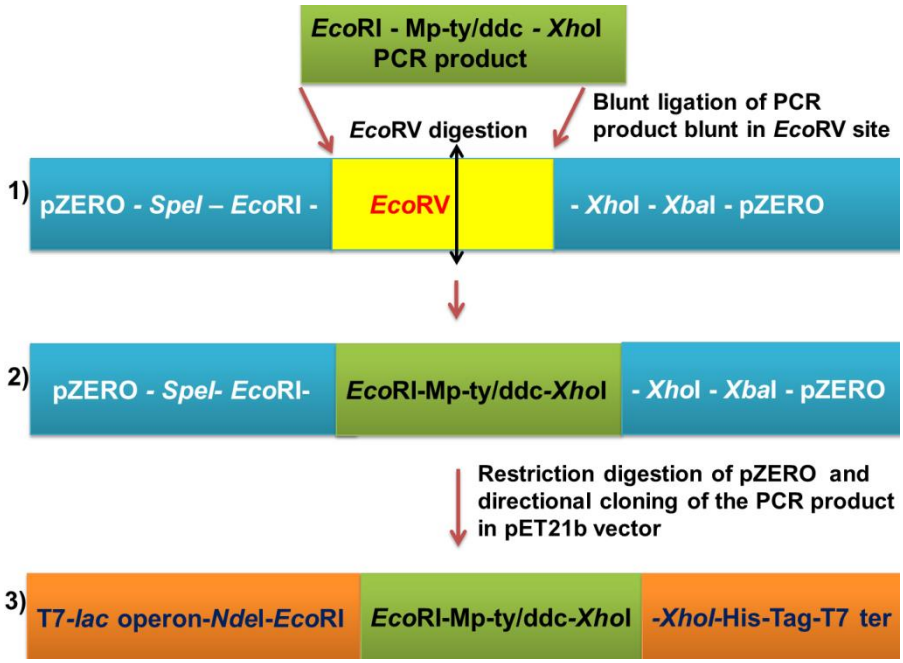


Figure 5.3 A schematic diagram showing the steps involved in building the pET21b-Mp-TY/DDC recombinant expression vector. The 1.45 kb Mp-ty/ddc ORF was cloned in the *Eco*RV site of pZERO plasmid vector (1-2) and the transgene was confirmed by sequencing before it was directionally cloned in a pET21b vector plasmid (3) for protein expression analysis using bacterial cells.

5.2.1.2: Induction of Mp-TY/DDC fusion protein expression in *E. coli.*

The effective IPTG concentration for induction of DOPA/tyrosine decarboxylase fusion protein (Mp-TY/DDC) expression was determined by conducting an assay for IPTG/bacterial growth culture temperature as follows: LB medium (100 ml) supplemented with 50 mg l^{-1} ampicillin was inoculated with an overnight fresh single bacterial colony of BL21 (DE3) (Novagen) or Tuner 2 (Novagen); harbouring a protein expression vector carrying an Mp-ty/ddc cloned gene (pET21b-Mp-ty/ddc). The negative control experiment was set as described above but the BL21 (DE3) or Tuner 2 cells harboured a pET21b plasmid without a transgene clone. The bacterial cultures were grown at temperature assays of; 18, 22, 25, 30 and 35 ˚C with shaking at 225 rpm in a rotary shaker (Gallenkamp cooled orbital incubator, UK) until the $O.D_{600nm}$ was 0.6 measured using a UV/VIS Spectrophotometer model Sp8-400, (Pye Unicam, UK). IPTG assay of; 0.1, 0.2, 0.3, 0.4 and 0.5 mM was added to the bacterial cultures in order to determine the effective concentration required for induction of the recombinant protein expression. The bacterial cultures were further incubated at the respective temperature assays (18 – 35 ˚C) with shaking at 225 rpm in a rotary shaker (Gallenkamp cooled orbital incubator, UK). During the bacterial growth, 1 ml samples were corrected at hourly intervals for 18 h. The samples were then centrifuged at 8,000 xg using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany) for 3 minutes and pellets kept at -80 ˚C prior cell lysis. The bacterial pellet were resuspended in a buffer; Bis-Tris (Sigma) (50 mM; 200 µl; pH 7) and EDTA (1 mM) was added to stop protease activity. The protein concentration of the samples was determined by conducting a Bradford assay as described in Section 5.2.1.3 below. An equal volume of 2 X protein loading dye (4% SDS, 20%

glycerol, 10% β-mercaptoethanol, 0.004% Bromophenol blue and 0.125 M Tris-HCl; pH 6.8) was added and the mixture was boiled for 6 minutes. The samples were then briefly centrifuged for 30 seconds to minimise the condensation effect. Recombinant protein expression was determined by analysing 20 ng of total extract protein by SDS-page gel electrophoresis described in Chapter 2 Section 2.2.20.

5.2.1.3: Bradford – BSA Protein assay.

The protein concentration of the protein produced by the bacterial cultures was determined by following the method described in the Bradford BSA microassay protocol (Bio-Rad, USA) as follows: BSA (10 mg ml^{-1}) was used to prepared standard BSA protein samples at concentrations (mg ml^{-1}) of; 2, 1.5, 1.0, 0.75, 0.25 0.125 and 0. The standard BSA (10 µl), blank (experimental control) (10 µl) and protein extract samples (10 µl) of unknown concentration were added to separate 2 ml cuvettes containing mixture of double distilled water (800 µl) and Bradford reagent (200 µl). The samples were incubated for 5 minutes at room temperature (22˚C) before the respective absorbance ($O.D_{595\ nm}$) for each was measured using a UV/VIS Spectrophotometer model Sp8-400 (Pye Unicam, UK). A standard curve of $O.D_{595}$ nm values (Y-axis) against protein concentration in µg ml^{-1} (X-axis) was plotted. The concentrations for the protein extract samples were determined using the standard curve. In cases where the samples were diluted, the protein concentrations determined from the standard curve was adjusted by multiplying the dilution factor used.

5.2.1.4: Expression and detection of recombinant protein in the soluble and insoluble fractions of bacterial host cells.

This experiment was to determine whether recombinant protein (Mp-TY/DDC) was expressed in the cytosol (as soluble protein fraction) or in globular membrane bodies (as insoluble protein fraction) of the bacterial host cells. The experiment was performed by following in the method by Sambrook et al., (1989) with modifications as described below.

A single bacterial colony of BL21 (DE3) or Tuner 2 bacterial cells harbouring a pET21b-Mp-ty/ddc protein expression plasmid (or pET21b plasmid for the experimental control) was inoculated on 5 ml LB media supplemented with 50 mg l^{-1} Ampicillin and grown overnight at 22 ˚C in a rotary shaker at 225 rpm. The overnight culture was then inoculated on 100 ml LB media supplemented with 50 mg l^{-1} Ampicillin and grown at 22 ˚C in a rotary shaker at 225 rpm up to $O.D_{600}$ nm of 0.6. The bacterial culture was then supplemented with 0.4 mM IPTG to induce expression of the Mp-TY/DDC recombinant protein and was then allowed to continue growing at 22 ˚C with shaking at 225 rpm as described above. Samples (50 ml) were collected after 1 and 2 hours and were centrifuged at 3,000 xg using a centrifuge (Beckman Coulter Avanti J-E, USA) at 4 ˚C for 10 minutes to remove the medium. The bacterial pellets were resuspended in Bis-Tris buffer (Sigma) (50 mM; 5 ml; pH 7.2) and EDTA (1 mM) and were then lysed using lysozyme (Sigma) (2 mg ml^{-1}) which was added and incubated with the mixture for 20 minutes on ice. The bacterial cells were further lysed by sonication at 10,000 Amps using a Sonicator (Model-XL 2020, Pharmacia Biotech, Sweden) for 8 seconds at a time followed by cooling on ice and the process was continued until the bacterial mixture formed a

clear solution. The bacterial solution was then centrifuged at 8,000 xg using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany) for 10 minutes. The supernatant (soluble protein fraction) was put in a new tube whereas the pellet (insoluble protein fraction) was resuspended in Bis-Tris buffer (Sigma) (50 mM; 5 ml; pH 7.2) and EDTA (1 mM). The absorbance for protein samples was taken at $0.D_{595}$ nm using a UV/VIS Spectrophotometer model Sp8-400, (Pye Unicam, UK). The protein samples (10 µg) were then analysed by SDS page gel electrophoresis as described in Chapter 2 Section 2.2.20 to determine whether the recombinant Mp-TY/DDC was expressed in the soluble or insoluble protein fraction. This experiment provides vital data because enzymatically active proteins are reported to be in the cytosol (soluble protein fraction) (Sambrook et al., 1989). However, this experimental method could not adequately detect fusion proteins expressed at very low concentrations. In light of the above, a Western blot analysis was performed to investigate the possibility of Mp-TY/DDC fusion expression in the soluble fraction at a very low concentration as described in Section 5.2.1.5 below.

5.2.1.5: Western blot analysis of Mp-TY/DDC fusion protein expression.

Western blot (Immunoblot) analysis refers to size-based separation of proteins by SDS gel electrophoresis followed by transfer onto a membrane and detection of specific proteins using an antibody or a protein stain (Sambrook et al., 1989; Dale and von Schantz, 2002). Immunoblot detection by antibodies is very sensitive and hence was used to detect the very low concentrations of Mp-TY/DDC fusion protein expressed in the soluble fraction of protein extracts from transformed bacteria cells as described below.

Bacterial cultures (50 ml) were grown and induced as described in Section 5.2.1.4 and the cells were pelleted at 3,000 X g for 10 minutes using a 4 ˚C centrifuge (Beckman Coulter Avanti Model J.E, USA). The bacterial pellet was then resuspended in 5 ml of lysis buffer; Bis-Tris (50 mM; pH7) and EDTA (1 mM) and were lysed using lysozyme (1 mg l^{-1}) for 20 minutes on ice. The cells were further lysed by sonication as described in Section 5.2.1.4. The lysate was spun at 8,000 X g for 5 minutes using a 4 ˚C centrifuge (Beckman Coulter Avanti Model J.E, USA) and the supernatant (soluble fraction) was collected. The protein concentration was determined from the standard graph of BSA as described in Section 5.2.1.3. Protein samples (20 µg) was analysed by SDS page as described in Chapter 2 Section 2.2.20. The protein samples were then transferred onto a hybond membrane (Amersham, USA) for probing with an antibody as described below. A western blot cassette (Bio-Rad, USA) was placed with the gray side down, on a lean surface. A fiber pad (Bio-Rad) of same size as the gel was pre-wetted in protein transfer buffer (10% Methanol; 24 mM Tris; 194 mM Glycine) was placed on the gray side of Western blot cassette (Bio-Rad, USA). A sheet of Whatman filter paper (3 mm) of the same size as the gel and pre-soaked in transfer buffer was placed on the fiber pad. Then the SDS page gel pre-washed in transfer buffer was placed on the filter paper and on top of it a pre-wetted hybond membrane (Amersham, USA) was carefully placed. The gel sandwich was completed by placing a piece of filter paper on the membrane. Care was taken to remove air bubbles. The last fiber pad was then placed on top the filter paper. The Western blot cassette was then closed firmly using latch. The cassette was placed in the electrophoretic tank together with a frozen bio-ice cooling unit. The tank was completely filled with the protein transfer buffer. A magnetic stirrer was added to maintain uniform buffer temperature and ion

distribution in the tank. The protein transfer was performed at 100 V; 350 mA for 1 hour after which the blot sandwich was unclamped and the side facing the membrane blot was marked. The target proteins were detected using their respective specific antibodies as described below. The membrane was blocked from non-specific binding by antibodies by immersing it in Tris-saline base buffer (TBS) (150 mM NaCl; 10 mM Tris, pH 8), supplemented with 5% non-fat dry milk powder and 0.05% Tween-20 and overnight at 4 ˚C with gentle shaking. The membrane blot was then washed in TBS supplemented with 0.05% Tween-20 (TBS-T) for 10 minutes, three times. The membrane blot was then incubated with a primary antibody; His-Tag antibody, mAb, Mouse (0.2 µg ml^{-1}) (GenScript, USA) in TBS-T supplemented with 1% BSA for 1 hour at room temperature. Consequently the membrane blot was washed in TBS-T supplemented with 0.05% Tween-20 for 10 minutes, three times before incubation with a secondary antibody; Alkaline phosphatase-conjugated goat anti-mouse IgG (Promega) (0.2 µg ml^{-1}) for 1 hour at room temperature. The membrane was again washed in TBS-T for 10 minutes, three times before it was developed as described in the method for alkaline phosphate detection as follows. NBT: [70% (v/v) Dimethylformamide; 66 µl], BCIP [100% (v/v) Dimethylformamide; 33 µl], was added to alkaline phosphatase (AP) detection buffer: [NaCl (100 mM); $MgCl_2$ (5 mM); Tris (100 mM); pH 9.5; 9.9 ml]. The resulting solution was poured over the membrane blot placed in a shallow basin and was incubated for 30 seconds to allow the secondary antibody to bind to the primary antibody. The reaction was stopped by rinsing the membrane blot in water or EDTA (0.5 mM) and the membrane blot was scanned.

5.2.1.6: *In vivo* Mp-TY/DDC enzyme activity assay.

In vivo Mp-TY/DDC enzyme activity assay was performed to investigate the putative enzymatic properties for the Mp-TY/DDC fusion protein extract *in vivo.* This involved extraction of the soluble protein fraction from transformed bacterial cells expressing the Mp-TY/DDC fusion protein as described in Section 5.2.1.4. The Mp-TY/DDC fusion protein was then probed for enzymatic activity by treatment with putative substrates; L-DOPA and L-tyrosine and other related compounds such as dopamine. The experiment was performed as described in the method by Facchini and De Luca, (1994) with modifications as follows. A single colony of the transformed bacteria; BL21(DE3) and Tuner 2 (Novagen, USA), harbouring protein expression plasmid (pET21b-Mp-TY/DDC), which carries an Mp-ty/ddc transgene insert were separately inoculated on 200 ml LB medium supplemented with Ampicillin (50 mg/l). A control experiment was set up as described above but the bacteria harboured a pET21b plasmid which had no transgene clone. The bacteria cultures were grown at 22 ˚C with shaking at 225 rpm in a rotary shaker (Model G25, New Brunswick Scientific co.inc, USA) until the $O.D_{600}$ was 0.6. Expression of the Mp-TY/DDC fusion protein was induced by addition of IPTG to a final concentration of 0.4 mM. After 1 and 2 hours of induction by IPTG, 100 ml culture media samples were taken and the cells collected using a 4 ˚C centrifuge (Beckman Coulter Avant Model J.E, USA) at 3,000 X g for 5 minutes. The bacterial cells were resuspended in Bis-Tris (200 mM; 5 ml, pH 7.2) and lysed by sonication (Model-XL 2020, Pharmacia Biotech, Sweden) for 4 - 8 seconds at a time followed by cooling on ice. This process was continued until the bacterial mixture formed a clear solution. The cell debris was removed by centrifugation at 8,000 X g for 5 minutes at 4 ˚C. The assay mixture for decarboxylase activity contained Bis-Tris (50 mM; pH 7.2), EDTA (1 mM), PLP (25

µM), an assay of either cold L-DOPA, tyrosine or dopamine (0.25, 0.5, 0.75 and 1 mM), and fusion protein extract (250 and 500 µl) in a total volume of 1 ml. All reactions were incubated for 1 hour at 35 ˚C with constant agitation at 100 rpm. The reactions were stopped by addition of 0.2 M formic acid and agitated for an additional 1 hr. The supernatant was collected using microcentrifuge (Heraeus Instruments Biofuge 13, Germany) at 8,000 X g for 3 minutes at room temperature. Triplicate samples (1 ml) of the supernatant were analysed by Reverse phase-HPLC (Thermo Separation products, USA) to determine the bioconversion products as described Chapter 3 Section 3.3.3.2. The enzyme assays thus served to determine whether the Mp-TY/DDC fusion protein extract exhibited the expected decarboxylase activity *in vivo.* Besides the *in vivo* enzyme assay, it was also prudent to determine whether Mp-TY/DDC fusion protein exhibited decarboxylase activity *in situ* using bacterial host cells as described in Section 5.2.1.7 below.

5.2.1.7: Precursor additions to bacteria cell cultures.

To determine whether Mp-TY/DDC fusion protein exhibited decarboxylase activity *in situ*, transformed bacteria culture were precursor-fed and then the bioconversion products were analysed by RP-HPLC as described below. Bacterial cultures were grown and induced as described in Section 5.2.1.6. After 1 and 2 hours of induction with IPTG, the bacterial cells were pelleted by centrifugation at 3,000 xg for 5 minutes using a 4 ˚C centrifuge (Beckman Coulter Avanti model J.E, USA). The bacterial cells were then resuspended in Bis-Tris (50 mM; pH 7.2), EDTA (1 mM) and exogenous L-DOPA (1 mM) or L-tyrosine (1 mM) or dopamine (1 mM) were added to the respective assays. The energy required for Mp-TY/DDC enzymatic

hydroxylation of L-tyrosine to L-DOPA in bacterial cells is derived from oxidation of NADH (Lee and Xun, 1998). Glycerol (5%) was thus added to the L-tyrosine-fed bacterial culture to maintain the reducing potential inside the cells to regenerate NADH (Lee and Xun, 1998). Cultures were grown at 22 ˚C with shaking at 225 rpm for 2, 4, 6 and 8 hour in the presence of exogenous substrates. Subsequently, medium samples were collected after removal of cells by 4 ˚C centrifugation (Beckman Coulter Avanti Model J.E, USA) at 3,000 xg for 10 minutes. Cell pellets and medium samples were frozen separately at -80 ˚C until analysed. The medium sample (1 ml) was analysed by Reverse phase-HPLC (Thermo Separation products, USA) as described Chapter 3 Section 3.3.3.2 to determine the bioconversion products. *In situ* decarboxylase activity by Mp-TY/DDC fusion protein in bacterial host cells was confirmed in cases when bioconversion of L-DOPA to dopamine or L-tyrosine to tyramine occurred (Facchini, 2001).

5.3: RESULTS.

5.3.1: Designing and building the Mp-TY/DDC-pET21b expression construct.

A 1.45 kb PCR product of Mp-ty/ddc ORF (Figure 5.4) was successfully isolated from pZERO-T carrying 1.727 kb Mp-ty/ddc putative full-length sequence using Mp-ty/ddc gene specific primers $F_{(PET\text{-}DC)}$ and $R_{(PET\text{-}DC)}$ (See Section 5.2.1.1). The 1.727 kb Mp-ty/ddc full-length sequence data was prior to this experiment confirmed as described in Chapter 4 Section 4.3.5.

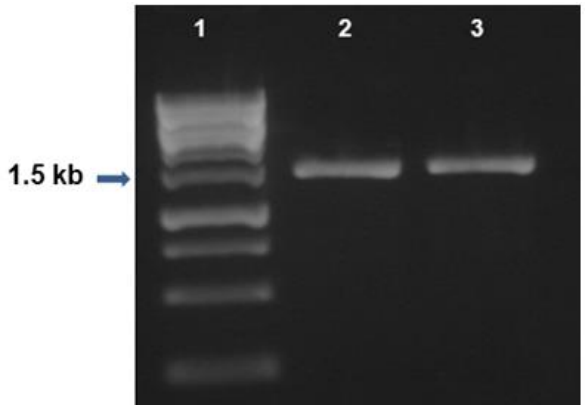
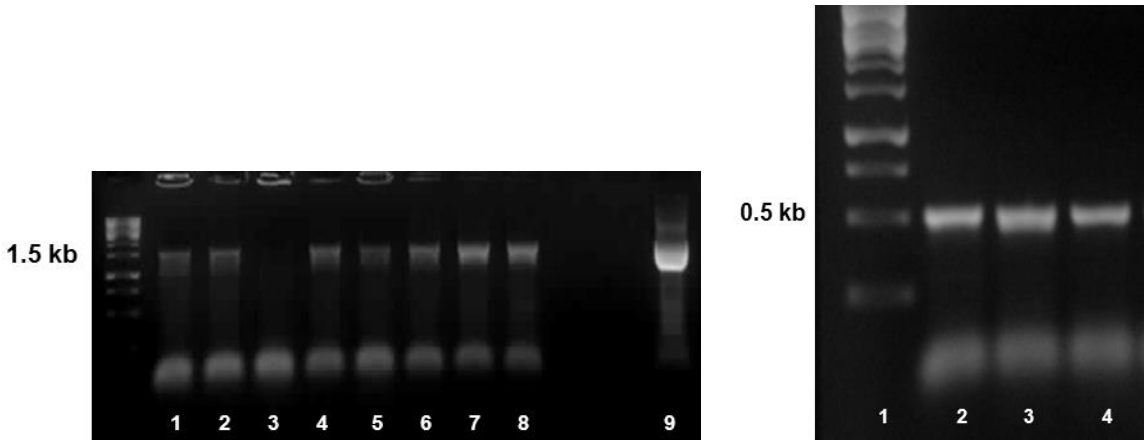


Figure 5.4 Shows 1.45 kb PCR products of Mp-ty/ddc ORF isolated from pZERO-T carrying 1.727 kb putative length of Mp-ty/ddc.

Initially the PCR product was blunt ligated into the *Eco*RV site of pZERO vector. Following transformation into *E. coli* DH5α cells, 7 positive colonies were identified by colony PCR (Figure 5.5).



(a) (b)

Figure 5.5 Colony PCR products of Mp-ty/ddc in were amplified using the (a) T7 and Sp6 primers (lanes 1, 2, 4 - 8). (b) Mp-ty/ddc gene specific primers (5'-ctctggtgagggtggtgg-3' and 5'-gtaagctgcatctacgtgga-3') (lanes 2-4). The positive control colony PCR was performed a pZERO-T-Mp-ty/ddc plasmid template using T7 and Sp6 primers (lane 9).

Plasmid DNA was extracted from the positive clones and the insert size was further confirmed by *Eco*RI and *Xho*I restriction digestion which released a 1.45 kb fragment (Figure 5.6).

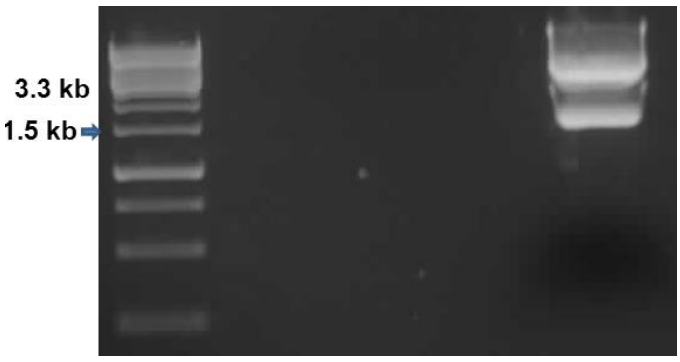
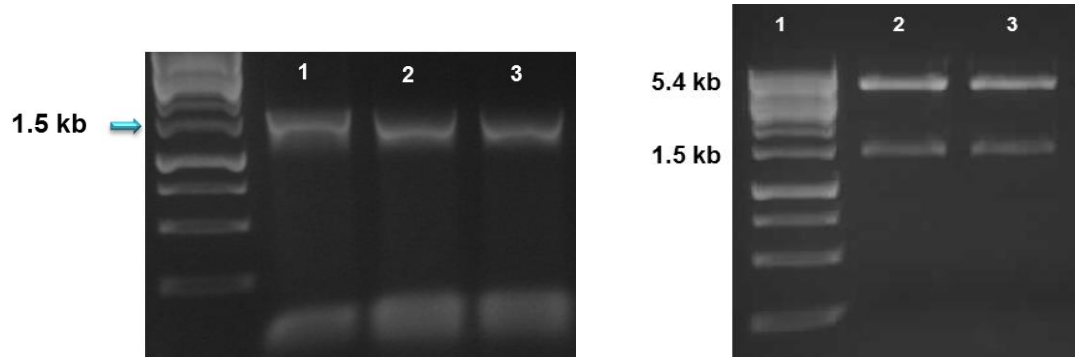


Figure 5.6 Shows 1.5 kb Mp-ty/ddc fragment released from pZERO-Mp-ty/ddc by *Eco*RI and *Xho*I.

The restriction fragment was then directional cloned in *Eco*RI and *Xho*I sites of the protein overexexpression vectors (pET21b) using BL21(DE3) (Novagen, USA) and Tuner 2 (Novagen, USA). Colony PCR products (1.5 kb) was obtained (see Figure 5.7) upon using gene specific primers; 5'-TAGAATTCATTGATTTCCTTGCTG-3' and 5'-ATCTCGAGCCTAACAGAGAATTGG-3' at annealation temperature of 58 ˚C.



(a) (b)

Figure 5.7 Shows (a) Colony PCR products (1.5 kb) amplified from *E.coli* strain BL21(DE3) transformed with the pET21b-Mp-tyddc plasmid (lanes 1-3). (b) Shows 1.45 kb (~1.5 kb) of the cloned Mp-ty/ddc gene recovered from the 5.4 kb pET21b plasmid by *Eco*RI and *Xho*I restriction digestion.

Sequencing confirmed in frame fusion of the 1.45 kb Mp-ty/ddc ORF into the pET21b protein expression vector. The translation start codon on the pET21b vector was in frame with the N-terminus for the Mp-TY/DDC sequence (Figure 5.8 a). Like wise the C-terminal for the Mp-TY/DDC sequence was in frame with the His-tag domain and the translation stop codon on the pET21b vector (Figure 5.8 b). The expressed protein was therefore a fusion protein produced using bacterial host cells; BL21(DE3) and Tuner 2 (Novagen,USA) as described in Section 5.2.1.4.

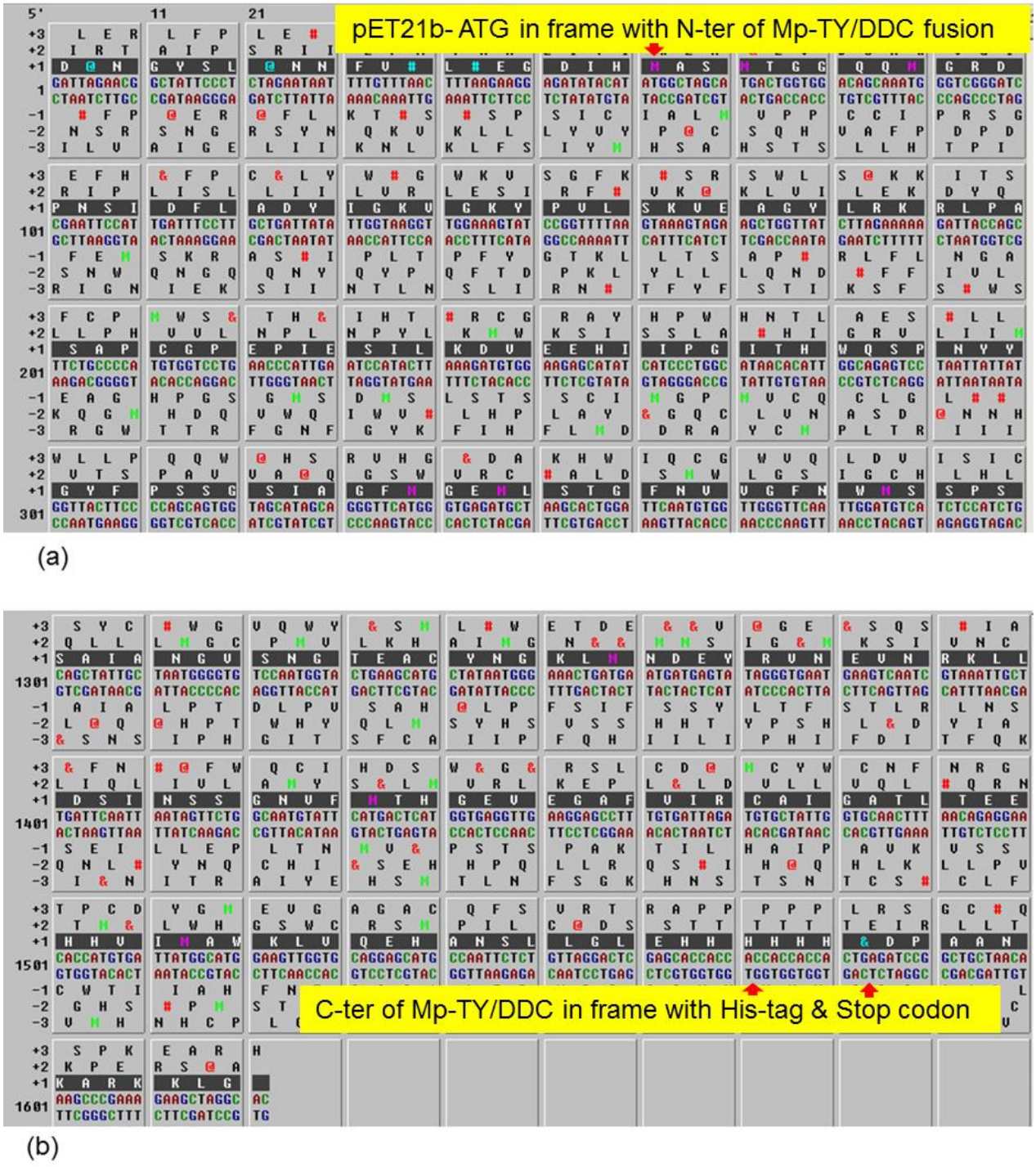


Figure 5.8 Protein sequence shows (a) the N-terminal methionine on the pET21b vector is in frame with the N-terminus for the Mp-TY/DDC sequence. (b) The C-terminal sequence for Mp-TY/DDC is in frame with the His-tag domain and the translation stop codon on the pET21b expression vector.

