Towards the biofortification of banana fruit for enhanced micronutrient content

By

Priver Namanya
Bsc (Hons.), Msc (Botany)

Centre for Tropical Crops and Biocommodities

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Abstract

In Uganda, vitamin A deficiency (VAD) and iron deficiency anaemia (IDA) are major public health problems with between 15-32% of children under 5 years of age showing VAD and 73% being anaemic. This is largely due to the fact that the staple food crop of the country, banana, is low in pro-vitamin A and iron, therefore leading to dietary deficiencies. Although worldwide progress has been made to control VAD and IDA through supplementation, food fortification and diet diversification, their long term sustainability and impact in developing countries such as Uganda is limited. The approach taken by researchers at Queensland University of Technology (QUT), Australia, in collaboration with the National Agricultural Research Organization (NARO), Uganda, to address this problem, is to generate consumer acceptable banana varieties with significantly increased levels of pro-vitamin A and iron in the fruit using genetic engineering techniques. Such an approach requires the use of suitable, well characterised genes and promoters for targeted transgene expression. Recently, a new banana phytoene synthase gene (*APsy*2a) involved in the synthesis of pro-vitamin A (pVA) carotenoids was isolated from a high β-carotene banana (F'ei cv Asupina). In addition, sequences of banana ferritin, an iron storage protein, have been isolated from Cavendish banana. The aim of the research described in this thesis was to evaluate the function of these genes to assess their suitability for the biofortification of banana fruit. In addition, a range of banana-derived promoters were characterised to determine their suitability for controlling the expression of transgenes in banana fruit.

Due to the time constraints involved with generating transgenic banana fruit, rice was used as the model crop to investigate the functionality of the banana-derived *APsy*2a and ferritin genes. Using *Agrobacterium*-mediated transformation, rice callus was transformed with *APsy*2a +/- the bacterial-derived carotene desaturase gene (*CrtI*) each under the control of the constitutive maize poly-ubiquitin promoter (ZmUbi) or seed-specific rice glutelin1 (Gt1) promoter. The maize phytoene synthase (*ZmPsy*1) gene was included as a control. On selective media, with the exception of ZmUbi-*CrtI*-transgenic callus, all antibiotic resistant callus displayed a

yellow-orange colour from which the presence of β-carotene was demonstrated using Raman spectroscopy. Although the regeneration of plants from yellow-orange callus was difficult, 16 transgenic plants were obtained and characterised from callus transformed with ZmUbi-*APys*2a alone. At least 50% of the T1 seeds developed a yellow-orange coloured callus which was found to contain levels of β-carotene ranging from 4.6-fold to 72-fold higher than that in non-transgenic rice callus. Using the seed-specific Gt1 promoter, 38 transgenic rice plants were generated from *APsy*2a-*CrtI*-transformed callus while 32 plants were regenerated from *ZmPsy*1-*CrtI*-transformed callus. However, when analysed for presence of transgene by PCR, all transgenic plants contained the *APsy*2a, *ZmPsy*1 or *CrtI* transgene, with none of the plants found to be co-transformed. Using Raman spectroscopy, no β-carotene was detected *in-situ* in representative T1 seeds.

To investigate the potential of the banana-derived ferritin gene (*BanFer*1) to enhance iron content, rice callus was transformed with constitutively expressed *BanFer*1 using the soybean ferritin gene (*SoyFer)* as a control. A total of 12 and 11 callus lines independently transformed with *BanFer*1 and *SoyFer*, respectively, were multiplied and transgene expression was verified by RT-PCR. Pearl's Prussian blue staining for *in-situ* detection of ferric iron showed a stronger blue colour in rice callus transformed with *BanFer*1 compared to *SoyFer.* Using flame atomic absorption spectrometry, the highest mean amount of iron quantified in callus transformed with *BanFer*1 was 30-fold while that obtained using the *SoyFer* was 14-fold higher than the controls. In addition, ~78% of *BanFer*1-transgenic callus lines and ~27% of *SoyFer*-transgenic callus lines had significantly higher iron content than the non-transformed controls.

Since the genes used for enhancing micronutrient content need to be expressed in banana fruit, the activity of a range of banana-derived, potentially fruit-active promoters in banana was investigated. Using *uid*A (GUS) as a reporter gene, the function of the Expansin1 (MaExp1), Expansin1 containing the rice actin intron (MaExp1a), Expansin4 (MaExp4), Extensin (MaExt), ACS (MaACS), ACO (MaACO),

Metallothionein (MaMT2a) and phytoene synthase (APsy2a) promoters were transiently analysed in intact banana fruit using two transformation methods, particle bombardment and *Agrobacterium*-mediated infiltration (agro-infiltration). Although a considerable amount of variation in promoter activity was observed both within and between experiments, similar trends were obtained using both transformation methods. The MaExp1 and MaExp1a directed high levels of GUS expression in banana fruit which were comparable to those observed from the ZmUbi and *Banana bunchy top virus*-derived BT4 promoters that were included as positive controls. Lower levels of promoter activity were obtained in both methods using the MaACO and MaExt promoters while the MaExp4, MaACS, and APsy2a promoters directed the lowest GUS activity in banana fruit.

An attempt was subsequently made to use agro-infiltration to assess the expression of pVA biosynthesis genes in banana fruit by infiltrating fruit with constructs in which the ZmUbi promoter controlled the expression of *APsy*2a +/- *CrtI*, and with the maize phytoene synthase gene (*ZmPsy*1) included as a control. Unfortunately, the large amount of variation and inconsistency observed within and between experiments precluded any meaningful conclusions to be drawn.

The final component of this research was to assess the level of promoter activity and specificity in non-target tissue. These analyses were done on leaves obtained from glasshouse-grown banana plants stably transformed with MaExp1, MaACO, APsy2a, BT4 and ZmUbi promoters driving the expression of the GUS gene in addition to leaves from a selection of the same transgenic plants which were growing in a field trial in North Queensland. The results from both histochemical and fluorometric GUS assays showed that the MaExp1 and MaACO promoters directed very low GUS activities in leaves of stably transformed banana plants compared to the constitutive ZmUbi and BT4 promoters.

In summary, the results from this research provide evidence that the banana phytoene synthase gene (*APsy*2a) and the banana ferritin gene (*BanFer*1) are functional, since the constitutive over-expression of each of these transgenes led to

increased levels of pVA carotenoids (for *APsy*2a) and iron content (for *BanFer*1) in transgenic rice callus. Further work is now required to determine the functionality of these genes in stably-transformed banana fruit. This research also demonstrated that the MaExp1 and MaACO promoters are fruit-active but have low activity in non-target tissue (leaves), characteristics that make them potentially useful for the biofortification of banana fruit. Ultimately, however, analysis of fruit from field-grown transgenic plants will be required to fully evaluate the suitability of pVA biosynthesis genes and the fruit-active promoters for fruit biofortification.

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List of Abbreviations

Abbreviation	Description
4-MU	4-methylumbelliferone
BCA	bicinchoninic acid
cDNA	complementary deoxyribonucleic acid
CTAB	centyltriethylammonium bromide
cds	coding sequence
cv	cultivar
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EAHB	East African highland bananas
EDTA	ethylene-diamine tetraacetic acid
FAAS	flame atomic absorption spectrometry
gDNA	genomic deoxyribonucleic acid
GOU	Government of Uganda
Hepes - N	-(2-hydroxymethyl) piperazine-N'-2-ethane sulfonic acid
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
*hpt*II	hygromycin phosphotransferase
ID	identity
IDA	iron deficiency anaemia
MS	Murashige and Skoog
MUG	4-Methylumbelliferyl-β-D-glucuronide
NIH	National Institute of Health
*npt*II	neomycin phosphotransferase
pBIN	binary plasmid vector - pBINPLUS
pCAM	binary plasmid vector - pCAMBIA
PCR	polymerase chain reaction
pDNA	plasmid deoxyribonucleic acid
Pipes	piperazine-N,N'-bis(2-ethanesulfonic acid)
pVA	pro-vitamin A
PVP	polyvinylpyrrolidone
RAE	retinol activity equivalents
RDA	recommended daily allowance
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
TAE	tris-acetate EDTA
Taq	*Thermus aquaticus*
TE	tris-ethylene-diamine tetraacetic acid
UBOS	Uganda Bureau of Statistics
uidA	GUS (β-glucuronidase)

USDA	United States Department of Agriculture
v	version
VAD	vitamin A deficiency
WHO	World Health Organisation
X-GLUC	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
Units	**Description**
˚C	degrees Celsius
cm	centimetre(s)
dwt	dry weight
g	gram(s)
g	relative centrifugal force
hr	hour(s)
(k)bp	(kilo)base pairs
kCal	kilo calories
kg	kilogram(s)
L	litre(s)
M	molar
mg	milligram(s)
min	minute(s)
mL	mili-litres(s)
MW	Molecular weight
mW	milliWatts
nm	nanometers
OD	optical density
pmol	picomoles
rpm	revolutions per minute
s	seconds
V	volts
v/v	volume to volume
w/v	weight to volume
λ	lambda
µg	microgram(s)
µL	microlitres
µm	micrometre
µM	micromolar
Institution names	
CTCB	Centre for Tropical Crops and Biocommodities
NARO	National Agricultural Research Organisation
QUT	Queensland University of Technology

Declaration

"The work contained in this thesis has not been previously submitted to meet the requirements of an award at this or any other higher education institution. To the best of my knowledge and behalf, the thesis contains no material previously published or written by another person except where due reference is made"

Signature _____

Date 16/04/2010

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Chapter 1 Literature Review

1.1 The challenge of micronutrient malnutrition in humans

Plants are the principal source of essential nutrients, including carbohydrates, proteins, mineral nutrients and vitamins, required for the healthy functioning of the human body. However, there is no single food source that contains all the nutrients in sufficient amounts to meet the required dietary allowances in a single serving. Therefore, a combination of different foods is often consumed to balance nutritional requirements. As a result, dependence on a simple diet composed of a single staple food causes diseases associated with nutrient deficiency. Micronutrients such as minerals and vitamins, although usually required in very small amounts, are vital for human health and their deficiency remains a worldwide cause of ill health.

1.1.1 Vitamin A deficiency and associated health implications

Individuals lacking sufficient vitamin A in their body suffer from night blindness which is the earliest symptom of vitamin A deficiency (VAD). Night blindness is due to progressive degeneration of mucous membranes in the eye. However, prolonged deficiency results in drying of conjunctiva, while acute cases of deficiency lead to adverse effects such as eye ulcers, inflammation and interior infection leading to irreversible blindness. VAD is frequently reported among women and children in less developed countries particularly Sub-Saharan Africa and South East Asia where an estimated 250,000 - 500,000 children go blind each year (WHO 2003; Bouis *et al.,* 2003b). Moreover, affected children have an increased risk of infection from other diseases such as diarrhoea and measles. The World Health Organisation (WHO) reported that half of the affected children die within 12 months of losing their sight (WHO 2003). Similarly, VAD causes night blindness in women during pregnancy and is responsible for nearly 600,000 maternal deaths due to problems related to child-birth each year. This deficiency has also been associated with elevated transmission of HIV from mother to child. It has been estimated that overcoming VAD could potentially decrease global child mortality by 23% (WHO 2003). Micronutrient undernutrition results in substantial increases in mortality and oveall disease burden. Globally, vitamin A, iron and zinc deficiencies are reported to have the largest percentage disease

burden amongst the micronutrients that are known to have a negative impact on public health (Black *et al.,* 2008; Stein 2010).

Vitamin A deficiency is a public health problem in Uganda with up to 15-32% of the children under 5 years and women of reproductive age showing VAD (UBOS 2007). Studies conducted on VAD children have shown that 15% of the affected children had acute respiratory infection while up to 41% and 25% had developed fever and diarrhoea, respectively, in the 2 weeks preceding data collection. These data reflect the high burden of infection associated with VAD.

1.1.2 Iron deficiency and associated health implications

Dietary iron exists as heme iron or non-heme iron. Heme iron is found in animal foods and is primarily derived from the haemoglobin and myoglobin found in meat while non-heme iron is derived from plant and dairy products. Unlike heme iron which is well absorbed non-heme iron consists mainly of iron salts which are bound to foods and therefore must be hydrolysed or solubilised prior to absorption into the body (Theil and Brait 2004). This depends largely on the presence of absorption enhancers or inhibitory substances in the meal. For example, reducing agents such as ascorbic acid and cysteine enhance iron absorption while compounds such as phytates inhibit iron absorption (Gurzau *et al.,* 2003). Similarly, vitamin A has been reported to enhance both iron metabolism and absorption into the red blood cells (Bloem 1995; Semba and Bloem 2002; Sonja *et al.,* 2005).

Dietary iron absorption occurs in two steps: absorption from the intestinal lumen into mucosal cells, and transfer from the mucosal cells to the plasma where the iron is bound to storage proteins such as ferritin and hemosiderin. This process is regulated by a complex enzyme-mediated mechanism for maintaining homeostasis and is strongly influenced by dietary components which bind iron in the intestinal lumen. In general, the amount of bioavailable iron (i.e. proportion of ingested iron that is absorbed) from plant foods ranges from 1 to 20% (Frossard *et al.,* 2000); (Gurzau *et al.,* 2003) while absorption of heme iron goes up to 30% (Trost *et al.,* 2006). Ultimately, the total iron

content in the body depends on the amount absorbed which is determined by the composition of diet.

Total iron in the body exists as stored iron (bound to ferritin and hemosiderin), transport iron (in transit to tissues) and functional iron (bound to hemoglobin, myoglobin and enzymes). Individuals whose storage, transport and functional iron become severely depleted, particularly during periods of increased demand for iron, suffer from iron deficiency anaemia (IDA). Common periods of high demand for iron include pregnancy, periods of blood loss such as childbirth or surgery, or iron demand as a result of insufficient iron intake/absorption (Trost *et al.,* 2006). Lack of sufficient dietary iron is the major cause of anaemia. In general, iron deficiency is characterised by reduced capacity to work and decreased resistance to fatigue. In pregnant women, severe iron deficiency anaemia is associated with intra-uterine growth retardation, foetal and maternal morbidity and mortality, premature births, low birth weights and increased risk of infection. Young children, whose mothers were anaemic during pregnancy, suffer from impaired brain function resulting in poor learning ability, decreased physical activity levels and behavioural problems during adolescence (WHO 2003; Black *et al.,* 2008).

Iron deficiency has been ranked as the world's leading nutritional disorder affecting both developed and developing countries (WHO 2000b). However, the prevalence of anaemia in developing countries has been reported at three to four times higher than that in developed countries (HarvestPlus 2003). Worldwide, IDA exerts the heaviest overall toll in terms of ill health, premature death and lost earnings. The prevalence of iron deficiency and anaemia is influenced by age, gender, diet, physiological and pathological factors. As many as 4-5 billion people are iron deficient with over 30% of the world’s population considered anaemic. In developing countries, anaemia affects all age groups with more than 50% of children and pregnant women, 45% of the elderly and 30% of adult men reportedly anaemic (WHO 2003; IFPRI 2005; WHO 2005; Stein 2010). In addition to iron deficiency, anaemia is frequently worsened by malaria (in the tropics), worm infections, physiological status and chronic infections such as HIV, particularly where medical care is limited (UBOS 2007).

In Uganda, 73% of the children under five years, 64% of pregnant women and 49% of the women of reproductive age are anaemic (UBOS 2007). However, the occurrence of iron deficiency was found to be higher than the prevalence of anaemia. For example, in women, the prevalence of iron deficiency with or without anaemia has been reported at 87.5% suggesting inadequate dietary intake as the main factor underlying anaemia in women in Uganda (UBOS 2007). In general, the prevalence of iron deficiency anaemia was lower in urban areas compared to rural areas.

1.1.3 Current approaches for combating micronutrient malnutrition: The case of vitamin A and iron

Common approaches to combating VAD include dietary interventions such as supplementation, food fortification, consumption of pro-vitamin A rich foods and biofortification of food crops. Supplementation involves the use of vitamin tablets in the form of pharmacological formulations, while food fortification aims at incorporating nutrients into processed foods such as breakfast cereals, margarine, sugar and salt. Other interventions promoted by WHO in developing countries include prolonged breast feeding time coupled with immunisation programs. These programs have been adopted in up to 61 countries worldwide and have resulted in reduced mortality amongst children (Semba *et al.,* 1997; Melikian *et al.,* 2001; Semba *et al.,* 2005; WHO 2005). In addition, supplementation with multiple micronutrients (i.e. iron, folic acid, vitamin A, iodine and zinc) has been reported to reduce early infant mortality especially in undernourished and anaemic women (SUMMIT 2008).

Iron deficiency anaemia can also be prevented by increasing the consumption of iron rich foods. As in the case of vitamin A, control strategies for iron deficiency include the use of dietary supplements mainly in the form of pharmaceutical iron rich tablets (ferrous sulphate) and fortified foods. In less developed countries, programs that promote the use of diet supplements often target pregnant women and to a less extent, young children (WHO 2000a). However, prevention of iron deficiency and anaemia require multi-disciplinary initiatives in combination with public health initiatives to control other causes of anaemia such as infection from hookworms and

malaria. The potential problem of using readily available ferrous sulphate tablets is the risk of overdose which may lead to iron overload.

An analysis of the cost-benefit economics of iron and vitamin A supplementation as well as food fortification, in terms of cost per life saved and productivity gained, has shown that the population- wide effects are greatly increased by fortification compared to supplementation (Hunt 2002). Although both approaches are a quick remedy to address micronutrient malnutrition, they have not been successful in developing countries due to limitations with their widespread distribution. Many such programs are also short term donor funded projects. As a result, their success as well as long term impact has been limited by lack of adequate and sustainable funding to support continuity. In addition, there are major weaknesses in national distribution systems while political instability hinders implementation in some countries. Although diet diversifications and use of fortified foods is desirable, these are expensive and therefore often unaffordable or even unavailable to communities whose daily dietary nutrient source is staple food (Bouis *et al.,* 2003b; Zhu *et al.,* 2007). Moreover, due to low purchasing power, poor families may consider buying fortified foods a luxury. All these issues taken together make such approaches unsustainable in the long term.

In Uganda, the National Anaemia Policy of Uganda mandates that, if the prevalence of anaemia is at least 40%, children under five years of age should receive appropriate supplementary iron tablets per day depending on their age. However, only 5.5% of the children below 5 years are reported to receive iron supplements (UBOS 2007). Some of the barriers to the effective implementation of the anaemia policy include inefficiencies in the health system such as availability/distribution of logistics, performance of health workers or antenatal care providers and awareness among pregnant women especially in rural communities (Mwadime *et al.,* 2002). In addition, iron deficiency is aggravated by infections such as malaria which accounts for up to 70% of out-patients and 50% in-patients below 5 years. There is limited information to show that integrated management of childhood illness using iron supplements is safe and efficacious in the context of a high burden of malaria and iron deficiency (UBOS 2007).

In the case of VAD, although the Uganda national vitamin A policy requires that all children from 6 months to 5 years receive bi-annual supplementation, only 36% of the children receive vitamin A capsules. As such, in addition to social marketing of the supplementation programme, strengthening the promotion of vitamin A rich foods such as the orange- fleshed sweet potatoes has been highly recommended for Uganda.

In recent years, efforts in science and biotechnology have focused on the production of staple crops with improved essential nutrient content through plant breeding, a process known as biofortification. Bearing in mind the magnitude of micronutrient malnutrition and the limitations of existing control measures, efforts towards production of crops with improved nutrient content is an attractive option to explore. To this effect, biofortification of staple food crops through plant breeding offers the opportunity to create a public good with comprehensive benefits for both producers and consumers. The strategy has great potential as a long term, affordable option and is expected to have positive effects on human health (Bouis 2003a).

1.1.4 Biofortification as a strategy to meet the challenge of micronutrient malnutrition

Current strategies for biofortification of crops include the use of both conventional breeding and transgenic approaches. Improvement of nutrient content through conventional breeding is based on exploiting the genetic variation that exists in crop species which can be utilised to introduce new alleles through cross breeding. Conventional breeding has great potential and its success is especially associated with the green revolution, where the rate of increased production of crops such as rice, wheat and maize in developing countries outpaced population growth such that the prices of rice and wheat fell by 30-40% between 1970-1979 (Bouis 2003a). Several international organisations of the Consultative Group on International Agricultural Research (CGIAR) have embarked on programs to study the genetic variation of micronutrient content and the feasibility of breeding programs for nutrient enhancement. Conventional breeding uses inherent properties of the crop, has far reaching impact on communities and has fewer regulatory constraints compared to genetically modified varieties (Zhu *et al.,* 2007). However, for staple food crops like

bananas where cultivated varieties are infertile and vegetatively propagated, cross breeding is limited. Early banana breeding reports show that the development of hybrid bananas by conventional breeding is hindered by long cropping cycles, complex genetic constitution, the trisomic pattern of gene inheritance and low female fertility which makes successful application of classical breeding methods limited (Vuylsteke *et al.,* 1998). This indicates that it is likely to take many years to develop an acceptable banana hybrid using conventional breeding methods. Therefore, for a crop like banana, the potential of genetic engineering is an invaluable alternative and complementary solution to conventional breeding efforts. Through genetic engineering, well characterised novel traits can be directly introduced into the plant genome and their expression can be targeted to specific plant parts such as fruits, seeds and leaves using tissue-specific promoters. In addition, genetic engineering has the advantage that characterised traits can be directly introduced into elite varieties and the process is not limited by the existing gene pool of a specific crop (Zhu *et al.,* 2007).

Biofortification requires an initial investment into research at a central position for product development with additional costs to cover nutrition safety and efficacy tests, the costs of dissemination and subsequent evaluation of both nutritional safety and impact (Hunt 2002). There are reasons to expect high returns from such an investment because, once this is done, the benefits do not disappear. Rather, not only are there nutritional benefits but agricultural productivity may also increase. Moreover, the benefits can be extended to other countries and communities with minimum investment in adaptive breeding and dissemination. This is possible as long as there is an effective domestic agricultural research infrastructure (Hunt 2002; Bouis *et al.,* 2003b). Since there are no expected recurrent costs especially for the farmers, biofortification is a strategy that has the potential for far-reaching impacts in poor rural communities where nutrient malnutrition is the main problem. Biofortification of staple foods in particular, has great potential as a long term, affordable option for alleviating nutrient deficiencies (Bouis *et al.,* 2003b). Although transgenic approaches can be undermined by political, social economic issues and intellectual property constraints, food security and malnutrition remain a major focus for developing countries.

Therefore, viable strategies available to the scientific community to improve nutritional value of foods continue to be explored.

1.2 Biofortification for improved pro-vitamin A carotenoids in plants

1.2.1 Pro-vitamin A carotenoids, their sources and health benefits

Carotenoids form a large class of lipophilic molecules synthesised by plants, algae and bacteria. In plants, carotenoids are synthesised and sequestered in plastids where they accumulate in chloroplasts (in photosynthetic membranes in association with light harvesting and reaction centre complexes) and in chromoplasts of fruits and petals (Sandmann 2001). Since animals and humans do not synthesise carotenoids in their bodies, food remains their principal source. The main dietary source of pro-vitamin A (pVA) carotenoids, particularly β-carotene, is the yellow or orange coloured foods such as carrots, some varieties of sweet potato, mangoes and papaya. Other plant sources include dark-green leafy vegetables such as spinach and lettuce. Recently, high β-carotene banana varieties, belonging to the F'ei group of bananas, were reported in Micronesia (Englberger *et al.,* 2003a; Englberger *et al.,* 2003b; Englberger *et al.,* 2003c). Vitamin A (in the form of fatty acid retinyl esters) can also be obtained from animal and dairy products particularly eggs, milk and liver. When consumed, pVA carotenoids are converted into retinol, the active form of vitamin A, in the gut wall and the liver. The common carotenoid precursors of vitamin A found in foods are β-carotene, α-carotene and β-cryptoxanthin (De Pee and West 1996). Among these, β-carotene is the most effectively converted to retinol and therefore is most nutritionally useful. Alpha carotene and β-cryptoxanthin are also converted to retinol but at only half the efficiency of β-carotene. Other carotenoids, such as lutein, lycopene and zeaxanthin, have no vitamin A activity but have other health promoting properties such as prevention of age related muscular degeneration. Lycopene is a potent antioxidant and is believed to have positive cardiovascular effects as well as possible effects in preventing prostate cancer (Giovannucci *et al.,* 1995).