Analysis of the Mp-TY/DDC fusion protein sequence on the Protein data bank (PDB) (http://www.rcsb.org/pdb/home/home.do) drew very significant alignments in 13

unique sequences of dopa decarboxylase. In addition, despite the lack of Mp-TY/DDC data on the PDB, a crystal structure analysis of Mp-TY/DDC sequence on PDB drew significant similarity with the human (*Homo sapiens*) aromatic dopa decarboxylase (Structure reference: 3RBF on PDB) (Figure 5.9). Consequently analysis of the Mp-TY/DDC protein sequence using BLAST on the NCBI data bank, drew 41% homology with the human (Homo sapiens) aromatic dopa decarboxylase.

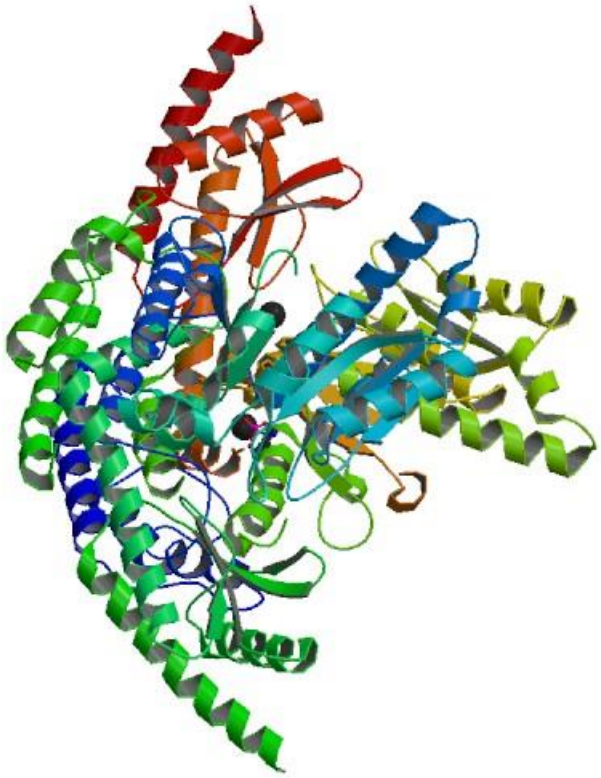
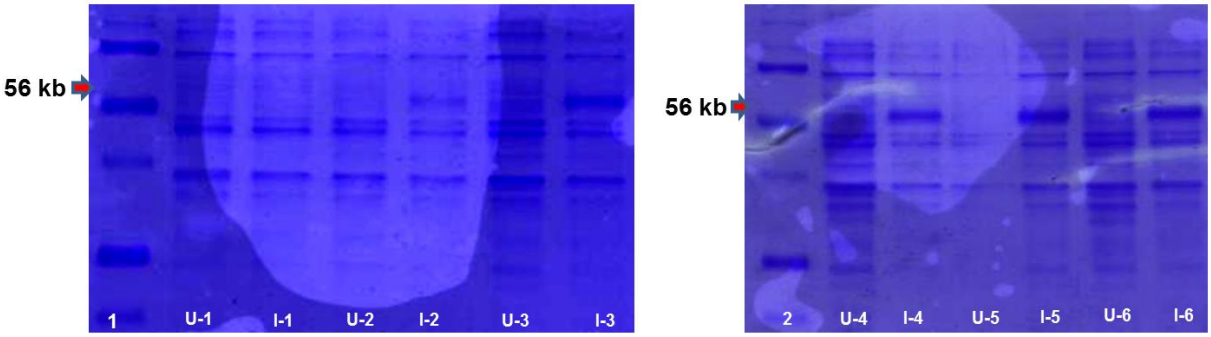


Figure 5.9 Crystal structure of Human aromatic L-amino acid decarboxylase (AADC) in apo form. The AADC showed very significant alignments with the 13 Mp-TY/DDC conserved entities with the PDB (Structure ref:3RBF).

5.3.2: DOPA/tyrosine IPTG induction assays.

The next step was to induce expression of the Mp-TY/DDC to assess its enzyme activity. As described in Section 5.2.1.2 transgenic protein expression was induced using IPTG and proteins were visualised on Coomassie stained SDS-PAGE gels. In an initial experiment cultures were grown at 22 ˚C, induced with 0.5 mM IPTG and sampled over an 18 h period. After 3 h of induction a band of 56 kD corresponding to the expected size of the Mp-TY/DDC protein was clearly seen in the insoluble fraction of induced but not uninduced cultures (Figure 5.10 a). Protein levels increased up to 5 h after induction but no further induction was seen after 5 hours (Figure 5.10 b).



(a) (b)

Figure 5.10 A band of recombinant Mp-TY/DDC (56 kd) was clearly visible in the insoluble protein fraction expressed after 3 hours of induction with 0.5 mM IPTG, at 22 ˚C in *E. coli* BL21 (DE3). Induction was performed for 18 h and the samples for analysis were taken at 1 h (U-1, I-1), 2 h (U - 2, I - 2), 3 h (U - 3, I - 3), 4 h (U - 4, I - 4), 5 h (U - 5, I - 5), where U represents “un induced” while I is for Induced samples. (Gels after 7 - 18 h of induction were not shown here).

The results showed that induction conditions for expression of the Mp-TY/DDC fusion protein in the insoluble protein fraction was achieved by: Culturing the

BL21(DE3) bacterial cells in LB media at 22 ˚C with shaking at 225 rpm up to - O.D_{600} of 0.6, followed by induction using 0.5 mM IPTG for 3 - 5 hours. However, for the expressed protein to be enzymatically active, it should be expressed in the soluble protein fraction found in the cytosol (Sambrook et al., 1989). The next step therefore was to determine the conditions for Mp-TY/DDC fusion protein expression in the soluble fraction as described in Section 5.3.3 below.

5.3.3: Expression and detection of Mp-TY/DDC fusion protein in the soluble fraction (Cytosol).

Putative DOPA/tyrosine decarboxylase was isolated from *M. pruriens* as described in Section 5.2.1.1, cloned and expressed in pET21b plasmid using bacterial cultures of BL21(DE3) and Tuner 2 (Novagen, USA) as described in Section 5.2.1.2. However, Sambrook et al., (1989) reported that functional fusion proteins are expressed in the soluble fraction (cytosol) and that IPTG concentration and culture temperature influence nature of the fusion protein. Consequently, an IPTG assay (0.1 - 0.5 mM) was performed at 18 ˚C, 22 ˚C, 25 ˚C, 30 ˚C and 35 ˚C to determine the concentration which could influence expression of the Mp-TY/DDC in the soluble fraction. The results obtained from analysis by SDS page gel electrophoresis showed no expression of the 56 kd Mp-TY/DDC protein fusion in the soluble fraction for all IPTG assay concentrations at temperatures; 18 ˚C, 25 ˚C, 30 ˚C and 35 ˚C. However, the 56 kd Mp-TY/DDC fusion protein was expressed in the control experiment of insoluble fraction at IPTG concentrations of 0.3 - 0.5 mM (Figure 5.11).

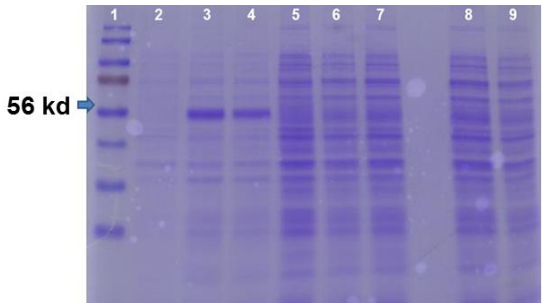


Figure 5.11 SDS page gel shows the 56 kd Mp-TY/DDC fusion protein expression in the IPTG induction assay at 35 ˚C using *E.coli* BL21 (DE3) culture. Lane 1 is the protein size marker (Bio-Rad), lane 2 (un induced insoluble fraction), lane 3 (insoluble fraction induced by 0.4 mM IPTG), lane 4 (insoluble fraction induced by 0.3 mM IPTG), lane 5 (un induced soluble fraction), lane 6 (soluble fraction induced by 0.3 mM IPTG for 1 h), lane 7 (soluble fraction induced by 0.3 mM IPTG for 2 h), lane 8 (soluble fraction induced by 4 mM IPTG for 1 h), lane 9 (un induced soluble fraction).

On the other hand, a very faint band which corresponds to the predicted molecular weight of Mp-TY/DDC fusion protein seemed to appear in the soluble fractions of the 0.3 mM IPTG assays induced for 1 and 2 hours at 22 ˚C (Figure 5.12; lanes 6 and 7 respectively).

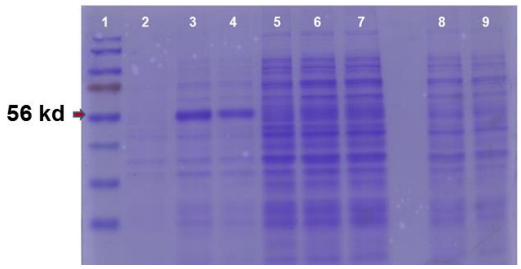


Figure 5.12 SDS page gel shows the 56 kd Mp-TY/DDC fusion protein expression in the IPTG induction assay at 22˚C using *E.coli* BL21 (DE3) culture. Lane 1 is the protein size marker (Bio-Rad), lane 2 (un induced insoluble fraction), lane 3 (56 kd Mp-TY/DDC insoluble fraction induced by 0.4 mM IPTG), lane 4 (56 kd Mp-TY/DDC insoluble fraction induced by 0.3 mM IPTG), lane 5 (un induced soluble fraction), lane 6 - 7 (a faint 56 kd soluble fraction Mp-TY/DDC induced by 0.4 mM IPTG for 1 h and 2 h respectively), lane 8 (soluble fraction induced by 0.3 mM IPTG for 1 h), lane 9 (un induced soluble fraction).

Following lack of substantial detection of Mp-TY/DDC fusion protein expression in the soluble fraction of *E.coli* BL21(DE3) cultures through induction by IPTG assays at different temperatures as described in Section 5.2.1.4. The experiments were repeated as described above but using a different bacterial strain, Tuner 2 (Novagen,USA). The *lac* permease (*lac* Y) mutation characteristic of Tuner 2 (Novagen, USA) bacterial strain made its membranes to be uniformly permeable to IPTG and hence more responsive to varying IPTG assay concentrations during induction of fusion protein expression experiments (Tuner 2 user manual, Novagen, USA). The Tuner 2 bacteria harbouring a pET21b-Mp-TY/DDC plasmid however did not express explicitly the 56 kd fusion protein in the soluble fraction of all cultures in the IPTG assay based on analysis by SDS page electrophoresis (see Chapter 2 Section 2.2.20). In addition, the Mp-TY/DDC fusion protein expression in the insoluble fraction of Tuner 2 cultures assays was weak when compared to that obtained from coressponding BL21(DE3) cultures based on analysis by SDS page electrophoresis (Figure 5.13).

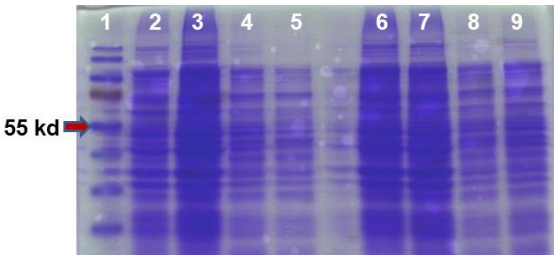


Figure 5.13 SDS page gel shows the 56 kd Mp-TY/DDC fusion protein expression in the IPTG induction assay at 22 ˚C using Tuner 2 bacterial culture. Lane 1 is the protein size marker (Bio-Rad), lane 2 (insolube fraction induced by 0.3 mM IPTG), lane 0.3 (un induced insoluble fraction), lane 4 (soluble fraction induced by 0.3 mM IPTG for 2 h), lane 5 (un induced soluble fraction), lane 6 - 7 (insoluble fraction induced by 0.3 mM IPTG for 1 and 2h respectively), lane 8 (un induced soluble fraction), lane 9 (soluble fraction induced by 0.3 mM IPTG for 2 h).

Similarly induction by an IPTG assay at temperatures of 25 ˚C, 30 ˚C and 35 ˚C did explicitly result in Mp-TY/DDC fusion protein expression in the soluble fraction based on analysis by SDS page gel electrophoresis (Figure 5.14).

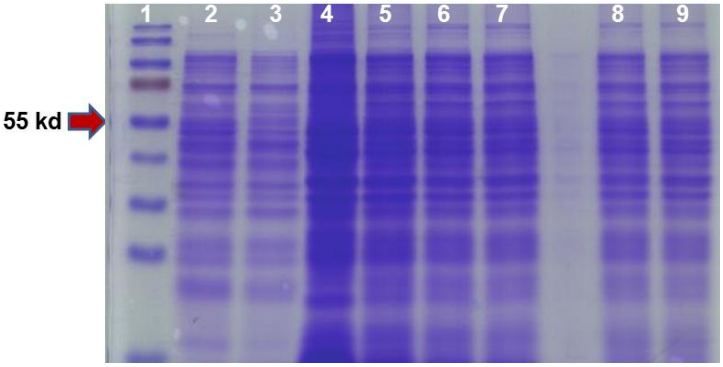


Figure 5.14 SDS page gel shows the 56 kd Mp-TY/DDC fusion protein expression in the IPTG induction assay at 35 ˚C using Tuner 2 bacterial culture. Lane 1 is the protein size marker (Bio-Rad), lane 2 (56 kd Mp-TY/DDC induced by 0.3 mM IPTG in insolube fraction), lane 3 (insoluble fraction induced by 0.4 mM IPTG), lane 4 (un induced insoluble fraction), lane 5 (insoluble fraction induced by 4 mM IPTG), lane 6 (soluble fraction induced by 0.4 mM IPTG for 2 h), lane 7 (soluble fraction induced by 0.4 mM IPTG for 2 h), lane 8 (un induced soluble fraction), lane 9 (soluble fraction induced by 0.3 mM IPTG for 1 h).

In all, the IPTG assays revealed expression of a 56 kd protein band which corresponds to the predicted molecular weight of Mp-TY/DDC fusion protein. The 56 kd putative Mp-TY/DDC fusion protein band was observed slightly more clearly in the soluble fractions of BL21(DE3) cultures induced with 0.4 mM IPTG for 2 h at 22 ˚C (Figure 5.11). In order to explicitly demonstrate expression of the 56 kd Mp-TY/DDC fusion protein, a Western blot analysis which enables amplification of expression signals of lowly expressed proteins was performed as described in Section 5.2.1.5.

5.3.3.1: Detection of Mp-TY/DDC fusion protein in the soluble fraction (Cytosol) by Western blot analysis.

The Western blot analysis was performed to investigate whether the Mp-TY/DDC fusion protein was expressed in the soluble fraction produced by transformed bacterial cells as described in Section 5.2.1.5. Western blot analysis is very sensitive and was performed it because it could detect very low quantities of expressed fusion protein in the soluble fraction of bacterial fusion protein extract unlike SDS page electrophoresis (See Chapter 2 Section 2.2.20). The 56 kd Mp-TY/DDC fusion protein expression signal was detected in the soluble fraction IPTG induced *E.coli* BL21 (DE3) harbouring a pET21b-Mp-ty/ddc plasmid was detected by Western blot analysis (Figure 5.15). A similar experiment on the control bacterial samples harbouring a pET21b plasmid with no insert did not show the Mp-TY/DDC fusion protein expression. However, no Mp-TY/DDC fusion protein signal could be detected in the soluble fraction of Tuner 2 b harbouring a pET21b-Mp-TY/DDC protein expression plasmid when analysed by a Western blot.

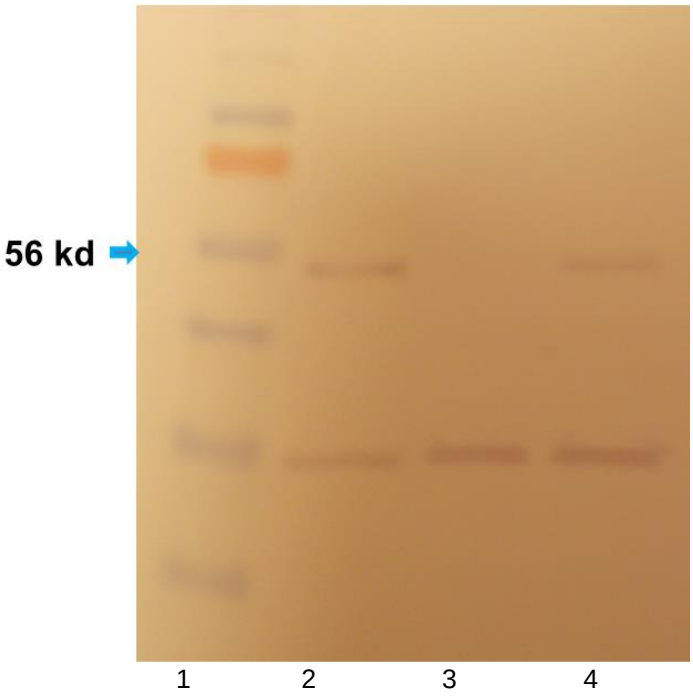
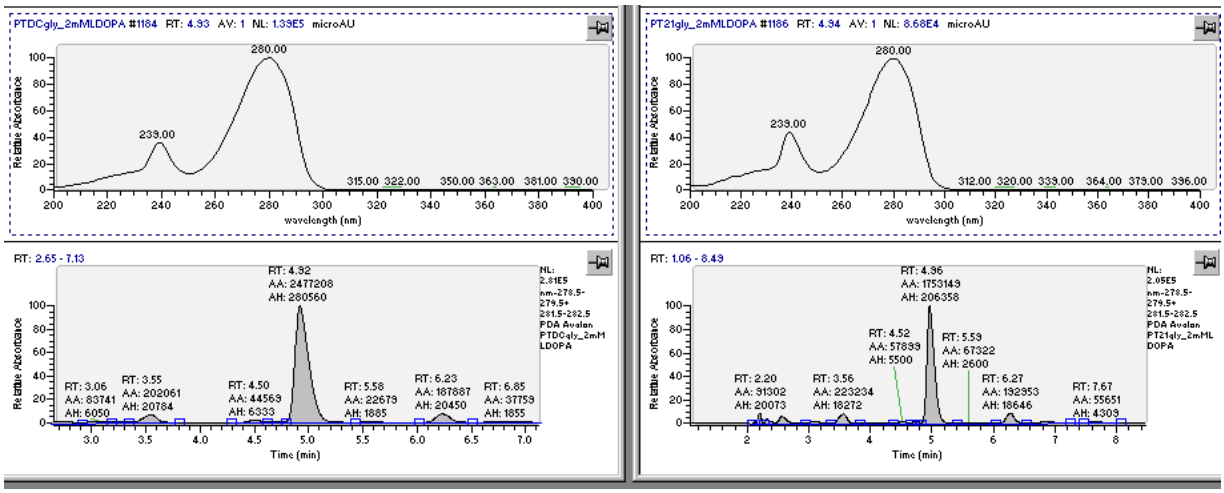


Figure 5.15 Western blot shows 56 kd Mp-TY/DDC fusion protein expression signals Lane 1 is a protein size marker, lane 2 is positive control sample of insoluble protein fraction of induced bacteria. Lane 3 shows no Mp-TY/DDC expression signal in the un induced bacterial cells and lane 4 shows a 56 kd Mp-TY/DDC fusion protein expression in the soluble fraction.

5.3.4: *In vivo* Mp-TY/DDC enzyme activity assay.

The *in vivo* enzymatic potential for the Mp-TY/DDC fusion protein in the soluble fraction of total bacterial protein extract was investigated by incubating the putative enzyme with putative substrates; L-DOPA and tyrosine (See Section 5.2.1.6). The bioconversion products generated by *in vivo* enzyme assays for L-DOPA or tyrosine were analysed by Reverse phase-HPLC as described in Section 5.2.1.6. However, analysis by Reverse phase-HPLC on the assay media for L-DOPA, L-tyrosine or dopamine substrates incubated with *in vivo* Mp-TY/DDC fusion protein revealed no bioconversion products were formed. In addition, substrate concentrations in the IPTG induced *in vivo* enzyme assays were identical to those for un induced assays.

Figure 5.16 shows the chromatogram and concentration of L-DOPA substrate (1 mM) was identical in both IPTG induced and the control experiment un induced *in vivo* enzyme assays before and after the experiment. L-DOPA had a retention time of 4.3 minutes and absorption spectra of 280 nm. Hypothetically, L-DOPA was supposed to be bioconverted to dopamine (absorption spectra, 279 nm) whereas for L-tyrosine assays, tyramine was the anticipated *in vivo* bioconversion product but was also not detected in assays from any of the IPGT induced samples. Similar results were obtained for IPTG induced assays performed at culture temperatures of 22 ˚C, 25 ˚C, 30 ˚C and 35 ˚C.



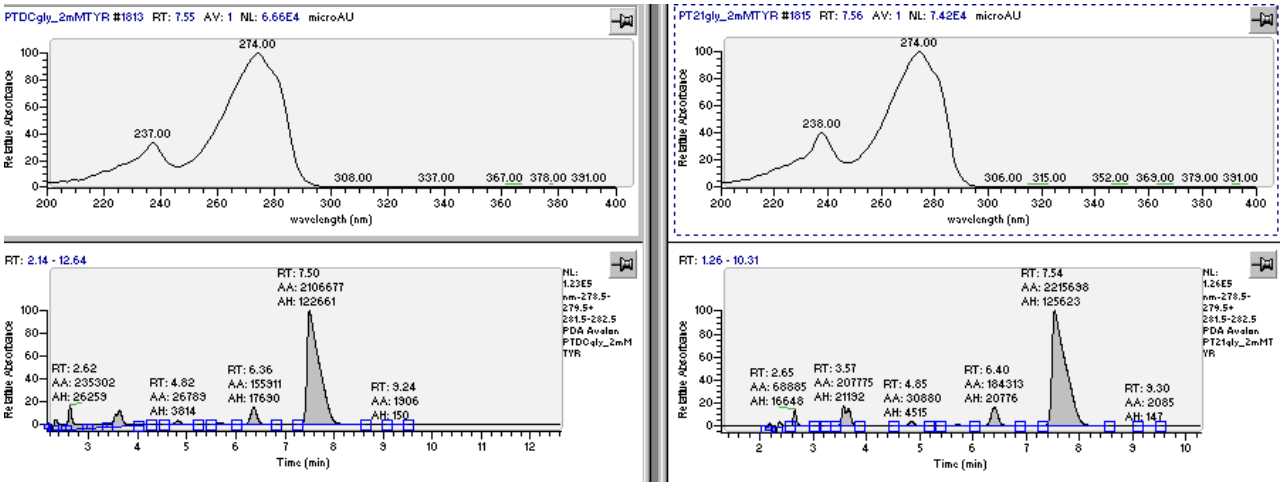
(a) (b)

Figure 5.16 Reverse phase-HPLC chromatograms for L-DOPA (1 mM) substrate for *in vivo* enzyme assays of; (a) IPTG induced (b) un induced samples after the bioconversion experiment. The L-DOPA concentration in IPTG induced and un induced assays were identical after the bioconversion experiment. L-DOPA had a retention time of 4.3 minutes and absorption spectra of 280 nm. The experiment was performed at 22 ˚C.

5.3.5: *In situ* Mp-TY/DDC enzyme activity assay.

5.3.5.1: Precursor additions to bacteria cell cultures.

To test whether the 56 kd Mp-TY/DDC fusion protein visualised on the SDS-PAGE gels was an active DOPA/tyrosine decarboxylase, *in situ* bioconversion assay were performed using transformed bacterial host cells as described in Section 5.2.1.7. The transformed bacterial culture; BL21(DE3) and Tuner 2 (Novagen, USA) harbouring a pET21b-Mp-ty/ddc plasmid, were precursor fed with exogenous putative substrates of L-DOPA (1 mM), L-tyrosine (1 mM) and dopamine (1 mM) in separate assays. However, analysis by RP-HPLC (See Chapter 3 Section 3.3.3.2) of the bacterial culture medium before and after the experiments showed no bioconversion products were produced at the end of the experiment. In addition, the concentration of the precursor fed into the culture medium was identical in the IPTG induced and the control experiment un induced assay samples. Similar results were obtained for assays performed at culture temperatures; 22, 25, 30 and 35 ˚C. Figure 5.17 shows chromatograms of L-tyrosine in culture media of induced and un induced samples was identical after the bioconversion experiment.



(a) (b)

Figure 5.17 Reverse phase-HPLC chromatograms for exogenous L-tyrosine (1 mM) precursor fed to transformed bacterial cultures of; (a) IPTG induced (b) un induced assays. The L-tyrosine substrate concentrations were identical for induced and un induced samples after the bioconversion experiment. L-tyrosine had a retention time of 7.5 min and absorption spectra of 274 nm.

5.4: DISCUSSION.

The aim of this chapter was to express *in vivo* and to characterise the putative DOPA/tyrosine decarboxylase (Mp-TY/DDC) protein from *Mucuna pruriens*. DOPA/tyrosine decarboxylase is one of the aromatic amino acid decarboxylases (AADC) found in plants and animals, and share significant sequence homology but exhibit different substrate specificities (Facchini, 2001). Animal AADC demonstrate preference for L-DOPA but to a relatively low extent also accept a few other substrates such as L-tyrosine, tryptophan derivatives and phenylalanine. On the other hand, plant AADCs exhibit distinct substrate specificity (Facchini and De Luca, 1994). In *Cytisus scoparius* for example, DOPA decarboxylase accepts only L-DOPA as a substrate whilst another AADC (tryptophan decarboxylase) in *C. roseus* exhibits substrate specificity for tryptophan (Facchini and De Luca, 1994). Mp-TY/DDC showed very significant homology with DOPA/tyrosine decarboxylase for *Glycine max* and *Papaver somniferum* (Chapter 4, Figure 4.2). In addition, fractionation analysis by SDS page (See Chapter 2 Section 2.2.20) of heterologously expressed total insoluble protein revealed the Mp-TY/DDC fusion protein had a molecular weight of 56 kd (Figure 5.11). The above preliminary findings suggest the Mp-TY/DDC from *M. pruriens* is a DOPA/tyrosine decarboxylase. However, to draw a conclusive identity of Mp-TY/DDC, the enzyme properties of Mp-TY/DDC were investigated. This involved heterologous expression of Mp-TY/DDC using bacterial cells and determination of the *in vivo* bioconversion potential of the total soluble protein upon treatment with different putative substrates as described in Section 5.2.1.6. However, based on fractionation analysis of the total soluble protein extract by SDS-page electrophoresis (See Chapter 2 Section 2.2.20), the 56 kd Mp-TY/DDC

fusion protein expression band could not be detected or at least clearly (Section 5.3.4). However, a Western blot analysis performed as described in Section 5.2.1.5 revealed expression of the 56 kd Mp-TY/DDC fusion protein in the total soluble protein extract heterologously expressed by bacterial culture (See Section 5.3.3.1). Expression of Mp-TY/DDC in the total soluble protein extract was a pre-requisite for studies on the bioconversion potential of Mp-TY/DDC, because the enzymatically active proteins are expressed in the soluble protein fraction (Sambrook et al., 1989). However, no Mp-TY/DDC enzyme activity was detected upon treatment of the total soluble protein extract neither with L-DOPA, L-tyrosine substrates nor with related compounds such as dopamine. In contrast, DOPA/tyrosine decarboxylases such as from *P. somniferum* which drew homology with Mp-TY/DDC are reported to bioconvert L-DOPA and L-tyrosine to dopamine and tyramine respectively (Facchini and De Luca, 1994; Facchini, 2001). Similarly, bacterial cultures harbouring the pET21b-Mp-ty/ddc plasmid for Mp-TY/DDC expression did not register bioconversion activity when precursor-fed with either L-DOPA or tyrosine. The observation did not conform with reports on bioconversion of L-DOPA and L-tyrosine to dopamine and tyramine respectively using bacterial cultures harbouring a plasmid containing a cloned DOPA/tyrosine decarboxylase gene (Facchini and De Luca, 1994; Lee and Xun, 1998). The above observations raised questions on the identity of the Mp-ty/ddc gene coding for the Mp-TY/DDC protein, despite showing significant homology with plant DOPA/tyrosine decarboxylases. However, the predicted crystal structure of Mp-TY/DDC based on analysis using the PDB is similar to that for human DOPA decarboxylase responsible for conversion of L-DOPA to dopamine (Figure 5.10). The finding strongly supports the view that Mp-TY/DDC is a DOPA/tyrosine decarboxylase based on the conserved 3 - dimensional structure, in

addition to amino acid homology. Another possible reason for Mp-TY/DDC not to show enzyme activity could be born out fact that it was heterologously produced in very small quantities which could not be detected clearly by SDS page protein electrophoresis (See Chapter 2.2.20). However, the Western blot analysis capable of detecting very low concentrations of protein fusion expression in the total soluble protein extract was used to successfully detect the low quantities of Mp-TY/DDC expressed (Sambrook et al., 1989). It is therefore likely that Mp-TY/DDC was heterologously expressed in very low quantities which were below the threshold for a viable enzyme action. Besides the above, it was also observed that putative full-length amino acid sequence of Mp-TY/DDC was approximately 20 amino acids shorter at the N-terminal when compared to similar protein sequences which drew very significant homology (See Chapter 4, Figure 4.2). The observation suggests probably the Mp-TY/DDC sequence is not entirely complete at the N-terminal and possibly this could have an effect on the either the protein folding properties resulting in the observed lack of enzyme activity (Dale and von Schantz, 2002).

In a nutshell the possible reasons for the apparent lack of enzyme activity by Mp-TY/DDC are diverse. However, the strong coherent evidence based on amino acid homology and the predicted crystal structure analysis on PDB suggests

Mp-TY/DDC from *M. pruriens* is likely to be a DOPA/tyrosine decarboxylase.

CHAPTER 6.0: TRANSGENICS.

6.1: INTRODUCTION.

6.1.1: Genetic transformation of legumes.

M. pruriens is a leguminous crop in family Fabaceae. Legumes are known harbour symbiotic *Rhizobium* bacteria which help to improve soil fertility by fixing atmospheric nitrogen into the soil (Bajaj, 1990). In addition, several leguminous plants such as *Phaseolus vulgaris*, *Glycine max, M. pruriens* and *Pisum sativum* are of high economic significance because they produce large quantities of nutritious protein and oil (Cho et al., 1995). In light of the above, there is increasing demand for leguminous crops than can be met by conventional agriculture. This has led to research on developing micropropagation strategies for growing leguminous crops throughout the year and independent of climatic seasons.

Micropropagation requires the sterile culture of explants which are induced to form new shoots and roots through culture media. These contain a source of fixed carbon (usually sucrose), essential mineral ions and a supply of vitamins and/or amino acids (Dahleen and Bregitzer, 2002). A widely used medium in plant tissue culture is the Murashige and Skoog (M.S. medium) developed by Murashige and Skoog (1962). To induce the production of shoots and roots, in order to regenerate whole plantlets, plant growth regulators are also included in the culture medium. The choice and concentration of growth regulators used for this purpose is dependent on the species and the explant used, but in general involves a balance of auxins and cytokinins. Unfortunately regeneration of leguminous crops has proved challenging (Halpin,

2005; Eapen et al., 2008). However Faisal and Anis, (2006) reported micropropagation of *M. pruriens* using nodal explants grown on half-strength M.S medium supplemented with the cytokinin 6-benzylaminopurine (BA at 5.0 µM), and the auxin α-Naphthalene acetic acid (NAA at 0.5 µM). For *in vitro* root regeneration, however, they then used M.S medium supplemented with auxin indole-3-butyric acid (IBA at 1.0 µM).

Like most large seeded legumes, *M. pruriens* has been variously reported to produce significant levels of phenolic compounds (Pras et al., 1993; Rajeshwar et al., 2005). However during tissue culture, phenolic substances tend to become oxidised (Arnaldos et al., 2001). This is because when cells are damaged as is the case during generation of explants, the contents of cytoplasm and vacuoles are mixed as well as exposing phenolic compounds to air (Laukkanen et al., 1999). Oxidised phenolic compounds may inhibit enzyme activity culminating in the darkening of the culture medium and subsequent lethal browning of explants. Liquid media can be used to reduce phenolic oxidation (Compton et al., 1986 and Laukkanen et al., 1999). In addition, frequent subculturing, enriching the media with antioxidants such as citric acid, ascorbic acid, polyvinyl pyrolidone (PVP) and activated carbon, which are added into it can reduce phenolic oxidation and contribute to whole plant regeneration from explants (Toth et al., 1994).

Tissue culture techniques for regeneration of plant explants are also needed in most protocols for generating transgenic plants (Walden and Wingender, 1995). For many crops, transformation can be achieved using the soil-borne gram negative bacterium *Agrobacterium tumefaciens* (Birch, 1997) and this is the most widespread method for transformation of legumes (Yamada, et al., 2001), although direct gene transfer has

also been reported for some species. For example, Russell et al., (1993) reported transformation of *Phaseolus vulgaris* by direct gene transfer into the apical meristem of seedlings. *Agrobacterium*-mediated transformation has advantages over direct DNA delivery techniques. These include the frequency of stable genomic integration, usually of single gene copy number and the transfer of relatively large segments of DNA (McCormac et al., 1998*).*

A. tumefaciens infects a range of dicot plants, causing formation of crown galls or tumours close to the site of infection. The tumour provides the bacterium with unusual amino acids (opines) which are an important source of carbon and nitrogen, at the expense of the plant host (Hellens and Mullineaux, 2000; Zupan et al., 2000). The genes required to induce tumour formation and to initiate opine biosynthesis in the infected host plants are transferred from the *Agrobacterium* (Hellens and Mullineaux, 2000; Broothaerts et al., 2005). *Agrobacterium* has a tumour-inducing (Ti) plasmid on which is a discrete T-DNA region delimited by 25 bp imperfect repeats on both sides (RB and LB) and approximately 35 virulence (*vir*) genes clustered in a *vir* region. The combined action of *vir* genes achieves the delivery of the T-DNA to the nucleus of the host plant, and it is in this T-DNA that genes for inducing tumour and opine biosynthesis are contained and which despite being of bacterial origin, have evolved to function only in plant cells (Dillen. et al., 1997; Hellens and Mullineaux, 2000). Most Ti plasmids commonly used in *Agrobacterium*-mediated transformation have been modified to remove their oncogenic genes from the T-DNA. In their place, desired genes have been introduced in the T-DNA region to be transferred into the plant genome. The gene substitution in the T-DNA region does not impede the ability of the *vir* genes to transfer the T-DNA from the plasmid

into the plant genome during transformation but does prevent formation of tumours (Hellens and Mullineaux, 2000; Gelvin, 2000). *Agrobacterium*-mediated plant transformation success has been enhanced by using a range of binary Ti vector plasmids and a range of disarmed *Agrobacterium* strains. In the binary vector system, the *vir* region resides on the disarmed Ti plasmid resident in the *Agrobacterium* strain and the T-DNA is on a separate binary Ti vector plasmid but still the *vir* genes are able to achieve the transfer of the T-DNA into the plant cell nucleus thereby increasing flexibility in manipulation. The Ti vectors replicate in both *E. coli* and in *Agrobacterium,* which is advantageous since most *in vitro* gene manipulation techniques use *E. coli* (Hellens and Mullineaux, 2000). Genes encoding resistance to certain antibiotics are often cloned on the Ti vector plasmid hence *Agrobacterium* transformed with the Ti vector plasmid can be selected using the antibiotic. Furthermore, *Agrobacterium* strains are marked with antibiotic resistance genes that are either on the chromosome or are Ti-plasmid localised. This enables growth of *Agrobacterium* on the antibiotic selection medium (Hellens and Mullineaux, 2000; Zupan et al., 2000). Suitable restriction enzyme sites and origin of replication sites (ORI) according to the research requirements are cloned on varied Ti vector plasmids to enable replication and isolation of the cloned genes. The pCAMBIA series of plasmids are a good example of Ti vector plasmids designed for T-DNA transfer into plants using *vir* genes on a disarmed Ti-plasmid in *Agrobacterium* (Hajdukiewicz et al., 1994).

The transformation of *Vigna aconitifolia* by direct DNA transfer to protoplasts using the heat shock method was one of the early reports on transformation of legumes (Kohler et al., 1987). However, since then research progress on transformation of

legumes has not matched that for cereals and many other crops (Birch, 1997; Dillen et al., 1997; Bent, 2000). This is partly due to the difficulties in regeneration of legumes as discussed above although, transformation of soybean (*Glycine max*) has been very successful, and 81% of soybean now grown is transgenic (http://www.isaaa.org). Despite this, transformation, especially of large seeded leguminous species, tends to show high levels recalcitrance (Veltcheva et al., 2005; Dillen et al., 1997; Yamada et al., 2001). A comprehensive proof of successful plant transformation requires the transgene(s) to be correctly expressed in both the primary transformants and their offspring generations (Dillen et al., 1997). There is no published report of a definitive genetic transformation of *M. pruriens* to date. Very recently, Sathyanarayana et al., (2012) reported a preliminary attempt for *Agrobacterium*-mediated transformation of *M. pruriens* using nodal explants, however although PCR was used to verify the presence of transgenes, expression and stability were not tested.

As well as the choice of plant growth regulators and culture medium for regeneration, the choice of *Agrobacterium* strain and explant for plant transformation is also very important. The most widespread used explants for legume transformation and plant regeneration include; cotyledonary nodes, leaf, epicotyl, and somatic embryogenesis but they tend to vary from one species to another (Dillen, et al., 1997; Yamada et al., 2001). Successful methods for transformation of legumes have used a range of *Agrobacterium* strains, examples are shown in Table 6.1. Yamada et al., (2001) reported that during transformation of *Vigna angularis*, each of the *Agrobacterium* strains EHA105 and AGLI, had a transformation efficiency of approximately 85% whereas that for LBA4404 was 50%.

Table 6.1 Range of *Agrobacterium tumefaciens* strains used for transformation of legume species transformation.

Species	*Agrobacterium* strain	reference
Phaseolus acutifolius	C58CIRif	Dillen et al., (1997)
Vigna angularis	EHA105, AGLI and LBA4404	Yamada et al., (2001)

6.1.2: Gene silencing constructs for plants.

RNA interference (RNAi) is widely used in functional genomics studies to suppress gene function in plants and it is based on the principle of post-transcriptional gene silencing (PTG) (Helliwell and Waterhouse, 2003; Wang and Xu, 2008).

RNAi technology involves transformation of a plant with T-DNA carrying an antisense copy of a plant gene targeted for expression silencing. During transcription, the antisense mRNA anneals to the target sense mRNA resulting in formation of dsRNA (Helliwell and Waterhouse, 2003; Wang and Xu, 2008). The dsRNA stimulates the dicer enzyme to break down the long dsRNA into short fragments of approximately 20 nucleotides referred to as the short interfering RNA (siRNA). Each siRNA unwinds into two single-stranded (ss) ssRNA: the passenger strand which is degraded and the guide strand which becomes incorporated into the RNA-induced silencing complex (RISC) (Hanon, 2002; Helliwell and Waterhouse, 2003). The guide strand in the RISC anneals to the complementary sequence on the target mRNA molecule and this induces the Argonaute catalytic component of RISC to

cleave the mRNA thereby silencing the target gene expression (Hanon, 2002; Helliwell and Waterhouse, 2003; Wang and Xu, 2008) (Figure 6.1).

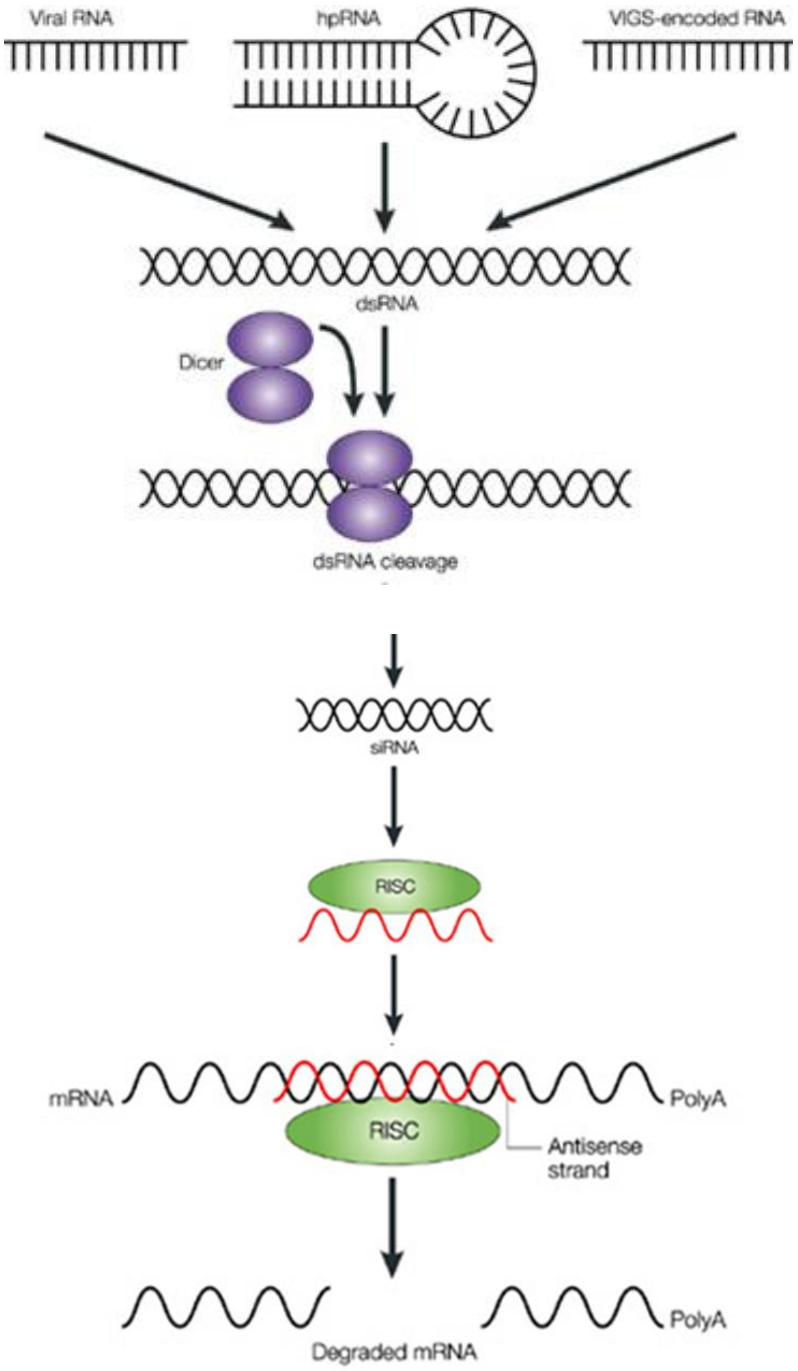


Figure 6.1 Schematic diagram describing the principle and mechanism of gene-silencing in plants. Diagram was obtained from <http://www.nature.com/nrg/journal/v4/n1/fig_tab/nrg982_F1.html>.

RNAi has been used successfully for manipulating metabolic pathways although issues of feedback control, redundancy and shared regulatory pathway control can arise (Cappel and Cristou, 2004).

6.1.3: Aims, objectives and approaches used in Chapter 6

The ultimate aim of this work was to use an RNAi construct based on the *M. pruriens* Mp-ty/ddc gene sequence obtained as described in Chapter 4 to silence the endogenous gene. The hypothesis is that this would result in increased levels of L-DOPA due to a reduction in its breakdown by the MP-TYDC enzyme.

The first hurdle was to develop a protocol for genetic transformation of *M. pruriens*. The approach was to use protocols for genetic transformation of other legumes as models or with modification to develop a robust protocol for genetic transformation of *M. pruriens*.

For the silencing approach, the next objective was to design and build an Mp-ty/ddc RNAi construct and use it to transform *M. pruriens*. The construct to be used for transformation carried an *npt*II transgene which would enable transgenics to grow on kanamycin selection nutrient medium. A kanamycin assay was therefore performed to determine the effective concentration for selection of *M. pruriens* transgenic plants. Since the aim was to increase L-DOPA levels, an L-DOPA assay was also performed to determine the maximum concentration that could be supplemented in the nutrient medium without affecting the health and growth of *M. pruriens* explants.

Finally the aim was to determine whether the silencing construct was effective in any transgenic lines obtained by measuring Mp-ty/ddc gene expression using semi-quantitative PCR.

6.2 MATERIALS AND METHODS.

6.2.1 Kanamycin assay on *M. pruriens* explants.

Dry seeds of *M. pruriens* were surface sterilised in 70% ethanol for 30 seconds followed by 1% sodium hypochlorite for 15 minutes and two washes with sterile distilled water. The seeds were plated onto basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 30 g l^{-1} sucrose and 8 g l^{-1} agar (Duchefa Biochemie) (pH 5.8). Seeds were then germinated in the dark. Two weeks after plating, the cotyledonary nodes and apical meristem were cut into pieces about 10 mm long with a scalpel blade. *M. pruriens* explants were micropropagated on M.S medium supplemented with 5 µM BA, 0.5 µM NAA and kanamycin concentrations (mg l^{-1}) of; 0, 10, 25, 50, 100, 200 and 500. The choice of concentration range used was based on those for selection of transgenic plants in family Fabaceae to which *M. pruriens* belongs (Dillen et al., 1997). The explants were grown at 25˚C under cool white fluorescent light (16/8 light regime, 50 - 60 µmol m^{-1} s^{-2}). The explants were transferred to fresh M.S medium at two week intervals to minimise the effect of gradual degradation of the kanamycin selection.

6.2.2: L-DOPA assay on *M. pruriens* explants.

Dry seeds of *M. pruriens* were surface sterilised, sown and grown as described in Section 6.2.1. The M.S medium was supplemented with L-DOPA concentrations (mg l^{-1}) of; 0, 5, 10, 25, 50, 100, 200 and 500. A total of 4 - 5 seeds were grown per tissue culture box in a triplicate sets for each L-DOPA concentration assayed. The maximum concentration of L-DOPA tolerated by *M. pruriens* seedlings during growth was assessed as that at which at least 90% of seedlings survived after 30 days growth.

6.2.3: Designing and building an Mp-ty/ddc gene silencing construct.

6.2.3.1: Plasmids and bacterial strains used.

The Mp-ty/ddc gene silencing construct was built by assembling the entire Mp-ty/ddc ORF (1.5 kb) in antisense orientation into the prepared restriction sites in the T-DNA region of a pGREEN-CO58 vector plasmid (Novagen, USA) as described in Section 6.2.3.1.1 below. PGREEN-CO58 plasmid backbone has a kanamycin resistance gene for bacterial selection and an *npt*II gene in the T-DNA region controlled by a CAMV35S promoter. The pGREEN-CO58 plasmid harbouring the Mp-ty/ddc antisense construct was co-transformed with a pSOUP plasmid (Novagen, USA) into *Agrobacteria* strains, GV3101, LBA4404 and EHA101 (Novagen, USA) as described in Section 6.2.3.1.1 below. The pSOUP plasmid has virulence genes ("*vir*") which

direct transfer of the T-DNA region from the pGREEN plasmid to the plant genome when the transgenic *Agrobacteria* infects plant cells during the plant transformation experiments (Hellens and Mullineaux, 2000) as described below. The *npt*II gene in the T-DNA region conferred the transgenic plants with kanamycin resistance while the Mp-ty/ddc antisense in the T-DNA was expressed by the CAMV35S to generate antisense transcripts which annealed to the sense Mp-ty/ddc transcripts generated by the plant genome. The double stranded RNA transcripts generated initiated dicer action to destroy all transcripts with sequences similar to that of Mp-ty/ddc.

A. tumefaciens strains used for plant transformations are detailed in Table 6.2

Table 6.2 *Agrobacterium* strains I used for the experiment

Strain	Type	Genotype	selection	Helper plasmid
GV3101	Nopaline	Cured	Rifampicin (chromosomal)	None
EHA101	Nopaline	pEHA101(pTiBo542DT-DNA)	Rifampicin (chromosomal DNA) and Kanamycin (Ti plasmid)	None
LBA4404	Octopine	pAL4404	Rifampicin (chromosomal DNA), Spectinomycin and Streptomycin (Ti plasmid)	None

6.2.3.1.1: Assembly of the Mp-ty/ddc antisense construct.

Mp-ty/ddc primers; *Eco*RI-F_{DC}: 5'-TAGAATTCCATTGATTTCCTTGCTG-3' and *Xho*I-R_{DC}: 5'-ATCTCGAGTCCTAACAGAGAATTGGCATGC-3' were designed as described in Chapter 2 Section 2.2.5.1 from sequence position 41 and 1,489 bp from the 5' end of the Mp-ty/ddc gene sequence obtained in Chapter 4. This corresponds to the putative full-length ORF for the Mp-ty/ddc. *Eco*RI and *Xho*I restriction sites were incorporated at the 5' ends of *Eco*RI-F_{DC} and *Xho*I-R_{DC} primers respectively, to facilitate directional cloning of the Mp-ty/ddc ORF in cloning vectors and in antisense orientation in Mp-ty/ddc gene-silencing construct.

The PCR reaction mixture contained; Phusion HF buffer (5 X; 10 µl), dNTPs (10 mM; 1 µl), *Eco*RI-F_{DC} (10 µM; 2.5 µl), *Xho*I-R_{DC} (10 µM; 2.5 µl), DNA (80 ng; 2.5 µl), Phusion DNA polymerase (1U; 0.5 µl) and sterile water (31 µl). Proof-reading phusion taq polymerase (Sigma) was used in the PCR to enhance specific amplification and to produce blunt ended PCR products. Thermocycling was performed using a PTC-100 thermocycler (MJ Research Inc., Waltham, USA) and the cycling conditions were; 98 ˚C; 30 seconds, 35 cycles (98 ˚C; 10 seconds, 61 ˚C; 10 seconds, 72 ˚C; 45 seconds) and 72 ˚C, 5 minutes. The PCR product was then blunt end ligated in the *Eco*RV site of a prepared pZERO plasmid and was cloned as described in Chapter 2 Section 2.2.13. The transgenic plasmid was purified from bacterial cells as described in Chapter 2 Section 2.2.14. The cloned 1.5 kb Mp-ty/ddc phusion PCR product was recovered from the cloning plasmid by serial restriction digestion using *Spe*I and *Not*I enzymes. The digestion reaction contained: plasmid DNA (2 µg), Yellow Tango buffer (Thermo Scientific) (2 X; 4 µl), BSA (10 X;

4 µl), *Spe*I (3 U) and the volume was made up to 40 µl with sterile water. The reaction mixture was incubated at 37 ˚C for 3 hours after which the linearised plasmid was recovered from the digestion reaction by column purification as described in the Chapter 2 Section 2.2.8. The linearised plasmid DNA was then digested using a *Not*I restriction enzyme. The digestion reaction contained: DNA (1.5 µg), Orange Tango buffer (ThermoScientific) (2 X; 4 µl), BSA (10 X; 4 µl), *Not*I (2.5 U) and the volume was made up to 40 µl using sterile water. The reaction was then incubated at 37 ˚C for 3 hours after DNA fragments were separated and analysed on ethidium bromide stained agarose gel electrophoresis as described in Chapter 2 Section 2.2.7. The *Spe*I - *Not*I fragment DNA restriction fragment contained the cloned 1.5 kb Mp-ty/ddc gene and was purified from the gel as described in Chapter 2 Section 2.2.9. The 1.5 kb Mp-ty/ddc fragment was then ligated in the *Spe*I - *Not*I restriction sites of a pre-digested pGreen-CO58 plasmid. In the pGreen-CO58 plasmid, the Mp-ty/ddc was in antisense orientation and between the CaMV35S promoter and a Tnos terminator of the T-DNA region (Figure 6.2).

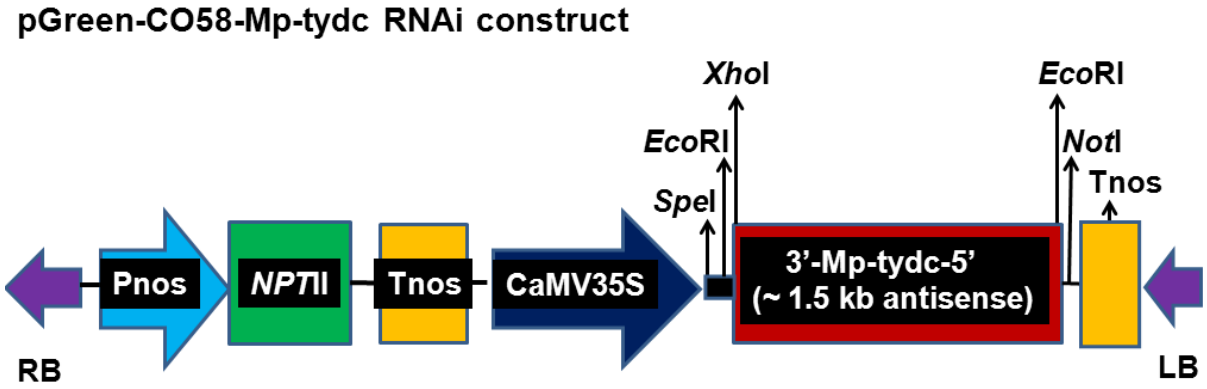


Figure 6.2 Map of the Mp-ty/ddc gene silencing cassette cloned in the T-DNA region (Right border, RB and left border, LB) of a pGreen-CO58 plasmid vector. The CaMV35S promoter and a Tnos terminator control expression of the 1.5 kb antisense Mp-ty/ddc, whereas *npt*II reporter gene expression is controlled by a Pnos promoter and a Tnos terminator.

6.2.3.2: Transformation and handling of *Agrobacterium* cells.

Competent cells of *Agrobacterium* were prepared as described in Chapter 2 Section 2.2.12. Transformation of *Agrobacterium* was performed using either a UV gene-pulser or a liquid nitrogen freeze-thaw method.

Transformation of competent *Agrobacterium* cells using the gene-pulser was performed according to the method described by Shen and Forde (1998) and Mattanovich et al., (1989) as follows: plasmid DNA (40 ng/µl) or a mixture of pGREEN (20 ng; 1 µl) and pSOUP (20 ng; 1 µl) was added to competent cells of *A. tumefaciens* (50 µl) in a 1.5 ml microcentrifuge tube. The mixture was then transferred to a 1 ml electroporation cuvette and the DNA was transformed into the bacterial cells by electroporation using a gene-pulser (Bio-Rad, USA) at 400 Ohms, 2.5 V and at a time constant greater than 6.5 milliseconds. LB-broth (0.9 ml) was immediately added to the transformed bacterial cells and this was followed by incubation on a rotary shaker at 225 rpm for 1.5 h. Aliquots (50 – 100 µl) of the bacterial culture were plated onto solid LBA mediuim (LB; 10 g l^{-1} agar, pH 7) supplemented with kanamycin (50 mg l^{-1}), and rifampicin (50 mg l^{-1}). In addition, gentamycin (20 mg l^{-1}) and streptomycin (25 mg l^{-1}) were added to LBA media plates for GV3101 and LBA4404 respectively. The kanamycin was used for selection of bacterial cells transformed with the *npt*II transgene whereas the rifampicin, gentamycin and streptomycin selected for their respective antibiotic resistance genes on the bacterial chromosomal DNA. The plates were incubated at 28 ˚C for 48 hours when single colonies of transgenic bacteria formed.

On the other hand, transformation of competent *Agrobacterium* cells using the liquid nitrogen freeze-thaw method was essentially as described by Sambrook et al. (1989)

as follows: A 100 µl aliquot of *Agrobacterium* competent cells in a 1.5 ml Eppendorf tube was taken from a -80˚C freezer and thawed on ice for about 5 minutes. Then plasmid DNA (0.5 – 1 µg; < 20 µl) was added to the cells and tube was frozen in liquid nitrogen for 1 minute before it was allowed to thaw for 5 minutes at 37 ˚C. LB medium (1 ml) was then added to the cells before the tube was incubated at 28 ˚C for 4 hours with shaking at 225 rpm in a rotary shaker (Incubator shaker Model G25, New Brunswick Scientific Co Inc, USA). The tube was then centrifuged in a microcentrifuge (Heraeus Instruments Biofuge 13, Germany) at 3000 X g for 2 minutes. The supernatant was discarded while the pellet was resuspended in LB medium (100 µl). For transformations with pSOUP and pGREEN into *Agrobacterium* strain GV3101, aliquots (50 µl) were plated on LBA supplement with kanamycin (50 mg l^{-1}), rifampicin (50 mg l^{-1}) and gentamycin (20 mg l^{-1}). The plates were incubated at 28 ˚C for 48 hours when single colonies of transgenic bacteria formed.

For long term storage of transformants a single colony of transgenic bacteria was inoculated in LB medium (5 ml) supplemented with kanamycin (50 mg l^{-1}), rifampicin (50 mg l^{-1}) and gentamycin (20 mg l^{-1}) and the culture vial incubated at 28 ˚C up to $O.D_{600}$ of 0.8. 30% glycerol stocks of the transgenic bacterial culture were prepared and stored at 80 ˚C.

Before each plant transformation experiment, a needle head size loop of transgenic bacteria were streaked on a plate of LBA supplemented with kanamycin, rifampicin and gentamycin selection. The plate was incubated at 28 ˚C for 48 h to produce single colonies of transgenic bacteria which were immediately used in plant transformation experiments as described in Sections 6.2.3.3 and 6.2.3.4.