Pro-vitamin A carotenoids are primarily responsible for visual functions in the human body. However, pVA carotenoids occur in various forms and their health benefits extend into multifunctional roles such that specific carotenoids are responsible for

different health promoting effects. For example, the hydrocarbon carotenoids such as β-carotene and lycopene which are mainly derived from the yellow and orange coloured fruits and vegetables are related to a reduced risk of site-specific cancers and heart disease; whereas oxygenated carotenoid types such as lutein and zeaxanthin which are abundant in dark-green leafy vegetables, are essential for visual functions such as protecting eye tissues (Yeum and Russel 2002). While vitamin A is primarily important for visual functions, it is also useful for other body functions such as normal cell division and differentiation, bone marrow growth and reproduction, regulation of the immune system as well as enhancing the functioning of the blood lymphocytes. Furthermore, vitamin A protects the body against bacterial, fungal and viral infections by promoting healthy surfaces to the lining of the respiratory system, urinary system, intestinal tracts and maintaining the integrity of the skin (Ross 1998).

1.2.2 Bioavailability of pVA carotenoids and recommended dietary allowances for Vitamin A

Recommended dietary allowance (RDA) values represent the average daily dietary intake levels of a nutrient which is required to meet dietary requirements of healthy individuals in a particular age and gender group. RDA for vitamin A is measured in retinol activity equivalents (RAE), a measure that is dependent on the bioavailability of pVA carotenoids. The bioavailability defines the percentage of ingested pVA carotenoids that is converted into metabolically active retinol available for storage and utilisation (Van Lieshout *et al.,* 2003). The Food and Agricultural Organisation (FAO) and WHO reports from the 1960's suggested that in oil, 6 μg of β-carotene carries the activity of 1 μg of retinol, while a ratio of 12 μg of β-carotene: 1 μg of retinol had been suggested for complex foods. However, more recent studies based on the factors influencing bioavailability of β-carotene and its subsequent bio-conversion into retinol suggest that 21 μg of β-carotene are required to produce 1 μg of retinol (West 2000; West *et al.,* 2002).

Some of the factors that affect bioavailability of pVA carotenoids include food preparation methods, carotenoid properties (such as their polarity), the complexity of food composition and interactions with other food compounds. In general, the

bioavailability of oxygenated (and therefore more polar) carotenoids is relatively higher than that of the less polar hydrocarbon carotenoids (Berg 1999; Yeum and Russel 2002; Bouis *et al.,* 2003b). In addition, since carotenoids are generally found complexed with protein in nature, release from the food matrix is an important initial step in the absorption process. Furthermore, iron deficiency has been reported to impair vitamin A metabolism by decreasing mobilization of retinol from liver stores and perhaps also by decreasing its absorption (Sonja *et al.,* 2005). Consequently, the RDA for vitamin A intake has been estimated at 1300 µg of RAE for lactating mothers, 770 µg for pregnant women, 700 µg for adult females, 900 µg for men and 400-500 µg for children below 14 yrs (National Academy of Sciences 2004; NIH 2005; USDA 2005).

1.2.3 Biosynthesis of pVA in plants

Extensive studies have been carried out in plants and bacteria to understand the carotenoid biosynthetic pathway. In plants, it has been shown that four enzymes, namely, phytoene synthase (*Psy*), phytoene desaturase (*Pds*), ζ-carotene desaturase (Z*ds*), and lycopene cyclase (*Lcy*), are required to complete the biosynthesis of β-carotene from an intermediate compound geranyl geranyl diphosphate (GGPP) (Sandmann 1994; Cunningham and Gantt 1998; Sandmann 2001). GGPP is a 20-carbon compound that is assembled from four molecules of a 5-carbon compound, isopentyl pyrophosphate (IPP), which in itself serves as a central metabolite for the synthesis of many other isoprenoid compounds in plants. Isomerisation of IPP to its allylic isomer, dimethylallyl pyrophosphate (DMAPP), the initial activated substrate for formation of the long chain polyisoprenoid compound GGPP, is a reversible reaction catalysed by IPP isomerase. GGPP is a precursor substrate for the synthesis of β-carotene as well as other carotenoid compounds such as tocopherols (vitamin E), phylloquinones (vitamin K1) and plastoquinones (Sandmann 2001).

The first reaction specific to the pathway of carotenoid biosynthesis involves the condensation of two C20 molecules of GGPP to produce the C40 carotenoid phytoene, and this reaction is catalysed by phytoene synthase (Fig 1.1), the enzyme that irreversibly directs GGPP substrate towards the formation of downstream products. Subsequently, *Pds* and *Zds* catalyse the conversion of phytoene to lycopene. However,

in non-photosynthetic bacteria, phytoene is converted to all-*trans* lycopene by a single phytoene desaturase enzyme – *CrtI* (Sandmann 1994). The cyclisation of lycopene is performed by two different lycopene cyclases (*Lcyε* and *Lycβ*) (Cunningham and Gantt 1998). Cyclisation by *Lycε* leads to α-carotene and its oxygenated derivative lutein, while cyclisation by *Lycβ* leads to β-carotene and the derived xanthophylls. The chemical structure of β-carotene is shown (Fig 1.2)

1.2.4 Transgenic approaches to improve pVA carotenoids in crops

Transgenic approaches have been effectively used to modify carotenoid content in plants to enhance their nutritional value (Zhu *et al.,* 2007; Aluru *et al.,* 2008; Jayaraj *et al.,* 2008; Apel and Bock 2009; Naqvi *et al.,* 2009; Kim *et al.,* 2010). In canola (*Brassica napus),* seed specific over-expression of a plastid targeted bacterial phytoene synthase (*CrtB)* from *E. uredovora* led to a 50-fold increase in the content of total carotenoids (Shewmaker *et al.,* 1999). In related studies in tomato, constitutive expression of the bacterial *CrtI* encoding the enzyme phytoene desaturase led to a 3-fold increase in β-carotene content representing up to 45% of total carotenoids (Romer *et al.,* 2000). This demonstrated a shift in carotenoid product in tomato which is a principal dietary source of lycopene.

In rice, where enhancement of pVA carotenoids, particularly β-carotene, is more advanced, a carotenoid pathway was engineered into the rice endosperm to develop "Golden Rice" (Ye *et al.,* 2000). Ordinarily, the rice seed endosperm does not contain any carotenoids but, by introducing the *Psy* gene from daffodil (*Narcissus pseudonarcissus)* and a bacterial desaturase *CrtI* gene from *E. uredovora,* the rice-seed was able to synthesize α-carotene, β-carotene, zeaxanthin and lutein (Ye *et al.,* 2000). The functional expression of the entire pathway led to carotenoid production in the Japonica rice cultivar Taipei 309. These studies demonstrated the integration of an entire and functional metabolic pathway in an alien background through transgenesis.

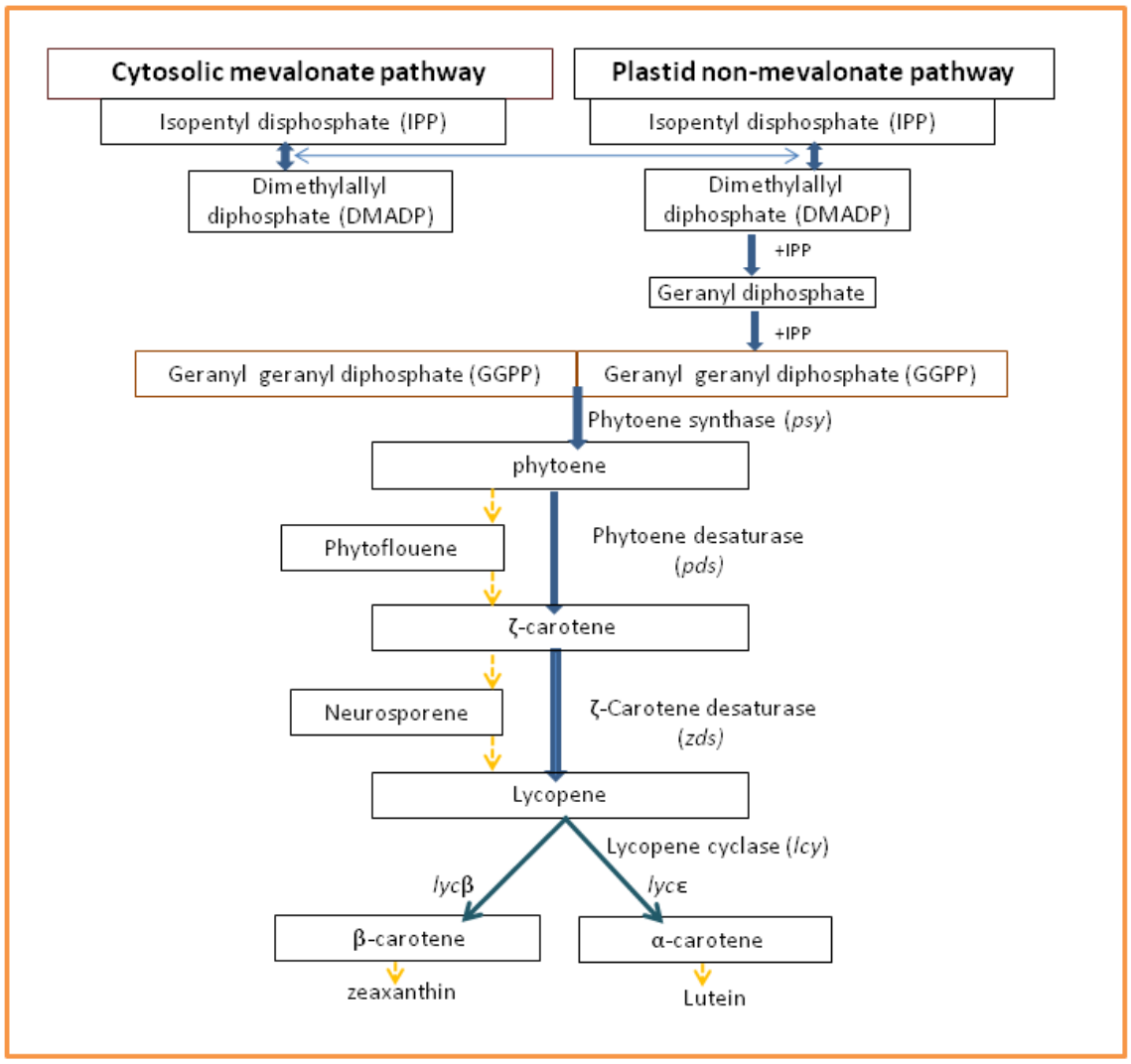


Figure 1.1 Biosynthetic pathway for pro-vitamin A carotenoids in plants. [Adopted from Sandmann 1994, 2001 and Cunningham and Grant 1998].

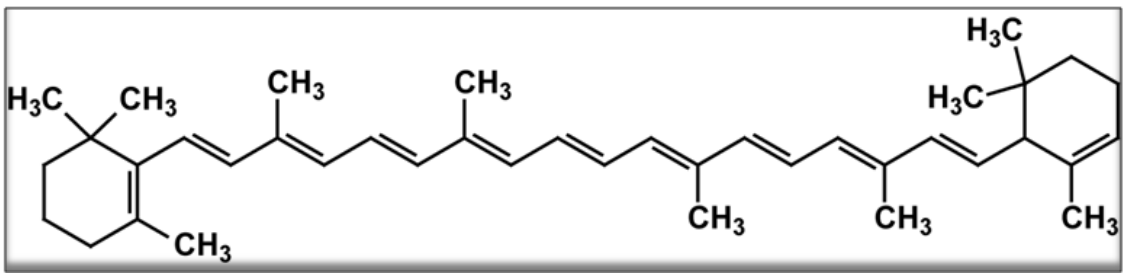


Figure 1.2 Chemical structure of β-carotene (studentsguide 2008).

In these experiments carried out in rice, the bacterial desaturase (*CrtI*) was able to perform both steps required for phytoene desaturation to obtain lycopene in plants. Both *Psy* and *CrtI* were able to complete the biosynthesis of β-carotene as well as the formation of further downstream xanthophylls without the need to introduce *Lcyb.* These concepts were then extended successfully to selected Indica rice cultivars that are well established in a number of countries in South East Asia (Datta *et al.,* 2003). In subsequent experiments in rice, altering the source of the *Psy* transgene, i.e. from different plant species (daffodil, tomatoes, pepper, carrot, rice and maize) revealed differences in carotenoid accumulation (Al-Babili and Beyer 2005; Paine *et al.,* 2005). Transformation of rice with *Psy* from daffodils produced 1.6 μg/g of carotenoid content in the rice endosperm (Ye *et al.,* 2000) whereas the use of *Psy* from maize (variety B73) resulted in increased amounts of carotenoid content in rice endosperm up to 37 μg/g (Golden Rice 2), of which 31 μg/g (84%) was β-carotene (Al-Babili and Beyer 2005; Paine *et al.,* 2005). In these experiments, no phytoene was detected in the transgenic plants indicating that *CrtI* was able to complete the desaturation of all phytoene produced. These results indicated that the source of the *Psy* transgene is essential in the generation of high levels of β-carotene content.

In related studies, the expression of *Psy* from *E. uredovora* under the control of a tomato-derived fruit-specific polygalacturonase (pgs) promoter has been demonstrated in the tomato fruit (Fraser *et al.,* 1994). The bacterial *Psy* enzyme was targeted to the chromoplast using the phytoene synthase-1 transit sequence, resulting in a 2-4 fold increase in total fruit carotenoids in transformed plants. In these experiments, the activities of other enzymes in the pathway were not significantly altered by the presence of the bacterial *Psy* gene. In earlier studies, expression of antisense RNA to the *Psy* gene of tomato was found to reduce the accumulation of carotenoid content in tomato fruits by as much as 97% without a noticeable effect on carotenoids in leaves (Bramley *et al.,* 1992). These findings implicated *Psy* as the enzyme that directs substrates to carotenoid synthesis and therefore is a potential target for transgenic studies in plants.

1.3. Biofortification for iron improvement in plants

1.3.1 Dietary iron, sources and functions in the human body

Iron is a vital constituent of plant and animal life. It is required in processes such as respiration, photosynthesis and for cellular functions including DNA synthesis, hormone production and electron transport. Iron is an essential component of haemoglobin which contains approximately 2 g of iron in an average adult body while a small amount is stored in circulating storage proteins such as transferitin (Gómez-Galera *et al.,* 2010). In addition, it is required for the synthesis of myoglobin and enzymes necessary for respiration, immune defence and thyroid function (Frossard *et al.,* 2000). Despite its absolute requirement, free iron can react with oxygen and generate reactive oxygen species such as superoxides which are destructive to cells (Cuerinot and Y 1994; Curie and Briat 2003). In order to supply sufficient iron for cell metabolic functions while avoiding toxic levels, multicellular organisms have evolved balanced mechanisms for iron homeostasis (i.e. iron regulation) during uptake, compartmentalisation, partitioning to various organs and storage. In this respect, iron transport is a major determinant of iron homeostasis. In most human diets, plants are the principal source of iron, mainly the dark-green leafy vegetables. Other foods that are rich in iron include meat, milk and eggs. Assuming a normal balanced diet, the RDA for iron is 10 mg/day for children and lactating mothers, 8 mg/day for adult males, 18 mg/day for adult females and 27 mg/day for pregnant women (National Academy of Sciences 2004).

1.3.2 Iron uptake in plants

In the soil, iron occurs in insoluble complexes as ferric iron (Fe^{3+}) which is toxic and insoluble and therefore not readily available to plants and other organisms unless it is reduced to ferrous iron (Fe^{2+}). Therefore, iron needs to undergo a reduction step before it can be transported from the soil through to the plant's edible parts. At neutral pH and alkaline environments, Fe^{3+} has a low solubility. As a result, plants encounter limitations of available iron to meet the critical needs for plant growth processes. Therefore, iron uptake by plants requires a preliminary step of either chelation or acidification in the rhizosphere (Curie and Briat 2003; Briat *et al.,* 2007). Plants have evolved strategies to increase their ability for iron uptake from the soil in a controlled manner while avoiding iron overload within the cells which leads to oxidative stress.

Plants utilise two distinct mechanisms known as Strategy I or Strategy II to mobilise iron from the rhizosphere. In general, dicotyledonous plants and non-graminaceous monocotyledonous plants utilise strategy I while all other monocotyledonous plants utilise Strategy II for iron uptake across the rhizosphere into their root cells. In some plants (eg. rice), both strategies are utilised (see below).

Strategy I plants excrete ions (protons) or organic acids into the rhizosphere, creating an acidic environment suitable for iron solubility into roots (Schmidt 2003; Jeong and Connolly 2009). This is achieved using the ATPase proton pump to excrete protons across the plasma membrane into the rhizosphere in response to an onset of iron limitation (Schmidt 2003; Jeong and Connolly 2009). Evidence to support the ATPase proton pump mechanism has been demonstrated in *Arabidopsis* where the expression of the AHA7 gene (*Arabidopsis* H+ ATPase) is up-regulated in response to iron deficiency (Colangelo and Guerinot 2004; Jeong and Connolly 2009). Similarly, studies in cucumber have shown that the CsHA1 proton ATPase is involved in the strategy I response for iron uptake (reviewed by (Jeong and Connolly 2009). In related studies, it has been demonstrated that low soil pH favours the reduction of ferric iron to ferrous iron (Fe^{3+} to Fe^{2+}), making it more soluble and ready to be transported into the plant (Briat and Lobreaux 1997). In addition to acidification of the rhizosphere under iron limiting conditions, Strategy I plants also induce the expression of ferric chelate reductases which act by reducing chelated ferric iron (Fe^{3+}) into soluble ferrous iron (Fe^{2+}) at the surface of root hairs particularly when iron is limiting. The reduction of Fe^{3+} to Fe^{2+} by ferric reductase oxidase (FRO2) has been shown to be an obligatory step both in iron uptake and being the primary factor in making iron available for absorption by the strategy I plants (Yi and Guerinot 1996; Frossard *et al.,* 2000).

In *Arabidopsis*, the FRO2 gene encodes an iron-deficiency inducible ferric chelate reductase which transfers electrons across the plasma membrane to reduce Fe^{3+} chelates to form Fe^{2+}in the soil (Robinson *et al.,* 1999). In order to perform this activity, FRO2 has intra-membranous binding sites for heme and cytoplasmic binding sites for nucleotide co-factors (FAD and NADPH) that donate and transfer electrons. The function of FRO2 in reducing ferric iron has been demonstrated in *Arabidopsis* where a

FRO2 loss of function mutant (*frd*1) showed a defect in iron uptake when iron was supplied as Fe^{3+}-chelate and showed extreme chlorosis when grown on iron-deficient media (Robinson *et al.,* 1999). Subsequently, it was shown that FRO2 complements the *frd*1 phenotype thereby providing evidence that FRO2 encodes the ferric chelate reductase involved in the iron-deficiency response (Robinson *et al.,* 1999; Jeong and Connolly 2009).

Although FRO2 is primarily expressed in the outer layer of root cells in response to iron deficiency in the soil (Connolly *et al.,* 2003), some studies have shown that other members of the FRO family, such as FRO1, are expressed throughout the root as well as leaves thereby suggesting an additional role in uptake and distribution of iron within the plant. In *Arabidopsis*, there are several FRO genes, some of which (FRO6, FRO7 and FRO8) are expressed in green tissues, while others (FRO3) are induced by iron limitation both in shoots and roots where function is limited to the vascular cylinder (Jeong and Connolly 2009).

In response to iron deficiency, strategy II plants (mainly grasses) release compounds known as phytosiderophores (PS) which are secondary amino acids synthesised from methionine, that chelate and solubilise inorganic Fe^{3}+ in the soil (Curie and Briat 2003). PS iron chelators are responsible for chelation and acquisition of iron from soil. PS forms Fe-PS complexes which are subsequently transported into the roots via the yellow-stripe like (YSL) metal transporters.

1.3.3 Iron transport in plants

In non-graminaceous and dicotyledonous plants that utilise the strategy I mechanism of iron acquisition from the soil, transport of iron across the root membrane is carried out by an iron regulated transporter (IRT1) from the ZIP family of metal transporters. It has been shown that IRT1 is localised in external cell layers of the roots and its role as the major transporter for metal uptake has been demonstrated by characterisation of *irt*1 mutants in *Arabidopsis* which showed severe sensitivity to iron deficiency (Vert *et al.,* 2002).

Although iron translocation through the plant to the various plant organs is poorly understood, recent identification of transporter genes of the YLS family (Curie *et al.,* 2009) explains the mode of iron loading and trans-loading throughout the plant. Once taken up by the plant, iron reacts with organic ligands thereby forming stable iron complexes that are subsequently loaded into the xylem sap and translocated throughout the plant via the transpiration stream (Curie and Briat 2003). A potential ligand involved in iron circulation is nicotianamine (NA) which is a precursor of phytosiderophores. NA has been shown to form stable complexes with iron in the phloem (Maas *et al.,* 1988) while xylem iron is complexed to citrate (Maas *et al.,* 1988; Curie and Briat 2003; Bauer and Hell 2006; Walker and Connolly 2008; Curie *et al.,* 2009). The trans-loading of iron complexes throughout the plant and distribution into tissues and organelles require specific trans-membrane transporters whose nature also depends on the type of Fe-substrate. Members of the YSL membrane transporters play the important role of taking up the iron-nicotianamine (Fe-NA), iron citrate (Fe-Citrate) and iron-phytosiderophore (Fe-PS) complexes and transporting them throughout the plant between vascular tissues to eventually support plant growth, gametogenesis and embryo development (Curie and Briat 2003; Briat *et al.,* 2007; Curie *et al.,* 2009). YSL genes have been observed in vascular tissue throughout the plant, thus supporting their role in long distance transportation of metals, particularly iron. However, although all plants produce NA, only grasses can transform NA into PS which then chelates Fe^{3+} resulting into Fe^{3+}-PS complexes in the soil. The YSL transporter then takes up the resulting Fe^{3+}-PS complex into the root cystol. The transport activity of YSL membrane transporters has been tested by heterologous expression of ZmYS-1 (from maize) in *fet*3*fet*4 yeast mutants defective for both low and high affinity metal transport. ZmYS-1 was able to restore growth of *fet*3*fet*4 mutants in the presence of Fe^{2+} or Fe^{3+} complexed to NA(Curie *et al.,* 2001; Curie *et al.,* 2009).

Unlike other grasses, rice has been shown to utilise both strategy I and II mechanisms to acquire iron from its environment (Fig 1.3). Rice can acquire ferrous iron directly via IRT-like transporters such as the *Oryza sativa* iron regulated transporters OsIRT 1 and OsIRT2 (Walker and Connolly 2008; Jeong and Connolly 2009). In addition, it has been shown that OsYSL15 (*Oryza sativa* yellow stripe like), a gene that is closely related to

ZmYS1, is expressed in the root epidermis and up-regulated under iron limitation. OsYSL15 is therefore believed to contribute to iron acquisition into the roots of rice. Similar YS1-like sequences have been identified in the genome of *Arabidopsis,* a dicotyledonous plant although their direct function in iron transport has not been confirmed. However, since NA is produced by all plants, it is thought that the YLS genes in dicots contribute to long distance circulation and distribution of metals within the plant (Curie *et al.,* 2009).