6.2.3.3: Transformation of *M. pruriens* using the *Agrobacterium*-mediated method for transformation of *Vigna angularis.*

A method for transformation of *M. pruriens* was developed based on the protocol for *Agrobacterium*-mediated transformation of *V. angularis* (Yamada et al., 2001). *M. pruriens* seeds ("90 day" cultivar) were obtained from Echo seed bank (Florida). *M. pruriens* seeds were surface sterilised and plated as described in Section 6.2.1 and then grown in the dark at 22 ˚C. Meanwhile, a single colony of the transgenic *A. tumefaciens* strains; EHA105, LBA4404 and AGLI harbouring the pCAMBIA2300 plasmid (pC23.35SECGFPHS), was freshly prepared as described in Section 6.2.3.3. The bacteria were grown at 28 ˚C overnight in liquid LB broth containing kanamycin (100 mg l^{-1}). They were then centrifuged at 8,000 X g for 3 minutes using microcentrifuge (Biofuge 13, Heraeus Instruments, Germany) and the pellet was resuspended to a final OD_{600} of 0.2 in M.S liquid medium containing glucose (15 g/l). After ten days growth, the elongated epicotyls of etiolated seedlings were cut into pieces about 10 mm long with a scalpel blade. Then the transgenic bacterial suspension (2 µl) was pipetted onto the wounded sites of each explant. About 20 to 30 explants were plated sideways on M.S medium supplemented with BA (10 mg l^{-1}) and acetosyringone (100 µM). After 2 days of co-cultivation at 22 ˚C in the dark, explants were washed twice with M.S liquid medium. Excess liquid on the plants was withdrawn with sterile filter paper. Explants were plated on M.S medium containing BA (1 mg l^{-1}), kanamycin (100 mg l^{-1}), and 300 mg l^{-1} timentine (in place for lilacillin (500 mg l^{-1}), and incubated at 25 ˚C under cool white fluorescent light (16/8 light regime, 50 - 60 µmol m^{-1} s^{-2}). The explants were transferred to fresh medium at two week intervals.

6.2.3.4: New protocol *for Agrobacterium*-mediated transformation of *M. pruriens.*

Dry *M. pruriens* seeds were surface sterilised and plated as described in Section 6.2.1. On day 13 of seed germination, a single colony of transgenic *A. tumefaciens* strain GV3101 harbouring an Mp-ty/ddc gene-silencing construct plasmid (Figure 6.2), was grown overnight at 28 ˚C with shaking at 225 rpm in liquid LB broth containing kanamycin (100 mg l^{-1}), rifampicin (50 mg l^{-1}) and gentamycin (20 mg l^{-1}). On the day scheduled for the plant transformation, 5 ml of the overnight culture of *Agrobacterium* was used to inoculate LB medium (50 ml) and was incubated at 28 ˚C on a rotary shaker at 225 rpm until it reached an $O.D_{600}$ of 0.7. The bacterial cells were then pelleted at 8,000 X g for 3 minutes using a microcentrifuge (Biofuge 13, Heraeus Instruments, Germany). Finally the bacterial cells was resuspended to a final OD_{600} of 0.6 in M.S liquid medium containing glucose (15 g l^{-1}) and were ready for use in plant transformation. Germinated seedlings were aseptically excised, the cotyledonary nodes and apical meristems were cut into pieces of about 10 mm long with a scalpel blade. The *Agrobacterium* culture (2 µl) was then pipetted onto the wounded sites of each explant. About 8 explants were micropropagated sideways per Petri dish (90 mm diameter; 15 mm height) on M.S medium supplemented with BA (50 µM or ~ 10 mg l^{-1}), NAA (0.5 µM) and acetosyringone (100 µM). After 2 days of co-cultivation at 22 ˚C in the dark, explants were washed twice with M.S liquid medium to remove *Agrobacterium*. Excess liquid on the explants was withdrawn with a sterile filter paper. The explants were plated on M.S shooting medium containing BA (50 µM), NAA (0.5 µM) and kanamycin (50 mg l^{-1}). In order to eliminate *Agrobacterium* from explants after transformation, timentine (350 mg l^{-1}) was added to the shooting medium. The explants were incubated at 25 ˚C under

cool white fluorescent light (16/8 light regime, 50 - 60 µmol m^{-1} s^{-2}). The explants were then transferred to fresh medium at two week intervals in order to reduce the effect of phenolic oxidation. When adventitious shoots developed and after more than 2 leaves emerged from shoots, each shoot was excised and transferred to liquid M.S medium supplemented with NAA (16.2 µM), kanamycin (100 mg l^{-1}), and timentine (350 mg l^{-1}). Rooted shoots were excised and repeatedly selected on the fresh medium at two week intervals.

6.2.3.4.1: Root induction Auxin assay.

Half-strength M.S liquid medium supplemented with NAA (15.6 µM; 3 ml) was transferred to pre-autoclaved Pyrex test tubes (16 X 100 mm). A single shoot explant of *M. pruriens* was aseptically excised from the basal callus and transferred to M.S liquid medium. A sterile Whatman paper (3 mm) fitted with a hole was used to keep the shoot explant in a vertical orientation in the tube and floating above the M.S liquid medium. However, the base of the stem explant was dipped in M.S liquid medium. The experimental set for root induction above was repeated in triplicate sets using M.S liquid medium supplemented with NAA concentrations of; 15.58, 15.6, 15.62, 15.64 and 15.66 (µM). The best NAA concentration was determined from the ability to induce the highest percentage of rooting in the explants.

6.2.3.5: Detection of the *npt*II transgene in putatively transformed *M. pruriens* by PCR.

Total DNA was isolated from *M. pruriens* leaves by the method described in Section 2.2.1.2. PCR analysis was conducted at Tm of 49 ˚C to screen for transformed explants as described in Section 2.2.6. The primer set; 5'-CTTGGGTGGAGAGGCTAT-3' and 5'-AGAACTCGTCAAGAAGGC-3' was designed and used to amplify of 749 bp domain of the *npt*II transgene coding sequence from genomic DNA extracted from transgenic plants (Beck et al., 1982).

6.2.3.6: Screening for *Agrobacterium* contamination in putative transgenic DNA.

Putative transgenic DNA samples which tested positive for the *npt*II transgene by PCR, were further screened for post co-cultivation *Agrobacteria* contamination. This was because the *Agrobacterium* strains used for plant transformation were carried an *npt*II gene on the pGREEN plasmid they harboured and this could produce false *npt*II positive PCR from untransformed plants. Screening explants DNA for contamination with *Agrobacterium* was based on the principle that the *Agrobacteria* also a harboured a helper plasmid (pSOUP) which carry a tetracycline resistance gene. The helper plasmid DNA is not transduced in to the plant genome during plant transformation and hence detection of the tetracycline resistance gene in plant DNA would confirm contamination with *Agrobacterium*. In the event of post co-cultivation contamination of explants with *Agrobacterium*, then the detection of the *npt*II transgene by PCR would not be sufficient evidence for plant transformation. In light of the above, tetracycline resistance gene primers; 5'-

CGGCCTCAACCTACTACTGG-3’ and 5’-TTGAAGCTGTCCCTGATGG-3’ designed to PCR amplify 0.5 kb of the tetracycline resistance gene on the pSOUP plasmid, were used to screen by PCR for *Agrobacterium* contamination in DNA as described in Chapter 2 Section 2.2.6. The DNA template was extracted from putative transgenics plants as described in Section 2.2.1.2.

6.3: RESULTS.

6.3.1: Developing a new protocol for genetic transformation of *M. pruriens.*

6.3.1.1: Assay to determine the effective kanamycin concentration for selection of the kanamycin resistant transgenic explants.

A kanamycin assay was performed to determine the minimum but effective concentration for selection of *M. pruriens* kanamycin resistant explants (Section 6.2.1). Following 14 days of culture of untransformed *M. pruriens* on kanamycin-containing medium, mortality was assessed. On 10 mg l^{-1} and 25 mg l^{-1} kanamycin selection, 85% and 10% of the explants respectively were healthy (Table 6.1). In contrast all explants on 50, 100, 200 and 500 mg l^{-1} kanamycin selection had died by day 14 of the experiment (Table 6.1). At day 21 all the explants on kanamycin selection had died and only the positive control explants grown on M.S medium without kanamycin were healthy and green (Figure 6.3). A kanamycin concentration of 50 mg l^{-1} was therefore the minimum assay concentration that effectively selected against all *M. pruriens* explants lacking the kanamycin resistance transgene within 14 days of experiment (Figure 6.3).

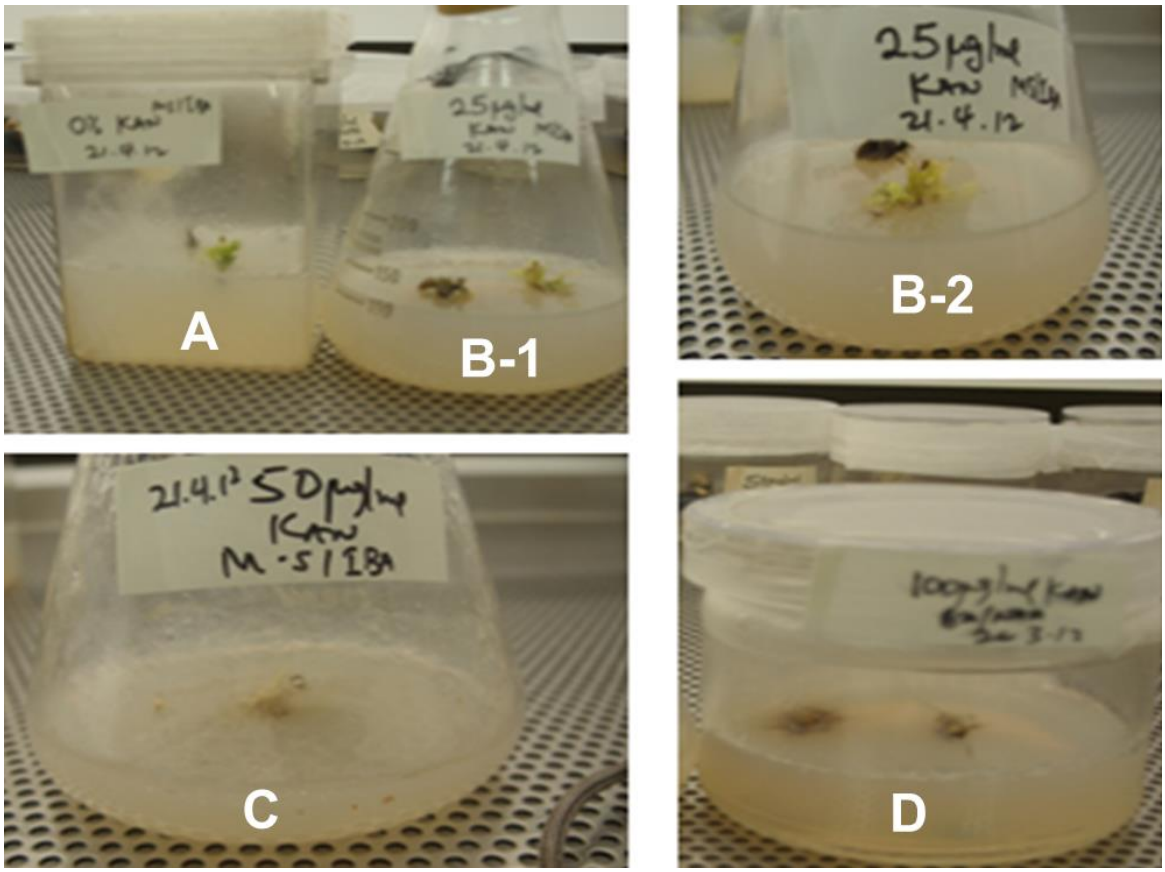


Figure 6.3 *M. pruriens* explants after 21 days of culture; explants in tissue culture box (A) grown on M.S medium with no kanamycin selection were healthy and green (positive control experiment), whereas explants grown on 25 mg l^{-1} kanamycin selection medium (B) and (C) had signs of necrosis. Explants grown on 50 and 100 mg l^{-1} kanamycin selection (D) had died by day 21 of the assay.

Table 6.1 Results for the kanamycin bioassay on untransformed *M. pruriens* explants.

Kanamycin conc. (µg/ml)	**No. of healthy plants after micropropagation for 1, 14, 21 and 30 days.**				
	1 d	**14 d**	**21 d**	**30 d**	% on day 30
0	10	10	10	10	100
10	20	17	0	0	0
25	20	2	0	0	0
50	20	0	0	0	0
100	20	0	0	0	0
200	10	0	0	0	0
500	10	0	0	0	0
Total	140				

6.3.1.2: Bioassay on L-DOPA.

An L-DOPA bioassay was performed to determine the maximum concentration which could be supplemented in the M.S medium and tolerated by *M. pruriens* seedlings as described in Section 6.2.2. *M. pruriens* seedlings were able to grow well on M.S medium supplemented with below 100 mg l^{-1} L-DOPA. However, L-DOPA at 200 mg l^{-1} was lethal to the *M. pruriens* seedlings (Figure 6.4; Table 6.2).

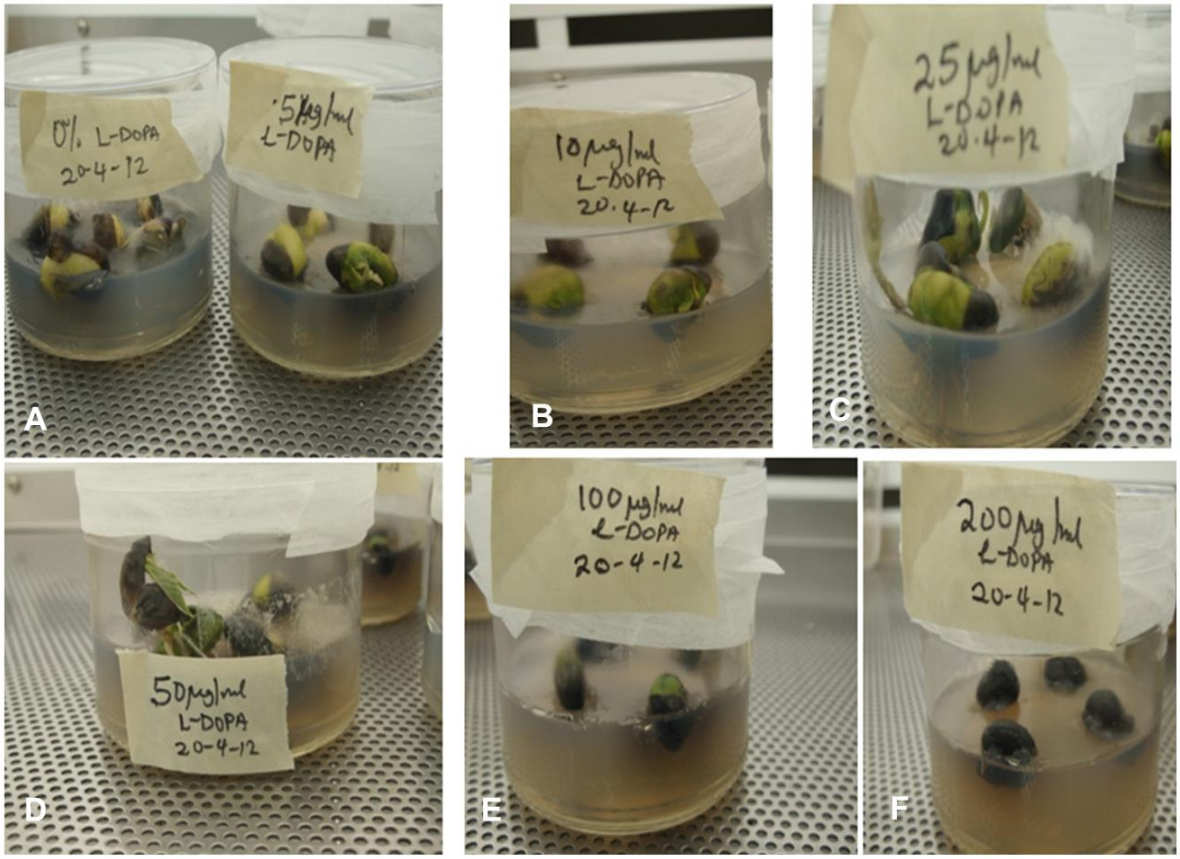


Figure 6.4 Bioassay to determine the concentration range of endogenous L-DOPA that *M. pruriens* seedlings can tolerate during growth. At day 21 of the assay, the seedlings growing on M.S media supplemented with L-DOPA concentrations of; A (0, 5 µg l^{-1}), B (10 µg l^{-1}), C (25 µg l^{-1}) and D (µg l^{-1}), germinated well. The seedlings in the assay E (100 µg l^{-1}) show reduced germination rates when compared to those in A, B and C. On the other hand seedlings in assay F (200 µg l^{-1}) died.

Table 6.2 Results of L-DOPA bioassay on untransformed *M. pruriens* seedlings

L-DOPA concentration (mg l^{-1})	**No. of healthy green and germinating seedlings on day:**			
	1	14	30	% on day 30
0	10	10	10	100.0
5	10	10	10	100.0
10	15	15	10	66.7
25	15	15	15	100.0
50	15	15	15	100.0
100	10	08	08	80.0
200	10	0	0	0.0
500	10	0	0	0.0
Total No. of plants	104			

6.3.1.3: Transformation of *M. pruriens* using a protocol for *V. angularis* as a model.

Attempts were made to transform *M. pruriens* by the method of Yamada et al., (2001) using epicotyl explants as described in Section 6.2.3.3. The level of kanamycin (50 mg l^{-1}) used for selection was based on the result for kanamycin assay described in Section 6.2.1. Three different *Agrobacterium* strains; LBA4404, AGLI and GV301 were used in the *M. pruriens* transformation experiments with a view of determining the most efficient strain. A cytokinin assay was also conducted to determine the optimal BA concentration required to enhance *M. pruriens* transformation efficiency. The assay was performed because the ratio of concentration of BA to acetosyringone significantly affects the efficiency of plant transformation (Yamada, et al., 2001). BA concentrations of 1, 3, 5 and 10 mg l^{-1} were used to supplement the

co-cultivation medium with a view of determining the effective concentration for *M. pruriens* transformation.

In general very few putatively transformed epicotyl explants survived on kanamycin selection for 2 months and of these no shoot regeneration occurred, while the greatest majority developed a lethal browning and died off. Most of the explants grown on media supplemented with 1 - 3 mg l^{-1} BA and kanamycin selection developed a lethal browning and died before reaching the 8 week stage. The number of putatively transformed explants which survived on kanamycin selection for 8 weeks was significantly increased with the increase in cytokinin concentration from 5 mg l^{-1} to 10 mg l^{-1} BA (Figure 6.5).

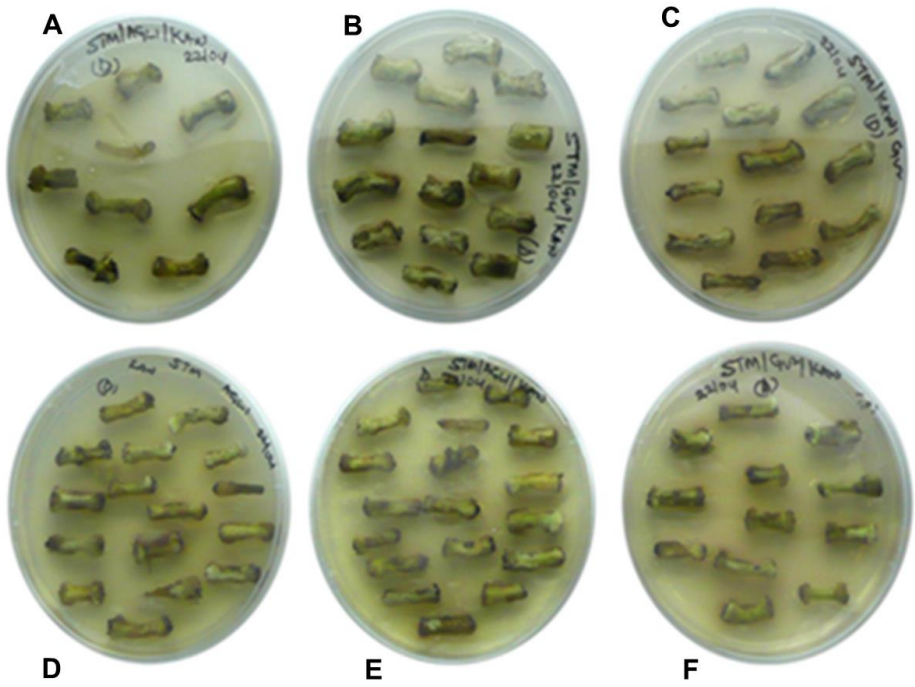


Figure 6.5 Putatively transformed *M. pruriens* epicotyl explants at 4 week stage after co-cultivation on M.S medium supplemented with 50 mg l^{-1} kanamycin selection and an assay of BA concentrations (mg l^{-1}) of; 3 (A and F), 5 (C and D), 10 (B and E). The explants in the 10 mg l-1 BA (B and E) assay grew relatively larger and had better survival rates.

M. pruriens transformation using LBA4404 showed a highest putative transformation rate of 8.9% which was achieved by explants co-cultivated on M.S supplemented with BA at 10 mg l^{-1}. A lower number (6.7%) of putative transformants was obtained on 5 mg l^{-1} BA. These putative transgenic calli remained healthy on kanamycin (50 mg l^{-1}) selection 8 weeks after co-cultivation but without shoot or root regeneration (Table 6.3).

Table 6.3 Efficiency of LBA4404 and BA concentration (1-10 mg l^{-1}) supplement to co-cultivation medium in the transformation of *M. pruriens.*

Transformed using LBA4404	No of stem explants. (Day 1)	No of calli (8 weeks)		Positive control explants (Day 1)	Positive control calli (8 weeks)	
BA (mg l^{-1}) in co-cultivation medium		No.	%	No.	No.	%
1	45	0	0.0	6	2	33.3
3	45	0	0.0	6	3	50.0
5	45	3	6.7	6	6	100.0
10	45	4	8.9	6	5	83.3
Total explants	180			24		

Transformation using AGLI showed a highest putative transformation rate of 17.8%, again achieved by explants co-cultivated on M.S supplemented with BA 10 mg l^{-1}. With this experiment there was a clear dose/response relationship for BA concentration throughout the concentration range tested. Again putative transgenic

calli formed were healthy on kanamycin (50 mg ml^{-1}) selection 8 weeks after co-cultivation but without shoot or root regeneration (Table 6.4).

Table 6.4 Efficiency of AGLI and BA concentration (1-10 mg l^{-1}) supplement to co-cultivation medium in the transformation of *M. pruriens.*

Transformed using AGLI	No of stem explants. (Day-1)	No of calli (8 weeks)		Positive control explants (Day-1)	Positive control calli (8 weeks)	
BA (mg l^{-1})		No.	%		No.	%
1	45	2	4.4	6	3.0	50.0
3	45	3	6.7	6	4.0	66.7
5	45	5	11.1	6	5.0	83.3
10	45	8	17.8	6	5.0	83.3
Total explants	180			24	17.0	

A better maximum transformation arte was obtained using GV3101 with a maximum transformation rate of 24.4% again achieved on with 10 mg l^{-1} BA, and again the dose-response for BA was very clear, but again there was no shoot or root regeneration even though calli remained healthy on kanamycin (50 mg ml^{-1}) selection for 8 weeks (Table 6.5).

Table 6.5 Efficiency of GV3101 and BA concentration (1-10 mg l^{-1}) supplement to co-cultivation medium in the transformation of *M. pruriens.*

Transformed using GV3101	No of stem explants (Day-1)	No of calli (8 weeks)		Positive control explants (Day-1)	Positive control calli (8 weeks)	
BA (mg l^{-1})		No.	%		No.	%
1	45	3	6.7	6	2.0	33.3
3	45	4	8.9	6	2.0	33.3
5	45	7	15.6	6	5.0	83.3
10	45	11	24.4	6	5.0	83.3
Total explants	180					

Hence *M. pruriens* transformation efficiency was progressively higher with the different *Agrobacterium* strains with GV3101, AGLI and LBA4404 (Table 6.3-6.5). Similarly, the cytokinin assay results showed that *M. pruriens* transformation efficiency with all the Agrobacterium strains tested was progressively higher with increasing BA concentrations up to 10 mg l^{-1}.

Having established conditions that appeared to give the best levels of transformation, a larger scale *M. pruriens* transformation experiment was performed in triplicate sets using the using the most efficient *A. tumefaciens* strains: AGL1 and GV3101 supplemented with 10 mg l^{-1} BA. After 8 weeks co-cultivation of the epicotyl stem explants, the majority had suffered lethal browning and died off on kanamycin selection. However, 18.8% (Table 6.6) and 23.4% (Table 6.7) of the AGLI and

GV3101-mediated putatively transformed explants respectively survived on kanamycin selection (50 mg l^{-1}) 8 weeks after co-cultivation. The putatively transformed explants also showed a remarkable increase in size but no shoot or root regeneration occurred.

Table 6.6 AGLI-mediated transformation of *M. pruriens* using epicotyl stem explants micropropagated on MS medium co-cultivation medium supplemented with 10 mg/l BA.

Transformed using AGLI (10 mg l^{-1} BA)	No of stem explants. (Day-1)	No of calli (8 weeks)		Positive control explants (Day-1)	Positive control calli (8 weeks)	
		No.	%		No.	%
Set-A	140	28.0	20.0	20	16.0	80.0
Set-B	140	27.0	19.3	20	13.0	65.0
Set-C	140	24.0	17.1	20	18.0	90.0
Total explants	420	79.0		60	47.0	
Mean		26.3	18.8		15.7	78.3

Table 6.7 GV3101-mediated transformation of *M. pruriens* using epicotyl stem explants micropropagated on MS co-cultivation medium supplemented with 10 mg l^{-1} BA.

Experimental set series (GV3101) (10 mg l^{-1}BA)	No of Stem explants. (Day-1)	No of calli. (8 weeks)		Positive control explants (Day-1)	Positive control calli. (8 weeks)	
	No.	No.	%		No.	%
Set-A	140	39.0	27.9	20	20.0	100.0
Set-B	140	32.0	22.9	20	19.0	95.0
Total explants	280	71.0		40	73.0	
Mean		35.5	23.4		36.5	97.5

6.3.1.4: Genetic transformation of *Mucuna pruriens* following a newly developed protocol.

Given the poor performance in terms of shoot and root development obtained with the protocol for *V. angularis* transformation, a new protocol was developed. In the new protocol for *M. pruriens* transformation I developed, instead of using epicotyl stem explants, I tested different tissues such as leaf, cotyledonary nodes and apical meristem as explants, since in different legume species transformation success had been obtained using these different tissues (see Section 6.1). In addition, the MS co-cultivation medium was supplemented with NAA (0.5 µM) and BA (50 µM) as described in Section 6.2.3.4. The auxin NAA was included to initiate and promote

shoot and root regeneration (Walden and Wingender, 1997; Marion, 2001) while a higher level of cytokinin BA was added to culture media because in the previous experiment (see Section 6.3.1.3), explants survival rates and size increased with an increase in BA concentration. Furthermore, M.S liquid medium supplemented with auxin, NAA was used to induce rooting and to reduce phenolic oxidation in the explants (Laukkanen, et al., 1999). The *Agrobacterium* culture used for the transformation experiment was grown to the exponential stage at $O.D_{600}$ of 0.6 – 0.8 during which the bacterial population in the cultures is young and relatively small but rapidly growing due sufficient nutrient supply and proper adaptation of cells to culture conditions (Sambrook et al., 1989). *Agrobacterium* strain GV3101 harbouring a pGREEN-CO58-Mp-ty/ddc-antisense plasmid was used in all subsequent plant transformation experiments because it had yielded the better survival rates for the explants than LBA4404 and AGLI in the previous experiments (See Section 6.3.1.3).

6.3.1.4.1: *M. pruriens* transformation using leaf explants.

An attempt to use leaf lamina as explants for transformation of *M. pruriens* succeeded in generating 17.4% putative transgenic calli which significantly increased in size during kanamycin selection. However, no shoot or root formation occurred (Figure 6.6). The greatest majority of the explants turned lethal brown and died off (Figure 6.6; Table 6.8).

Figure 6.6 Putatively transformed leaf explants on biweekly sub-culture to M.S medium supplemented with 50 µM kanamycin after 6 weeks. A few explants formed brown callus (ringed explant) but the majority tended to quickly develop a lethal browning.

However, it was observed that exceptionally large green calli were generated by the untransformed leaf explants micropropagated on M.S medium without kanamycin selection (Figure 6.7). The untransformed explants were micropropagated to serve as a tissue culture experimental control for *M. pruriens* transformation.

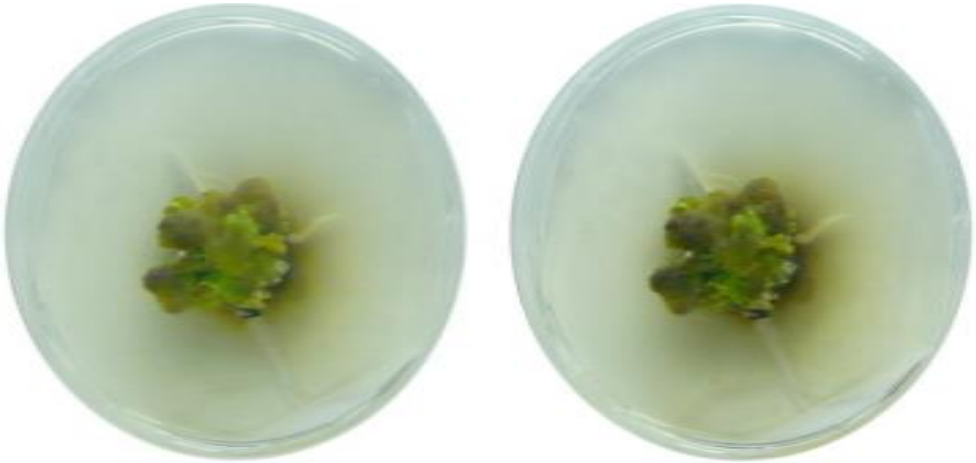


Figure 6.7 Large 6 weeks old calli derived from untransformed leaf explants of *M. pruriens* grown on M.S media without selection. The experiment was a positive control for tissue culture of transgenic *M. pruriens.*

Table 6.8 Summary of results obtained for the transformation of *M. pruriens* using leaf explant tissue.

Experimental set series (GV3101)	No of leaf explants. (Day 1)	No of calli. (6 weeks)		Non-transgenic control (Day 1)	Non-transgenic calli (6 weeks)	
	No.	No.	%		No.	%
Set-A	140	30.0	21.4	20	20.0	100
Set-B	140	19.0	13.6	20	19.0	95.0
Set-C	140	24.0	17.1	20	17.0	85.0
Total explants	420			60		
Mean		24.3	17.4		18.6	93.3

6.3.1.4.2: *M. pruriens* transformation using cotyledonary nodal explants.

91.2% *M. pruriens* explants regenerated shoot systems on kanamycin selection (50 mg l^{-1}) upon *Agrobacterium* (GV3101)-mediated transformation of cotyledonary nodal explants (Table 6.9). Co-cultivation assay on M.S medium supplemented with BA (50 µM), NAA (0.5 µM), 100 µM acetosyringone produced the highest percentage putatively transformed explants which later regenerated the shoot system on kanamycin selection (Figures 6.8-6.10; Table 6.9).

(a) (b)

Figure 6.8 Cotyledonary nodal explants of *M. pruriens* regenerating shoots on M.S media 2.5 weeks after co-cultivation with *Agrobacterium* (a) Positive control explant regenerating on growth media with no selection. (b) Putatively transformed explant regenerating on growth media supplemented with 100 µg ml^{-1} kanamycin.

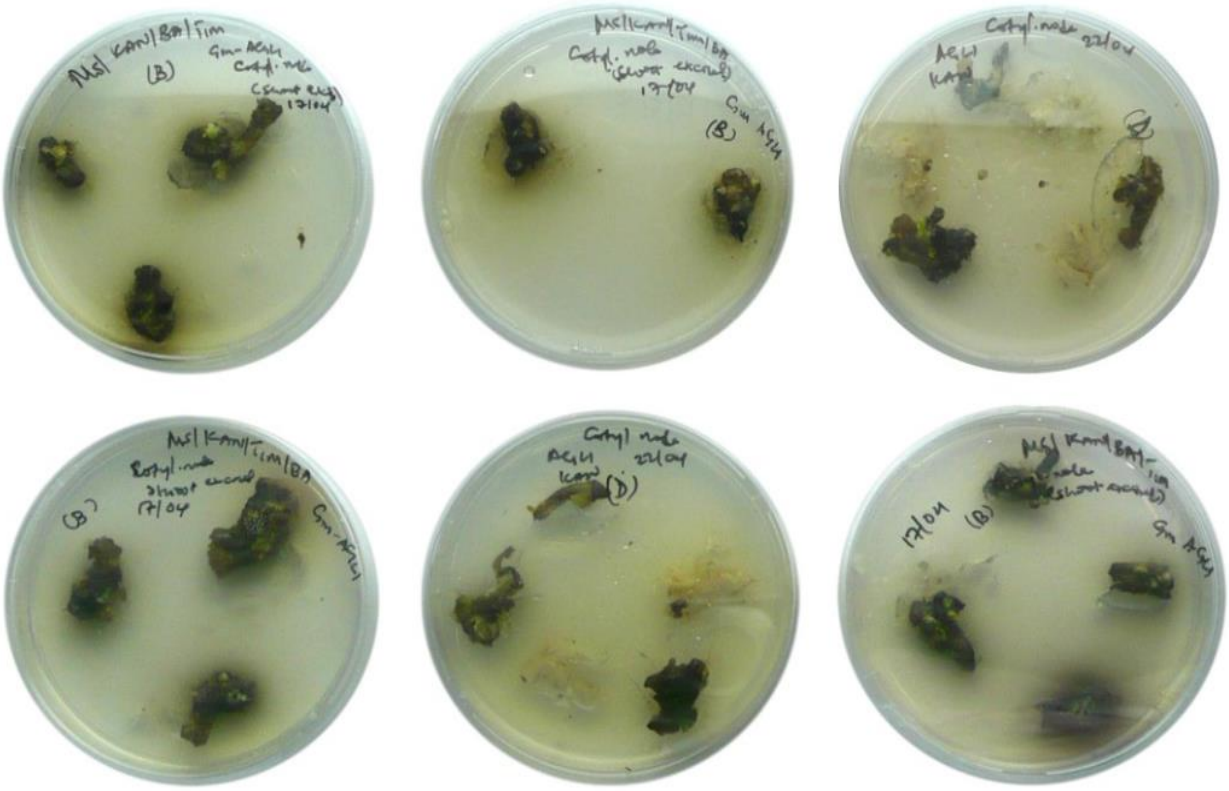


Figure 6.9 Putatively transformed cotyledonary explants of *M. pruriens* starting to regenerate shoots 3 weeks after co-cultivation on MS media supplemented with kanamycin selection.

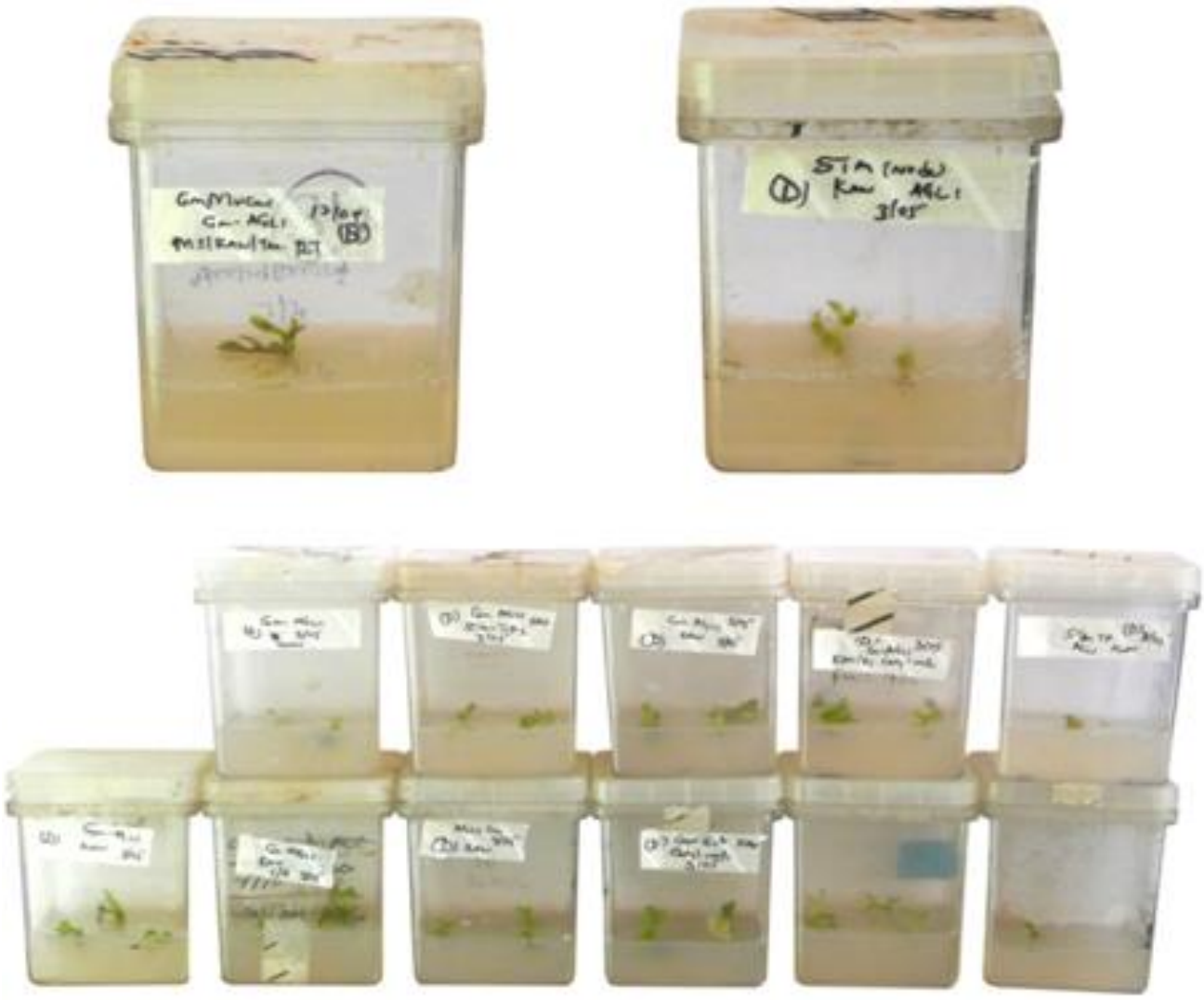


Figure 6.10 Putatively transformed 30 day old cotyledonary node *M. pruriens* explants regenerating shoots on M.S medium supplemented with IBA (1.0 µM) and 100 µg/ml kanamycin selection.

Table 6.9 *Agrobacterium* strain, GV3101-mediated transformation of *M. pruriens* using cotyledonary nodal explants.

Experimental set series (GV3101)	No of cotyledonary nodal explants (Day-1)	No of shoots (8 weeks)		Non-transgenic control (Day-1)	Non-trangenic shoots (8 weeks)	
	No.	No.	%		No.	%
Set-A	140	130.0	92.9	20	19.0	95.0
Set-B	140	129.0	92.1	20	19.0	95.0
Set-C	140	124.0	88.6	20	17.0	85.0
Total explants	420	380.0		60		
Mean		127.7	91.2		18.3	91.7

6.3.1.4.3: *M. pruriens* transformation using epicotyl apical bud explants.

Regeneration rates were also high using *M. pruriens* epicotyl bud explants with 85.5% regenerating shoot systems on kanamycin selection (50 mg l^{-1}) (Figure 6.11; Table 6.91). However, attempts to induce root regeneration in the explants by micropropagation on M.S solid supplemented with the auxin IBA (1.0 µM) were not succesful.

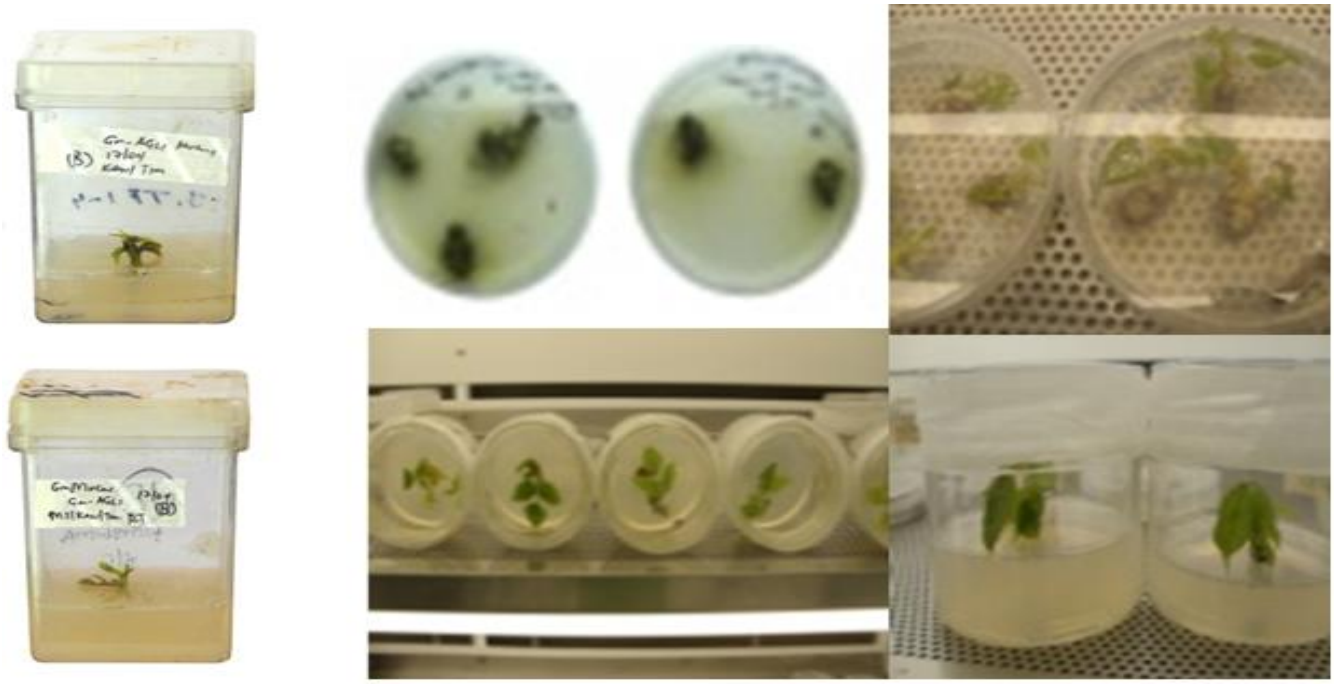


Figure 6.11 Putatively transformed 2 month old epicotyl apical bud shoot explants subcultured M.S medium supplemented with IBA (1.0 µM) and 100 µg ml^{-1} kanamycin selection.

Table 6.91 *Agrobacterium* strain GV3101-mediated transformation of *M. pruriens* using cotyledonary nodal explants.

Experimental set series (GV3101)	No of epicotyl apical bud explants. (Day-1)	No of transgenic shoots (8 weeks)		Non-transgenic control (Day-1)	Non-transgenic shoots (8 weeks)	
	No.	No.	%		No.	%
Set-A	140	110.0	78.6	20	17	85.0
Set-B	140	115.0	82.1	20	17	85.0
Set-C	140	134.0	95.7	20	18	90.0
Total explants	420	359.0		60		
Mean		119.7	85.5		17.3	86.7

6.3.1.4.4: *Mucuna pruriens* regenerating roots on liquid MS media.

An auxin (NAA) assay was performed using half strength M.S liquid medium supplemented with a range of NAA concentrations from 15.6 - 16.6 µM in an effort to induce root development in the cotyledonary nodal and hypocotyl tip explants of *M. pruriens* as described in Section 6.3.1.4.1. The NAA concentration range used for the assay was selected based on report by Sathyanarayana et al., (2012) that a concentration of 16.2 µM NAA induced rooting in *Mucuna pruriens* var utilis. Successful root development was achieved in two of the triplicate *M. pruriens* explants growing on half-strength M.S medium supplemented with NAA (16.2 µM) but not in any of the other explants in the NAA assay (Figure 6.12; Table 6.92).

Given the above positive result, 420 cotyledonary nodal and 420 hypocotyl tip explants of *M. pruriens* (140 explants for each experimental set trial) were transformed and MS supplemented with NAA (16.2 µM) was used as the rooting media. However, no root regeneration occurred in all subsequent experimental trials. It was however observed that although the seed stock used in the root induction assay had better regeneration potential, they produced seeds of low regeneration potential and these were used in the subsequent transformation experiments. The low regeneration potential could possibly have contributed to the lack rooting by the putative transgenic shoot explants.

Figure 6.12 Micropropagated *M. pruriens* regenerating root system on half-strength M.S liquid media supplemented with NAA (16 µM).

Table 6.92 *Agrobacterium* strain GV3101-mediated transformation of *M. pruriens* using cotyledonary nodal explants.

M.S liquid media supplemented with NAA (µM) (Non-transgenic explants)	No. of cotyledonary nodal explants. (Day-1)	No. of rooted Explants (4 weeks)
15.6	3	0
15.8	3	0
16.0	3	0
16.2	3	2
16.4	3	0
16.6	3	0
Total explants	18	

6.3.1.4.5: Detection of the *npt*II transgene in putatively transformed *Mucuna pruriens* by PCR.

To confirm that explants growing on kanamycin had indeed been successfully transformed, PCR was performed using primers for the *npt*II (neomycin phosphotransferase) kanamycin resistance gene on genomic DNA extracted from the explants as described in Section 6.2.3.5. The results obtained (Figure 6.13) show that the *npt*II transgene was detected by PCR analysis in 8 out of 10 genomic DNA samples extracted from leaf explants of putatively transformed *M. pruriens.*

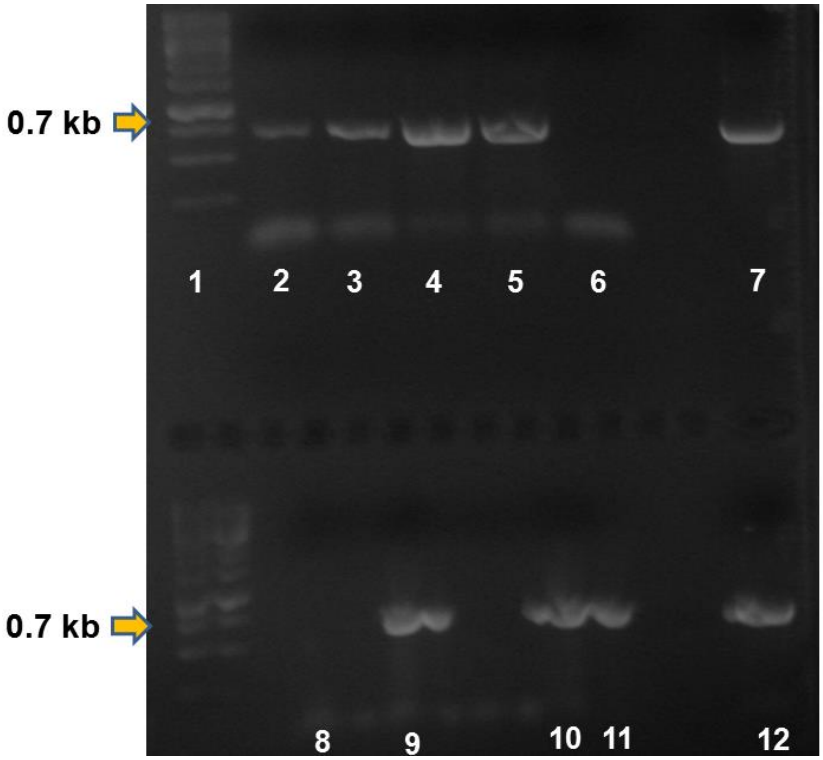


Figure 6.13 Gel electrophoresis shows the 0.749 kb *npt*II transgene PCR product (lanes 2, 3, 4, 5, 9, 10, 11 and 12) amplified from genomic DNA extracted from putative transgenic *M. pruriens*. Lane 6 is shows no *npt*II transgene in the genome of untransformed "wild type" *M. pruriens* (negative control) whereas lane 7 shows the approximately 0.749 kb *npt*II transgene PCR product amplified from a pGREEN-CO58 plasmid (Positive control).

Further validation for successful transformation of *M. pruriens* was by screening for the absence of *Agrobacterium* contamination on the surface of leaf explants and hence in the extracted DNA. Absence of *Agrobacterium* contamination was confirmed by PCR analysis to detect the tetracycline resistance (TetR) marker gene on the pSOUP plasmid using DNA extracted from the putatively transformed explants. No PCR product was obtained for the 0.5 kb TetR gene in the DNA samples of putatively transformed *M. pruriens* which had earlier tested positive for the *npt*II transgene (Figure 6.14). However, the tetracycline resistance gene was PCR amplified from a pSOUP plasmid, used as a positive control for the PCR (Figure 6.14).

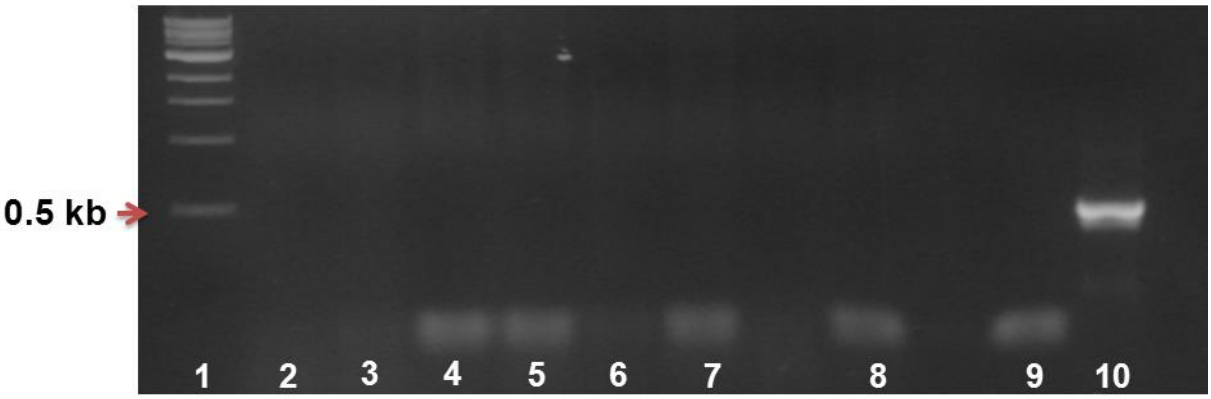


Figure 6.14 Gel electrophoresis shows a 0.5 kb TetR gene PCR product isolated from a pSoup plasmid (lane 10) as serves as a positive control for the PCR. However, detection of the tetracycline resistance gene by PCR analysis in genomic DNA extracted from putative transgenic *M. pruriens* was negative (lanes 2 - 8) and similarly, the gene could not be detected in the genomic DNA of untransformed *M. pruriens* (lane 9). Lane is a 1 kb DNA ladder (NEB).

6.4: DISCUSSION.

The aim of the work described in this chapter was to develop a protocol for genetic transformation of *M. pruriens.* A further aim was to silence or knock down expression of the Mp-ty/ddc gene with the aim of increasing L-DOPA levels through a reduction in its metabolism, and this required transformation of the *M. pruriens* with an RNAi construct for Mp-ty/ddc.

Firstly two experiments were conducted to establish the optimum level of kanamycin for selection of transgenics, and the maximum level of L-DOPA that would still allow normal seedling growth. The optimum kanamycin concentration obtained for selection of transgenic *M. pruriens* was 50 mg l^{-1}. A comparative study on transformation of other Fabaceae family species showed that the effective kanamycin concentration for selection of transgenic explants expressing *the npt*II gene was species dependent but ranged from 50 – 300 mg l^{-1} (Dillen et al., 1997; Marion, 2001; Yamada et al., 2001). For example Dillen et al., (1997) used 300 mg/l kanamycin to select transgenic *Phaseolus acufolius A*. Gray while Yamada et al. (2001) used 100 mg l^{-1} kanamycin to select transgenic *Vigna angularis.* The optimal concentration for selection of transgenic *M. pruriens* explants is therefore at the lower end but within the range used in similar plant transformation experiments of Fabaceae.

The L-DOPA assay revealed that levels of up to 50 mg l^{-1} did not affect germination and growth. However, the slower growth rate observed in L-DOPA (100 mg l^{-1}) assays suggest the plantlets were beginning to experience physiological stress due

to high L-DOPA levels both endogenously produced in the plant cells and that exogenously supplemented to M.S medium. Thus if the silencing construct was effective, then DOPA/tyrosine decarboxylase would not be encoded and consequently the L-DOPA produced by plant tissues would not be converted to dopamine. Since the gene manipulated affects the post L-DOPA synthesis stage, the Mp-ty/ddc gene silencing strategy may serve to conserve the amount of L-DOPA produced by the plant but may not affect the biosynthesis rate. However, there is a possibility that accumulation of L-DOPA in the plant tissues beyond a threshold of 200 mg l^{-1} for *M. pruriens* seedlings or explants (See Section 6.2.1.2) could initiate a negative feedback and stop L-DOPA biosynthesis. However, it has been observed that mature *M. pruriens* plant tissues especially seeds produce far more L-DOPA than the threshold concentration for seedlings or explants (Siddhuraju and Becker, 2001). Mature *M. pruriens* plants are more likely to be tolerant to enhanced L-DOPA accumulation in growing seed tissues as a result of successful silencing of the ty/ddc gene unlike the seedlings or explants which have leaves with a low threshold for exogenous L-DOPA. Clearly the L-DOPA assay experiment only tested the effect of exogenous L-DOPA and it would be useful to determine endogenous threshold levels, as uptake may not be complete. Another factor that would need to be tested is the regeneration of the explants producing higher DOPA levels, which was not tested here due to lack of time.

A number of approaches have been used to transform legumes (See Section 6.1.1). Critical parameters seem to be the choice of explant, regeneration media and choice of *Agrobacterium* strain. All of these important parameters were tested here. The hypocotyl, cotyledonary node, hypocotyl tip and leaf explants were tested but

successful plantlet regeneration and detection of the *npt*II transgene was achieved by using cotyledonary nodes and hypocotyl tip. The hypocotyl and leaf explants only succeeded in forming calli, thus the success of the new protocol may be due at least in part to the choice plant tissue used as explant for transformation. Some leguminous plants such *Vigna angularis* regenerated from hypocotyl stem explants (Yamada et al., 2001). Some species such as *Phaseolus vulgaris* required direct gene transfer into the apical meristem of seedlings by particle bombardment (Russell et al., 1993). On the other hand *Arachis hypogeum was* reported to be transformed by *Agrobacterium* using wounded embryonic axes as well as somatic leaf explants (McKently et al., 1995; Cheng et al., 1996). In contrast, *M. pruriens* could only form calli from leaf explants but without cell re-differentiation for organogenesis or shoot regeneration. A particular problem encountered was the lethal browning of explants caused by oxidation of phenolic compounds which are produced in large quantities especially in explants of higher plants like *M. pruriens* (Toth et al., 1994; Laukkanen et al., 1999; Arnaldos et al., 2001).

The best nutrient media for induction of root development in *M. pruriens* here was determined to be M.S liquid media supplemented with 16.2 µM NAA (Figure 7.3; Table 6.92) and as reported by Sathyanarayana et al. (2008). However, the explants in the consequent large scale experiments did not develop roots. The possible reasons for lack of root development are diverse. For instance unhealthy seed stock could generate unhealthy explants which in turn show poor genetic transformation and regeneration potential (Veltcheva et al., 2005).

Among the factors reported to significantly influence higher plant transformation efficiency is the strain of *Agrobacterium* used (Yamada et al., 2001). The results obtained in my study show that transformation efficiency obtained with *A. tumefaciens* strain GV3101 was higher than that obtained using AGLI, and the least efficiency was observed in LBA4404. The *Agrobacterium* strains used here differ in their genotype with respect to their virulence genes (See Table 6.2).

Yamada et al., (2001) reported maximum plant transformation efficiency when using higher concentrations of cytokinin and transformation inducer (acetosyringone). Dillen et al. (1997) too reported high transformation rates in *Arachis hypogea* upon using co-cultivation medium supplemented with high BA concentration (13 µM). Similarly, I obtained higher transformation rates in assays supplemented with higher cytokinin concentrations of BA (10 mg l^{-1}) and transformation rates gradually decreased with a decrease in BA concentration. Cytokinin induces cell division in plants and thus higher concentration could be required to sustain rapid cell division of the transgenic cell to form callus and to re-differentiate to regenerate the whole plant (Birch, 1997; Hellens and Mullineaux, 2000; Yamada et al., 2001). On the other hand acetosyringone induces the *Agrobacterium* to infect the plant cells and in due process transfer transduce the T-DNA into the plant genome. Both acetosyringone and BA play synergistic roles in ensuring gene transduction and cell proliferation to regenerate plant parts (Hellens and Mullineaux, 2000).

However, despite the difficulties encountered with rooting of *M. pruriens* explants, the success of transformation was based on kanamycin resistance and PCR analysis for presence of transgene, *npt*II as described in Section 6.2.3.5. The explant was also

screened for no *Agrobacterium* contamination as described in Section 6.2.3.6, before being confirmed as transgenic based on detection the *npt*II transgene by PCR analysis.

The PCR results were very encouraging, indicating that the *M. pruriens* had taken up the *nptII* gene but that the positive PCR result was not due to residual *Agrobacterium* growth since the PCR result for TetR was negative. Further work would be required to establish whether the transgene is being expressed and whether it is heritable. This requires the transgenic plants to root, grow to maturity and produce seeds. The new generation seeds would then be grown and DNA would be extracted from the seedlings and screened by PCR for presence of *npt*II transgene. Similarly, the successful silencing of the Mp-ty/ddc would further be confirmed by determining the gene expression profile in both the parent and the succeeding generation of *M. pruriens* plants.

In all I developed two methods for transformation of *Mucuna pruriens* using a cotyledonary node and a hypocotyl tip explants.

CHAPTER 7: GENERAL DISCUSSION.

The aim of the work presented in this thesis was to isolate and characterise genes related to L-DOPA metabolism from the legume *Mucuna pruriens,* and to attempt to manipulate levels of L-DOPA in this species through development of a transformation system.

This species was chosen because unlike most other L-DOPA producing plants, *M. pruriens* produces fairly high quantities (Wichers et al., 1993; See Table 1.0). This would give the best chance of success for manipulations to increase further L-DOPA production in tissues of *M. pruriens* to the target yield of 10 mg l^{-1} required for commercial pharmaceutical extraction (Hellwig et al., 2004; Xu et al., 2012). The disadvantage of *Mucuna pruriens* is that it has not been extensively studied to date hence extensive gene sequence information and a plant transformation system were not available at the start of the project.