1.3.4 Accumulation of iron in plants

Excess iron can be toxic to plant tissues especially in the form of Fe^{3+}. Therefore, it has to be chelated into soluble forms (Fe^{2+}) and its deposition in the various plant parts is tightly balanced in order to minimise accumulation of toxic levels in the plant system (Grusak and DellaPenna 1999). In soil, Fe^{3}+ is first reduced to Fe^{2}+ before it can be taken up by plants. However, excess forms of Fe^{2}+ can react with reduced forms of oxygen thereby catalysing the formation of highly reactive free radicals which damage the cells and cellular components (Briat and Lobreaux 1997). As a result, excess iron is chelated by molecules such as citrate from which iron can be transferred to ferritin molecules for safe storage in various plant parts until needed (Laulhere and Briat 1993). Ferritin belongs to a class of multimeric proteins and is composed of 24 subunits forming a protein shell with an iron core in its central cavity that is capable of storing up to 4500 iron atoms (Fig 1.4) (Briat and Lobreaux 1997; Briat *et al.,* 1999). *In vitro* studies have demonstrated that, during iron deposition into ferritin, iron is first released by reduction from a chelating molecule. When iron crosses the ferritin shell, it is oxidised via the ferro-oxidase activity of ferric subunits (Laulhere and Briat 1993; Briat and Lobreaux 1997). Iron stored in ferritin is released in a controlled manner when needed. As such, ferritin acts as an iron buffer inside the cell by storing iron and making it available in a non-toxic and bioavailable form. Apart from leaves where the bulk of iron is stored in the form of ferritin in chloroplasts where it is engaged in both photosynthetic processes and correct development of the photosynthetic apparatus, ferritin proteins are localised in plastids, in particular in non-green plastids such as etioplasts and amyloplasts. In seeds, ferritin is a major long term iron storage protein. During germination, iron stored in the seed as ferritin is remobilised to provide

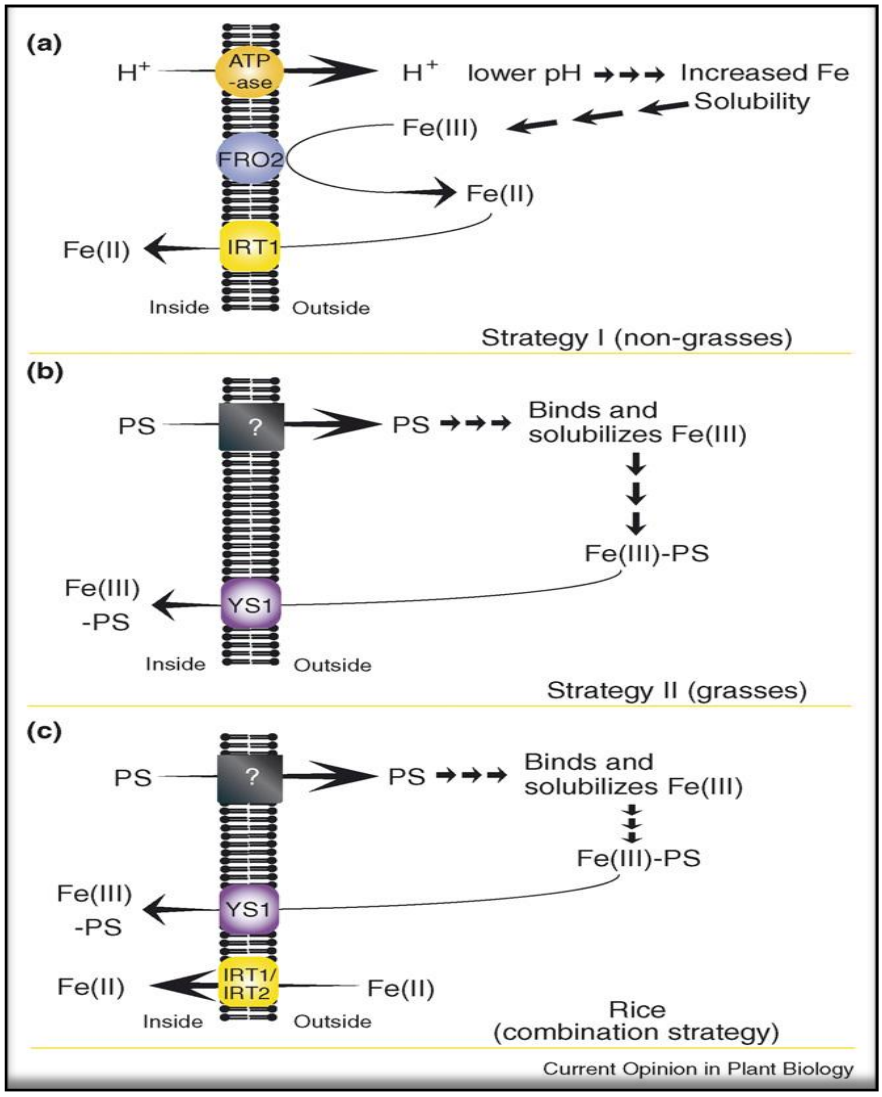


Figure 1.3 Strategies for iron uptake from the soil. (a) Strategy I. Acidification of the rhizosphere to solubilise Fe(III) provides the substrate for the enzyme FRO2, which in turn provides Fe(II) for transport into the cell by IRT1. (b) Strategy II. Phytosiderophores (PS) are produced by the root cells, and are released into the rhizosphere by an unknown mechanism. PS bind to Fe(III) in the soil, and the resulting Fe(III)–PS complexes are taken up into root cells by the transporter YS1. (c) Rice uses the typical grass Strategy II mechanism, but can also take up Fe(II) directly through OsIRT1 and OsIRT2 (Walker and Connolly 2008). (For the purpose of this illustration, Fe(II) is the same as Fe2+ while Fe(III) is the same as Fe3+ as represented in text).

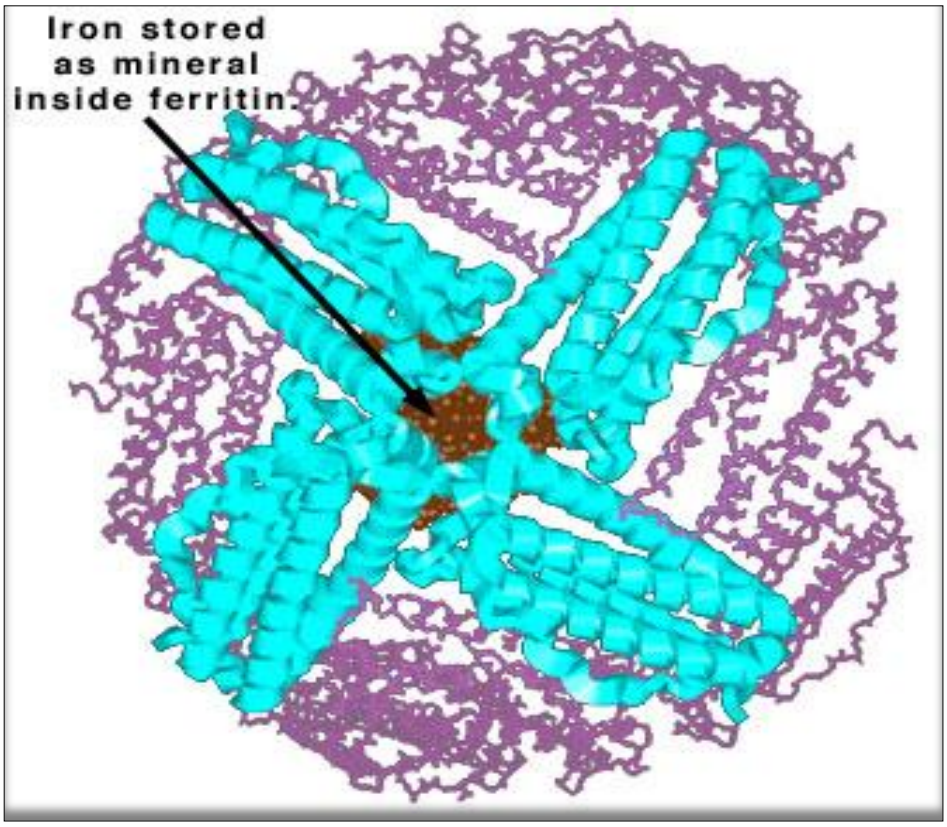


Figure 1.4 A three-dimensional representation showing the structure of ferritin, the iron-storage protein. Ferritin has a spherical shape and iron (brown) is stored as a mineral (Fe^{3+}) in the central cavity (Casiday and Frey 1999)

soluble iron for the formation of iron-containing proteins and supporting leaf development in young plants (Briat *et al.,* 1999; Curie and Briat 2003). However, the synthesis and abundance of ferritin is tightly controlled at the transcriptional level by cellular iron status (Briat *et al.,* 1999) which is determined by developmental and environmental factors such as excess iron or oxidative stress. For example, an increase in abundance of ferritin has been observed in developing and senescing leaves as well as maturing seeds (Briat and Lobreaux 1997). As such, the function of iron is based on the correlation between localisation of the ferritin iron storage protein and their expression in response to environmental and developmental stages (Briat and Lobreaux 1997; Briat *et al.,* 1999). When stored in ferritin, iron cannot react with oxygen. Therefore, in addition to being the major long term storage protein and a transient buffer for iron-dependent processes, ferritin performs the secondary function of protecting cells against oxidative stress (Briat *et al.,* 2007). The function of ferritin to protect cells against free iron-induced oxidative stress has been demonstrated in *Arabidopsis* whereby the loss of function of four *Arabidopsis* ferritin genes (*AtFer* 1-4) not only led to high levels of reactive oxygen species but also increased the activity of enzymes that are involved in their detoxification (Ravet *et al.,* 2009).

1.3.5 Transgenic approaches to improve iron content in edible plant parts

Transgenic approaches for improvement of iron in plants aim at improving the plant's ability to acquire iron from the soil, and to transport and store it in edible parts. As discussed previously, the proteins involved in the process of iron uptake, transport and storage in plants have been identified and isolated. In a number of studies, these proteins have been utilised to improve the iron content of crop plants. Isolation of the ferric chelate reductase gene (FRO2) from *A. thaliana* (Robinson *et al.,* 1999) paved the way for the generation of crops with improved iron quality and increased growth on iron-deficient soils. In *A. thaliana,* over-expression of FRO2 under the control of the constitutive CaMV 35S promoter resulted in transgenic plants that grew better under low iron conditions compared to control plants. Earlier related studies had also demonstrated that, when iron is limiting, the reductase activity on the surface of the root epidermal cells increases (Cuerinot and Y 1994). In addition, constitutive expression of *Arabidopsis* FRO2 in soybean also increased reductase activity both in

roots and leaves (Vasconcelos *et al.,* 2006). This reinforces the concept that reduction of Fe^{3+} to Fe^{2+} is a critical step in iron uptake.

In addition to iron reduction, transport across the plasma membrane to root epidermal cells is a critical step in iron uptake by plants. The iron root transporter (IRT1) is the major plasma membrane iron transporter, responsible for high affinity metal uptake especially in response to deficiency. IRT1 belongs to a group of eukaryotic metal transporters called the ZIP family (Curie and Briat 2003). It is only found in root epidermal cells and no other protein has been identified that compensates for its function (Vert *et al.,* 2002). This was demonstrated by elimination of IRT1 activity in *A. thaliana* by T-DNA insertion which led to severe leaf chlorosis and failure of mutant plants to grow. However, over-expression of the IRT1 transgene resulted in an accumulation of IRT1 protein in roots when iron was limiting (Connolly *et al.,* 2002), suggesting that IRT1, as well as FRO2, are both low iron-inducible proteins. In addition, iron uptake in *A. thaliana* has been improved by over-expressing genes encoding Fe^{3+} reductases in addition to iron transporters necessary for high affinity uptake from the soil and phloem transport (Connolly *et al.,* 2003).

Alternative transgenic approaches to increase the iron content of edible plant parts involve increasing the concentration of metal binding proteins such as ferritin or lacto-ferritin or increasing compounds that promote absorption (Ye *et al.,* 2008; Djennane *et al.,* 2010; Gómez-Galera *et al.,* 2010; Lin *et al.,* 2010). Ferritin genes have been isolated and sequenced from soybean (Ragland *et al.,* 1990) and other plants including French bean, pea and maize. Over-expressing plant ferritin to increase iron concentration has been demonstrated in rice where transgenic plants over-expressing the soybean ferritin gene under the control of the rice seed storage protein glutelin promoter (GluB-1) accumulated 3 fold more iron in their seeds compared to seed of untransformed rice plants (Vasconcelos *et al.,* 2003). In related experiments where the same combination of ferritin was used to transform an Indica rice cultivar, not only was there an increase in iron, but also an increase in seed zinc content up to 1.5 fold. In addition, endosperm-specific expression of the French bean-derived ferritin gene (Spence *et al.,* 1991) led to a 2-fold increase in the rice-seed iron content (Lucca *et al.,* 2002).

In some studies, constitutive expression of the soybean ferritin gene led to increased levels of iron in leaves but not seed. This has been demonstrated in rice (Drakakaki *et al.,* 2000) and in tobacco (Goto *et al.,* 1998) with a 2 fold and 1.3 fold increase in iron content obtained in leaves of transgenic plants, respectively, but not in seed. In addition, constitutive expression of soybean ferritin has been shown in lettuce leaves (Goto *et al.,* 1999; Goto *et al.,* 2000) with up to 1.7 times more iron in transgenic lettuce leaves compared to controls. Furthermore, when soybean ferritin was constitutively over-expressed in tobacco (Van Wuytswinkel *et al.,* 1998), an up-regulation of ferric reductase activity was observed. This reinforces the concept that the control of iron uptake is not solely controlled locally by the root but that there is a likely long-range shoot-to-root signal transmission pathway that integrates feedback events within the plant (Curie and Briat 2003). This suggests that over-expressing ferritin probably increases the plant's storage capacity for iron, a demand that induces the plant to behave as if it were growing under iron-limiting conditions. Furthermore, transgenic attempts have been made to increase phytase activity in the grain and reduce phytate biosynthesis in order to improve the bioavailability of iron (Brinch-Pedersen *et al.,* 2007).

1.4 Bananas and their nutritional significance

Cultivated edible bananas are derived from two species of the *Musa* genus, *M. acuminata* Colla and *M. balbisiana* Colla, resulting in eight genome groups classified as AA, AB, AAA, AAB, ABB, AABB, AAAB, ABBB with the letters A and B representing contributions from *M. acuminata and M. balbisiana*, respectively (Simmonds and Shepherd 1955). Bananas (*Musa* spp.) are grown in over 120 countries and consumed by up to 400 million people making banana the fourth most important staple food crop in world production after rice, wheat and maize (Ammar-Khodja 2000; FAO 2004; INIBAP 2004d). East African Highland bananas (AAA-EAHB), a unique type of cooking banana endemic in the East African region, are a major staple food in East and Central African countries including Uganda, Rwanda, Burundi, parts of the Democratic Republic of Congo, Kenya and Tanzania where an estimated 90% of total production is used for domestic consumption (Ammar-Khodja 2000; INIBAP 2004d). Amongst these countries,

Uganda grows and consumes the largest proportion at 220-250 kg/person/yr and is ranked the largest consumer in the world (Ammar-Khodja 2000; Kizza *et al.,* 2004). Approximately 70% of the bananas grown in Uganda are the AAA-EAHB cooking type belonging to the Lujugila-Mutika subgroup, also known as 'Matooke' (Karamura 1998).

In general, bananas contain 23% carbohydrate, 1% protein and 0.3% fat providing 116 Kcal of energy per 100 g of flesh (roughly equivalent to the flesh provided by one banana). Bananas are rich in other nutrients particularly vitamin C at 8.7 mg/100 g, potassium at 3580 μg/g and vitamin B in the form of thiamine, riboflavin, niacin and pyridoxine excluding vitamin B12 (USDA 2005). However, most cultivated varieties, and in particular the AAA bananas, have low levels of micronutrients particularly vitamin A, iron and iodine, vitamin E. As such, major diseases associated with micronutrient are reported amongst communities who rely heavily on these bananas for food (McIntyre *et al.,* 2001; Bachou and Labadorious 2002).

Some banana varieties are considered a source of pVA carotenoids, especially β-carotene. However, β-carotene content is highly variable between *Musa* spp. Commercial dessert banana was estimated to contain 21-26 μg β-carotene per 100 g of raw fruit (Holden *et al.,* 1999; USDA 2005). In more recent analyses, Cavendish fruit grown in Hawaii has been reported to contain an average of 55.7 μg β-carotene and 84.0 μg α-carotene per 100 g of fresh tissue (Wall 2006). In Cavendish bananas growing in Australia, 50-64 μg β-carotene and 93-123 μg α-carotene per 100 g of fresh tissue has been reported (Englberger *et al.,* 2007). In the Federated States of Micronesia, there are bananas varieties with high levels of pVA carotenoids where up to 2780 μg β-carotene per 100 g of raw fruit has been measured in one of the high β-carotene banana cultivars, *Uht en yap,* with a characteristic deep orange-colored flesh (pulp) (Englberger *et al.,* 2003b). Similarly, high levels of β-carotene up to 1412 μg/100 fresh fruit has been estimated from a yellow-orange fleshed variety, Asupina (Fe'i) grown in Australia (Englberger *et al.,* 2007). Englberger also observed a corresponding decrease in the intensity of pulp colour with decreasing levels of carotenoid content. Unfortunately, the banana varieties that have high levels of β-carotene have not been selected for cultivation, probably due to cultural preferences. As previously discussed

(section 1.2.2), the RDA for vitamin A intake is estimated at 1300 µg of RAE for lactating mothers, 770 µg for pregnant women, 700 µg for adult females, 900 µg for men and 400-500 µg for children below 14 yrs. Considering a ratio of 12 µg of β-carotene: 1 µg of retinol, bananas such as the Australian Cavendish contain 134 β-carotene equivalents/100 g fresh fruit, only providing 11µg RAE.

Available data for micronutrient nutrient content tends to vary depending on the geographical location of the fruit sampled, sampling procedure, sample size and method of estimation. For example, the iron content in commercial banana, probably Cavendish, has been estimated at 0.26 mg per 100 g of fresh fruit (USDA 2005). However, in other studies, iron content in Cavendish collected from three locations in Hawaii was estimated to be in the range 0.62 –1.0 mg per 100 g of fresh fruit (Wall 2006). The RDA for iron is 10 mg/day for children and lactating mothers, 8 mg/day for adult males, 18 mg/day for adult females and 27 mg/day for pregnant women (section 1.3.1).

1.5 Banana biofortification - strategy for alleviating micronutrient malnutrition

Many of the banana cultivars in cultivation have been selected over-time and have evolved deep cultural significance within communities such that farmers/consumers have passionately established preferences that extend to specific varieties that are already integrated into the farming system. In such a situation, the major barrier would be accepting a new cultivar that may not be agronomically suitable and cannot be put to the same use. Previous attempts to introduce new crops in already established subsistence cropping systems in Uganda have consistently failed (Carswell 2003). Besides being a staple food crop, bananas such as EAHB are also a regional cash crop. Therefore, rather than introduce new crops, the nutrient quality of staple food crops can be improved by increasing amounts of necessary micronutrients in edible plant parts through biofortification. In an already established cropping system, a biofortified staple crop would ensure the delivery of adequate micronutrients through a daily, normal diet without recurrent costs. For several reasons biofortification through genetic engineering appears to be the most suitable approach for bananas. Firstly, most popular cultivated varieties are essentially sterile making their conventional

breeding impossible. Secondly, they form no viable pollen, are vegetatively propagated and therefore there is limited risk of gene flow into the environment or other plants. Finally, with recent advances in banana transformation methods and the availability of a number of gene sequences, development of new banana varieties through transgenic approaches is possible.

1.5.1 Strategies for improving pVA carotenoids in banana

High β-carotene bananas have been identified and characterised (Englberger *et al.,* 2003b). The alternative quick option would be to introduce these high β-carotene varieties in communities that mainly depend on banana for food. However, as discussed above, this approach is limited especially where subsistence cropping systems are already established. Transgenic approaches therefore offer viable alternatives to introduce novel genes that promote iron uptake or biosynthesis of pVA in varieties already established within the existing farming system. Based on the knowledge generated from the development of Golden Rice, a similar transformation approach can be used for banana. The sequences for *Psy* from daffodil *(N. pseudonarcissus)* (Ye *et al.,* 2000) and *CrtI* from bacteria *(E. uredovora)* (Misawa *et al.,* 1990) have been isolated and are available in public gene banks. This sequence information, together with PCR-based techniques, has been used to isolate *Psy* homologues from crop plants such as maize (Paine *et al.,* 2005). Subsequently, maize *Psy* and the bacterial *CrtI* have been shown to increase carotenoid content in rice (Paine *et al.,* 2005). Furthermore, using the available sequence information, the banana homologues of *Psy* have been isolated and sequenced from the high β-carotene F'ei bananas [cv Asupina] at QUT with the aim of transferring the sequences into cooking banana for the improvement of pVA content of the fruit (Mlalazi 2010).

1.5.2 Strategies for improving bananas for increased iron content

Unlike β-carotene, there are no reported iron-rich banana varieties. Therefore, improving iron content in banana fruit can only rely on the application of biotechnological techniques. The existing knowledge of iron uptake from the soil and storage can be exploited in order to improve iron content in banana. In soil, iron exists in the form of Fe^{3+} which is toxic and insoluble and therefore not readily available to

plants until it is chelated and reduced to forms that can be taken up and transported into the plant (as described in section 1.3.2). Three main genes involved in this process, FRO2, IRT1 and plant ferritin, whose functions have been demonstrated in other crops, can be tested in banana through genetic transformation. The full-length sequence of the soybean (*Glycine max* cv. Williams 82) ferritin gene is available (Ragland *et al.,* 1990). Similarly, the full-length sequences of *A. thaliana* (cv. Landsburg) FRO2 (Robinson *et al.,* 1999) and IRT1 (Eide *et al.,* 1996) genes are available in public databases. Synthesis of cDNA encoding these genes can be done from mRNA derived from soybean cotyledons (for soybean ferritin), and from the roots of *A. thaliana* (for FRO2 and IRT1) using RT-PCR with specific primers. Similarly, cDNA encoding native banana ferritin, FRO2 and IRT1 can be isolated using degenerate and gene-specific primers. Scientists at QUT have isolated and sequenced the banana ferritin (Rosier - PhD thesis in preparation). Therefore, with appropriate promoters, this and other target sequences can be over-expressed individually or in combination, to assess their potential for enhancement of iron content in banana.

1.6 Banana transformation and generation of transgenic plants

Transformation and generation of true-to-type transgenic plants requires a well established cell and tissue culture and regeneration system. In the last two decades, advances in banana biotechnology have led to the development of different methods for plant regeneration for a range of banana varieties. In particular, embryogenic cell suspension cultures have been established and regenerated through somatic embryogenesis from highly proliferating meristems (Dhed'a *et al.,* 1991), zygotic embryos (Marroquin *et al.,* 1993) and immature male flowers (Côte *et al.,* 1996; Navarro *et al.,* 1997; Becker *et al.,* 2000). Genetic transformation of banana has been reported using electroporation of protoplasts (Sagi *et al.,* 1994) and co-cultivating wounded meristems (May *et al.,* 1995; (Tripathi *et al.,* 2008). However, eletroporation of protoplasts is constrained by the difficulty and time consuming procedures for generation of protoplasts while transformation of meristems is limited in value due to the potential of generating chimeric plants. Therefore, these two systems are not routinely used. Successful transformation and regeneration of true-to-type transgenic banana plants with foreign genes has been achieved in different cultivars using particle bombardment (Sagi *et al.,* 1995; Becker *et al.,* 2000) and *Agrobacterium*-mediated

transformation (Ganapathi *et al.,* 2001; Khanna *et al.,* 2004; Ghosh *et al.,* 2009). Furthermore, Khanna (2004) reported an improved *Agrobacterium*-mediated procedure in banana demonstrating higher transformation effi ciencies by incorporating centrifugation steps. Although both transformation methods are well established and routinely applied, particle bombardment provides lower transformation efficiency and is limited to the availability of a particle gun and expensive gold particles. *Agrobacterium*-mediated transformation is the preferred method due to its ability to produce both high transformation efficiencies and low copy number transgene insertion into the genome.

Immature male flowers are the most commonly used explant to induce callus for establishment of embryogenic banana cell suspensions. However, it takes at least 9-12 months to induce embryogenic callus and obtain a cell suspension in volumes sufficient for ascertaining regeneration potential and to carry out a transformation experiment. The banana transformation process takes at least 8-10 months from the time of transformation to regenerating transgenic shoots *in-vitro* (Côte *et al.,* 1996; Becker *et al.,* 2000) and the banana vegetative cycle takes at least 11 -16 months from planting up to fruit formation. Therefore, it would take at least 3 yrs before a transgenic banana fruit could be obtained. The function of selected target genes and promoters for banana transformation ideally needs to be evaluated in a model crop with relatively shorter generation before introducing them into banana. Also, to ensure expression of proteins involved in uptake, synthesis and accumulation of iron or pVA carotenoids, tissue-specific (root or fruit) promoters are required. The use of native genes and promoters is a desirable strategy to ensure transgene compatibility with the crop's genetic makeup and preserving the genetic integrity of transformed cultivars. As part of the Grand Challenges in Global Health (GCGH) program, genes encoding ferritin and enzymes in the carotenoid pathway, as well as a range of promoters have been isolated from other banana cultivars, with the aim of transferring them into East African highland bananas (EAHB). Some of these target genes and promoters have not yet been functionally characterised. Previous studies have demonstrated that rice can accumulate carotenoids (Ye *et al.,* 2000; Paine *et al.,* 2005) and iron (Drakakaki *et al.,* 2000; Vasconcelos *et al.,* 2003) following transgene expression. Therefore, rice offers

an ideal monocot system for initial studies to characterise target genes. In addition, promoters can be analysed by transient assays and/or by analysis of stably transformed banana plants (Dugdale *et al.,* 2000; Dugdale *et al.,* 2001; Mlalazi 2010) .

1.7 Rice as a model system for evaluation of monocot genes

Rice has a short life cycle and is relatively easy to transform through *Agrobacterium*-mediated transformation of mature seed-derived callus (Hiei *et al.,* 1994; Nishimura *et al.,* 2006). Transformation of rice callus has been used by several authors to transform Indica, Japonica and Javanica rice varieties (reviewed by (Shrawat and Lorz 2006). Consequently, a large number of different rice varieties have been transformed with genes for a range of agronomic traits including herbicide resistance, salt tolerance, disease resistance, seed quality improvement (reviewed by (Tyagi *et al.,* 1999), root vigour (Agarwal *et al.,* 2007) and oxidative stress resitance (Zhao and Zhang 2006). In addition, enhancement of micronutrient iron or pVA carotenoids and protein has been evaluated in rice leading to the development of varieties with improved nutritional value (Ye *et al.,* 2000; Paine *et al.,* 2005; Li *et al.,* 2009). Because rice provides an efficient transformation system, it is considered a model plant for the study of gene function in monocotyledonous plants just as tobacco and *Arabidopsis* are used as models to study dicotyledonous plants. Again, rice has been used to evaluate the function of genes and promoters from monocotyledonous plants such as maize, wheat, barley and regulatory elements from different rice varieties (Tyagi *et al.,* 1999).

1.8 Promoters for banana biofortification

1.8.1 Sources of banana fruit-related promoters

Banana is a climacteric fruit whose postharvest physiology is characterised by a green-storage phase followed by production of ethylene which triggers the conversion of starch into sugars during ripening (Pathak *et al.,* 2003; Bapat *et al.,* 2010). Other changes during ripening include softening of the fruit pulp and peel due to cell wall degradation, a decline in polyphenols, increase in the activity of enzymes that catalyse the formation of aromatics and increase in both protein synthesis and accumulation (Medina-Suarez *et al.,* 1997; Giovannoni 2001; Asha *et al.,* 2007). There is limited information about banana promoters and their role in controlling the ripening

processes and gene expression in the fruit. However, several studies have reported the identification and isolation of transcripts from genes associated with fruit ripening (Holdsworth M J *et al.,* 1987; Clendennen and May 1997; Medina-Suarez *et al.,* 1997; Trivedi and Nath 2004; Asha *et al.,* 2007; Mlalazi 2010). The promoter sequences controlling the expression of these fruit- or ripening-related genes could be utilised in an attempt to identify promoters that would be useful for transgenic expression studies in banana fruit.

Expansins are one group of proteins (α- and β-family) that have been shown to participate in fruit ripening in addition to several other processes during plant development including cell wall expansion, organogenesis and seed germination (Cosgrove 2000; Lee *et al.,* 2001; Trivedi and Nath 2004). Transcripts of expansin (MaExpa1) were isolated from banana fruit (Cavendish, cv Robusta) and shown to be both ethylene regulated and expressed only during ripening (Trivedi and Nath 2004)**.** Four related expansin genes (*MaExp*A2, *MaExp*A3, *MaExp*A4 and *MaExp*A5) isolated from banana (*Musa acuminata* (AAA), cultivar Harichal) are reported to be expressed differentially during fruit development and ripening (Asha *et al.,* 2007). In our laboratory at QUT, a range of phytoene synthase (*Psy*) genes including *Psy*1, *Psy*2a and *Psy*2b which are involved in the biosynthesis of pVA carotenoids, have been isolated from Cavendish banana and the high β-carotene F'ei banana variety, Asupina (Mlalazi 2010). Of these genes, the *Psy*2a was preferentially obtained from banana fruit suggesting fruit-preferred expression. In related research, the promoters associated with the banana-derived *Psy* genes were isolated and made available for analysis in this research.

The 1-aminocyclopropane-1-carboxylate (ACC) synthase catalyzes the rate-limiting step in ethylene biosynthesis by converting *S*-adenosylmethionine to ACC, which is subsequently oxidized by ACC oxidase (ACO) to form ethylene (Yang and Hoffman 1984)**.** In many plant species, ACC synthase (ACS) is encoded by a multiple gene family, whose expression is regulated developmentally and environmentally (Wang *et al.,* 2005). Members of the ACS gene family such as the *At*ACS4, *At*ACS5 and *At*ACS7 have been indentified in *Arabidopsis* and were found to be expressed at different stages of growth and development in a range of plant tissues (Wang *et al.,* 2005). However all

their promoters were found in reproductive organs and shown to be responsive to ethylene and auxin treatment (Wang *et al.,* 2005; Tang *et al.,* 2008)**.** In banana up to nine clones of the banana ACS have been indentified, of which only the MaACS1 isoformwas expressed during fruit ripening (Huang *et al.,* 2006)**.** An increase in transcripts of the CMe-ACS1 gene from melon fruit have previously been shown to increase during ripening at 5 days after harvest and shown to be ripening induced (Miki *et al.,* 1995). The mRNA of two ACS genes, CMeACS2 and 3 isolated from melon fruit have also been shown to increase during ripening but their expression was earlier, at 3days, than CMeACS1 (Ishiki *et al.,* 2000)**.** In other studies in banana, transcript accumulation of patterns and protein levels of MaACS and MaACO genes have been shown to be responsive to ethylene treatment (Choudhury *et al.,* 2008). In addition, GCC-box putative ethylene responsive elements (ERE) were identified in banana fruit which have been previously reported to be *cis*-acting ERE in the promoters of many ethylene inducible genes (Sato *et al.,* 1996).

The ACO (1-aminocyclo-propane-1-carboxylate oxidase) gene, another ripening-related gene encodes a key enzyme in the ethylene biosynthesis pathway, was first identified from tomato (Holdsworth M J *et al.,* 1987). Homologues of the ACO gene have been identified in other fruits such as kiwifruit (MacDiarmid and Gardner 1993) and apple (Ross *et al.,* 1992), and its expression was shown to be induced upon the application of exogenous ethylene. Using *Agrobacterium*-mediated infiltration in combination with the GUS reporter gene, the function of the ACO promoter has been demonstrated in different fruits including tomato (Blume and Grierson 1997), apple (Atkinson *et al.,* 1998) and peach (Ruperti *et al.,* 2001; Rasori *et al.,* 2003), with promoter activity shown to be linked to ethylene responsiveness.

Extensin forms one of the most abundant cell wall proteins found in higher plants and is commonly associated with phloem tissues. However, studies in different plants have shown that extensin gene expression and localization can apparently vary from plant to plant and among cell and tissue types, presumably in accord with the different functions of different cell types and tissues (Showalter 1993). In banana fruit, the peel appears to share ripening-related events with the pulp including softening and starch

degradation, processes that generate stress in the fruit and pulp (Seymour 1993). A number of ripening related genes including ACO and extensin have been identified in the banana peel (Drury *et al.,* 1999). The extensin-like sequence isolated from the peel was shown to be up-regulated during ripening, probably due to the stress caused by the swelling of the pulp. In contrast, extensin from the pulp was found to be down-regulated during ripening and this response may reflect the biochemical events designed to weaken or change the structure of the cell wall (Medina-Suarez *et al.,* 1997; Drury *et al.,* 1999). Transcripts of extension have also been identified in citrus fruits, where they have been shown at high levels during fruit development and decrease levels towards the fruit ripening stage (Kita *et al.,* 2000). In these studies, the accumulation of extensin transcripts was shown to be related to morphological and developmental changes in the rind of the citrus fruit before ripening. Other transcripts of extensin, Ext1.4, have been identified in tobacco (Hirsinger *et al.,* 1997). The promoter region of Ext 1.4 has been studied in transgenic tobacco plants and found to occur in various tissues where mechanical stress exists and in cells proliferating under hormonal control (Hirsinger *et al.,* 1999). Furthermore, studies in *Arabidopsis* have shown that the extensin gene is developmentally regulated and auxin responsive (Merkouropoulos *et al.,* 1999).

Metallothioneins (MTs) are cysteine-rich polypeptides that are involved in metal detoxification and homeostasis in both prokaryotes and eukaryotes. A type-3 metallothionein (MT-3)-like gene (*Cit*MT45) has been identified from *Citrus ushiu* and its promoter was shown to confer preferential expression in the citrus fruit tissues (Endo *et al.,* 2007). Three members (MT2A, MT2B and MT3) of the MT-like gene family have been identified from ripening banana fruit (Liu *et al.,* 2002). The three members were found to express differentially in various organs but were more abundant in reproductive than in vegetative tissue. In addition, expression of MT members appeared to be regulated by ethylene whereby transcripts of the MT2A were found to attain peak abundance during banana ripening i.e. at the onset of ethylene production and gradually decreased during the advanced stages of fruit ripening (Liu *et al.,* 2002). Similar observations and ethylene responsiveness have been reported for a type2-MT gene from banana (Clendennen and May 1997). In contrast, transcripts of MT3 whose

transcripts were very low in young fruits, were detected in later stages of fruit development and increased in advanced stages of fruit ripening (Liu *et al.,* 2002). The abundance of metallothionein transcripts in the pulp and peel of mature banana fruit and during ripening suggested that the MT genes are mainly associated with the fruit.

The promoter elements of genes whose transcripts are predominantly expressed during fruit development and ripening would seem likely candidates for use in fruit expression studies to regulate the production of proteins of interest in transgenic banana fruit. In preliminary studies at QUT, the activity of a number of banana-derived promoters related to fruit development or ripening genes such as MaACO, MaExp1, APsy2a and MaMT2a have been tested in banana fruit by particle bombardment and shown to be fruit-active (Jason Geijskes – personal communication) and (Mlalazi 2010). In this study, some of these promoters were selected for further analysis using transient assays in banana and in stably transformed banana plants.

1.8.2 Other promoters used in transgene expression studies in banana

Different promoters have been previously used in banana transformation studies. The constitutive cauliflower mosaic virus (CaMV 35S) promoter has been the most commonly used (Sagi *et al.,* 1995; Dugdale *et al.,* 1998; Becker *et al.,* 2000; Khanna *et al.,* 2004; Ghosh *et al.,* 2009). In addition, the maize poly-ubiquitin promoter (Christensen *et al.,* 1992; Christensen and Quail 1996) has been used to demonstrate reporter gene expression and/or stable transformation of banana via particle bombardment (Becker *et al.,* 2000; Arinaitwe *et al.,* 2004; Becker and Dale 2004) or *Agrobacterium*-mediated transformation (Khanna *et al.,* 2007). Other promoters that have been used in transgenic expression studies in banana include the banana actin promoter (ACT1) which demonstrated strong near-constitutive activity in banana leaves, roots and floral tissues (Hermann *et al.,* 2001).

Furthermore, the activity of viral promoter sequences has been analysed for reporter gene expression in banana. Of these, promoter fragments from the Australian *banana streak badnavirus* (BSV) showed high-level expression of either the green fluorescent protein (GFP) or the β-glucuronidase (GUS) reporter gene (*uid*A) in transient expression systems and/or vegetative plant cells (Schenk *et al.,* 2001). Other viral promoter sequences have been isolated from the *banana bunchy top virus* (BBTV) DNA and

shown to direct reporter gene expression in banana tissues (Dugdale *et al.,* 2000). In additional studies, inclusion of intron fragments to the promoter sequence significantly enhanced the activity of the BBTV-derived BT6.1 promoter to levels similar to that of the constitutive CaMV 35S promoter (Dugdale *et al.,* 2001).

1.8.3 Analysis of promoter activity by transient expression

Although the analysis of promoter activity in stably transformed plants is considered ideal, in many cases, the generation of stably transformed plants is difficult, time consuming and expensive. As a result, alternative approaches based on transient expression assays are frequently used to provide a quick, preliminary assessment of promoter activity. Such assays rely on the use of reporter genes as a means of assessing tissue specificity and quantifying promoter activity.

1.8.3.1 Reporter genes

The establishment of transient transformation procedures relies on the use of reporter genes such as the *uidA* gene encoding β-glucuronidase (GUS) derived from *E. coli* (Jefferson *et al.,* 1987b) or the gene encoding green fluorescent protein (GFP) derived from the jellyfish, *Aequorea victoria* (Chalfie *et al.,* 1994). Both of these gene products are easy to visualise, thus allowing quick detection of transformation events after a transformation experiment, either in transient or stable expression assays. The GFP reporter gene has been shown to be a powerful tool in assessing transient or stable transformation and in monitoring sub-cellular localisation of protein (Leffel *et al.,* 1997).

The GUS gene is the most commonly used reporter gene in plant transformation systems for transient assays. GUS activity can be easily detected by both qualitative and quantitative methods using histochemical and fluorometric assays, respectively (Jefferson 1987a; Jefferson *et al.,* 1987b). Although the detection of GUS activity in routine plant transformation is used to verify transient or stable transformation, GUS has widely been used as a reporter gene to evaluate the function of promoter sequences and their potential applications in target plant tissues (Rigau *et al.,* 1993; Atkinson *et al.,* 1998; Humara *et al.,* 1999; Basu *et al.,* 2003; Rasori *et al.,* 2003; Hellens *et al.,* 2005). The resulting GUS protein can then be detected and measured from cells

of transiently transformed tissue or in stably transformed plants. This reporter gene system offers many advantages over other systems including (i) lack of endogeneous GUS activity in most plants (Jefferson *et al.,* 1987b) and (ii) GUS is very stable, with an optimum detection in the pH range of 5.2 ≥ 8.0 with the most activity in the presence of reducing agents such as mercaptoethanol, dithiothreitol (DTT) or metabisulphite (Jefferson *et al.,* 1987b; Serres *et al.,* 1997; Côté and Rutledge 2003). Further, many improvements have been made to GUS assays over the years making them more widely applicable to different plant species (Côté and Rutledge 2003; Fior *et al.,* 2009).

The most commonly used substrates for histochemical and fluorometric GUS assays are 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) and 4-Methylumbelliferyl-β-D-glucuronide (MUG), respectively. The GUS enzyme interacts with X-Gluc to produce a blue insoluble precipitate at the site of enzyme activity. Similarly, MUG is not fluorescent until cleaved by GUS to form the fluorescent 4-Methylumbelliferone (MU) (Jefferson 1987a; Cervera 2004). During extraction of GUS protein from plants tissues, adsorbent compounds such as polyvinylpyrrolidone (PVP) (Jefferson 1987a), or Dowex (Loomis *et al.,* 1978) are often included to remove interfering compounds such as secondary products (e.g. phenolics) which bind to proteins and interfere with protein recovery.

1.8.3.2 Particle bombardment

Particle bombardment is a well established method of transformation in many laboratories, and its utility to evaluate the function of promoters has been reported in a number of plant species. In bananas, for example, transient particle bombardment methods have been used to evaluate the activity of a *Banana bunchy top virus*-derived promoter (BT6.1) in banana embryogenic cell suspensions (Dugdale *et al.,* 2001). In addition, particle bombardment has been used to demonstrate transient evaluation of heterologous promoters in strawberry fruit (Agius *et al.,* 2005), in cotyledons of *Pinus pinea* (Humara *et al.,* 1999), in leaves and roots of the creeping bent grass (Basu *et al.,* 2003) and in tobacco protoplasts (Rigau *et al.,* 1993).

1.8.3.3 *Agrobacterium*-mediated infiltration

Although particle bombardment is well established and routinely used in many research laboratories, there is an increase in the use of *Agrobacterium*-mediated transient assays because it requires no specialised equipment and intact plant tissues/organs can be transformed. The usefulness of transient GUS expression by the *Agrobacterium*-mediated infiltration (agro-infiltration) method has been demonstrated for promoter analysis in a range of tissues such as tobacco leaves (Yang *et al.,* 2000; Hellens *et al.,* 2005; Lee *et al.,* 2007) and tomato fruit (Atkinson *et al.,* 1998; Rasori *et al.,* 2003). Furthermore, agro-infiltration has been used to study transient gene expression in a variety of fruits including apple, pear, peach, strawberry, orange and tomato (Spolaore *et al.,* 2001; Wroblewski *et al.,* 2005). However, these assays were shown to be limited to species and tissues that are biologically and physiologically amenable to *Agrobacterium* infection (Wroblewski *et al.,* 2005). A range of physiological factors affecting the efficiency of *Agrobacterium*-mediated transformation in a transient expression assay include the density and volume of the *Agrobacterium* suspension, age and type of tissue, the time period post-infection before assay, bacteria-host compatibility and additives such as acetosyringone (Wroblewski *et al.,* 2005; Orzaez *et al.,* 2006; Li *et al.,* 2007; Hasan *et al.,* 2008).

In addition to the analysis of promoter activity, agro-infiltration has also been used in plants to analyse the function of genes such as the heat shock proteins in tomato (Orzaez *et al.,* 2006) and the expression of the *A. thaliana* early flowering genes (Apetalai - AP1) in tomato (Hasan *et al.,* 2008). In other studies, agro-infiltration has been used as a valuable tool in determining expression and production of biologically active therapeutic proteins such as the active human interferon-beta (HuIFN-beta) genes in lettuce leaves (Li *et al.,* 2007). In similar studies, genes that encode the poly-γ-glutamate synthase (*pgs*) system were transiently expressed under the control of constitutive 35S promoter in tobacco leaves resulting in the synthesis of immunologically detectable γ-pgs protein levels (Tarui *et al.,* 2005).

Despite transient assays providing a rapid, preliminary assessment of promoter activity, conclusive quantitative analyses in stably transformed plants are ultimately required

since the results from transient analyses do not always correlate with those obtained in transgenic plants (Holtorf *et al.,* 1995; Hamilton *et al.,* 2000).

1.9 Project aims

One of the major activities of the Bill and Melinda Gates Foundation-funded banana biofortification project at QUT is the isolation of genes associated with the accumulation of iron or the biosynthesis of pVA carotenoids and the isolation of promoters suitable for directing transgene expression in fruit. A number of genes that play a major role in the accumulation of iron or the biosynthesis of pVA carotenoids have been isolated from *Musa* spp. Cavendish or the F'ei high β-carotene variety, Asupina, respectively. Further, a number of promoters associated with fruit ripening genes have been isolated from *Musa* spp. Cavendish. The major aim of this PhD project was to characterise selected banana genes that encode major proteins for the accumulation of iron and biosynthesis of pVA carotenoids. Due to the lengthy process of banana transformation and the long vegetative/cropping cycle before fruit formation, rice was used as an alternative model monocot crop to characterise the new banana genes. In addition, the activity of a range of banana-derived potentially "fruit-active" promoters was investigated for their ability to drive fruit-preferred transgene expression in banana using particle bombardment and *Agrobacterium*-mediated infiltration methods.

Chapter 2 General Materials and Methods

2.1. Preparation and transformation of bacteria with vector DNA

2.1.1. Preparing electro-competent *Agrobacterium tumefaciens* cells

Electro-competent cells of *Agrobacterium tumefaciens (*strain AGL1) were prepared as previously described (Dower *et al.,* 1988). In a 15 mL Falcon tube, 5-10 mL of YM media (Appendix 1) was inoculated with 100 µL of *Agrobacterium* cells [in 10% glycerol] and incubated overnight at 28°C on a rotary shaker at 200 rpm. An aliquot (2 mL) of the overnight culture was added to a 250 mL Erlenmeyer flask containing 50 mL of 2XYT media (Appendix 1) and the flask was incubated at 28°C on a rotary shaker at 200 rpm. When the cells were at the exponential growth stage an optical density OD_{600} = 0.5-0.6 was determined using a UV/Vis spectrometer - Lambda 35 Perkin Elmer), a 40 mL aliquot was transferred into 50 mL pre-chilled Falcon tubes and then centrifuged at 4000 x g at 4°C for 10 min in a bench top centrifuge (Universal 32-R Hettich Zentrifugen). The pelleted cells were washed twice in 20 mL of sterile cold MilliQ water in order to remove all salt residues. Subsequently, cells were washed once in filter-sterilised, cold 10% glycerol before re-suspension into ice cold 10% glycerol in a 1:3 ratio [pellet: glycerol]. Aliquots (100 µL) of cells were dispensed into 500 µL pre-chilled microcentrifuge tubes and stored at -80°C.

2.1.2. Preparation of *Escherichia coli* competent cells for transformation by heat shock method

E.coli cells (strain XL1-Blue) were made chemically competent as previously described (Inoue *et al.,* 1990). A frozen glycerol stock culture of *E. coli* cells was thawed on ice and a 100 µL aliquot of cells was inoculated into 4 mL of LB media (Appendix 1) containing 0.05 µM tetracycline to establish a starter culture. The cell culture was incubated at 37°C overnight with shaking at 200 rpm on a rotary shaker. From the overnight bacterial culture, 100 µL was re-inoculated into fresh 4 mL LB for 6-7 hrs at 37°C with shaking at 200 rpm. Subsequently, 1 mL of the culture was transferred into 250 mL of SOB media (Appendix 1) and the cultures shaken at 200 rpm at 18°C for approximately 40 hrs until an optical density between OD_{600} = 0.5 to 0.6 was reached. Subsequent operations were performed at 4°C. Aliquots of 50 mL bacterial cells were

transferred into pre-chilled 50 mL Falcon tubes and incubated on ice for 10 min before centrifugation at 4000 x *g* at 4°C for 10 min. The pelleted cells were gently re-suspended in 16 mL of chilled transformation buffer (TB) (Appendix 1) and left to stand on ice for 10 min before centrifugation at 4000 x *g* at 4°C for 10 min. The pellet was gently re-suspended in 3 mL of TB containing 7% DMSO. Cells were then incubated on ice for 10 min after which aliquots of 50 µL were transferred into pre-chilled 500 µL microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C. Such competent cells can be stored up to 1 month at -80°C.

2.1.3. Transformation of *Agrobacterium* by electroporation

An aliquot of 100 µL electro-competent *Agrobacterium* cells was thawed on ice and diluted with 100 µL 10% glycerol. To 50 µL of diluted cells, 1 µL (~ 200 ng) of plasmid[1] DNA was added and gently mixed by pipetting. The mixture was transferred to a sterile pre-chilled electro-cuvette before exposure to a quick electric pulse at 2800 V for 3-5 s in an electroporator (EC100, Thermo EC Apparatus Corporation). Cells were allowed to recover in 1 mL of SOC media (Appendix 1) at 28°C, 200 rpm for 1-2 hrs. Transformed cells were pelleted at 12879 x *g* for 5 min in a microcentrifuge. Most of the SOC media was removed leaving at least 250 µL and the pellet of cells was resuspended. Aliquots of 100 µL were placed on solidified YM (Appendix 1) media containing 25 mgL^{-1} rifampicin, 100 mgL^{-1} carbenicillin (selecting for AGL1) and appropriate antibiotic selection agents for the vector DNA. For each transformation, a digested linear vector was included as a negative transformation control while non transformed bacterial cells were included as negative selection control. Agar plates were incubated at 28°C for 2-3 days. Three transformed single colonies were selected and these were each re-cultured onto solidified YM with appropriate antibiotics to confirm the selection process. A fresh colony containing vector DNA was selected for preservation as described in section 2.2.3**.**

2.1.4. Transformation of *E.coli* by heat shock method

An aliquot of 50 µL of competent *E.coli* cells (2.1.2) was thawed on ice in a 1.5 mL microcentrifuge tube. To this, 5 µL of cloned recombinant plasmid DNA (from a ligation

[1] *pDNA from a standard miniprep method for preparation of pDNA for sequencing is clean enough for electroporation into Agrobacteria.*

reaction) was added and the mixture incubated on ice for 20-30 min. The mixture was subjected to heat shock at 42°C for 90 s followed by 5 min on ice. The cells were then inoculated into 500 µL of LB media (Appendix 1) and allowed to recover by gentle shaking (200 rpm) for 1 hr in 37°C. Bacterial cells were pelleted by centrifugation at 17530 x *g* in a microcentrifuge for 1 min, excess liquid media was removed and the cells were re-suspended in 200 µL LB media. Using a sterile loop, transformed cells were streaked onto 25 mL of solid LB-Agar media supplemented with the appropriate antibiotic agent and incubated at 37°C overnight. For pUC19 plasmids and pGEMT-4Z, 100 mgL^{-1} ampicillin was used, while 100 mgL^{-1} kanamycin was used for pCAMBIA 2300 and pBINPLUS plasmids that have the *npt*II selection marker gene. For each transformation, a digested linear vector was included as a negative transformation control, while non transformed bacterial cells were included as negative selection control. Solid LB-Agar plates were incubated at 37°C overnight. A freshly transformed colony containing vector DNA was selected for preservation as described in section 2.2.3.