In light of the above, my PhD research started with confirming the levels of L-DOPA in *Mucuna pruriens* (Chapter 3), which were indeed found to be fairly high compared to most other natural sources of L-DOPA (See Table 1.0). This encouraged me to work on the isolation and characterisation of enzymes involved in L-DOPA biosynthesis and metabolism. From work on better-studied species, the key genes involved are tyrosine hydroxylase (tyoh) and DOPA/tyrosine decarboxylase (ty/ddc) responsible for conversion of L-tyrosine to tyramine, and L-DOPA to dopamine respectively (Chattopadhyay et al., 1994; Figure 1.8). Attempts to isolate, clone and sequence the tyrosine hydroxylase gene (tyoh) using a degenerate primer PCR approach as described in Chapter 2 Section 2.2.5.1 was not successful for any of the

primers designed from the different conserved sequence regions of the protein sequences (See Appendix V.II). This was due to lack of adequate sequence data for plant tyoh on the NCBI data base for use to design appropriate degenerate primers (See Appendix V.II). However it was possible to isolate an almost complete sequence for a putative L-DOPA decarboxylase gene (Chapter 4) and characterise it (Chapter 5). This opened the possibility of using an antisense strategy (Sambrook et al., 1989) for down-regulation of this gene to increase L-DOPA levels since it was hypothesised that one reason for low levels of L-DOPA in plants is the activity of decarboxylases that metabolise it to dopamine. The next challenge was the design of a functional transformation system for *Mucuna pruriens* (Chapter 6).

So as a result of this work I have assembled many of the components needed to reach my original aims (See Section 1.4). In this chapter I will now assess in more detail how far my aims have been reached and the work needed to fully reach them. I will also assess how this work might be taken forward.

7.1: Expression of a putative DOPA/tyrosine decarboxylase in relation to levels of L-DOPA.

The sequence evidence revealed that Mp-TY/DDC drew 74% amino acid homology with DOPA/tyrosine decarboxylases (TY/DDC) from species of the same taxonomic family as *M. pruriens* (See Figure 4.23). In contrast, tyrosine decarboxylase (TYDC) drew 50% homology with Mp-TY/DDC for species in the same family (Fabaceae) with *M. pruriens*. Based on percentage homology, Mp-TY/DDC can then be proposed to be more likely a TY/DDC rather than a TYDC. The expression of the Mp-ty/ddc gene in *M. pruriens* was significantly higher in root and stem tissues than

in seed tissue while leaf tissues showed relatively very low Mp-ty/ddc expression (Figure 4.25). In contrast L-DOPA-content in *M. pruriens* seed tissues was 15-fold higher than that in stem and leaf tissues and 17-fold that in root tissues (Table 3.1). This almost converse pattern of gene expression and L-DOPA levels suggests that if the Mp-ty/ddc gene is indeed a functional L-DOPA decarboxylase, it may indeed be important in regulating levels of L-DOPA by conversion to dopamine. It may therefore be useful to measure levels of dopamine in tissues of *M. pruriens*. The low L-DOPA-content in leaves despite the low expression of Mp-ty/ddc suggests L-DOPA is produced at low concentrations in leaves. A similar gene, from *Papaver somniferum* Ps-ty/ddc was also expressed at low levels in leaves (Facchini and De Luca, 1995). In addition, Facchini and De Luca (1995) demonstrated a relationship between expression of ty/ddc homologues with presence tertiary derivatives of dopamine such as morphine and isoquinoline alkaloids in stem and root tissues of *P. somniferum*. However, the Ps-ty/ddc expression in seed capsule of *P. somniferum* was reported to be low despite presence of high levels of isoquinoline alkaloids (a tertiary derivate of dopamine) (Facchini and De Luca., 1995). Further analysis suggests the isoquinoline compounds were produced in the stems and roots of *P. somniferum* and were transported to the seed capsule (Facchini and De Luca, 1994). Thus at least in *P. somniferum* the dopamine is further metabolised, hence also in *M. pruriens* analysis of dopamine levels may not be sufficient to infer the regulatory activity of the Mp-ty/ddc enzyme.

In a bid to determine the relationship between Mp-ty/ddc gene expression and L-DOPA or dopamine biosynthesis, I performed enzyme activity assays as described in Chapter 5 Section 5.2.1.6 - 7. It was determined by SDS page fractionation

analysis that Mp-ty/ddc encoded a 56 kd Mp-TY/DDC protein fusion which was heterologously expressed in the total insoluble protein extract from bacterial culture although it was not clearly detected in the soluble fraction. However, heterologously expressed Mp-TY/DDC did not show enzyme activity upon treatment with putative substrates: L-DOPA and tyrosine, or with structurally related compounds such as a dopamine (See Chapter 5 Section 5.3.4-5). The possible causes for the lack of Mp-TY/DDC fusion enzyme activity are diverse and include production of insufficient quantities of soluble functional proteins (Sambrook et al., 1989; Wang et al., 2010). Low solubility of expressed proteins is likely to be due to be misfolding of the protein (Wang et al., 2010). The possible causes for protein misfolding are diverse but it could have been a result of over-expression leading to the aggregation of the majority of protein to aggregate in inclusion bodies in inactive form which is insoluble (Sambrook et al., 1989). The view is supported by the report that pET21b vector system I used for cloning and heterologous expression of Mp-TY/DDC was designed for protein over-expression (pET21b expression vector system user manual, Novagen, USA). However, despite several reports of proteins forming inclusion bodies when over-expressed, equally there reports of proteins have been over-expressed but did not form inclusion bodies (Liu et al., 2012). The notion that low levels of soluble protein production accounted for the apparent lack of enzyme activity exhibition by heterologous expressed Mp-TY/DDC fusion protein is further supported by the observation that soluble Mp-TY/DDC protein could not be detected by SDS page electrophoresis (Chapter 5, Figure 5.11) but was detected by western blot analysis (Figure 5.15). The western blot analysis is very sensitive and able to detect several-fold lower concentrations of proteins in solution than could be done by SDS page electrophoresis (Sambrook et al., 1989). Attempts were made to increase

the proportion of soluble protein by conducting assays for culture temperature and IPTG concentration as described in Chapter 5 Section 5.2.1.2. Further possible ways of improving recombinant Mp-TY/DDC protein expression include co-expression of molecular chaperones (proteins in *E.coli* which help in protein folding) at higher levels together with the heterologous proteins (Sambrook et al., 1989). In addition, fusion of NusA or Yeast small ubiquitin-related modifier (Yeast SUMO) with heterologously expressed proteins is reported to increase the protein solubility when compared to the His-tag fusion I used for Mp-TY/DDC expression (Wang et al., 2010). Further options include heterologous expression using eukaryotic systems such as *Saccharomyces cerevisiae*. This has the advantage of producing large quantities of recombinant protein directly into the culture media (Hellwig et al., 2004; Xu, et al, 2012). In addition, heterologous expression using eukaryotic systems enable post-translational modifications which are normally required by many eukaryotic genes but cannot be performed by *E.coli* because they lack the necessary enzymes. In addition, amino acid sequences for the cloned DOPA/tyrosine decarboxylases from other species in the family Fabaceae of *M. pruriens*, which drew very significant homology with Mp-TY/DDC, were 2 - 20 amino acids longer at the N-terminus (Figure 4.23). This suggested presence of an incomplete N-terminus of the cloned putative Mp-TY/DDC from *M. pruriens*, which may mean that the fusion polypeptide chain did not fold in the correct form and hence may account for the lack of solubility and enzyme activity (Sambrook et al., 1989). However, there are reports on heterologous expression of partial polypeptides in some proteins (Wang et al., 2010).

In light of the challenges involved in expressing the Mp-TY/DDC fusion protein, a further bid to verify whether the expressed Mp-TY/DDC was a DOPA/tyrosine

decarboxylase was to analyse the sequence on the Protein data bank (PDB) to draw similarities with characterised proteins on the data base. Mp-TY/DDC drew homologies and similarities with the human DOPA decarboxylase on the PDB (Figure 5.9). The fact that DOPA decarboxylase like DOPA/tyrosine decarboxylase, converts L-DOPA to dopamine despite being derived taxonomically from very distant species in animal and plant kingdoms respectively supported the suggestion that Mp-TY/DDC is likely to be a DOPA/tyrosine decarboxylase. In light of the above, a potential way to over-come the challenge of the putative missing amino acids on the Mp-TY/DDC would be by generation of a synthetic N-terminus based on sequence homology with other plant DOPA/tyrosine decarboxylases (Fujimori, 2009). A library would be constructed by allowing non-conserved residues to vary while keeping conserved ones the same. Another approach would be to sequence the transcriptome from a plant tissue which produces the highest levels of L-DOPA to identify a full length sequence for this gene. The advantage of obtaining transcriptome sequence is that other genes related to L-DOPA biosynthesis or metabolism might be identified by homology to known sequences (Sambrook et al., 1989). Besides DOPA/tyrosine decarboxylase, the other L-DOPA biosynthesis pathway, tyrosine hydroxylase (TYOH), is reported to control conversion of L-tyrosine to L-DOPA in plants (Chattopadhay et al., 1994; Facchini, 2001). However, given the lack of adequate sequence data for the tyrosine hydroxylase gene (tyoh) on publically available data bases, gene isolation was not possible.

Full length sequences could then be obtained by RACE if not already derivable from the transcriptome sequences and could then be tested *in vitro*. This would also be good strategy in the event that the Mp-TY/DDC is in fact a different gene from DOPA/tyrosine decarboxylase (Sambrook et al., 1989).

7.2: Putative transformation of *M. pruriens*.

After isolation, characterisation and analysis of the Mp-ty/ddc gene and gene product (Mp-TY/DDC) as discussed in Section 7.2, an Mp-ty/dcc gene RNAi construct was designed as described in Chapter 6, Section 6.2.9.2. The aim was to increase L-DOPA levels in plant tissues by knocking out or down-regulation of the DOPA/tyrosine decarboxylase which controls the conversion L-DOPA to dopamine (Facchini, 2001). A putative full-length ORF anti-sense was used in the construct to silence the Mp-ty/ddc because it is reported to be effective in some plants (Helliwell and Waterhouse, 2003; Wang and Xu, 2008). However, for dicots the most efficient gene-silencing model is the "hairpin RNAi construct" designed in such a way that the sense portion anneals to antisense upon transformation to appear like a dsRNA or hairpin loop in the transgenic plant cells. This activates dicer to cleave the RNA transcripts having similar sequences to that of the "hairpin" RNAi construct (Helliwell and Waterhouse, 2003). The immediate challenge for this work however, was that the gene silencing constructs required transformation into the plant (Helliwell and Waterhouse, 2003; Schmidt et al., 2012) but there were no reports on transformation of *M. pruriens.* In light of the above, efforts were geared towards establishing an effective transformation method for *M. pruriens* and after which the effectiveness of different gene-silencing models would be compared to determine the best model for optimising of L-DOPA synthesis in *M. pruriens*.

I therefore developed a protocol for *Agrobacterium*-mediated transformation of *M. pruriens* using cotyledonary nodal and stem tip explants (See Chapter 6 Section 6.2.3.4). The transformation efficiency was above 80% for both cotyledonary nodal and stem tip explants (See Table 6.8 and 6.9 respectively) based on kanamycin resistance (Figure 6.11) and transgene detection by PCR (Figure 6.13) observed in

putative transformed plants but absent in untransformed control plants. This suggests successful transformation (Hellens and Mullineaux, 2000). Absolute confirmation of successful plant transformation requires the transgene to be expressed both in the transgenic plant and its offspring generation. In my work, the transgene was confirmed as present in the transformed *M. pruriens* plants (See Figure 6.13). However a key difficulty encountered was in developing a root system and as consequence it was not possible to regenerate whole plants and to further confirm *M. pruriens* transformation by PCR screening for the *npt*II transgene genome of succeeding generations. In addition due to the long life-cycle (5 months) of *M. pruriens* (90 day cultivar), the long time required to do experiments, and a shortage in the supply of the parental stock of *M. pruriens* ("90 day" cultivar), the scope of my work did not therefore include studies on the offspring generation.

The advent of a transformation protocol for *M. pruriens* in this study opens up opportunities for *in vitro* molecular analysis studies on the biochemical pathways in the plant including manipulation to favour production of novel products such as L-DOPA. Specifically a key experiment is to confirm the down-regulation of the Mp-ty/ddc expression profile in *M. pruriens* explants putatively transformed with an Mp-ty/ddc RNAi cassette and how it relates to L-DOPA levels in different tissues of *M. pruriens* putatively transformed with the Mp-ty/ddc-RNAi cassette. The RNAi construct was built based on the hypothesis that Mp-ty/ddc gene codes for DOPA/tyrosine decarboxylase which is responsible for conversion L-DOPA to dopamine in *M. pruriens,* and hence silencing the Mp-ty/ddc would result in accumulation of L-DOPA. The above hypothesis was also based on the findings

obtained by Southern blot analysis (Chapter 4 Section 4.3.6) that Mp-ty/ddc was a single copy gene.

All *M. pruriens* ("90 day" cultivar) tissues tested had L-DOPA levels which are significantly lower than the L-DOPA tolerance threshold of 200 mg ml^{-1} L-DOPA concentration (Table 6.2). This therefore allows manipulation of the synthesis pathways to enhance L-DOPA production. However, as the commercial production requirements for natural products is 10 mg ml^{-1} (Xu et al., 2012), additional approaches would be required such as using transgenic cell culture approaches and heterologous expression using bacterial and yeast systems (Sambrook et al., 1989).

7.3: Future work.

The short term future work include: establishing a rooting system for the transgenic *M. pruriens* plants. The effects of the antisense construct could then be tested fully on the parental and the subsequent offspring generations of *M. pruriens*. This would then enable experiments to establish an appropriate RNAi system (antisense or "hairpin" RNAi) (Schmidt et al., 2012) for silencing the putative DOPA/tyrosine decarboxylase. The effect on the L-DOPA profile in different tissues of *M. pruriens* could then be established. Another key objective of future work would be to further verify the enzymatic activity of the cloned putative *M. pruriens* DOPA/tyrosine decarboxylase to establish whether it is indeed able to catabolise L-DOPA *in vitro*.

The long term future plans include resolving the possibility of a negative feed-back loop (Moreno et al., 2013) being activated to stop L-DOPA levels from increasing in

tissues of *M. pruriens* after silencing the DOPA/tyrosine decarboxylase responsible for conversion of L-DOPA to dopamine. One strategy would involve developing cell suspension cultures to secrete the L-DOPA produced into the culture medium which may not activate the negative feed-back loop on L-DOPA production in the plant cells (Xu et al., 2011). Production of natural products by using plant cell cultures has many advantages over using whole plant systems and these include: the rapid growth of cell suspension cultures with cell doubling occurring in some cases in a day, products that can be produced more consistently using controlled bioreactors and require fewer environmental regulatory compliance requirements (Xu et al., 2012). In addition, natural products produced by cell suspension cultures require simple purification from a well-defined culture media (Sharma et al., 2004). On the other hand, the yield for natural products derived from plant cell culture is currently too low (0.00001- 0.2 mg ml^{-1}) for commercial purposes. However, advances in molecular pharming have produced yields of several natural products and proteins beyond the 10 mg ml^{-1}threshold required for commercial purposes (Hellwig et al., 2004). Nevertheless, there is a need to further improve the product yield by 5 - 10 fold to meet the desired target profit margins required by the molecular pharming industry (Xu et al., 2012).

Besides silencing the Mp-ty/ddc gene in plant cell cultures, another approach to enhance L-DOPA production in *M. pruriens* would be by over-expression of tyrosine hydroxylase (Mp-tyoh) gene which is responsible for conversion of L-tyrosine to L-DOPA (Chattopadhyay et al., 1994). The Mp-tyoh gene could be isolated by construction of a cDNA library (Sambrook et al., 1989) or by more recent transcriptomic approaches. Studies could then be performed on over expression of

biosynthetic genes such as the Mp-tyoh gene *in vivo* using whole plant systems, cell cultures or *in vitro* by heterologous expression using bacteria and yeast systems as highlighted in Section 7.2 above. The relationship between the Mp-tyoh gene and L-DOPA production *in vitro* or *in vivo* would be determined by measuring the corresponding changes in L-DOPA profile by Reverse phase-HPLC as described in Chapter 3 Section 3.3.3.

Further work would involve identifying promoter regions for L-DOPA synthesis pathway genes of *M. pruriens*, whose entire nucleotide sequences and adjacent sequences to the gene would have been fully confirmed by sequencing. Sequences flanking each of the L-DOPA synthesis or metabolic pathway genes of interest could be tested by reporter gene/ or eGFP reporter fusions (Fujimori, 2009) to verify expression patterns. This would enable better *in situ* approaches for manipulation of the DOPA genes especially in cell-suspension cultures.

In conclusion, despite the many obstacles that need to be overcome, progress has been made in this work towards manipulating *M. pruriens* for the production of commercially interesting levels of L-DOPA. Although clearly this would require substantial investment of time and resources, there is a strong need to produce a product free from contaminating enantiomer and other by-products of chemical synthesis that result in unacceptable side effects for the use of L-DOPA in the treatment of Parkinson's disease. This becomes an increasingly pressing problem now in many countries with a growing elderly population.

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APPENDIX IV: ISOLATION AND CHARACTERISATION OF A *MUCUNA PRURIENS* PUTATIVE DOPA DECARBOXYLASE GENE (*Mp-ddc*).

IV.I: INVERSE PCR FOR ISOLATION OF THE FULL Mp-ty/ddc GENE.

IV.I.I: INTRODUCTION.

A 1,727 bp portion of Mp-ty/ddc was isolated from genomic DNA, leaf and seed cDNA of *M. pruriens* by a number of approaches. These included, degenerate PCR on genomic DNA template which was used to obtain the initial Mp-ty/ddc gene sequence. This was followed by 3'RACE on leaf cDNA template and this was used to isolate the 3'end of the Mp-ty/ddc gene, 5'RACE on seed and leaf cDNA which was used to obtain the 5'end sequence. However, based on sequence alignments with DOPA/tyrosine decarboxylases of closely related species to *M. pruriens*, the Mp-TY/DDC is probably missing the first 20 amino acids at the 5'end which could not be isolated by 5'RACE. Inverse PCR was therefore attempted to isolate this putatively missing sequence.

IV.I.II: MATERIALS AND METHODS.

IV.I.II.I: Principle of Inverse PCR and primer design.

Inverse PCR is a variant of PCR in which DNA of unknown sequence but flanking a known sequence can be amplified. The principle of inverse PCR is summarised in Figure IV.I.

The technique involves digestion by a restriction enzyme of genomic DNA containing the known sequence and its flanking region. The individual restriction fragments are converted into circles by intramolecular ligation, and the circularized DNA is then used as a template in the PCR. The unknown sequence is then amplified by two primers that bind specifically to the known sequence and point in opposite directions. The product of amplification reaction is a linear DNA fragment containing a single site for the restriction enzyme originally used to digest the DNA.

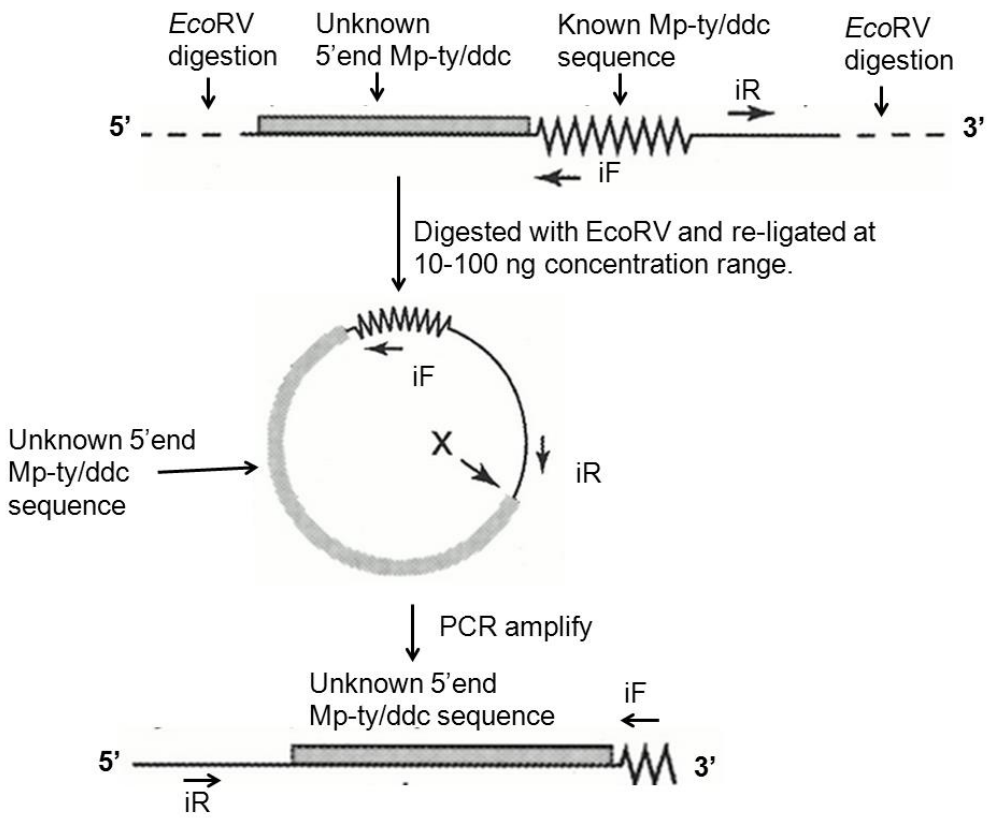


Figure IV.I Schematic diagram illustrating the principle of IPCR. DNA was digested by *Eco*RV on either side of the known and adjacent unknown sequence. At low DNA concentrations, the digested *Eco*RV sites at the 5' and 3' ends should re-ligate forming circular DNA joined at point X. Two opposite facing primers designed from the known sequence region were used to PCR amplify into the unknown sequence.

IPCR was used to amplify the putative missing 65 bp at the 5' end of the Mp-ty/ddc gene, isolated and sequenced in Chapter 4. The size of the complete Mp-ty/ddc gene sequence was estimated from the average size (1.8 kb) of the same gene in taxonomically closely related species to *M. pruriens* on the NCBI data bank (www.ncbi.nih.gov/BLAST/). Suitable restriction enzyme sites present both in the known Mp-ty/ddc gene sequence and the 5' UTR were determined by Southern analysis on genomic DNA of *M. pruriens* as described in Section 4.2.5. The Southern analysis result (Section 4.3.6) revealed that digestion of *M. pruriens*

genomic DNA using *Eco*RV produced a 4 kb signal when using the Mp-ty/ddc gene probe . The DNA fragment was released by double digestion with *Eco*RV with the first site at position 1.5 kb at the 3' end of the Mp-ty/ddc ORF shown in Figure D.2 and the second *Eco*RV site deduced to be in the 5'utr due to presence of the Mp-ty/ddc 4 kb hybridisation signal.

IV.I.II.II: Isolation of the 5'end sequence of Mp-ty/ddc by IPCR.

IPCR primers were designed as described in Section 2.2.5.1 from the known Mp-ty/ddc gene sequence portion. IPCR forward and reverse primers; (iF1): 5'-CTGATGGATGATGAGTATAGGGTG-3', and (iR1): 5'-GTGGAGAGAAGAGAGTCAGGAG-3' respectively, were positioned approximately 0.7 kb apart and in opposite orientation (Figure D.3 a) on the inverse PCR template described below. Nested forward and reverse IPCR primers; iF2: 5'-GATTATGGGCATGGAAGTTG-3' and iR2: 5'-GGAAATTTGCTGGATGGATC-3', respectively positioned approximately 0.9 kb apart (Figure D.3a) were used in an attempt to re-PCR amplify the IPCR product as described below.

The Inverse PCR template was generated from *M. pruriens* genomic DNA by double digestion using *Eco*RV (New England BioLabs Inc., UK). The DNA digestion reaction was set up in 1.5 ml Eppendorf tubes as follows; DNA (5 µg), 10 X NEB buffer 3 (15 µl) and *Eco*RV (4 units) (New England BioLabs Inc., UK) reagents were made up to 150 µl volume with sterile water. The DNA digestion reaction tubes were vortexed and then spun for a maximum of 10 seconds in a microcentrifuge (Biofuge 13, Heraeus. Instruments, Germany) and contents were collected at the bottom of

the tubes. The DNA was restriction digested overnight for 14 - 16 hours in a 37 ˚C incubator (Heratherm Incubator - ThermoScientific, Germany). The digested DNA (Figure IV.II) was then purified from the enzyme reaction mix by phenol-chloroform extraction followed by isopropanol precipitation as described in Section 2.2.17 and 2.2.18 respectively.

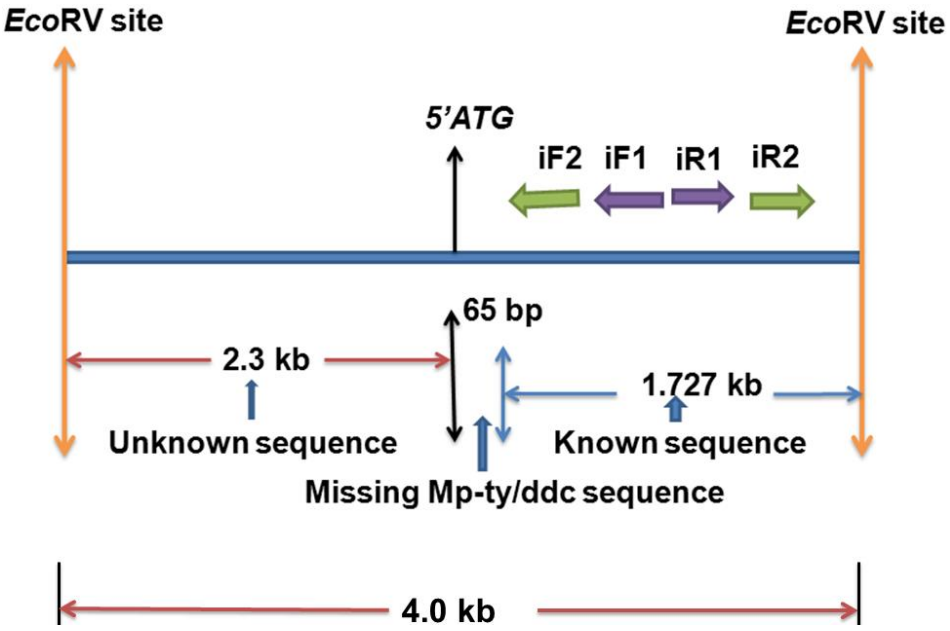


Figure IV.II A schematic diagram of the 4kb DNA fragment produced by *Eco*RV digestion of gDNA extracted from *M. pruriens.*

The recovered digested DNA was resuspended in sterile water to a stock concentration of 300 µg ml^{-1}. This was used to prepare low DNA concentrations of 0.1, 0.25, 0.5 and 1 µg ml^{-1} which were used to set up a series of re-ligation reactions using;

template DNA	(40, 100, 200 and 300) ng,
10X ligation buffer (Promega,	50 µl
T4 DNA ligase	(4 units) and
sterile water	to 400 µl

The ligation reaction was incubated for 12-16 hours at 16˚C (Figure IV.III a).

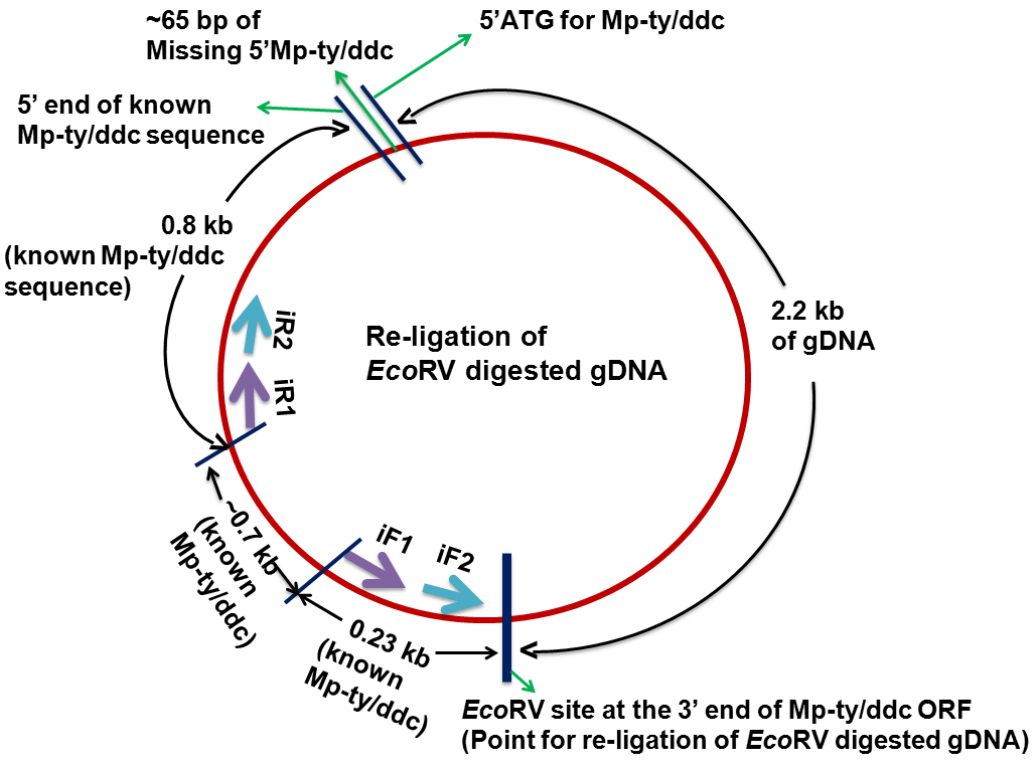


Figure IV.III(a) A schematic diagram of the 4 kb DNA molecule formed by re-ligation at low concentration of the *Eco*RV digested *M. pruriens* genomic DNA. The 3.3 kb DNA portion containing the 65 bp of unknown 5' end Mp-ty/ddc sequence was PCR amplified using IPCR primers iF1 and iR1.