2.2 Preparation of bacterial cultures

2.2.1. Preparation of *E.coli* suspension cultures

An aliquot of 100 µL from a glycerol stock of transformed *E.coli* cells or a single colony of freshly transformed *E.coli* was inoculated into 5 mL of LB media containing appropriate antibiotics and shaken (200 rpm) at 37°C overnight. Cells from overnight culture were harvested for plasmid DNA isolation (2.3.1).

2.2.2. Preparation of *Agrobacterium* suspension cultures

An aliquot of (100 µL) of transformed AGLI cells from a glycerol stock or a single colony of freshly transformed AGL1 was inoculated into 5 mL of YM media containing 25 mgL^{-1} rifampicin and 100 mgL^{-1} kanamycin to select for the selection marker gene *npt*II. The culture was shaken (200 rpm) at 28°C for 2-3 days. A portion (5 mL) of the culture was then inoculated into 20 mL of fresh LB media containing the appropriate antibiotics and shaken (200 rpm) overnight at 28°C. Cells were then harvested for transformation of plant tissues (2.5.2).

2.2.3. Preparation of glycerol stocks for preservation of bacterial cultures

An aliquot of 500 µL of bacterial culture in its exponential growth stage (2.2.1, 2.3.2) was mixed with an equal volume of 50% glycerol solution (v/v) in 2 mL cryovials and stored at -80°C until needed.

2.3. Manipulation of pDNA

2.3.1. Preparation of vector DNA from *E.coli* by alkaline lysis

An aliquot (1.5 mL) of overnight starter culture (2.2.1) was centrifuged at 18000 x *g* for 5 min and the bacterial pellet resuspended by vortexing in 150 µL of chilled TE buffer (Solution 1 Appendix 1) containing 10 µg of RNase A. Subsequent manipulation of cells was carried out at 4°C. To the resuspended cells, 300 µL of freshly made 0.2M NaOH/ 1% SDS alkaline lysis solution (Solution 2 Appendix 2) was added and left for a maximum of 5 min. To the lysate, 225 µL of ice-cold potassium acetate (Solution 3 Appendix 3) was added followed by immediate mixing by inversion and centrifugation at 18000 x *g*, 4°C for 5 min. Plasmid DNA was further extracted by the addition of an equal volume of 24:1 chloroform: isoamyl alcohol solution, mixed well by inversion before centrifugation at 18000 x *g* for 5 min. The supernatant was transferred to a fresh tube and pDNA was precipitated by the addition of an equal volume of 100% isopropanol followed by centrifugation at 18000 x *g* for 5 min. Precipitated plasmid DNA was washed twice in 70% ethanol, the pellet was dried under vacuum for 15 min before re-suspension in 50-100 µL of either DEPC-treated MilliQ water or TE buffer. An aliquot (5 µL) of resuspended pDNA was analysed by agarose gel electrophoresis to assess the quality of extracted pDNA.

2.3.2. Restriction digestion of plasmid DNA

Restriction digestion reactions were prepared in a 20 µL reaction mixture as described (Sambrook and Russel 2001) consisting of 1U enzyme per µg of pDNA and 1 X appropriate restriction buffer (depending on enzyme in use). Restriction digestions were carried out at 37°C for 1-2 hrs. Digested products were separated by agarose gel electrophoresis (2.3.3).

2.3.2.1 Dephosphorylation of vector pDNA

To avoid re-circularisation, digested pDNA of cloning vectors was treated with alkaline phosphotase enzyme (AP) to remove the 5' phosphate groups. One unit of AP and 1 X AP buffer were added to the 20 µL digestion reaction mixture (2.3.2) and incubated at 37°C for 30-60 min after which the AP was inactivated by incubation at 65°C for 10 min.

2.3.3. Agarose gel electrophoresis

Separation of nucleic acids was carried out in 1% agarose gels (for DNA) or 2% agarose gels (for RNA or DNA fragments less than 500 bp) in 1X TAE electrophoresis buffer (Appendix 1) premixed with 0.25X Sybr safe stain solution in DMSO (Sybr Safe™ DNA gel stain, Invitrogen). With the exception of PCR reactions performed with GoTaq® green master mix (Promega), 6X gel loading buffer (Appendix 1) was added to all samples before loading onto agarose gels. Gels were observed and recorded using a gel documentation system (Syngene G-Box with GeneSnap version 6.07 and GeneTools version 3.07).

2.3.4. Purification of DNA from agarose gels by Freeze 'N Squeeze (Bio-Rad)

Restriction digested DNA fragments or PCR products were separated by electrophoresis on agarose gel. DNA fragments of interest were visualised under UV light, excised from the stained gel and purified by the freeze and squeeze procedure as per manufacturer's instructions.

2.3.5. Ligation of DNA fragments

Ligation reactions for vector DNA and purified DNA fragments of interest were carried out at a concentration of 3:1 ratio (insert: vector) in a 10 µL reaction mixture containing 2X ligation buffer and 1U of T4 ligase enzyme (Roche). Ligation reactions were incubated at 14°C overnight.

2.3.6. Preparation of DNA for sequencing

Sequencing reactions were prepared in a 20 µL reaction mixture containing approximately 200 ng pDNA, 1 X sequencing buffer with 1/8X BDT (ABI PRISM BigDye™ Terminator v3.1 cycle sequencing ready reaction kit, Applied Biosystems) in a 3:1 ratio

(buffer: BDT) and 3.2 ρmoles (1 µL) of an appropriate sequencing primer. Thermocycling conditions for sequencing reaction were 96°C for 1 min, 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min with steps 2-4 repeated 30 times. Sequencing products were subsequently cleaned and precipitated using an ethanol/EDTA/sodium acetate precipitation method according to the BigDye terminator v1.1 Cycle sequencing Kit protocol (Applied Biosystems 2002). Briefly, the contents of each sequencing reaction were mixed with 2 µL of 3 M sodium acetate (pH 5.2) and 2 µL of 125 mM EDTA (pH 8.0) followed by the addition of two volumes of 100% ethanol. After incubation at RT for 15 min, the precipitated products were pelleted by centrifugation at 18000 x *g* for 20 min, washed in 70% ethanol and allowed to air dry. Genetic analysis (by capillary electrophoresis) was carried out by Griffith University sequencing facility, Queensland. Sequence data was analysed using Contig Express (Invitrogen Vector NTI Advanced™ V10).

2.3.7. Nucleic acid amplification

Oligonucleotides (primers) were acquired from either GeneWorks or Sigma Aldrich at a concentration of 100 µM. Primers were diluted to 10 µM for polymerase chain reactions.

2.3.7.1 Polymerase chain reactions (PCR)

PCR components were either mixed together in 50 µL reactions volume or prepared in 20 µL reaction volumes by using GoTaq® green master mix (Promega). The 50 µL reactions contained 100 µM dNTPs (Roche), 1X $MgCl_2$ buffer, 10 ρmoles of each forward and reverse primer, 2.5U *Taq* DNA polymerase (Roche) and MilliQ water. When prepared with GoTaq® green, reactions contained 1X GoTaq® green (Roche), 10 ρmoles of each forward and reverse primer and MilliQ water. Reactions were denatured at 94°C for 2 min prior to 34 cycles at 94°C for 30 s, appropriate annealing temperature (depending on primers in use) for 30 s and 72°C for 1 min per kilo base pair (kbp) of expected product. Finally, complete extension of products was carried out at 72°C for 4-7 min.

2.3.7.2 Reverse transcription PCR (RT-PCR)

Reverse transcription reactions were carried using either the Titan One tube RT-PCR Kit (Roche) or the 2-Step RT-PCR method using the Improm II™ RT-Kit (Promega) following manufacturer's instructions. When using the Titan One tube RT-PCR method, the reagents were made in two separate master mixes (1 and 2). Master Mix 1 contained 0.2 mM dNTPs, 5 mM DDT solution, 10 ρmoles each reverse and forward primer, 1 µg RNA and MilliQ water to 25 µL. Master Mix 1 was incubated at 68°C for 2 min, then on ice for 1 min before adding Master Mix 2. Master Mix 2 contained 1X RT-PCR $MgCl_2$ buffer, 1 µL enzyme mix, 10 U of RNase inhibitor and MilliQ water to a final volume of 25 µL. RT-PCRs were incubated at 50°C for 40 min, then 94°C for 4 min before 35 cycles of 94°C for 30 s, 50-55°C for 30s (depending on primer in use) and elongation at 68°C for 2 min. An extra 4-7 min was included to complete elongation of the amplicons.

Using the 2-Step RT-PCR method, reagents were again prepared in two Master Mixes (1 and 2). Master Mix 1 contained 10 ρmoles of cDNA OligoDT primer, 1 µg of RNA (2 µL RNA extract) and MilliQ water to a total volume of 5 µL. Master Mix 1 was denatured at 99°C for 1 min and placed in ice before adding Master Mix 2. Master Mix 2 consisted of 1X Improm II buffer, 25 mM $MgCl_2$, 10 mM dNTPs, 1 µL reverse transcriptase, 10U RNase inhibitor and MilliQ water to a total volume of 15 µL. The reaction mixture (20 µL) was equilibrated at 25°C for 5 min, and then cDNA synthesis was achieved by incubation at 42°C for 1 hr, followed by inactivation of reverse transcriptase at 70°C for 15 min. An aliquot of synthesised cDNA (2 µL) was then used in a PCR using gene specific primers as per 2.3.7.1 above.

2.4. Extraction of nucleic acids from plant tissue

2.4.1. Extraction of genomic DNA from rice tissue (leaf or callus)

When extracting DNA from rice callus, up to 350 mg of callus was placed into 2 mL cryovials and dried under vacuum in freeze drier (Flexi-Dry ™ MP, Microprocessor Control, FTS® Systems, Inc NY). The dried tissue was subsequently homogenised to a fine powder with a 2 mm sterile bead in a bead beater (Mini Bead-beater™, Biospec products). When extracting DNA from leaf, at least 1 g of fresh tissue was ground to a powder in liquid nitrogen with mortar and pestle. The ground tissue was added to 1 mL

of pre-warmed (65 °C) 0.8% CTAB DNA extraction buffer (Appendix 1) containing 1.4% (v/v) β-mercaptoethanol followed by incubation at 65 °C for 1 hr with mixing at 5–10 min intervals. Samples were allowed to cool to room temperature before centrifugation at 9000 x *g* for 15 min. DNA was extracted from the supernatant by two chloroform extractions with an equal volume of ice cold chloroform: isoamyl alcohol (24:1) followed by centrifugation at 4000 x *g* (in bench top centrifuge] or 9000 x *g* [in a microcentrifuge] for 5 min at 4°C. The supernatant was recovered and genomic DNA was precipitated by the addition of 2/3 volume of ice-cold isopropanol and incubation at -80°C for 30-45 min [or at 4 °C overnight] followed by centrifugation at 9000 x *g*, 4°C for 10 min. The pellet was washed with 70% ethanol, air dried and resuspended in 500 µL of TE buffer (Appendix 1). The DNA was incubated at 37 °C for 1 hr with 20 µg of RNase A and the reaction stopped by the addition of an equal volume of phenol: chloroform (1:1 v/v). After mixing, the sample was centrifuged at 9000 x *g* for 5 min at RT. The supernatant was recovered and DNA was precipitated by the addition of 2 volumes of 100% ethanol and 1/10 volume of 3 M NaAc and incubation at -80 for 30 min. The DNA was pelleted by centrifugation at 18000 x *g* for 10 min then washed twice with 70% ethanol, air dried and resuspended in 30-50 µL TE buffer. The quality and concentration of DNA was determined by agarose gel electrophoresis against a molecular weight DNA ladder (2-Log, Biolabs) and spectrophotometry by reading absorbance of DNA at 260 nm and 280 nm.

2.4.2. Rapid CTAB DNA extraction for quick screening of transgenic plants

Leaf tissue (~30-50 mg) in a 2 mL cryovial containing a lead bead (Wichester super hard chilled size BB approx. 5 mm) and 500 µL of 2% rapid CTAB buffer (Appendix 1) was homogenised for 1 min at a frequency of 30 rotations/s in a tissue lyser (Qiagen, Retsch® Tissue lyser II). Samples were spot spun before incubation at 65°C for 10 min. After cooling to RT, an equal volume of chloroform: isoamyl alcohol (24:1) was added followed by vigorous vortexing before centrifugation at 6000 x *g* for 5 min. The supernatant was recovered into a clean microcentrifuge tube to which an equal volume of isopropanol was added followed by centrifugation at 18000 x *g* for 5 min. The precipitated DNA was washed twice in 70% ethanol at 18000 x *g* for 5 min, the pellet

was air dried and resuspended in 100-200 µL of TE buffer (Appendix 1) or MilliQ water. From this rapid extraction procedure, 1-2 µL of DNA was used for PCR.

2.4.3. Extraction of total RNA from plant tissue

RNA extraction was carried out according to the procedures described by (Chang *et al.,* 1993; Asif *et al.,* 2000) with minor modifications. Plant tissue (at least 500 mg) was ground into a fine powder in liquid nitrogen using a mortar and pestle. The powdered tissue was placed in a 15 mL falcon tube containing 3 mL of pre-heated (65°C) RNA CTAB extraction buffer (Appendix 1) containing 2% β-mercaptoethanol before incubation at 65°C for 10-30 min with periodic mixing. The sample was then cooled on ice, after which an equal volume of chloroform was added. The sample was mixed thoroughly before centrifugation at 4000 x *g* at 4°C for 10 min in a bench top centrifuge. The supernatant was recovered and chloroform extraction was repeated. The aqueous upper phase was transferred to a fresh tube and RNA was precipitated by the addition of ¼ volume of 10M LiCl followed by incubation at 4°C overnight. Precipitated RNA was pelleted by centrifugation at 4000 x *g* in a bench top centrifuge or 18000 x *g* in microcentrifuge at 4°C for 20 min. The pellet was resuspended in 500 µL DEPC treated MilliQ water per gram of tissue. Aliquots of the resuspended RNA were transferred into a 1.5 mL microcentrifuge tube to which an equal volume of chloroform was added, mixed thoroughly before centrifugation at 18000 x *g* for 5 min at room temperature in a microcentrifuge. To precipitate the RNA, the aqueous upper phase was transferred into a fresh 1.5 mL tube to which 2 volumes of 100% ethanol was added and mixed thoroughly before incubation at -80°C for 45 min. Precipitated RNA was recovered by centrifugation at 4°C for 20 min at 18000 x *g* in a microcentrifuge. The pellet was dried in a vacuum desiccator for 10 min and re-suspended in 50 µL DEPC treated H_2O (2.4.3.1) per gram of plant tissue. Total RNA was treated with RNase free DNase (Promega) according to the manufacturer's instructions. Briefly, the DNase reaction contained about 1 µg of RNA, 1U of DNase and 1 X DNase reaction buffer in a total volume of 10 µL. The reaction was carried out at 37°C for 30 min, and terminated with 1 µL of DNAse stop solution after which DNase was inactivated at 65°C for 10 min. The quality of RNA was analysed by agarose gel electrophoresis and concentration was

determined by spectrophotometry at A_{260nm} and A_{280nm}. RNA samples were stored at -20°C when required for immediate use or stored at -80°C.

2.5. Transformation of rice callus

2.5.1. Induction of rice embryogenic callus

Embryogenic callus was induced from mature seeds of rice (*Oryza sativa* L. sp japonica cv Nipponbare). De-husked dry rice seeds were washed with 70% ethanol for 1 min, rinsed in distilled water and surface sterilised by immersion in a solution of 80% commercial bleach (4.0% hypochlorite m/v) containing one drop of Tween-20 for 30 min with gentle rotation. Sterile seeds were rinsed 3 times with sterile water and blotted dry on filter paper. Up to 12-15 seeds were placed onto solidified 2N6 callus induction media (Appendix 1) in 90 x 14mm Petri dishes and were incubated in the dark at 25°C for 3-4 weeks. Embryogenic callus was multiplied on solidified 2N6 solid media and subcultured every 3 weeks. Proliferating callus was pre-cultured on fresh 2N6 media 4 days before transformation.

2.5.2. Preparation of *Agrobacterium* suspension and transformation of rice callus

An *Agrobacterium* suspension culture was prepared as described in 2.2.2. The optical density (OD) of the overnight suspension culture was determined at 600 nm by spectrophotometry before centrifugation at 4000 x *g* for 10 min. The pellet was resuspended in bacterial re-suspension media (BRM) (Appendix 1) containing 100 µM acetosyringone to an OD_{600}= 0.50 followed by gentle agitation at 70 rpm for 3 hrs at room temperature.

Freshly subcultured callus (4 days old), each approximately 2.5 mm in size, was transferred into a 50 mL falcon tube to which 10 mL of 2N6 media (preheated to 45°C) was added. The mixture was incubated at 45°C in a water bath for 5 min after which the 2N6 media was removed and replaced with 10 mL of *Agrobacterium* suspension containing pluronic F68 detergent solution to a final concentration of 0.02% before centrifugation at 600 x *g* for 10 min in a benchop centrifuge. The tubes were left to stand undisturbed for 30 min, before removing the *Agrobacterium* suspension. The callus was blotted dry on filter paper before transfer to solidified co-cultivation media

(Appendix 1) supplemented with 100 µM acetosyringone in 90 x 14 mm Petri dishes. Cultures were incubated in the dark for 3 days at 23°C.

Transformed callus was rinsed 3-4 times in liquid 2N6 media containing 200 mgL^{-1} Timentin and blotted dry on filter paper. Dry callus was transferred onto solidified 2N6-S media supplemented with 200 mgL^{-1} Timentin and half the concentration of appropriate antibiotics (25 mgL^{-1} of geneticin to select for *npt*II or hygromycin to select for *hpt*II) for the first 2 weeks on selection. Subsequently, transformed callus was transferred every two weeks onto fresh 2N6-S selection media (Appendix 1) containing the full concentration (50 mgL^{-1}) of antibiotic selection agent. Cultures were incubated in the dark at 25°C and monitored for proliferation of resistant callus.

2.5.3. Regeneration of rice plants from transformed callus

After 6 weeks on selection media, individual proliferating antibiotic resistant clusters, representing putatively transformation events (also referred to as "callus lines"), were multiplied separately on 2N6-S media for 6-9 weeks with 3 weekly transfer onto fresh media (Nishimura *et al.,* 2006). Proliferating antibiotic resistant callus was transferred to solidified 2N6-R regeneration media (Appendix 1) containing 200 mgL^{-1} Timentin and 50 mgL^{-1} appropriate antibiotic selection in 100 mm X 20 mm Petri dishes. Cultures were kept in the dark for the first 7 days on regeneration media and later transferred into light at 25°C, under 16:8 light-dark photoperiod from where callus was monitored for shoot formation. Shoots with at least 3 well formed leaves were transferred to solidified rooting media supplemented with 200 mgL^{-1} Timentin and the appropriate antibiotic selection agent in 120 mm X 60 mm culture vessels Cultures were incubated at 25°C in the light and monitored for plant growth, elongation and root development.

2.5.4. Acclimatisation of rice shoots from *in-vitro* culture

Rice shoots (at least 5 week old) were removed from rooting media and the roots were washed under a gentle stream of tap water to remove all agar. Shoots were suspended in tap water in 30 mL polycarbonate tubes sealed with Parafilm™ to support the shoots. The shoots were covered with a plastic bag and kept at 25°C for 10-12 days until new roots started growing. At 7 days, the plastic bags were perforated to reduce

humidity and shoots were left in this state for another 3-5 days. Plants were transferred to potting soil mixture (seed raising mixture – Type: Searles) in 100 mm diameter garden pots and kept in a temperature/humidity controlled cabinet at 30°C, 70% external humidity under 14:10 photoperiod for 2-3 weeks. Acclimatised plants were grown up to maturity in 200 mm diameter garden pots, in premium potting mixture (Type: Searles) in the glass house. Plants were fertilised with AQUASOL soluble fertiliser once every 3 wks.

2.6. Transformation of raw banana fruits

2.6.1 Preparation of gold particles for particle bombardment

Micro carrier particles (250 mg gold-1.0µm, Bio-Rad) were weighed into a 1.5 mL microcentrifuge tube to which 1 mL absolute ethanol was added and vortexed well for at least 1 min before centrifugation for 5 min at 400 x *g* in a microcentrifuge. The supernatant was removed and the ethanol wash was repeated twice with vortexing at every wash step. Gold particles were then washed three times in 1 mL sterile MiliQ water with vortexing and centrifugation at 400 x *g* for 5 min. Washed gold particles were re-suspended by vortexing in 2 mL of sterile 50% (v/v) glycerol, dispensed into 25 µl aliquots in 1.5 mL tubes and stored at −20°C.

2.6.2 Preparation of fruit slices for particle bombardment

Freshly harvested mature banana fruit was kindly provided by Kurt Lindsay, Snake Gully Bananas, Rocksburg, SE Queensland. Fruit was prepared for particle bombardment as described (Mlalazi 2010) and Jason Geijskes (personal communication). Briefly, the undamaged outer surface of the banana pulp was used. The banana fruit was sliced into 3-4 centimetre cross-sections, a longitudinal slit was made through the surface of the peel and gently pulled off too expose the surface of the pulp undamaged. Parallel fruit sections approximately 2 cm X 2 cm x 5mm were cut from the undamaged sections of the fruit surface. For particle bombardment 2 freshly cut fruit slices were placed onto filter paper moistened with 1 mL of BL media in a Petri dish.

2.6.3 Particle bombardment – The procedure

Banana fruit slices were transformed by particle bombardment as described (Becker and Dale 2004). An aliquot of 25 μL (containing 3 mg of gold particles) was vortexed for 1 min before adding 2 μg plasmid DNA, 25 μl of 2.5 M $CaCl_2$ and 5 μl of 0.1 M spermidine. Gold particles were maintained on ice and kept in suspension by vortexing every 30 s for 5 min. Gold particles were then allowed to precipitate for 10 min before removing 22 μL of supernatant. The remaining suspension was mixed by vortexing and 5 μL aliquots of the pDNA-coated gold particles were used for transformation. Target tissue was placed at approximately 7.5 cm from the point of particle discharge and covered by a 210 μm stainless steel mesh baffle. Bombardment was carried out at a controlled helium pressure of 550 kPa and chamber vacuum of –84 kPa (25 mmHg). Transformed fruit slices were incubated in the dark at 25°C for 3 days to allow for transient GUS expression.

2.6.4 *Agrobacterium*-mediated infiltration of banana fruits

Four days before transformation, a suspension of *Agrobacterium* containing the respective binary vectors was prepared from glycerol stocks (2.2.2). The concentration of bacterial culture was adjusted to either OD_{600} = 0.5 or OD_{600} = 0.8 using spectrophotometry. Thereafter, the virulence of the *Agrobacterium* cells was activated by transferring an aliquot (5 mL) of the 3 day culture into 25 mL of BRM media containing 200 μM acetosyringone followed by gentle agitation for 3hours.

Preliminary transient GUS expression in banana fruits was carried out using fruits obtained from the supermarket alongside organic fruit from backyard gardens. Subsequently, green mature banana fruit (*Musa* spp. Cavendish) was obtained fresh from a farmer in North Queensland. Freshly harvested fruits were brought to the laboratory, washed in tap water and blotted dry. *Agrobacterium* suspension was introduced into the fruit through either the distal or proximal end of the fruit. Using a 1 mL disposable syringe and needle (size 21G; 0.8 mm X 38 mm), fruit was infiltrated with either 1 mL or 2 mL of *Agrobacterium* suspension containing vector DNA. Transformed fruits were then incubated in the dark at 23°C for either 3 or 4 days post infiltration (dpi). Fruits were sequentially sliced into transverse sections starting at the point of

injection along the fruit length and transient GUS activity was determined by histochemical assay as described in section (3.8.2).

2.7. Analytical methods for carotenoid and iron quantification

2.7.1 Analytical methods for carotenoid analysis

2.7.1.1 HPLC chemicals and solvents

Chemicals for extraction of carotenoids and solvents for sample preparation were all HPLC grade obtained from Sigma-Aldrich Pty. Ltd, or Lomb Scientific Pty Ltd, Australia. The calibration standard for β-carotene was obtained from Fluka™, while the internal standard, α-tocopherol acetate, was obtained from Sigma-Genosys®. Opaque amber glass HPLC vials (1.5 mL, 10 mm screw top vial, flat bottomed 12x32 mm) were used when analysing carotenoid extracts to minimise exposure to light.

2.7.1.2 Carotenoid extraction from rice callus and banana fruit

The procedure for extraction of carotenoids for quantification by HPLC was adapted from a method used by Prof. Peter Beyer Research group (at Centre for Applied Biosciences, University of Freiburg, Germany) for extraction of carotenoids and vitamin E from freeze dried banana fruits. When extracting from rice callus, fresh callus (300-400 mg) was weighed into 2 mL cryopreservation vials, snap frozen in liquid nitrogen and freeze dried under vacuum overnight. The dry weight was recorded, and two stainless steel beads(2 mm) were added into the cryovials then dry callus was homogenised for 1 min into a fine powder using a bead beater (Mini Bead-beater™, Biospec products). When extracting from banana fruit, fresh fruit was sliced into small pieces, freeze dried, and ground to fine powder in liquid nitrogen. Dried rice callus (25-50 mg) or a sub-sample (500 mg) of dry banana powder was used for carotenoid extraction.

The powdered tissue was transferred onto 15 mL falcon tubes to which 2 mL of acetone was added together with 100 µL (100 µg) of α-tocopherol acetate (Internal standard) from a working stock concentration of 1 mgL^{-1}. The mixture was thoroughly mixed by vortexing before centrifugation at 4000 x *g* for 5 min. Acetone extraction was repeated

three times. To separate the organic phase from the aqueous phase, 2 mL of PE: DE (2:1; v, v) consisting of petroleum ether (40-60°C fraction) and diethyl ether was added to the combined supernatant and mixed thoroughly by vortexing. To this, a solution of 1% NaCl (w/v) was added up to 14 mL and mixed well before centrifugation at 4000 x *g* for 5 min. Extraction of the organic phase was repeated with 1 mL of PE: DE. The upper organic phase was combined into a clean 15 mL falcon tube and dried in a vacuum desiccator in the dark. The dried organic phase was re-dissolved in 2 mL chloroform from which a 600 µL aliquot was used to determine the sample OD at 465 nm. For HPLC analysis, 600 µL of the organic phase was dispensed into a 1.5 mL HPLC vial covered with GC grade cap from which 20 µL were injected into the HPLC column. For the internal standard, four separate aliquots of 100 µL α-tocopherol acetate solution at 1 $mgmL^{-1}$ were dried in a vacuum desiccator and re-dissolved in 2 mL of chloroform out of which 20 µL were injected into the HPLC column.

2.7.1.3. Carotenoid separation and detection

Carotenoid separation was performed using a C30 reverse phase matrix with a methanol-tertmethyl butyl ether based mobile phase and detection of signal was monitored from a photodiode array detector (Fraser 2000, Ye et al., 2000). Using RP-HPLC (Agilent HP100 HPLC – Circa 2004) fitted with a photodiode array detector (DAD-Agilent 1100) the signal for β-carotene was monitored under visible light at 465 nm being the λ_{max} absorbance for β-carotene in chloroform. In addition, detection of α-tocopherol acetate signal was monitored at 287 nm under ultraviolet lamp (Fraser *et al.,* 2000) (Rodriguez-Amaya and Kimura 2004). Monitoring of spectra was done in the range 100-700 nm with a band width of 4 nm from the wavelength of maximum absorbance. The stationary phase consisted of a reverse-phase C30 column 250X4.6 mm, 3 µm (Waters YMC™ Ireland) attached to an S-5 4.0x23 mm guard cartridge (Waters YMC™ USA). All tubing for the injector and the column were 1/16”x0.05” solid peek tubing connected with short finger-tight peeking fittings (Alltech Associates, Australia). The mobile phase consisted of HPLC grade solvents C and D whereby solvent C was a mixture of Methanol: Tert methyl butyl ether (TMBE) in a 1: 1 ratio v/v. Solvent D consisted a mixture of Methanol: TMBE: Water in a ration of 5: 1: 1 v/v. The separation profile was 57% C and 43% D for 10 min; this was followed by a step of

100% C for 10 min, with an additional 100% C at 1.5 $mLmin^{-1}$ up to 13.10 min. This was followed by a 57% C, 43% D up to 29 min, with an extra 6 min at a flow rate of 1 $mLmin^{-1}$ up to 35 min. The flow rate was maintained at 1 $mLmin^{-1}$ for the first 20 min and last 6 min of elution while the rest of the time the flow rate was 1.5 $mLmin^{-1}$. Bracket injections were included in each HPLC sequence table after every 4 sample injections Bracket injections were drawn from alpha tocopherol acetate calibration sample at a concentration of 1 µg by injecting 10 µL from a 100 μgmL^{-1} standard solution.

2.7.1.4. Quantification of carotenoid content

Quantification of provitamin-A carotenoids (β-carotene) was based on the area under the peak at the wavelength providing maximum absorbance in chloroform i.e. 465 nm for β-carotene and 287 nm for α-tocopherol acetate using Chemstation software for Agilent HP100 HPLC – Circa 2004. Using Microsoft Office Excel, the amount of β-carotene per test sample was calculated from the area values against a calibration curve equation as well as the original sample weights and dilutions used. A correction factor was calculated using area under the curve for ISTDs. Subsequently, the correction factor was multiplied by the calculated carotenoid value to obtain the actual amount of carotenoid in each sample. ANOVA was used to compare mean β-carotene content between samples for at least 3 extracts per sample.

2.7.1.4.1 Calibration curve for alpha-tocopherol acetate

To prepare the standard solutions, a stock solution of alpha tocopherol acetate was prepared in chloroform at a concentration of 10 $mgmL^{-1}$. This was then diluted 1:100 to make a 100 μgmL^{-1} ISTD solution from which 2 µL, 4, µL, 10 µL and 15 µL containing an equivalent 0.2 µg, 0.4 µg, 1 µg, and 1.5 µg respectively were injected onto for HPLC column to generate a calibration curve for alpha tocopherol acetate. The calibration points selected covered the range of concentrations expected in the samples including 1 µg which was the amount of ISTD incorporated into the extracted samples.

2.7.1.4.2 Calibration curve for β-carotene

A stock solution of 100 μgmL^{-1} was prepared by dissolving 1 mg of β-carotene in 10 mL chloroform in 10 mL volumetric flask from which dilutions were made to prepare five standard solutions at concentrations 0.2 μgmL^{-1}, 0.5 μgmL^{-1}, 1 μgmL^{-1}, 2 μgmL^{-1}, and 4

μgmL^{-1} in 10 mL of chloroform. From each standard solution, an aliquot of 1 mL was dispensed into an HPLC vial for analysis. The five calibration points selected covered a range of concentrations that had an OD_{465} ≤1.00 whereby 465 nm is the λ_{max} for β-carotene in chloroform.

2.7.2. Analytical methods for iron quantification

2.7.2.1 Preparation of samples for CEM microwave digestion

Proliferating rice callus each putatively transformed callus line was pooled/harvested from 2N6 multiplication media and rinsed twice in 50 mL of sterile Milli-Q water to remove carry-over iron from the media. Callus was blotted dry on filter paper, transferred into 50 mL falcon tubes and snap-frozen in liquid nitrogen before being freeze dried overnight. Dry weight was recorded before grinding the callus into fine powder in mortar and pestle. Ground callus was stored in a desiccator. During preparation of samples for digestion, callus was handled with plastic or glass instruments only.

2.7.2.2 CEM Microwave digestion

A subsample (500 mg) of dry powder was weighed directly into digestion vessels (CEM Advanced composite digestion vessels). In addition, 500 mg of tobacco leaf powder was digested as standard reference material (contains 368±7 mg of Fe/kg of dwt; certified SRM # 1573a, certified by National Institute of Standards and Technology, US department of Commerce, Gaithernburg, MD 20899). Equal amounts (10 mL) of 70% HNO_3 (TraceSELECT grade, Fluka, Sigma was added into each vessel. An equivalent volume was added into the pressure control vessel (without sample). The vent in the cover of each digestion vessel was fitted with a rupture membrane (CEM). The set up of the microwave digester (MDS 2000, 630 Watt) as well as connecting the pressure control system was done according to the instrument instruction manual. Briefly, the digestion parameters included a five stage cycle with increasing pressure from 20, 40, 75, 125 and 150 psi for 10 min for every stage while maintaining microwave power at 45%, time at pressure (TAP) was set at 5 min and fan speed of 100 rotations per min. Digested samples were removed from the microwave at a pressure below 5 psi and

were allowed to completely cool to room temperature before transferring samples to an acid resistant container.

2.7.2.3 Preparation of standard solutions for iron

Standard solutions were prepared from a stock solution of iron nitrate ($Fe(NO3)_3.9H_2O$; trace select grade, Fluka/sigma at 10g of iron in Nitric acid). Four concentrations of the standard solution were prepared at 0.5, 1.0, 2.5, and 5 mgL^{-1} for the standard curve. First, 100 mL of a working solution was prepared at a concentration of 200 mgL^{-1}. To do this, 2 mL of a 10 gL^{-1} iron nitrate solution were taken into a 100 mL volumetric flask and topped up to 100 mL mark with 10% nitric acid. Subsequently, the working solution was diluted 1:20 (with 10% nitric acid) to make a 100 mL of a 10 mgL^{-1} solution out of which serial dilutions were made in 10% nitric acid to prepare 100 mL of each calibration solution at 0.5, 1.0, 2.5, and 5 mgL^{-1} in 100 mL volumetric flasks.

2.7.2.4 Preparation of digested samples for FAAS

Each digested sample was diluted 1: 5 in 10% nitric acid in a 25 mL volumetric flask. The diluted sample was transferred into a FAAS sample tube ready for analysis. The procedure for FAAS analysis of iron was carried out following the standard operating procedure of the instrument (Varian SpectrAA 220 FS; SPS-5) as detailed in Appendix 2). The light source was provided by a 10 mA hollow cathode lamp (Varian, SpectrAA hollow cathode lamp for AAS producing a wavelength of 248.3 nm) Data (absorbances) were recorded using Varian SpectrAA-220 software version 5.1, Varian Australia 2004.

2.7.2.5 Calculation of metal concentration in test samples

Absorbance values generated for each sample were analysed using Microsoft Office Excel 2007. The concentration of iron (per gram of tissue) was estimated based on the standard curve (2.7.2.3). The percentage extraction efficiency (EE) of iron was calculated based on the amount of iron extracted from the tomato leaf standard reference material (2.7.2.2) as below:

% EE = iron extracted x 100
368 mg/kg dwt (expected iron content in tomato reference leaf powder used).

The percentage EE value was then used to calculate a correction factor for estimation of actual iron concentration in test samples. Mean iron content was estimated from one spectroscopic measurement each from two digested extracts per callus line or mean of two spectroscopic measurements from one extract. Univariate analysis of variance (ANOVA) was used to compare mean iron content between individual callus lines/treatments. The final iron content in a transformed callus line was expressed as mg of Fe/kg dwt of tissue.

2.8 Transient GUS assays

2.8.1 Fluorometric assay

Fresh banana leaf tissue was collected from transgenic plants plants growing in a glasshouse at DPI-Redlands-Queensland. Samples from the field were kindly collected and delivered to the laboratory by CTCB-QUT scientists visiting the field trials at Innisfail, Northern QLD. For all plants from the glasshouse and the field, the first fully open leaf was sampled, kept at 4°C and brought to the laboratory. Leaves were washed in tap water and blotted dry. Five leaf discs (approx. 5 mm in diameter) were punched along the lamina and placed directly into histochemical stain. Another 15-20 leaf discs were punched into 2 mL cryovials and were freeze dried for use in fluorometric assay.

2.8.1.1 Protein extraction for MUG and BCA assay

The procedure used for MUG assay was adapted from the fluorometric assay procedure described by (Jefferson 1987a) for microcentrifuge tubes but scaled down to suit application to 96-well microtitre plate format. All buffers solutions were prepared as shown in Appendix 1.

Four banana leaf discs (approx. 2.8 mg dry weight), were macerated for 1 min at a frequency of 30 rotations/s in extraction tubes containing 2 stainless steel 2 mm beads (Qiagen). To extract soluble proteins, 180 µL of GUS extraction buffer supplemented with 27.2 mgmL^{-1} (dry weight) Dowex 1X2-400 resin and 500 nM DTT was added to the homogenized sample and mixed with slow agitation for 10 min at room temperature

before centrifugation for 5 min at 4000 x *g*. [Washed and wet Dowex 1X2-400 ion exchange resin was only included in the GUS extraction buffer at the stage of protein extraction. The supernatant was transferred into a pre-chilled 96 well microtitre plate and maintained on ice for all subsequent steps. From this sample plate, aliquots were taken for protein assay and 4-Methylumbelliferone (4-MU) assay.

Protein concentration was determined using the BCA^{TM} Protein assay Kit (Thermo Scientific) containing BCA^{TM} reagent A and B as well as a 2 mgmL^{-1} bovine serum albumin (BSA). A working solution was prepared by mixing 50 parts of reagent A with 1 part of reagent B i.e. 50 mL reagent A + 1 mL reagent B. BSA was diluted in GUS extraction buffer to make seven standard concentrations ranging from 0-1 mg mL^{-1}. A 10 µL sample, as well as 10 µL each of six BSA standards were transferred in triplicate into a 96 well plate to which 200 µL of working solution was added before incubation at 37°C for 30 min. The plate was cooled to room temperature for 10 min and absorbance was determined at 562 nm on a plate reader (AD 200C, Beckman Coulter with ADLD analysis software version 1.6). The amount of protein measured was calculated in mg/10 µL of sample against a BSA standard curve.

MUG assays were carried out in 96 well micro plates (Optoplate^{TM} 96, PerkinElmer micro plate for Life sciences Research) in triplicate in a 60 µL reaction volume. Using a multichannel pipette, 10 µL of sample was transferred into designated wells to which 20 µL extraction buffer was added followed by 30 µL of pre-warmed 4-MU substrate (2mM). For each sample, an extra well was included where 200 µL of stop buffer was added before adding the 4-MU substrate. This sample was designated as T0 (Time zero). Six standard solutions for 4-MU were prepared in the range 0-200 ρmoles. Alongside the test samples on the micro plate, 60 µL each of the 6 standard solutions were included in triplicate together with a negative control (blank well) containing 60 µL of buffer. The Microplate was covered with tape pad (Quiagen), incubated at 37°C for 30 min and reaction was stopped by adding 200 µL of stop buffer (Appendix 1) into all samples and standards except T0 wells. Fluorescence was determined using a Luminescence spectrometer [PerkinElmer LS50B] at excitation wavelength of 365 nm

and emission wavelength of 455 nm with a slit width of 5 nm. Enzymatic activity was calculated from fluorescence values against a standard curve of 4-MU in ρmoles. The final GUS activity determined and expressed as ρmoles of 4-MU/mg of protein produced/ min.

2.8.1.2 Washing Dowex 1X2-400 ion exchange resin

Dowex 1X2-400 resin was washed as described by (Loomis *et al.,* 1978). Briefly, 100 g of resin was placed onto a piece of miracloth [Quick filtration material for gelatinous grindates; Calbiochem®] which was placed into a funnel-flask filtration system connected to vacuum. Resin was washed in 1 L of 1 M HCl followed by a wash in 1-2 L of methanol containing 1% concentrated HCl until an $A_{260} \leq 1.00$ was detected in the elute. The absorbance of elute was measured against Methanol (containing 1% HCL) blank. A portion of wet Dowex was dried in an oven overnight and the percentage water content determined upon which an equivalent 27.2 $mgmL^{-1}$ [dry weight] was weighed out into GUS extraction buffer (2.8.1.1).

2.8.2 Histochemical assay

Histochemical GUS assay was carried out according to (Jefferson 1987a). Plant tissues were submerged into histochemical stain (Appendix 1) and incubated overnight at 37°C. To clear chlorophyll, leaf tissue was incubated in a solution of Ethanol: Acetic acid (3:1) overnight at room temperature. Stained tissue was stored in 70% ethanol.

Chapter 3 Evaluation of banana phytoene synthase (*APsy*2a) for enhancement of pro-vitamin A using rice as a model

3.1 Introduction

The production of staple foods with adequate nutrient content is a key factor in alleviating nutrient deficiencies which affect millions of people worldwide, particularly those in developing countries. Bananas, including cooking bananas such as the East African highland bananas (EAHB), are among the world's major staple food crops. Unfortunately, many cultivated varieties have very low levels of essential micronutrients, in particular pro-vitamin A (pVA). Two of the most commonly used strategies to increase the levels of micronutrients in plants are conventional and molecular breeding. Although banana varieties with high pVA content have been reported (Englberger *et al.,* 2003a; Englberger *et al.,* 2003b; Englberger *et al.,* 2003c), the use of conventional breeding to transfer these traits into cooking banana varieties is not a viable option due to the low fertility of commercially and agronomically important banana cultivars. In contrast, molecular breeding is a viable alternative strategy particularly with the availability of efficient and reliable banana transformation systems (May *et al.,* 1995; Sagi *et al.,* 1995; Becker *et al.,* 2000; Khanna *et al.,* 2004) and the availability of genes associated with pVA biosynthesis (Scolnik and Bartley 1996; Cunningham and Gantt 1998). Although not yet attempted in banana, such an approach has been successfully used to increase the carotenoid content of several different crops including carrot (Jayaraj *et al.,* 2008), maize (Aluru *et al.,* 2008), rice (Ye *et al.,* 2000; Datta *et al.,* 2003; Al-Babili and Beyer 2005; Paine *et al.,* 2005), tomato (Romer *et al.,* 2000; Apel and Bock 2009), tobacco (Busch *et al.,* 2002) and canola (Shewmaker *et al.,* 1999). The most successful example of using molecular breeding to increase the pVA content in crop plants has been the generation of Golden Rice (Ye *et al.,* 2000; Paine *et al.,* 2005).

The development of pVA-enhanced Golden Rice involved the transformation of rice premature embryos with a phytoene synthase (*Psy*) gene derived from daffodil in combination with a bacterial phytoene desaturase (*CrtI*) (Ye *et al.,* 2000). The

endosperm carotenoid content of Golden Rice was later improved 23-fold by replacing the daffodil *Psy* with maize *Psy* (Paine *et al.,* 2005). In an effort to identify banana-derived *Psy* genes for the generation of pVA-enhanced bananas, our group has recently isolated a new phytoene synthase gene (*APsy*2a) from a high β-carotene banana variety (F'ei cv. Asupina) (Mlalazi 2010). The use of native genes and promoters is a desirable strategy to ensure transgene compatibility with the crop's genetic makeup and therefore preserving the genetic integrity of transformed cultivars. Unfortunately, due to the lengthy time from banana transformation to transgenic fruit production, at least two to three years is required to assess the function of this gene in banana fruit. Rice was considered a suitable alternative plant to examine the functionality of *APsy*2a gene since (i) it has been used to study the function of pVA biosynthesis genes (Ye *et al.,* 2000; Paine *et al.,* 2005) and as a model plant to study gene function of monocotyledonous plants (Tyagi *et al.,* 1999), (ii) an efficient transformation system is available and (iii) the time taken from transformation to seed production is relatively short (at least 8-10 months) (Hiei *et al.,* 1994; Nishimura *et al.,* 2006).

The aim of the research described in this chapter was to evaluate whether the banana *APsy*2a gene is functional for the enhancement of pVA carotenoid content using rice as a model system and using the maize-derived *Psy* (*ZmPsy*1) as a control. The specific objectives were to: (i) to generate transgenic rice plants with either *APsy*2a or *ZmPsy*1, in combination with *CrtI*, under the control of the constitutive maize poly-ubiquitin promoter (ZmUbi) or the seed-specific glutelin1 promoter (Gt1), (ii) analyse transgene expression in the transgenic plant tissue and (iii) quantify the carotenoid content in transgenic plants.

3.2 Materials and methods

3.2.1 Expression vectors and transformation of rice callus

Embryogenic rice callus was transformed with genes for pVA biosynthesis under the control of either a constitutive or a seed-specific promoter. For constitutive expression, single-gene binary vectors in a pBINPLUS backbone (hereafter abbreviated as pBIN) (Fig 3.0A) containing *APsy*2a or *ZmPsy*1 or *CrtI* under the control of the constitutive maize poly-ubiquitin promoter (ZmUbi) were used. For seed-specific

expression, single-gene binary vectors in a pCA-GT1 backbone (Fig 3.0B) containing *APsy*2a, *ZmPsy*1 or *CrtI* under the control of the rice glutelin1 promoter were used. The binary vector pBIN contained the selectable marker gene, *nptII* (conferring resistance to geneticin), while pCA-GT1 contained the selectable marker gene, *hptII* (conferring resistance to hygromycin). The pCA-GT1 base vector was kindly provided by Prof. Peter Beyer (Centre for Applied Biosciences, University of Freiburg, Germany). All expression vectors used in this chapter (Table 3.1) were cloned by Dr. Jason Geijskes, CTCB, QUT-Australia.

Expression vectors were transformed into rice callus via *Agrobacterium*-mediated transformation as previously described (Chapter 2.5). Rice callus was transformed with the pBIN-ZmUbi-*APsy*2a or pBin-ZmUbi-*ZmPsy*1 vectors alone and co-transformed with pBIN-ZmUbi-*CrtI*. For seed-specific expression, all rice callus was co-transformed with pCA-Gt1-*APsy*2a or pCA-Gt1-*ZmPsy*1 and pCA-Gt1-*CrtI.* For the first 2 wks, selection of transformed rice callus was carried out on 2N6-S media (Appendix 1) supplemented with 200 mg L^{-1} timentin and 25 mg L^{-1} geneticin (for *npt*II) or hygromycin (for *hpt*II). Subsequently, transformed callus was transferred fortnightly onto fresh 2N6-S media containing the appropriate antibiotic (at 50 mg L^{-1}) and Timentin (200 mg L^{-1}). After 6 wks, proliferating callus lines were independently multiplied for an additional 9 wks with transfer to fresh media every 3 wks. Regeneration of transgenic plants was carried out on 2N6-R media supplemented with 50 mg L^{-1} of geneticin or hygromycin as previously described (Chapter 2.5 and Appendix 1).

3.2.2 Histochemical assay

Histochemical GUS assay was carried out as described (Chapter 2.8.2). Randomly selected clumps of transformed rice callus were assayed for transient GUS expression after 3 days of co-cultivation. In addition, GUS assay was carried out for a few clumps of transformed callus from antibiotic resistant/proliferating clusters 6 wks after transfer to selection media. Further, leaf tissue from putatively transformed plants was harvested from potted rice plants before transfer to the glasshouse and stained for GUS expression. Mature dry rice seed was analysed for GUS activity immediately after harvest.