Following ligation the DNA was purified by phenol-chloroform extraction followed by isopropanol precipitation as described in Section 2.2.17 and 2.2.18 respectively and stored at -20˚C. The 0.1 µg ml^{-1}DNA re-ligation reaction sample was used as a template for inverse PCR amplification. Separate experimental trials were performed for the 0.25, 0.5 and 1 µg ml^{-1}DNA re-ligation reaction samples as templates for their respective inverse PCR amplification. The inverse PCR reagents were set up in 0.5 ml PCR tubes as described in KOD Hot Start DNA Polymerase kit protocol (Novagen, UK) using;

2X Xtreme buffer	(25 µl),
dNTP mix	(2 mM; 10 µl),
iF1	(10 Mm**;** 15 µl)
iR1	(10 mM, 1.5 µl),
re-ligated template DNA	(0.1, 0.25, 0.5 and 1.0 µg ml^{-1}),
KOD Hot Start DNA Polymerase	(1.5 units) and
sterile water	to 50 µl

iF1 (10 Mm**):**; 15 µl) A negative control reaction was set up as described above with the exception of template DNA whereas for the positive control reaction, the DNA template was replaced with a pET21b plasmid containing a 1.5 kb Mp-ty/ddc transgene clone. The control and the experiment inverse PCR reagents were run on a PTC-100 thermal cycler (MJ Research Inc., Waltham, USA) using the following thermal cycling conditions: 94 ˚C, 2 minutes; 94 ˚C, 15 seconds; 34 cycles (60 ˚C, 30 seconds; 68 ˚C, 6 minutes); 68 ˚C, 10 minutes. 10 µl of inverse PCR reaction

samples (Figure IV.III b) were analysed by agarose gel electrophoresis as described in Section 2.2.7.

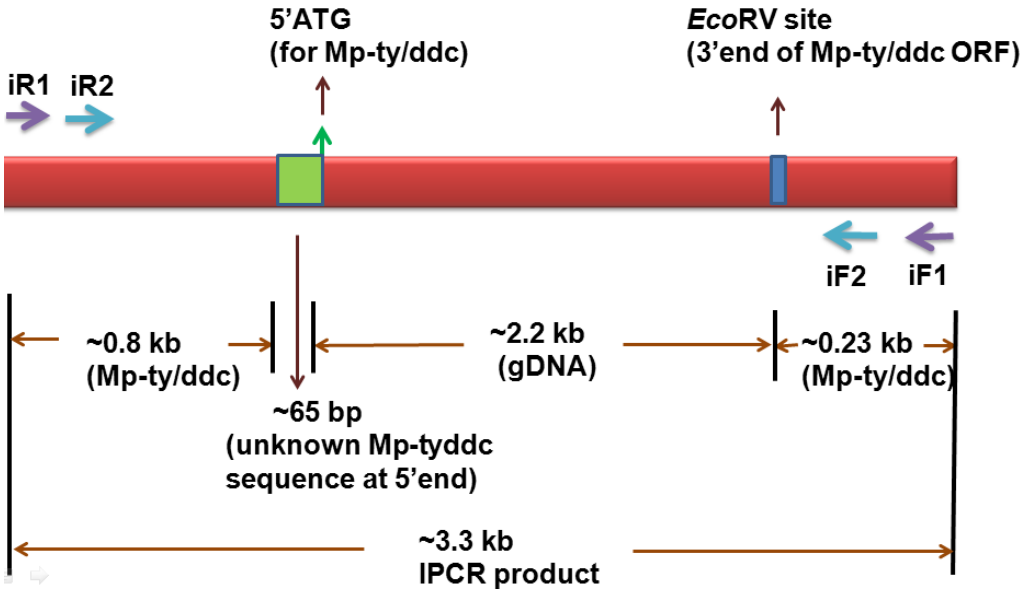


Figure IV.III (b) A schematic diagram of the 3.3 kb inverse PCR product amplified using primers; iF1 and iR2 from re-ligated *Eco*RV digested *M. pruriens* genomic DNA.

The primers were used to PCR amplify from the re-ligated DNA template, 3.3 kb IPCR product that should contain the approximately 65 bp of unknown sequence at the 5' end of Mp-ty/ddc (Figure IV.III b).

IV.I.III: RESULTS.

IV.I.III.I: Isolation of the 5' terminal sequence of the Mp-ty/ddc gene by inverse PCR.

A 3.3 kb inverse PCR product was isolated from the re-ligated circular DNA amplified using Forward and Reverse Mp-ty/ddc primers; iF1and 5'-iR1 as shown in Figure IV.IV (b). A band, of the expected size (4.5 kb) was obtained from the positive control.

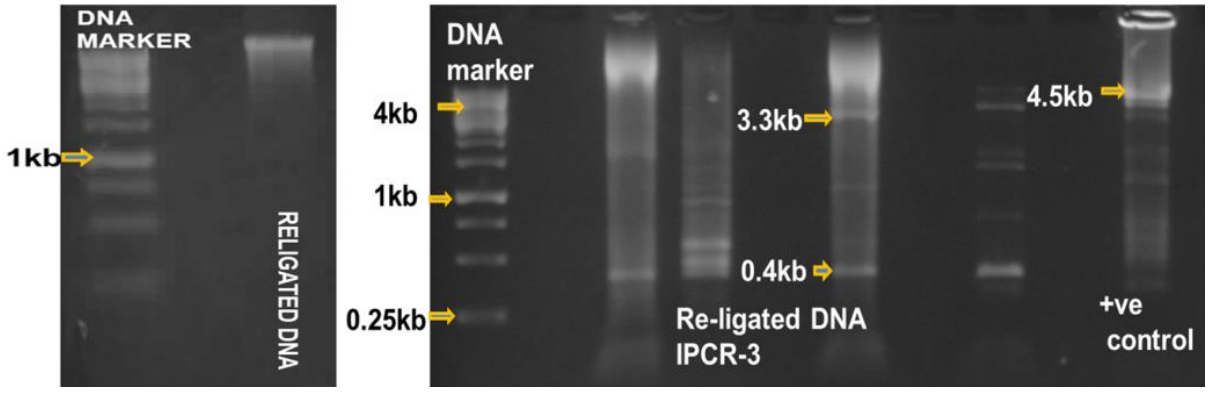


Figure IV.IV (a) Gel electrophoresis showing the *Eco*RV DNA fragment which was re-ligated by joining the *Eco*RV site at the 5' UTR to that at the 3' end of the Mp-ty/ddc gene (lane 2). Figure IV.IV(b) lane 2 shows a 3.3 kb DNA band which was generated by inverse PCR. Lane 3 shows the 4.5 kb Mp-ty/ddc IPCR product which was amplified from the pET21b-Mp-ty/ddc plasmid as a positive control.

The purified 3.3 kb inverse PCR product was however of too low concentration to be sequenced directly whereas attempts to re-amplify it using NEB Taq polymerase

(New England BioLabs, UK) and “nested” inverse PCR primers; iF2and iR2, were futile as shown by Figure IV.V.

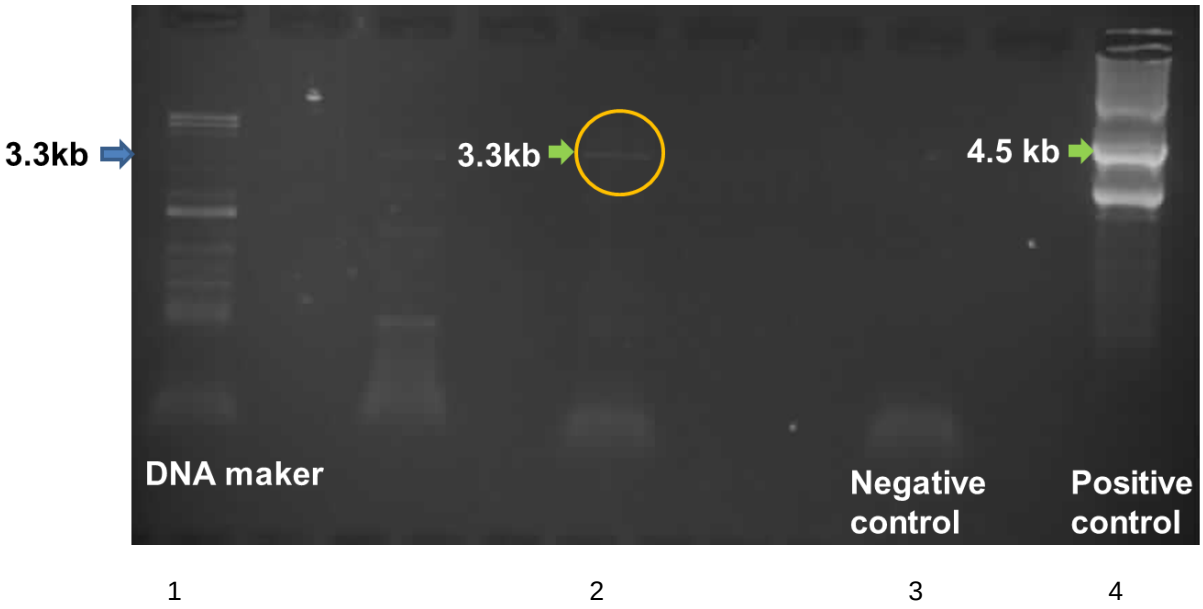


Figure IV.V Gel electrophoresis of inverse-PCR products: lane 2 shows a very weak 3.3 kb DNA band which was generated by IPCR using nested IPCR primers; iF2 and iR2. Lane 3 shows no product was obtained upon using water a template for IPCR (negative control), whereas in lane 4, a 4.5 kb Mp-ty/ddc DNA band was amplified from the pET21b-Mp-ty/ddc plasmid by IPCR and served as a positive control experiment.

IV.IV: DISCUSSION.

The predicted size of the re-ligation product for *Eco*RV digested genomic DNA of *M. pruriens* was 4 kb based on the Southern blot analysis (Section 4.3.6). The 4 kb re-ligated genomic DNA was used as a template for inverse PCR amplification. However, the size of the inverse PCR product was 3.3 kb because the primers; iF1 and iR1 used in IPCR, were designed to anneal at positions approximately 0.7 kb apart and in opposite orientations on the re-ligated circularised DNA template (Figure IV.iii a). The resulting first inverse PCR amplification cycle generated a 3.3 kb linearized DNA PCR product. This served as template for second and subsequent PCR re-amplification cycles to generate the 3.3 kb inverse PCR product obtained (Figure IV.iv b). However, efforts to re-amplify the IPCR product by nested PCR for direct sequencing or cloning were not successful. However this approach could be used in further efforts to obtain the 5' end of the gene.

IV.II: ISOLATION OF PUTATIVE TYROSINE HYDROXYLASE FROM *MUCUNA PRURIENS*.

IV.II.I: INTRODUCTION.

Tyrosine hydroxylase (TYOH) is the enzyme responsible for bioconversion of L-tyrosine to L-DOPA in plants (Pras et al., 1995; Taiz and Zieger, 2006). It is encoded by tyrosine hydroxylase gene(s) (tyoh) in plants and other organisms. The aim of this experiment was to isolate and sequence tyrosine hydroxylase gene(s) (Mp-tyoh) from *M. pruriens* with a longer term aim manipulating it. However, there was hardly any plant TYOH sequence on the NCBI and other publicly accessible data bases. In light of the above, I designed degenerate primers (see Chapter 2 Section 2.2.5.2) from conserved sequence regions of TYOH available even though they belonged to taxonomically species, which I used in attempts to isolate the Mp-tyoh by PCR as described in Chapter 2 Section 2.2.6.

IV.II.II: METHODS AND MATERIALS.

IV.II.II.I: Plant material.

M. pruriens seeds were procured from Echo seed bank (USA) and grown in the under greenhouse conditions as described in Chapter 2 Section 2.1.2.

IV.II.II.II: Extraction of genomic DNA.

Genomic DNA was extracted from young leaves of *M. pruriens* plants as described in Chapter 2 Section 2.2.1.

IV.II.II.III: Designing Degenerate Primers and PCR.

Degenerate primers were designed as described in Chapter 2 Section 2.2.5.2 and were used to isolate the Mp-tyoh gene(s) by PCR approach as described in Chapter 2 Section 2.2.6.

IV.II.II.IV: Gene cloning, Sequencing and Analysis.

This was performed as described in Chapter 2 Sections; 2.2.11 and 2.2.15 respectively.

IV.II.III: RESULTS.

This experiment was designed to isolate from *M. pruriens* genome, the tyrosine hydroxylase gene(s) which encodes enzymatic bioconversion of L-tyrosine to L-DOPA in plants. However, due to very limited sequence information for both the gene and *M. pruriens* on the NCBI and other public data bases, degenerate primers used in attempts to isolate the gene by PCR approach were designed from the few available sequences as described above despite being of diverse taxa. TYOH sequences for species; *Mythmna* (gi:3439252) *Felis* (gi:7774525), *D. rerio* (gi:1692347), *M. musculus* (gi:6678337),, *Apis* (gi:5858520), *Rattus* (gi:339681), *Tyr 4* (gi:6981652), *Tribolium* (gi:163751), *B. taurus* (gi:1495887), *S. aurata* (gi:1148421) , *G. gallus* (gi:6523293) , *Schistosoma* (gi:2613098), *Xenopus* (gi:1479041), *Tyr* (gi:163797), *Physcomitrella* (gi:1680449) *and Chlamydomonas* (gi:1594636) on the NCBI data bank were aligned using the genedoc software program (see Chapter 2 Section 2.2.5.2). Conserved amino acid sequences; DHPGF (position- 260) and YWFTVEFG (position 440) shown in the Figure IV.I below were used to design forward and reverse degenerate primers respectively used to isolate the Mp-tyoh by PCR.

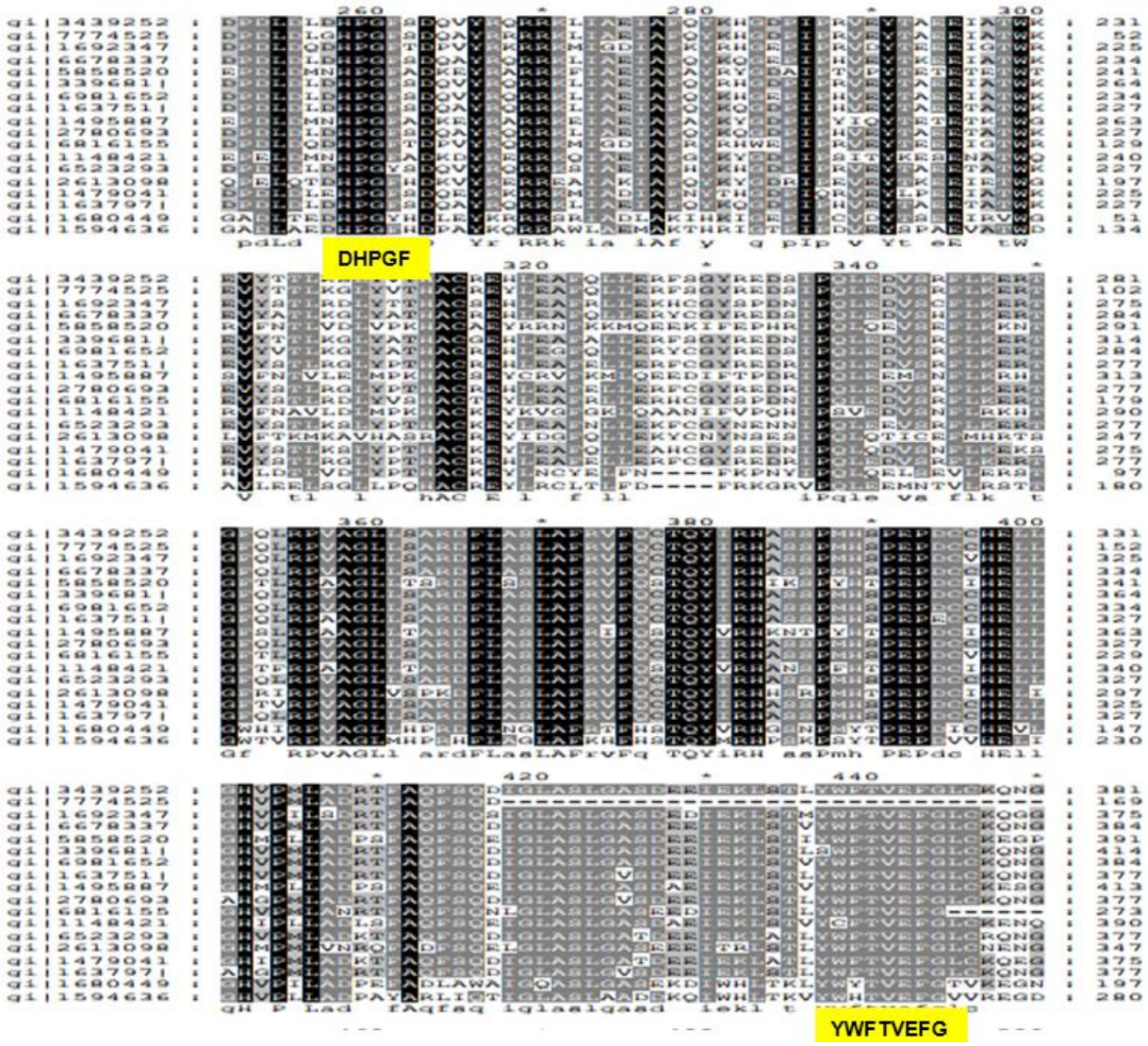


Figure IV.I Tyrosine hydroxylase protein alignments sequences for; *Mythmna* (gi:3439252) *Felis* (gi:7774525), *D. rerio* (gi:1692347), *M. musculus* (gi:6678337),, *Apis* (gi:5858520), *Rattus* (gi:339681), *Tyr 4* (gi:6981652), *Tribolium* (gi:163751), *B. taurus* (gi:1495887), *S. aurata* (gi:1148421) , *G. gallus* (gi:6523293) , *Schistosoma* (gi:2613098), *Xenopus* (gi:1479041), *Tyr* (gi:163797), *Physcomitrella* (gi:1680449) *and Chlamydomonas* (gi:1594636). Conserved amino acid sequence, DHPGF (position- 260) and YWFTVEFG (position 440) were used to design forward and reverse degenerate primers respectively.

A number of degenerate primers were designed but only one primer set; produced PCR product (Figure IV.II) despite performing a gradient PCR (49 - 65 ˚C). The PCR product however, was a stack of weak DNA bands. The DNA band corresponding to the expected size of PCR product (approximately 5.4 kb) was purified from the gel, cloned and sequenced as described in Chapter 2 Sections; 2.2.11 and 2.2.15.

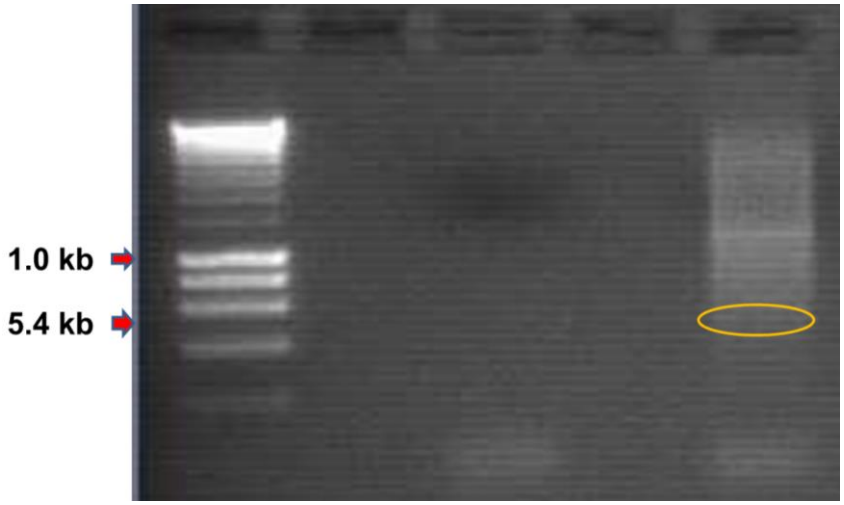


Figure IV.II shows a gel photograph of 0.4 kb PCR product (lane-3) stack of thin multiple DNA bands (smear-like) of varying bp size that were isolated by a PCR approach under standard conditions (Section 2.2.6) at Tm of 50 ˚C from genomic DNA of *M. pruriens* using degenerate primers for tyrosine hydroxylase gene; 5'- GA(T/C) CA(T/C) CC(T/C) GG(AGC) TT(TC)-3' and 5'-TA(TC) CCA (A/G)AA (AGC)GT (ACT)AC-3'.

Analysis of the sequence result using Blast against the NCBI data bank did draw any alignment for TYOH.

IV.II.IV: DISCUSSION.

The scarcity of plant tyrosine hydroxylase sequence on public data bases makes gene isolation using degenerate primers a less effective. Construction of a cDNA for could be the most suitable approach for isolation of the highly unconserved and tyrosine hydroxylase.

APPENDIX V: RECOMBINANT EXPRESSION AND ACTIVITY OF *M. PRURIENS* PUTATIVE DOPA DECARBOXYLASE GENE.

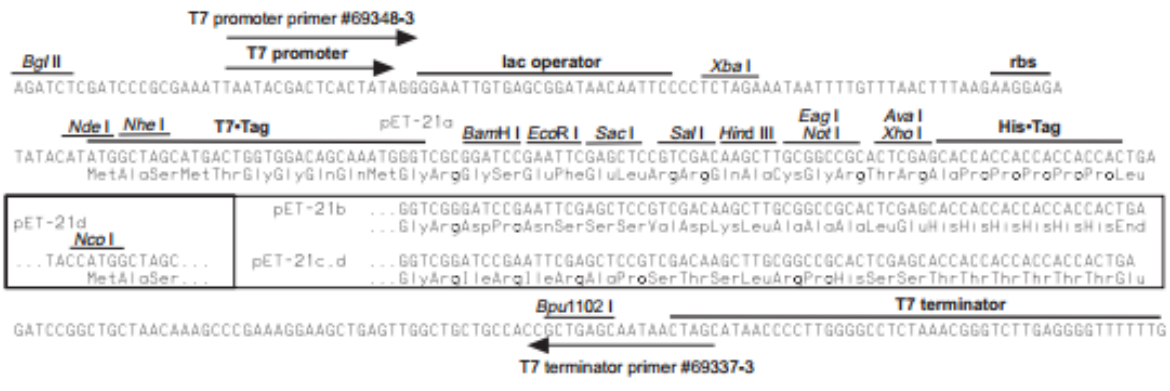
V.I: INTRODUCTION.

See chapter 5 Section 5.1.

V.II: METHODS AND MATERIALS.

The putative full Mp-ty/ddc ORF (1.5 kb) sequence was directional cloned in the *Eco*RI and *Xho*I restriction sites of pET21b (See Figure V.I a) and in frame for recombinant protein expression using bacterial cells as described in Section 5.3.4-5.

(a)



V.III: RESULTS.

The sequence result in Figure V.I (b) confirms that the 1.5 kb Mp-ty/ddc ORF was directionally cloned in frame for expression using pET21b expression vector.

(b)

GATTAGAACGGCTATTCCCTCTAGAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCT
AGCATGACTGGTGGACAGCAAATGGGTCGGGATCCGAATTCCATTGATTTCCTTGCTGATTATAT
TGGTAAGGTTGGAAAGTATCCGGTTTTAAGTAAAGTAGAAGCTGGTTATCTTAGAAAAAGATTACC
AGCTTCTGCCCCATGTGGTCCTGAACCCATTGAATCCATACTTAAAGATGTGGAAGAGCATATCA
TCCCTGGCATAACACATTGGCAGAGTCCTAATTATTATGGTTACTTCCCCAGCAGTGGTAGCATA
GCAGGGTTCATGGGTGAGATGCTAAGCACTGGATTCAATGTGGTTGGGTTCAATTGGATGTCAT
CTCCATCTGCCACTGAGCTTGAAGCCTTAGTCATGGATTGGCTTGGACAAATGCTGAAGCTCCCC
AAGACATTCCTTTTCTCTGGTGAGGGTGGTGGGGTGCTTTTGGGGACTACTTGTGAGGCCATTTT
GTGCACTTTGGTGGCTGCAAGGGAGAAAAAGCTTTCACAAGTTGGGAAGGAGAAGATAGGGAAG
CTTGTTGTGTATGCCTCTGATCAAACACACAGTGCACTTCAGAAGGCTGCTCAAATTGCTGGGAT
CCATCCAGCAAATTTCCGGGTCATCAAAACCAAGAGGTCAAGTTTCTTTGCTTTGTCTCCTGACT
CTCTTCTCTCCACCATTCTTTTGGATGTGGAGAATGGCTTGATTCCTTGTTTCCTATGTGCAACTG
TTGGCACTACTGCAATAGCCACCATTGATCCTGTGGGGCCATTGTGTAGTGTGGCCAAGGACTA
TGGCATTTGGGTCCATGTTGATGCTGCTTATGCAGGCTCAGCTTGCATTTGCCCTGAGTTTAGAT
ATTGCATTGATGGGGTTGAAGAGGCAAACTCCTTTAGCCTCAATGCTCATAAGTGGTTTTTGACC
AATTTAGCATGTTGTTGCCTTTGGGTGAAAGATCACACTGCCCTCACAAAATCCTTGTCAGTGGA
TCCTCCTTTCTTGAGGAACAAGGCTTCTGAGTCAAAGCAAGTGATTGACTACAAGGATTGGCAGA
TACCATTGAGTAGGAAATTTAATGCCCTCAAACTATGGCTTGTTCTTAGAAGCTATGGTGTTGAGA
ACCTTAGGAACTTCCTGAGAAACCATGTGCAAATGGCCAAAACTTTTGAAGGGCTGGTAAGGTTG
GATAAGAGGTTTGAGATTGTTGTGCCTCCAAAATTCTCTTTGGTTTGCTTTAGGATTGCACCATCA
GCTATTGCTAATGGGGTGTCCAATGGTACTGAAGCATGCTATAATGGGAAACTGATGAATGATGA
GTATAGGGTGAATGAAGTCAATCGTAAATTGCTTGATTCAATTAATAGTTCTGGCAATGTATTCAT
GACTCATGGTGAGGTTGAAGGAGCCTTTGTGATTAGATGTGCTATTGGTGCAACTTTAACAGAGG
AACACCATGTGATTATGGCATGGAAGTTGGTGCAGGAGCATGCCAATTCTCTGTTAGGACTCGA
GCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGAAGCTAGGCAC

Figure V.I (b) Sequence result for (a) the fusion of Mp-ty/ddc into pET21b. (b) Map of the pET21b expression vector the *Eco*RI and *Xho*I sites into which the Mp-ty/ddc sequence was cloned. The map also for shows the pET21b sequence coding the His-tag fusion protein, the *lac* operator, T7 promoter and T7 terminator.

Crystal structure analysis of the Mp-TY/DDC fusion protein on Protein data bank (PDB) showed the predicted protein structure was similar to that human (*Homo sapiens*) aromatic dopa decarboxylase (Structure reference:3RBF on the Protein data bank). The Mp-TY/DDC protein sequence (Figure V.I (a)) drew significant homology with 69.2% of the 13 connserved entities of Mp-TY/DDC showed 100% homology with the human DOPA decarboxylase on PDB (Figure V.II (b)). 76.9% of the 13 conserved entities in Mp-TY/DDC showed 100% homology with the human DOPA decarboxylase (Figure V.II c). Based on x-ray ressolution analysis, the Mp-TY/DDC was classified as a DOPA decarboxylase enzyme on PDB (Figuresd-h).