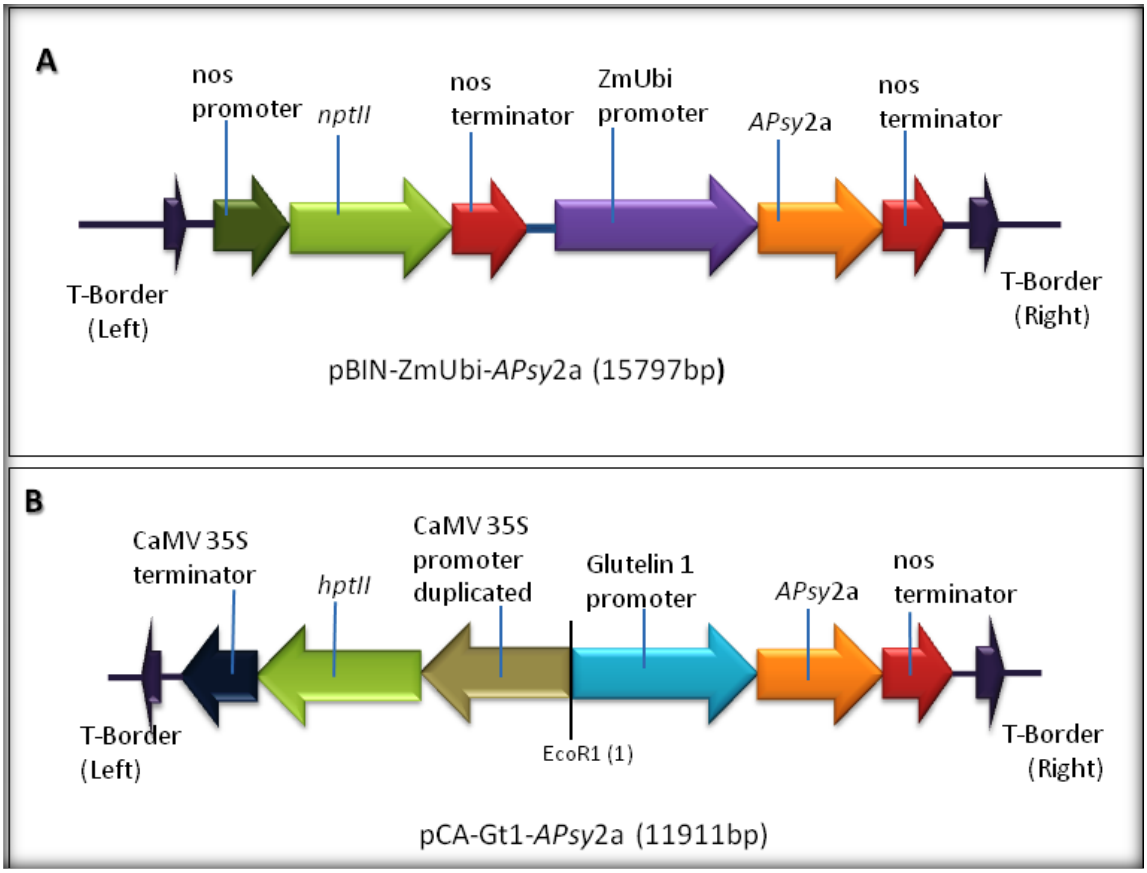


Figure 3.0 Schematic representation of the APsy2a binary expression vectors used for transformation of rice. pBIN-ZmUbi **(A)** and pCA-GT1 **(B)** vectors containing the banana phytoene synthase (*APsy*2a) gene are shown. The T-DNA region is between the right and left borders.

Table 3.1 List of binary expression vectors for transformation of rice

Vector ID	Expression vector details[a]
Constitutive expression vectors	
pBIN-ZmUbi-*APsy*2a	pBIN-ZmUbi-*APsy*2a-nos-nos-*nptII*-nos
pBIN-ZmUbi-*ZmPsy*1	pBIN-ZmUbi-*ZmPsy*1-nos-nos-*nptII*-nos
pBIN-ZmUbi-*CrtI*	pBIN-ZmUbi-*CrtI*-nos-nos-*nptII*-nos
pBIN-ZmUbi-GUS	pBIN-ZmUbi-GUS-nos-nos-*nptII*-nos
Seed-specific expression vectors	
pCA-Gt1-*APsy*2a	pCA-Gt1-*APsy*2a-nos-35S-*hptII*-35S
pCA-Gt1-*ZmPsy*1	pCA-Gt1-*ZmPsy*1-nos-35S-*hptII*-35S
pCA-Gt1-*CrtI*	pCA-Gt1-*CrtI*-nos-35S-*hptII*-35S
pCA-Gt1-GUS	pCA-Gt1-GUS-nos-35S-*hptII*-35S

[a] **vector backbone-promoter-gene-terminator-promoter-selectable marker-terminator**

3.2.2 PCR analysis of transgenic rice plants

Genomic DNA was extracted from rice callus or rice leaf tissue as previously described (Chapter 2.4.1 and 2.4.2). Screening of gDNA for the presence of the respective transgene was carried out by PCR in 20 µL reactions as described (Chapter 2.3.7.1) using gene-specific primers (Table 3.2). For callus lines that displayed different shades of colour intensity, approximately equal number of clumps for each colour was mixed into a composite sample for gDNA extraction.

3.2.3 RT-PCR analysis of transgenic rice plants

RNA was extracted from leaf tissue of putatively transgenic rice plants as previously described (Chapter 2.4.3), and was eluted in 50 µL of RNase-free water. Transcripts were detected by Titan One tube RT-PCR (Roche) as previously described (Chapter 2.4.4.2) using 2 µL of DNase-treated total RNA extract as template (Chapter 2.4.3). The absence of DNA in the RNA extracts was confirmed by standard PCR using β-actin primers (2.3.7.1) before RT-PCR (Chapter 2.3.7.2) with gene-specific primers (Table 3.2).

Table 3.2 List of primers used for PCR analysis of transgenic rice plants and callus

Primer name	Primer sequence 5'...3'*	Purpose/Transgene	Annealing (T°C)	Product size (bp)
*APsy*2a-*Bam*H1Fwd	GGATCCATGTCTGGCTCTATTGTTTGG	*PCR/APsy*2a	55	1200
*APsy*2a-*Xba*1-Rev	TCTAGATTATAGTGTTCCTGCAAATTTGG	*PCR/APsy*2a		
B73-SPF685-Fwd	CAAACGCCAGCTGCGCAC	*PCR/ZmPsy*1	55	685
B73-SPF685-Rev	CCGGTTCGTGACGACCCCTTTG	*PCR/ZmPsy*1		
CrtI-Fwd	GTCGACGAAACCAACTACGGTAATTGGTGC	*PCR/CrtI*	55	1500
CrtI-Rev	TCTAGATCATATCAGATCCTCCAGCATC	*PCR/CrtI*		
VirCFwd-AGL1	GCCTTAAAATCATTTGTAGCGACTTCG	PCR/VirC	62	738
VirCRev-AGL1	TCATCGCTAGCTCAAACCTGCTTTCTG	PCR/VirC		
β-actin Fwd	GGTGTYATGGTWGGKATGGG	PCR	50	1100
β-actin Rev	CCTCCAATCCAGACACTGTAC	PCR		

* Restriction site sequences within the primer are underlined

3.2.4 *In-situ* analysis of carotenoids in rice callus and seed by Raman spectroscopy

In-situ analysis of carotenoids was carried out using Raman spectroscopy. Raman measurements were performed using a Perkin-Elmer spectrum 2000 NIR-FT (Near InfraRed Fourier Transform) Raman Spectrometer fitted with a ND: YAG Laser emitting at 1064 nm in the range 10-750 mW. To generate a reference spectrum for β-carotene, 1 μg mL^{-1} of the standard compound (Sigma) was dissolved in carbon tetrachloride (CCl_4) and analysed. An aliquot of 500 μL β-carotene-CCl_4 solution was dispensed in a sample vial and mounted vertically onto the Raman spectrometer stage.

To analyse rice, a single seed or cluster of proliferating callus was placed into a sample holder and then mounted onto the Raman spectrometer stage. In order to analyse callus, two representative callus clumps (pale yellow or orange coloured) were selected from one putative transgenic callus line expressing pBIN-ZmUbi-*APsy*2a, pBIN-ZmUbi-*ZmPsy*1 or pBIN-ZmUbi-*CrtI*. For rice seeds, at least six seeds from plants expressing pCA-Gt1-*APsy*2a, pCA-Gt1-*Psy*1 or pCA-Gt1-*CrtI* were analysed. A single laser beam at 320 mW was run through each sample. The resulting excitation of carotenoid molecules and vibrations was measured by performing 64 scans at 320 mW of laser power with a spectral resolution of 8 cm^{-1} in the Raman shift range 200-3800 cm^{-1}. The

output data was processed into Raman spectra using Raman spectrum for Windows and Microsoft Office Excel. Raman peaks for β-carotene in transformed rice callus were identified against the spectrum of β-carotene standard. In these experiments, Raman spectroscopy was only used for *in-situ* detection of the presence of carotenoids in intact transgenic rice callus or seed while carotenoid quantification was carried out by HPLC.

3.2.5 Quantification of carotenoid in rice callus by HPLC

Up to 300-400 mg of fresh callus was freeze dried and total carotenoids were extracted using 2 mL acetone as described (Chapter 2.7.3.2). The organic phase was recovered using 1 mL PE: DE, dried under vacuum and re-dissolved in 2 mL chloroform of which 20 µL was injected onto the HPLC column. Carotenoid separation was performed using a C30 reverse phase matrix in a methanol–tertbutyl ether-based mobile phase. The β-carotene signal was monitored from a photodiode array detector at 465 nm. Estimation of β-carotene was determined against a calibration curve and standardised using an internal standard (alpha tocopherol acetate) as previously described (Chapter 2.7 and Appendix 3). An acceptable percentage recovery for internal standard was above 70%.

3.3 Results

3.3.1 Constitutive expression of *Psy* under the control of the ZmUbi promoter

3.3.1.1 Coloured rice callus – evidence for constitutive expression of *Psy*

Approximately 150 clumps of rice embryogenic callus were each transformed with either *APsy*2a, *ZmPsy*1 or *CrtI* alone, or were co-transformed with *APsy*2a and *CrtI* or *ZmPsy*1 and *CrtI*, all under the control of the maize poly-ubiquitin promoter (Table 3.1). For controls, 60 clusters were transformed with pBIN-ZmUbi-GUS while a further 60 clumps were used as non-transformed selection and regeneration controls. After 4-6 wks on selection media, antibiotic resistant clumps began to display a yellow-orange phenotype (Fig 3.1 A and B). Two (1.3%) callus clusters that had each been transformed with *ZmPsy*1 alone or co-transformed with *ZmPsy*1 and *CrtI* displayed a deep-orange colour while 17 (11.3%) callus clusters transformed with *APsy*2a alone displayed a yellow-orange colour. In addition, three (2%) callus clusters co-transformed with *APsy*2a and *CrtI* also displayed a yellow-orange colour. None of the three (2%) antibiotic resistant clusters transformed with *CrtI* alone or four (6.7%) resistant clusters transformed with GUS displayed any distinct colour difference from non-transformed controls.

Each antibiotic resistant cluster was subsequently referred to as a "callus line". In order to increase the quantity of the callus, all callus clumps in each line were subdivided and transferred to fresh 2N6-S selection media for 6-9 weeks (with transfer onto fresh media every three weeks). When the callus clusters from individual callus lines were cultured on selection multiplication media, it was observed that they differentiated into different shades of colour ranging from pale yellow to orange (Fig 3.1C). However, the intensity and proportion of the deep orange colour was greatest in lines transformed with *ZmPsy*1 alone or in combination with *CrtI*. Overall, these observations provide circumstantial evidence that the banana *APsy*2a gene was functional in rice callus.

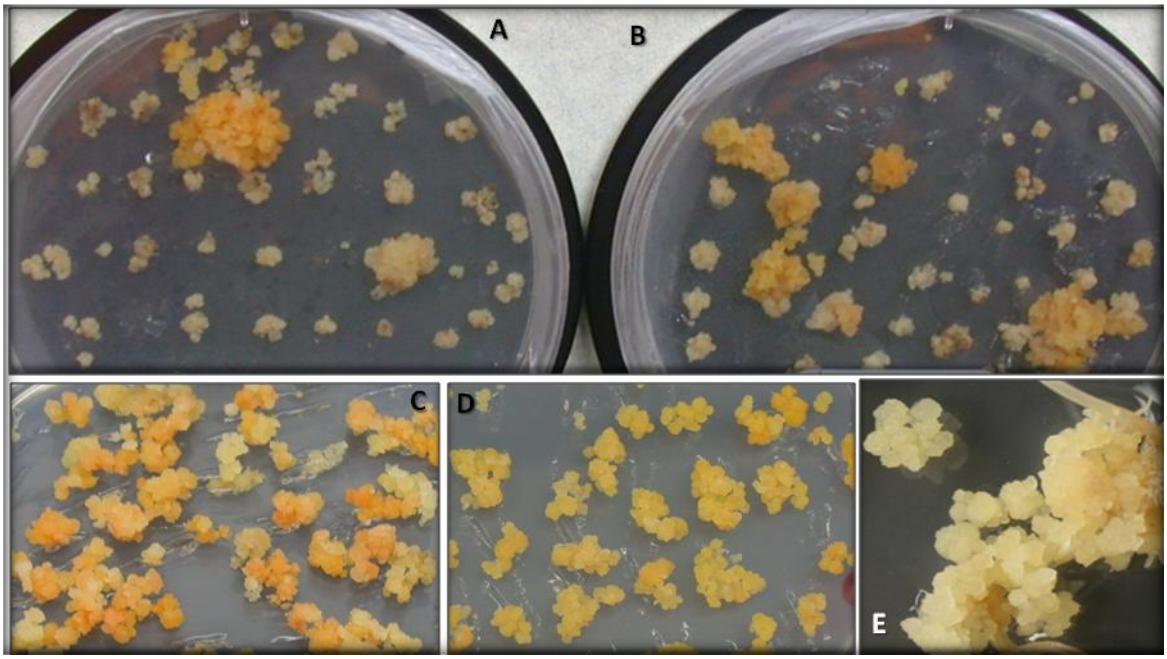


Figure 3.1 Antibiotic resistant coloured callus transformed with pBIN-ZmUbi-APsy2a or pBIN-ZmUbi-ZmPsy1 proliferating on selection media. Plates **A** and **B** show antibiotic resistant callus clusters from callus transformed with *ZmPsy*1 and**.** *APsy*2a, respectively, after 8 wks on selection media. Plate **C** and **D** show one representative callus line transformed with pBIN-ZmUbi-*ZmPsy*1 or pBIN-ZmUbi-*APsy*2a, respectively, after 3 wks on selective multiplication media. Plate **E** is non-transformed, cream-yellow callus growing on 2N6 without selection.

3.3.1.2 Analysis for β-carotene in transgenic callus using Raman spectroscopy

The presence of proliferating yellow-orange resistant callus clusters on selection media provided circumstantial evidence that constitutive expression of *Psy* resulted in the production of coloured carotenoids. At this stage of the experiment, no detailed quantitative analysis for pVA carotenoids was carried out. However, representative coloured clumps were analysed for β-carotene *in-situ* using Raman spectroscopy. Initially, a reference spectrum for β-carotene was generated by analysing 1 µg mL^{-1} of a pure β-carotene compound (Sigma) dissolved in carbon tetrachloride (CCl_4). As illustrated in Fig 3.2A, the wave positions for CCl_4 run from 200-800 cm^{-1} Raman shift while β-carotene displayed characteristic peaks in the range 1000-1500 cm^{-1} Raman shift. A closer examination of the spectrum for the β-carotene pure compound revealed three strong peaks at 1523, 1156 and 1003 cm^{-1} (Fig 3.2B) which correspond to the C=C, C-C and the methyl group, respectively. When rice callus was analysed for the presence of carotenoids by Raman spectroscopy, the characteristic β-carotene peaks were observed at wave positions 1521±2 for C=C, 1155 for C-C and 1005±2 cm^{-1} for the methyl group (Fig 3.3A). The intensity of the Raman shift was greatest in deep orange-coloured callus (mainly pBIN-ZmUbi-*ZmPsy*1 transgenics) compared to yellow-coloured callus (mainly pBIN-ZmUbi-*APsy*2a transgenics) (Fig 3.3A). No peaks were observed from non-transformed callus or callus transformed with *CrtI*. Similar to that reported in other studies, slight shifts in wave positions were observed in the rice callus from those for the pure β-carotene standard compound (Table 3.3).

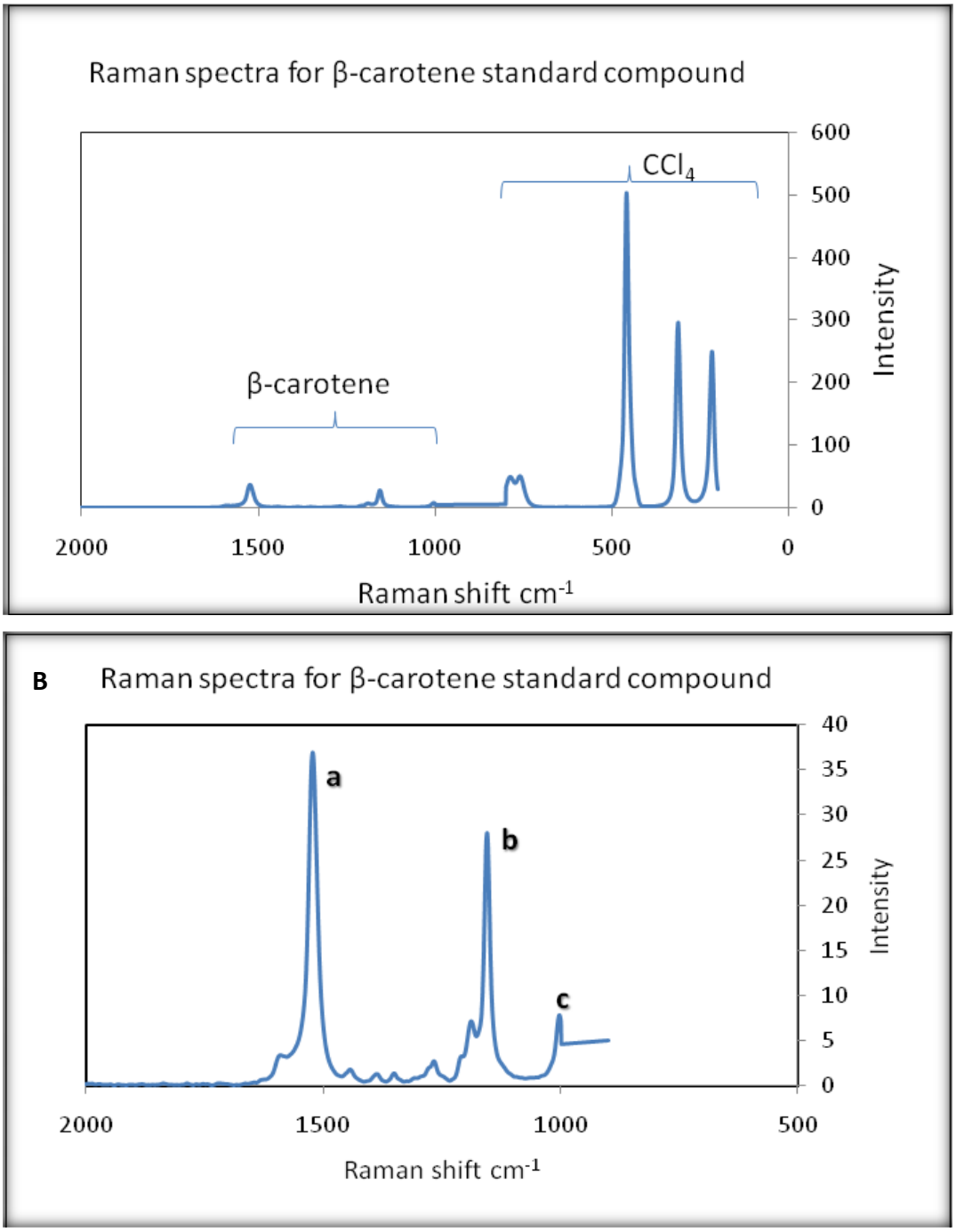


Figure 3.2 Raman spectra for β-carotene standard compound. Graph **A** shows the spectrum for β-carotene peaks in the range 1000 - 1550 cm^{-1} and peaks of carbon tetrachloride in the range 200 -800 cm^{-1} Raman shift. Graph **B** shows a close-up of the Raman spectra for β-carotene with peaks for C=C at 1523 cm^{-1} (**a**), C-C at 1156 cm^{-1} (**b**) and CH_3 (**c**) at 1003 cm^{-1} Raman shift

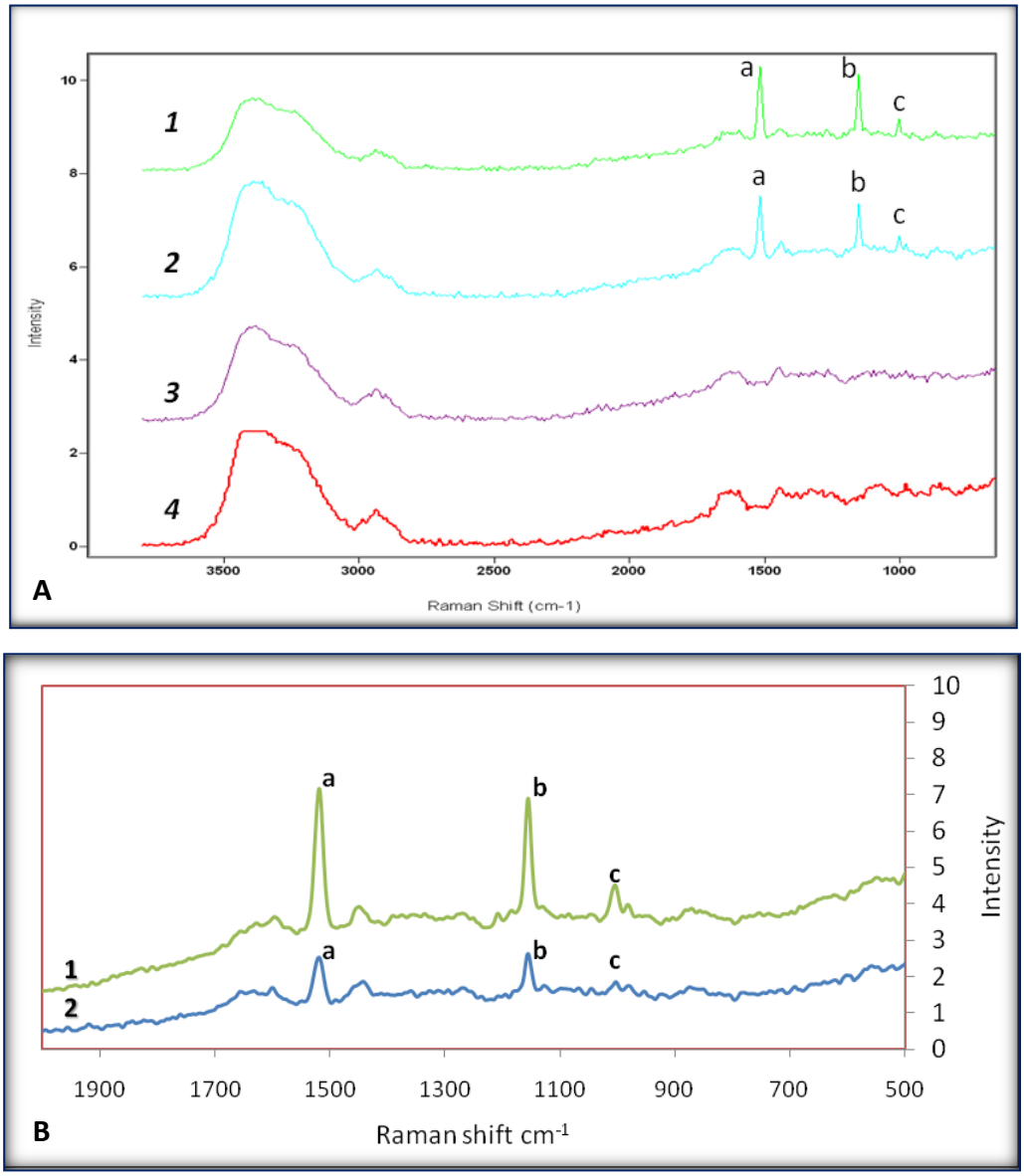


Figure 3.3 Raman spectra showing the presence of β-carotene in coloured transgenic rice callus. Graph **A** shows an example of spectra for callus expressing **1**. *ZmPsy*1 **2.** *APsy*2a **3**. *CrtI* all under the control of ZmUbi promoter and **4.** non-transformed wild-type callus with peaks at approximately 1521±2 (**a**), 1155 (**b**) and 1005±2 cm^{-1} (**c**) Raman shift. Graph **B** shows an example of the difference in the intensity of the Raman shift for the **1.** orange and **2**. yellow callus showing the three β-carotene peaks i.e. **a.** for C=C; **b**. for C-C and **c**. for CH_3 vibrations.