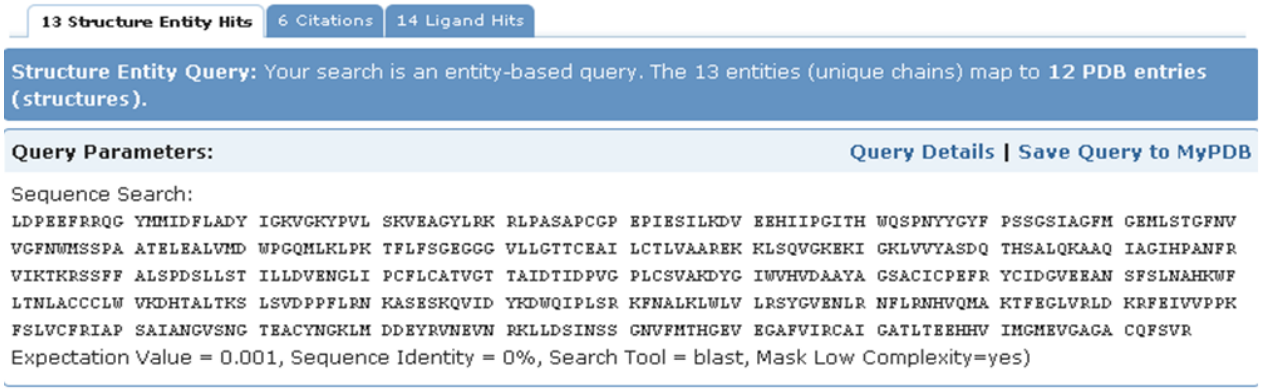
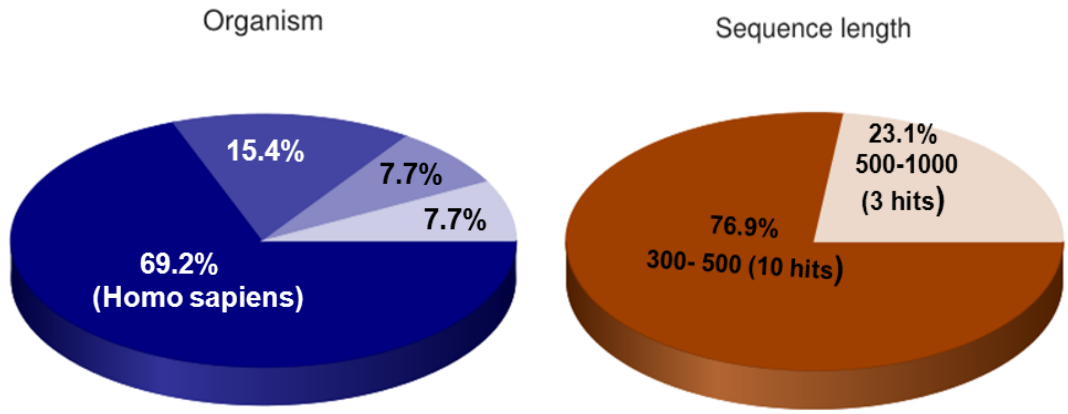
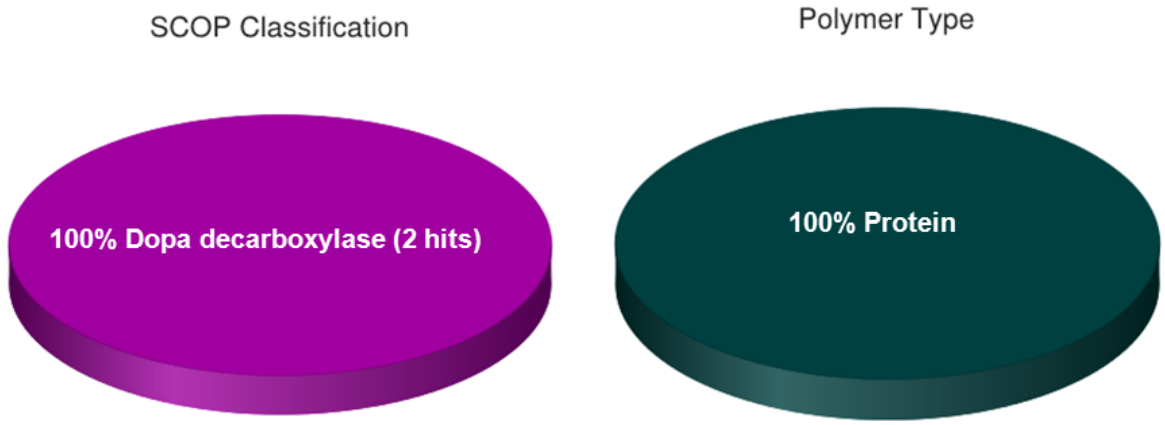


Figure V.I (a) Mp-TY/DDC protein sequence blast analysis on the Protein data bank (PDB) drew very significant alignments in 13 unique sequences of dopa decarboxylase.



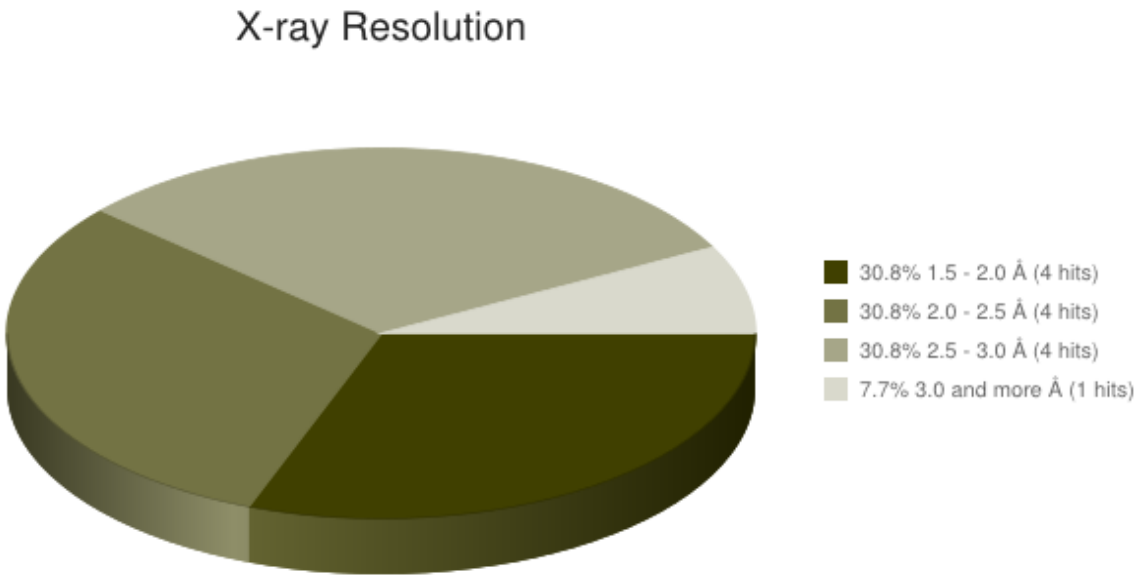
(b) (c)

Figure V.I (b) 69.2% of the 13 conserved entities in the Mp-TY/DDC sequence when blast analysed on the PDB very significant homology with dopa decarboxylase of Homo sapiens. (c) 76.9% of the 13 conserved entities in Mp-TY/DDC sequence show 100% homology with the human dopa decarboxylase.



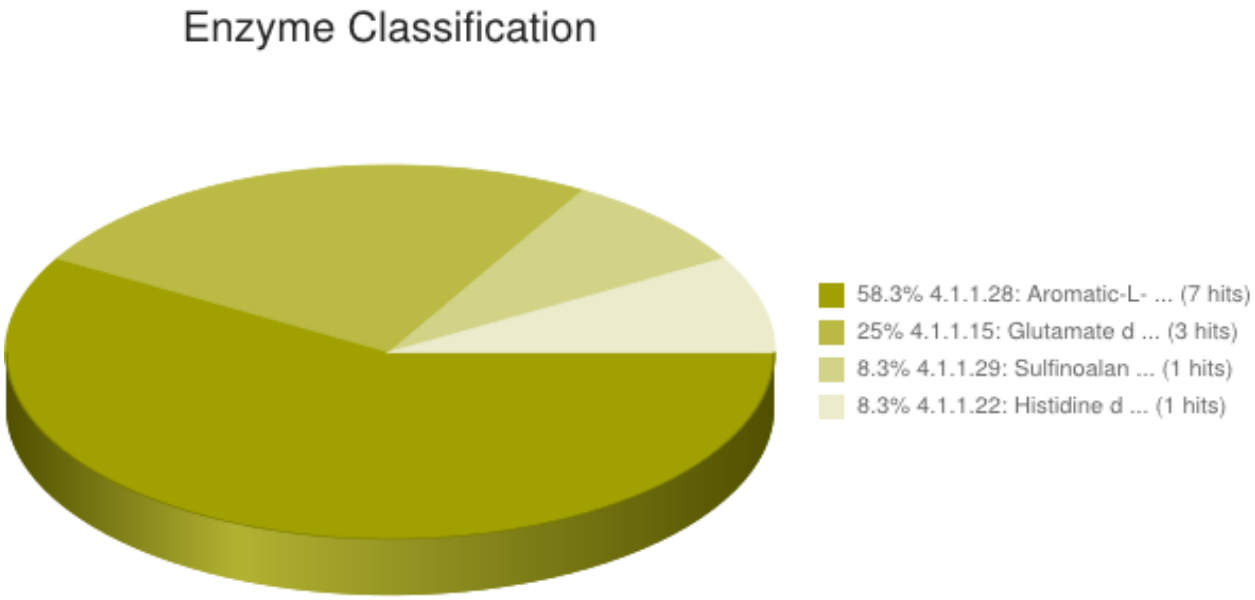
(d) (e)

Figure V.I (d) 2 of the 13 conserved entities for the Mp-TY/DDc and the PDB show 100% homology to dopa decarboxylase. (e) Polymer analysis for all the conserved entities between Mp-TY/DDC and PDB reveal the sequence is for a protein.



(f)

Figure V.I (f) X-ray resolution of the Mp-TY/DDC sequence on the PDB was characteristic for aromatic dopa decarboxylase.



(h)

Figure V.I (h) Enzyme classification of the Mp-TY/DDC sequence on the PDB shows 58% of the conserved sequence domains correspond to Aromatic DOPA decarboxylase.

Mp-TY/DDC sequence from *M. pruriens* plant showed 41% sequence homology with the human DOPA decarboxylase when analysed by blast on the NCBI data bank. The high sequence homology for the two taxonomic distant species suggests the gene plays the same role in both (Figure IV.I).

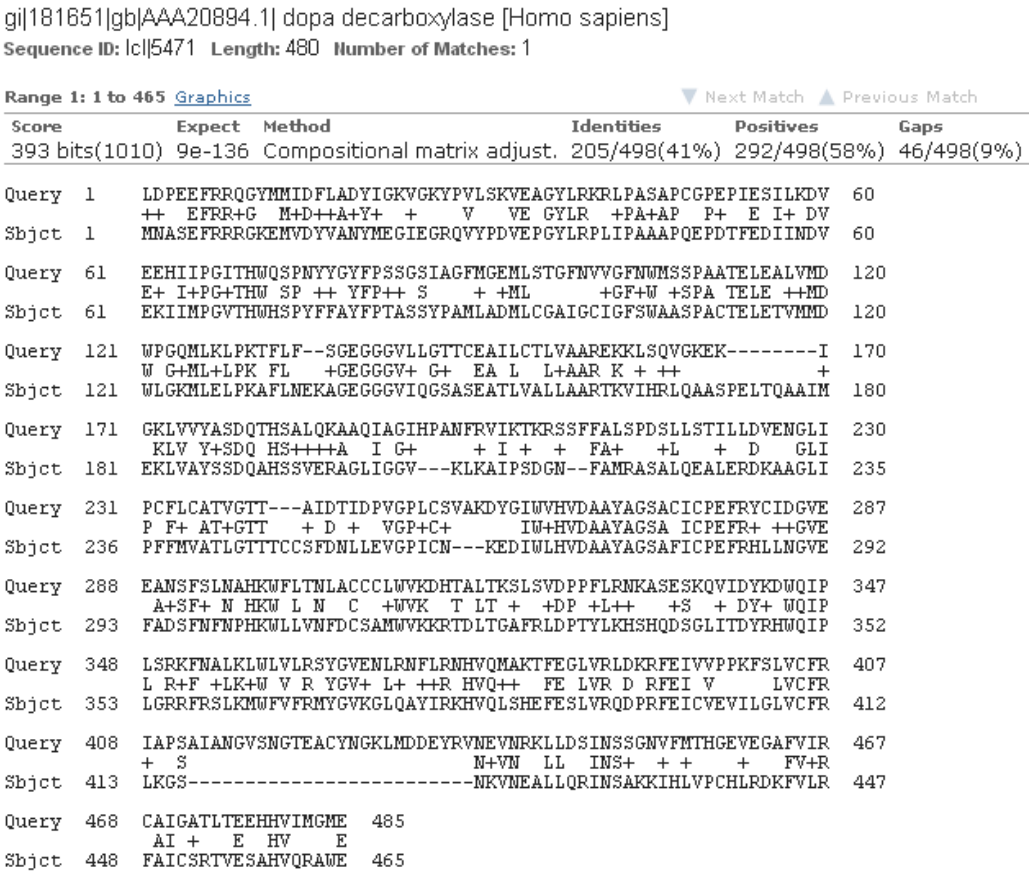


Figure IV.I Mp-TY/DDC alignment with the human DOPA decarboxylase on the NCBI data bank show 41% homology.

V.IV: DISCUSSION.

Analysis of the Mp-TY/DDC on the NCBI data bank and on PDB all drew significant alignments with plant DOPA/tyrosine decarboxylase and human DOPA decarboxylase respectively. The Mp-TY/DDC drew 74% sequence homology with TY/DDC of Glycine max which like *M. pruriens* belongs to the family Fabaceae but in addition drew 41% homology with human DOPA decarboxylase when analysed on the PDB. Based on the above, the Mp-TY/DDC is likely to be a DOPA tyrosine decarboxylase. However, the further characterisation to determine the enzyme properties is required to confirm the identity of the protein.

APPENDIX VI: TRANGENICS.

VI.I: INTRODUCTION.

Preliminary higher plant transformation experiments were performed on *Nicotiana tabacum* following the method by described by Draper et al. (1988) and Gallois and Marinho (1995) before I developed a new protocol for putative transformation of *Mucuna pruriens*. *N. tabacum* was chosen as a model for plant transformation training because it is reported to have high transformation efficiency and it is a dicot like my PhD study plant-*Mucuna pruriens* (Marion, 2001).

VI.II: METHODS AND MATERIALS.

VI.II.I: *Agrobacterium strains* and plasmids used.

A. tumefaciens strains AGL1, LBA4404 and GV301 were transformed with a pCAMBIA plasmid pC23.35S.ECGFPHS which carry transgenes; *npt*II and GFP genes in the T-DNA region. The *npt*II and GFP gene encode for kanamycin resistance and green fluorescence proteins respectively in the putatively transformed plants.

VI.II.II: *Agrobacterium*–mediated transformation of *Nicotiana tabacum* leaf discs.

The methods for preparation and use of *Agrobacterium tumefaciens* electrocompetent cells were based on Shen and Forde (1989), and Mattanovich et al. (1989) as described in Chapter 6 Section 6.2.3.2.

N. tabacum leaf explants were then transformed was then conducted following the method described by Draper et al. (1988) and Gallois & Marinho (1995) summarised below. Surface sterilised fully expanded tobacco leaves were sliced into small discs, immersed in MSO/LBA4404 culture for 20 minutes with gentle shaking. The leaf discs were then blotted with a sterile tissue and a total of 8 discs per plate were immersed adaxial side up in MSD4X2 media plates with no selection. The plates were sealed with a parafilm and incubated in 16 h light regime for 2 days before they were transferred to shooting media plates containing MSO /150 µg/µl cefotaxime/50 µg/µl kanamycin and 4.44µl 6-BAP. The cefotaxime was used to kill the *Agrobacteria* after leaf transformation, kanamycin was selection for the transformed explants while BAP induced shoot development. The callus appeared approximately after two weeks and shoot development occurred approximately after 4 weeks of post co-cultivation. The transformed shoots were excised at the base from the callus and only one shoot per leaf disc was transplanted into tissue culture boxes containing the rooting medium MSO/ 50 µg/µl kanamycin/150 µg/µl cefotaxime/5.37 µM NAA. NAA induced root formation. At 4 leaf stage, the shoots were cut between internodes and transferred to fresh MSO rooting medium with selection described above. The transformed plantlets were transferred to soil soon after the roots appeared and grown under greenhouse conditions.

VI.II.III: Detection of transgene *npt*II gene in putatively transformed plants by PCR method.

Total DNA was isolated by the method of Draper and Scott (1988) and used to investigate the presence of the transgene. Primer set for *npt*II gene was designed and used to isolate the 800 bp transgene DNA; Sequences are 5'-ATACCGTAAAGCACGA GG-3' and -ATCTCACCTTGCTCCTGC-3. Thermocycling was performed at 49˚C as described in Chapter 2 Section 2.2.6.

VI.II.IV: Detection of putatively transformed plants by GFP imaging.

The presence of the GFP in the transformants was detected by blue light excitation (Chiu et al., 1996). Root tips of the putatively transformed plants were observed under a fluorescent microscope with a filter set providing 455 - 490 nm excitation and emission above 515 nm.

VI.III: RESULTS.

VI.III.I: *Agrobacterium*-mediated transformation of *N. tabacum*.

The results for tobacco transformation were 95% successful, based on regeneration on antibiotic selection, PCR analysis and isolation of the *npt*II transgene from genomic DNA and image analysis of the green fluorescent protein product of transgene GFP observed in the root tips. Table VI.I shows the data obtained from transformation of *N. tabacum*.

Table VI.I Shows data for transformation of *N. tabacum.*

Trials	Leaf explants	Shoot explants on selection media.	Root regeneration on selection media	% Transformation
Set-A	80	75	75	93.8
Set-B	80	77	76	95
Set-C	80	75	75	93.8
Set-D	80	78	78	97.5
Total explants	320			95.03%

After 2 weeks of callus formation on kanamycin selection medium, shoots began to emerge by forming two cotyledonary leaves and a distinct stem was observed at four leaf stage. Figure VI.II shows shoot formation from putatively transformed calli.

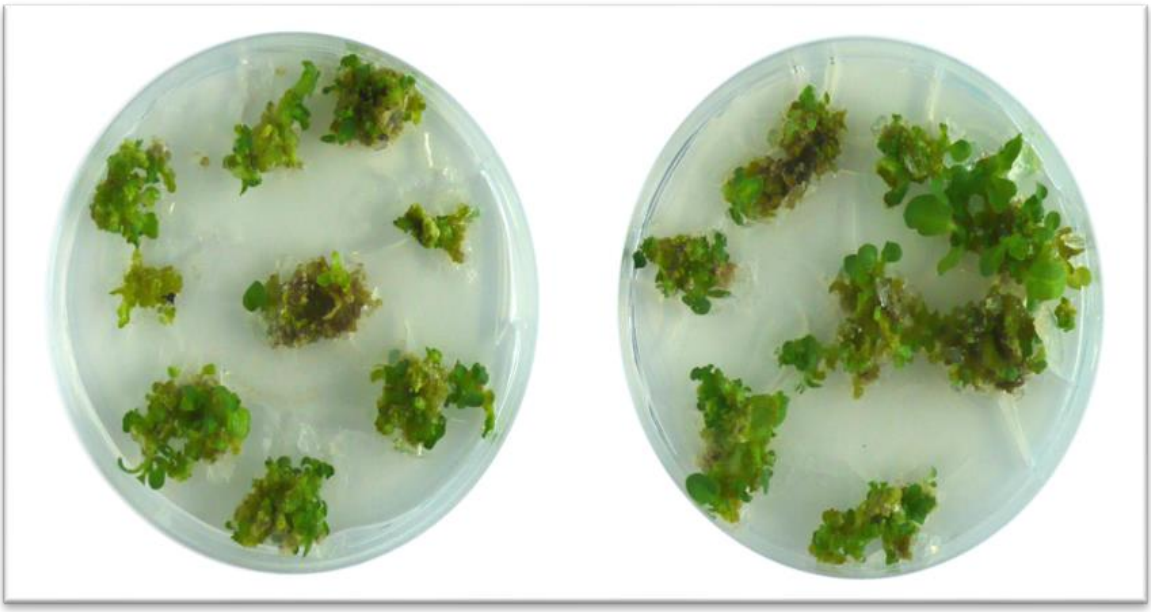


Figure VI.II (a) shows 2 weeks old putatively transformed *N. tabacum* calli regenerating shoot systems on kanamycin selection media.

The shoots excised from the calli continue to grow on M.S media supplemented with kanamycin and after 8 weeks, a distinct root system had been regenerated (See Figure VI.II (b).

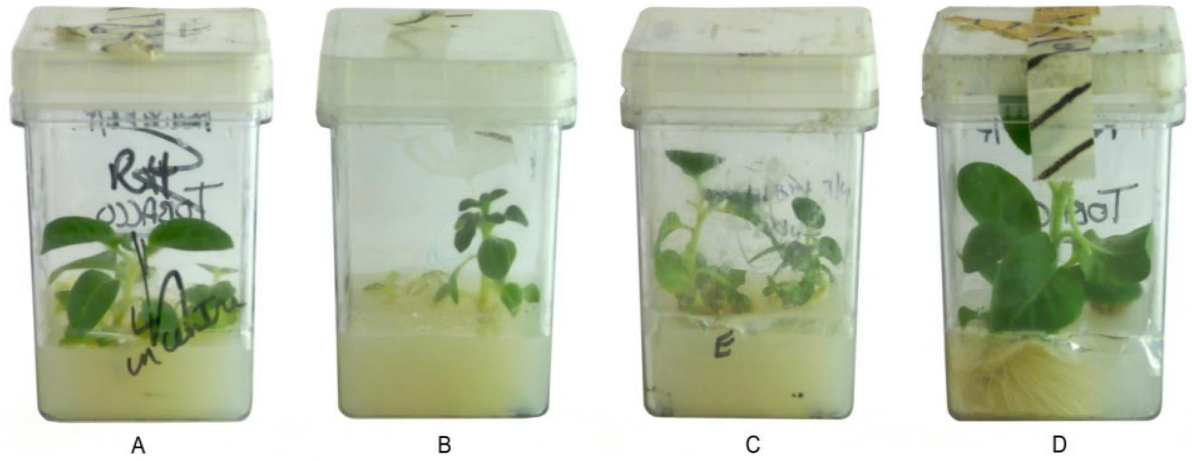


Figure VI.II (b) shows 7 weeks old putatively transformed *N. tabacum* explants on rooting media supplemented 50 mg l^{-1} kanamycin selection.

VI.III.II: Detection of presence of the *npt*II transgene in putatively transformed *N.tabacum* by PCR analysis.

The 749 bp kb *npt*II transgene was detected by PCR analysis in the genomic DNA extracted from putatively transformed *N. tabacum* by PCR analysis (See Figure VI.III).

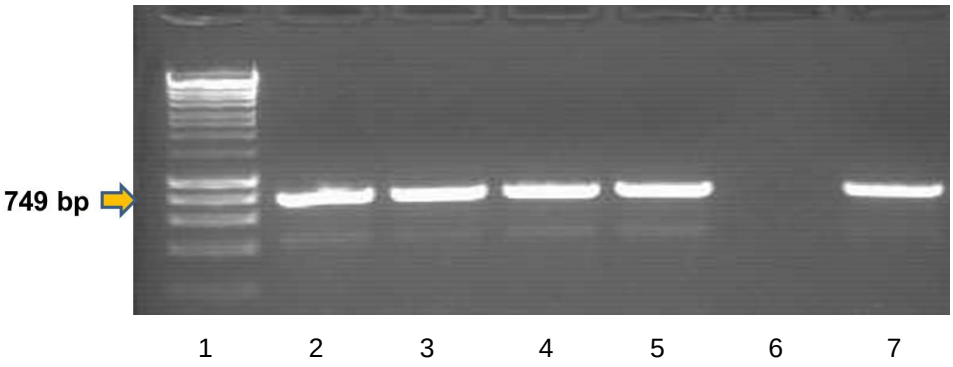
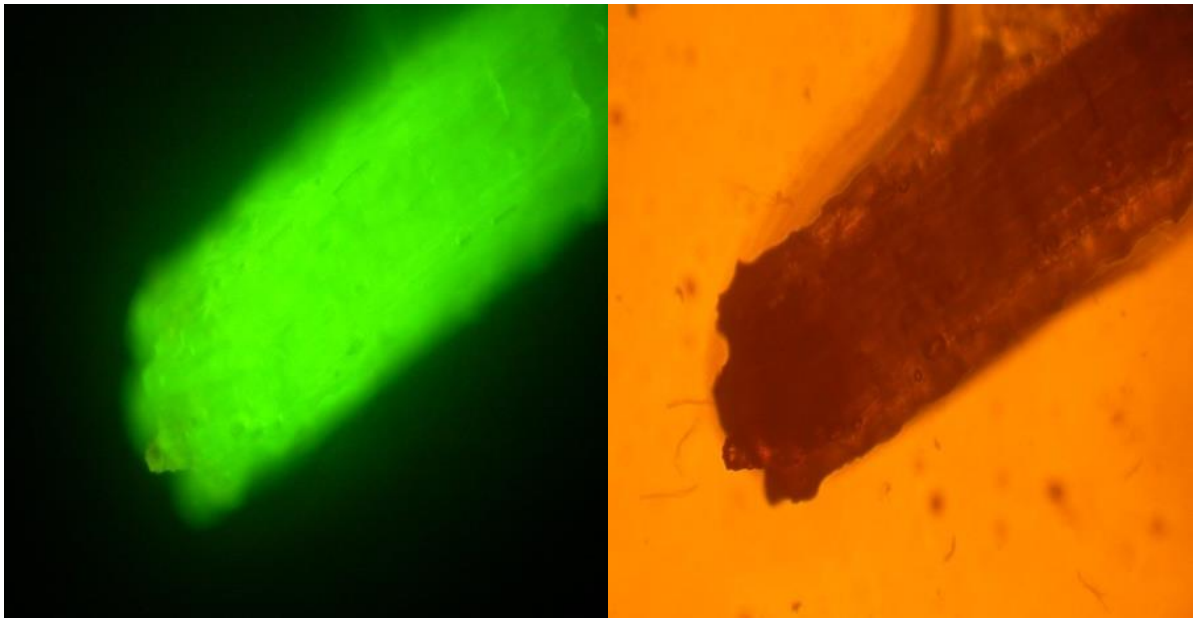


Figure VI.III shows 749 bp kb PCR product of transgene *npt*II isolated from genomic DNA extracted from different transformed tobacco plants (1, 2, 3, 4 and 5) and from pCAMBIA2300 plasmid DNA used as a positive control (lane 7) using primer set; 5'-CTTGGGTGGAGAGGCTAT-3' and 5'-AGAACTCGTCAAGAAGGC-3'. No PCR product was obtained for the negative control (lane - 6).

VI.III.III: GFP analysis of root tips of putatively transformed *N. tabacum.*

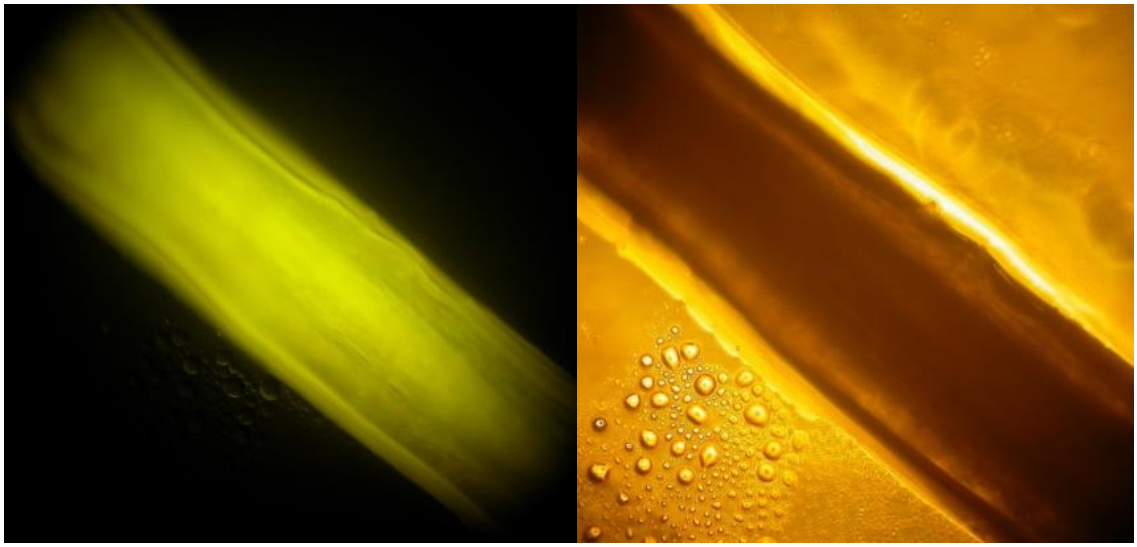
GFP Image analysis of the root tips of *N. tabacum* under blue light excitation of a GFP microscope showed green fluorescence for the putatively transformed plants (Figure VI.IV a).



(a) (b)

Figure VI.IV shows image analysis of root tips of putatively transformed *N. tabacum* using a GFP microscope. Figure (a) Green fluorescence was observed in the root tips under blue light excitation. Figure (b) Root tip of putatively transformed *N. tabacum observed* under normal light.

On the other hand, no green fluorescence was observed in the root tips of un transformed *N. tabacum* plants (Figure VI.V).



(c) (d)

Figure IV.V No green fluorescence was observed the root tips of un transformed *N. tabacum* (negative experimental control).

VI.IV: DISCUSSION.

The transformation of *N. tabacum* was confirmed by detection of the transgenes; *npt*II and GFP encoding gene by PCR and GFP image analysis respectively. The *npt*II transgene conferred kanamycin resistance to the transgenic plants which enabled them to grow on kanamycin selection (Hellens, R and Mullineaux). On the other hand, the GFP gene encoded for green fluorescence proteins observed in the root tips of putatively transformed *N. tabacum* plants (See Figure VI.IV). The high transformation efficiency of 95% (See Table VI.I) justified the choice of *N. tabacum* as a model for preliminary work on plant transformation before I developed a new protocol for putative transformation of *Mucuna pruriens*.