Table 3.3 Positions of Raman shift (cm^{-1}) for β-carotene in rice callus

Chemical group	β-carotene standard	*APsy*2a (ORC)	*APsy*2a (YC)	*ZmPsy*1 (ORC)	*ZmPsy*1 (YC)	Mean Raman shift (cm^{-1})	STDev
C=C	**1523**	1520	1524	1519	1519	1521	2
C-C	**1156**	1155	1155	1155	1155	1155	0
CH3	**1003**	1003	1008	1004	1004	1005	2

ORC = Orange callus, YC = Yellow callus

3.3.1.3 Regeneration of rice callus transformed with constitutively expressed transgenes

After 6 wks on selective multiplication media, the callus was transferred to regeneration media. Although shoot-structures began appearing in non-transformed callus as well as some transgenic lines after 2 wks, the regeneration of coloured callus into normal plants was difficult and appeared to be affected by the intensity of the colour of the transgenic callus. For example, very few shoots developed from deep orange-coloured callus transformed with either pBIN-ZmUbi-Zm*Psy*1 alone or co-transformed with pBIN-ZmUbi-*CrtI* (Table 3.4). Further, any "shoot-like" structures which did develop from this callus were orange and stunted with thick leaves or appeared bleached and devoid of chlorophyll. None of them developed into normal shoots, and the callus clusters subsequently became necrotic (Fig 3.4A and B) while on regeneration media. As such, no plants were regenerated from coloured callus transformed with either pBIN-Ubi-*ZmPsy*1 alone or co-transformed with pBIN-ZmUbi-*CrtI*.

In contrast, shoots did develop and plants were regenerated from some callus lines transformed with either pBIN-ZmUbi-*APsy*2a or pBIN-ZmUbi-*CrtI*. Out of 17 antibiotic-resistant callus lines transformed with pBIN-ZmUbi-*APsy*2a alone, a total of 76 plants were regenerated from five of these lines (Table 3.4). Ultimately, however, only 19 plants all derived from one line, were acclimatised to the glasshouse stage. Most of the plants regenerated from the other four lines did not produce roots when transferred to rooting media with selection, suggesting that they were escapes, while other plants died during the early stages of plant acclimatisation. Of the three antibiotic-resistant callus lines transformed with pBIN-ZmUbi-*CrtI,* 18 plants were regenerated from one line and 16 of these were successfully acclimatised to the glasshouse. As a control, 14 plants were regenerated from the non-transformed regeneration control callus and four of these were successfully acclimatised to the glasshouse.

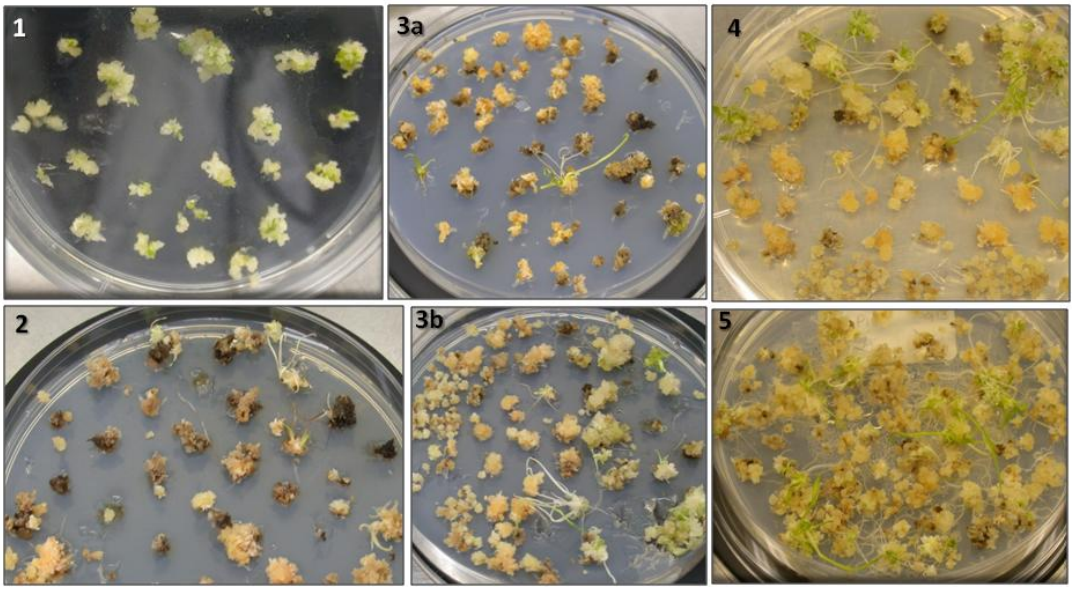


Figure 3.4A Regeneration of rice callus transformed with pBIN-ZmUbi-APsy2a or pBIB-ZmUbi-ZmPsy1. Plate **1** shows emergence of shoots from non-transformed control callus after 2 wks on regeneration media; Plate **2** shows *ZmPsy*1-orange callus with some necrotic clusters, bleached shoots, and no normal shoots were obtained from this callus. Plate **3a** and **3b** show *APsy*2a yellow-orange callus with limited regeneration but normal shoots were obtained; Plate **4** and **5** shows *APsy*2a-shoots regenerating from callus that was less intense in colour (i.e. yellow and or pale yellow callus).

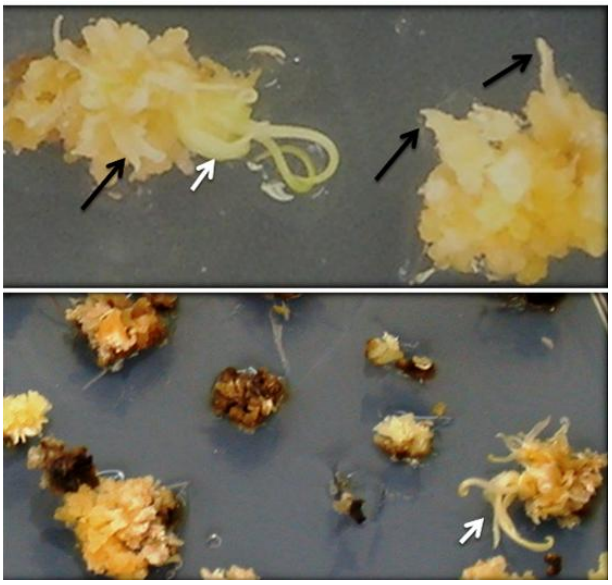


Figure 3.4B Phenotypic abnormalities during regeneration of coloured callus transformed with pBIN-Ubi-ZmPsy1. The black arrows indicate the coloured, thick "shoot-like" structures while the white arrow shows the thick leaf phenotype of a shoot that eventually did not develop into a normal plant. The white arrow (in the lower panel) shows an originally coloured shoot which did not accumulate chlorophyll, remained stunted and eventually became necrotic.

Table 3.4 Summary of rice embryogenic callus transformation and regeneration studies with pVA biosynthesis genes under the control of the ZmUbi promoter

Expression vector	No. of callus clumps transformed	No. of resistant callus clusters (lines)	No. of plants regenerated	No. of plants acclimatised	RT-PCR for transgene expression		Escapes	GUS assay	Plants with seed
					Positive for *APsy*2a	Positive for *CrtI*			
pBIN-ZmUbi-*APsy*2a	150	17	76 (5 lines)	**19 (1 line)**	**16**	na	**3**	na	**7**
pBIN-ZmUbi-*APsy*2a + pBIN-ZmUbi-*CrtI*	150	3	0	0	na	na	na	na	na
pBIN-ZmUbi-*ZmPsy*1	150	2	0	0	na	na	na	na	na
pBINZmUbi-*ZmPsy*1 + pBIN-ZmUbi-*CrtI*	150	2	0	0	na	na	na	na	na
pBIN-ZmUbi-*CrtI*	150	3	18 (1 line)	**16**	na	**0**	**16**	na	5
pBIN-ZmUbi-GUS	60	4	6	**6**	na	na	2	**4**	0
NT selection control	60	0	0	na	na	na	na	na	na
NT-regeneration control	60	na*	14	**4**	0	0	na	0	**4**

* Not applicable

3.3.1.4 Transplanting of putative transgenic rice plants and production of T1 seed

Regenerated shoots were taken from both the transformed callus clumps and non-transformed controls and acclimatised into soil as previously described (Chapter 2.5.4, Fig 3.5). Both transgenic and control *in-vitro* shoots in this experiment were generally weak and small (~8-10 cm) at the time of transplanting into soil. Despite regular fertilising, most plants grew poorly and had only developed single or 2-3 tillers by flowering stage. In general, all the plants produced only a small number of panicles and the seed set was very low.

3.3.1.5 Analysis of transgene expression in putative transgenic rice plants

Initially, attempts were made to analyse plants for the presence of the respective transgenes by PCR using primers specific to the *npt*I sequence (located on the pBIN backbone vector outside the T-DNA). This strategy was not possible, however, due to the presence of false positives resulting from the non-targeted amplification of the *npt*I sequence present within persistent residual *Agrobacterium* in transgenic plants. As such, the rice plants were analysed for transgene expression by reverse transcriptase (RT)-PCR. To detect the presence of gene-specific transcripts in each of the acclimatised plants, total RNA was extracted and contaminating DNA was removed by treatment with DNase. To ensure that amplicons were derived from RNA and not contaminating DNA, the DNase-treated extracts were initially used in a conventional PCR with β-actin primers; only those extracts from which no amplicons were observed were used for RT-PCR.

When the 19 putative ZmUbi-*APsy*2a-transgenic rice plants were analysed by RT-PCR using *APsy*2a-specific primers, a product of the expected size (1200 bp) was amplified from 16 plants (Fig 3.6; Table 3.4**).** In contrast, no products were amplified from any of the 16 putative ZmUbi-*CrtI*-transgenic rice plants analysed using primers specific for *CrtI* (Table 3.4). Further, no amplicons were ever obtained from RNA extracted from the non-transformed controls using primers designed to amplify the entire coding sequence of either *APsy*2a or *CrtI.*

Figure 3.5 Acclimatisation of putative transgenic rice plants derived from callus transformed with APsy2a or CrtI under the control of the ZmUbi promoter.
A. *In-vitro* rice plant on rooting media prior to acclimatisation; **B.** Rice plants 1 wk after transfer to soil; **C**. Rice plants ready for transfer to the glasshouse; **D.** Transgenic rice plants at flowering stage in the glasshouse; **E.** Mature transgenic rice plants at glasshouse developed a small number of panicles. **F.** Non-transformed control rice plants 3 wks after potting into soil.

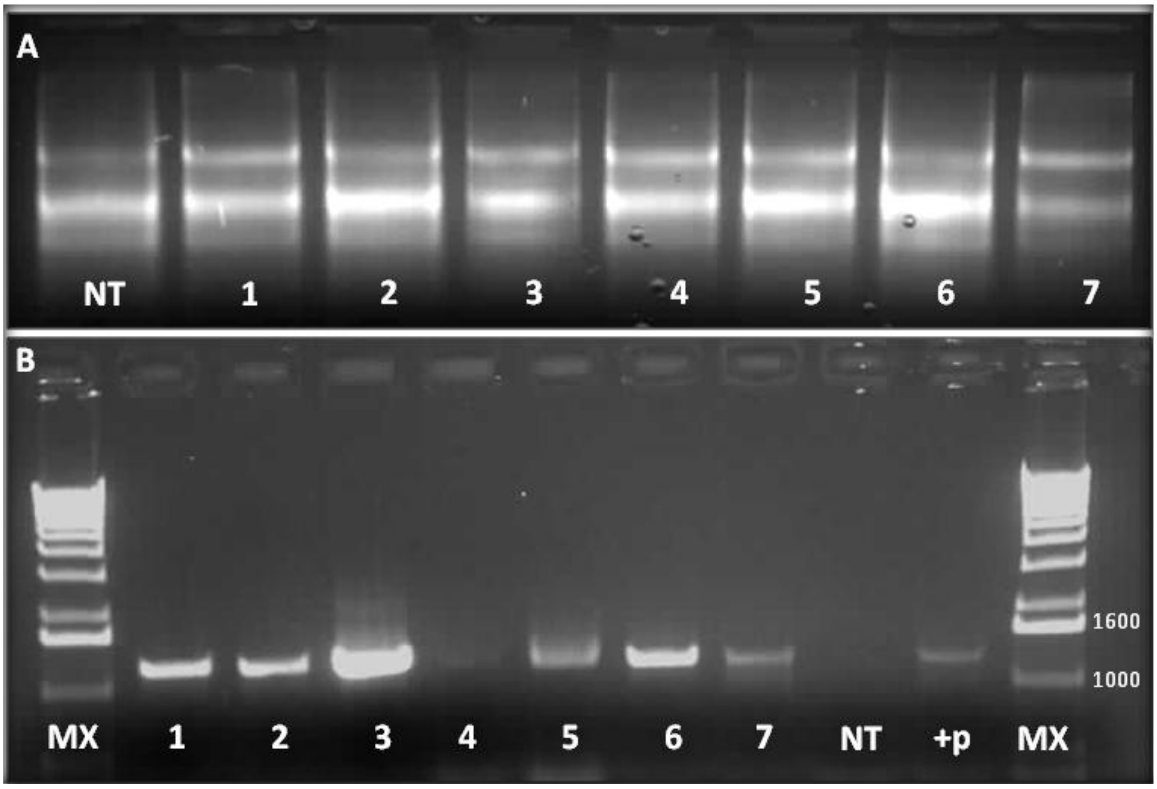


Figure 3.6 Representative agarose gel of RT-PCR products from putatively transgenic rice plants. RNA extracts from seven putative ZmUbi-*APsy2a*-transgenic plants (1-7) and a non-transformed (NT) control plant are shown in (**A)** and corresponding RT-PCR products using primers specific for the *APsy*2a gene **(B)**. The fragment at 1.2 kbp corresponds to the entire coding sequence of *APsy*2a, **+p** = plasmid (pBIN-ZmUbi-*APsy*2a) positive control, **MX** = Marker X (Roche Applied sciences).

3.3.1.6 Callus induction from T1 rice seed

Transgenic plants were acclimatised and grown to maturity in a glasshouse as reported in section 3.3.1.4. T1 seed was harvested when the panicles and seed husks were dry (after approx. 4-5 months from potting stage). However, seed set from both the transgenic rice plants and the controls was relatively low (ranging from 0-40 seeds) since a number of kernels did not fill with seed (Fig 3.7A**).** Out of the 16 ZmUbi-*APsy*2a-transgenic plants (from one line), only seven plants produced viable seed. Each of these plants was subsequently assigned an individual UAS number as an identifier, while seed derived from a plant was numbered as UAS with the plant number and an additional seed or callus number (e.g. UAS 2.1). Visually, the colour of T1 seed obtained from the transgenic plants was very similar to wild-type seed except for a few seeds that had a brown hue (Fig 3.7B**).** The four control plants expressing GUS did not produce any seed.

In order to obtain sufficient material to conduct further analyses, callus was induced from individual T1 seeds (Fig 3.8**)**. Of the seeds obtained from the seven ZmUbi-*APsy*2a-transgenic plants, only those from six plants produced embryogenic callus when placed on 2N6 callus induction media supplemented with 50 mg mL^{-1} geneticin (Table 3.5). Further, whereas the callus generated from seeds derived from two lines (UAS 6 and 10) was creamy-yellow in appearance, at least 50% of the callus produced from seeds derived from the remaining plants, with the exception of UAS 7, was yellow-orange (Table 3.5 and Fig 3.8). With plant UAS 7, only 14.3% of the callus produced was yellow (Table 3.5). The callus which developed from seeds of the non-transgenic control plants was creamy-yellow in colour (Fig 3.8).

The callus produced from each seed was subdivided and placed on 2N6 multiplication media (Appendix 1) supplemented with 50 mg L^{-1} geneticin. None of the creamy-yellow callus that was produced from any of the seeds, with the exception of UAS 10.4, proliferated further and all except that derived from UAS 10.4 eventually died. With the yellow callus, it was observed that the colour became heterogeneous over time and developed a mixture of different colours ranging from creamy-yellow to yellow to orange and often in varying degrees of colour intensity (Fig 3.9). Further, not all the coloured callus proliferated (Table 3.5).

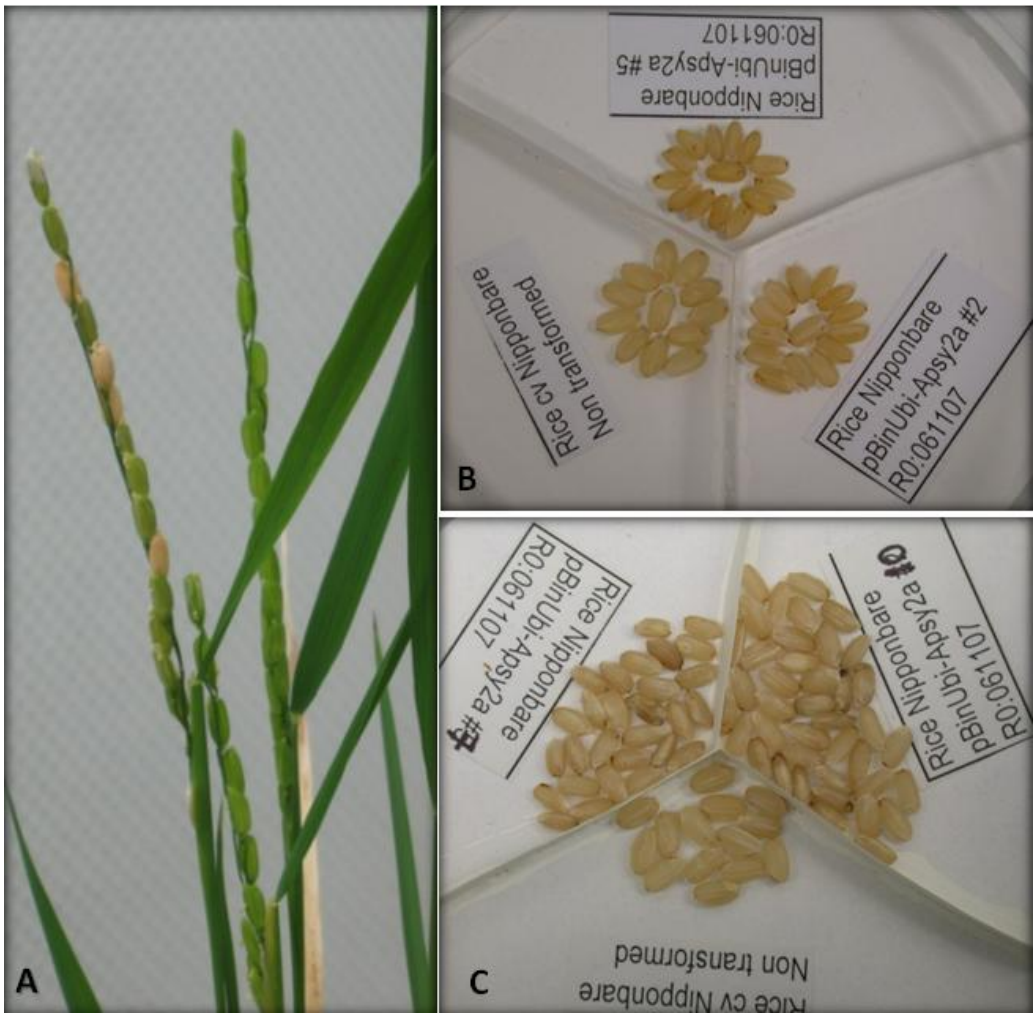


Figure 3.7 **T1 seed derived from rice plants expressing APsy2a under the control of the ZmUbi promoter. A**. Inflorescence showing incomplete seed filling of the panicle, **B.** T1 seeds from plant UAS 2 and UAS 5 exhibiting a brown hue; **C.** T1 seed from plant number UAS 7 and UAS 10 were indistinguishable from wild-type T1 seeds**.**

Table 3.5 T1 seed harvested from rice plants transformed with pBIN-ZmUbi-*APsy*2a

Plant No	# of seeds harvested	# of seeds producing callus	# of seeds with yellow callus	# of seeds with cream-yellow callus	# of callus lines proliferating on selection media
UAS#2	17	10	5	5	2
UAS#4	1	0	0	0	0
UAS#5	16	12	7	5	6
UAS#6	4	2	0	2	0
UAS#7	27	21	3	18	2
UAS#8	2	2	1	1	1
UAS#10	40	6	0	6	1
NT (WT)	32	12	0	12	-

UAS = Plant ID number for putative rice plants transformed with pBIN-ZmUbi-*APsy*2a
(UAS 2.4 represents plant number 2; seed or callus number 4

Figure 3.8 Induction of embryogenic callus from T1 transgenic rice seed expressing APsy2a under the control of the ZmUbi promoter. 1a. Wild-type rice seed; **1b.** T1 rice seed, **2a** and **2b**. Initial stages of callus induction showing orange roots/callus or cream-yellow root/callus (2a) developing from individual T1 seeds derived from same plant (UAS 2); **3.** Representative coloured embryogenic callus induced from one seed (UAS 2.5) after 3 wks of proliferating on 2N6-S media supplemented with geneticin; **4.** Cream-yellow embryogenic callus induced from wild-type seed on 2N6 media without selection.

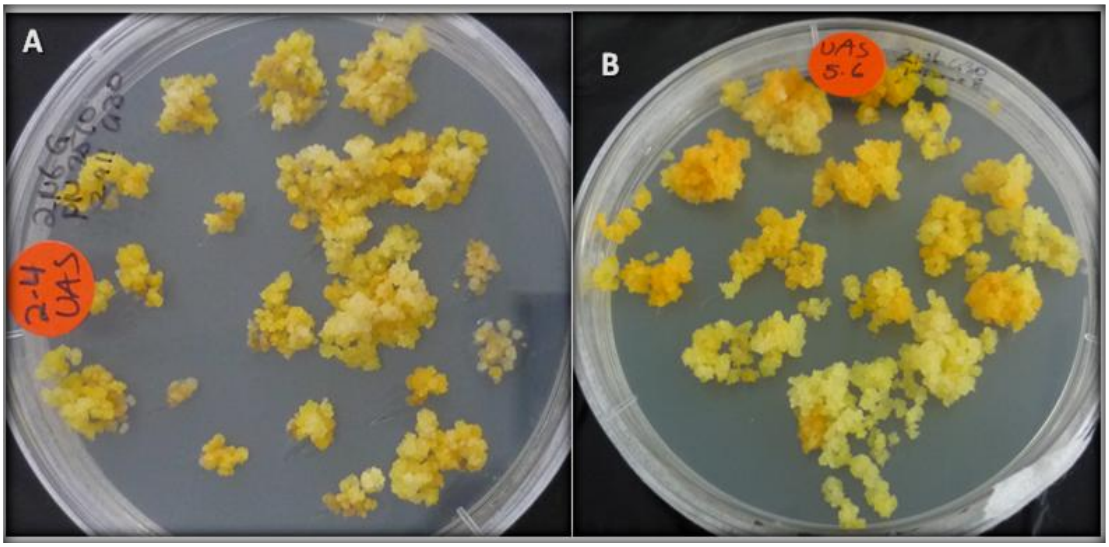


Figure 3.9 Different shades of yellow-orange colour in T1 callus expressing APsy2a under the control of ZmUbi promoter. Representative plates show callus derived from T1 seed UAS 2.4 (**A**) and UAS 5.6 (**B**).

3.3.1.7 PCR analysis of T1 callus to detect presence of transgene

Since the T1 callus originating from the seeds of ZmUbi-*APsy*2a-transgenic rice plants was heterogeneous in colour, a composite sample containing approximately equal portions of callus clumps that were representative of the different colours, were picked from a plate of proliferating callus, mixed and used for gDNA extraction. To confirm the presence of the transgene in the proliferating callus, genomic DNA was extracted and used in a PCR with primers specific for the *APsy*2a transgene. A band of the expected size (1.2 kbp) was amplified from all proliferating callus thereby confirming the presence of the *APsy*2a transgene (Fig 3.10).

3.3.1.8 HPLC for quantification of carotenoid content in T1 callus

The previous results from Raman spectroscopy indicated that the constitutive expression of *APsy*2a led to enhanced levels of β-carotene in rice callus. In order to quantify these levels, total carotenoids were extracted from the 12 proliferating callus lines (11 yellow-orange and one creamy-yellow, Table 3.5) which were derived from T1 seeds and which tested positive for *APsy*2a transgene. These were analysed by HPLC. Extracts from callus derived from non-transgenic rice seeds were included in the HPLC analyses for comparative purposes. Very small amounts (0.002 µg/mg dwt) of pVA carotenoids were detected in extracts derived from the non-transgenic callus. In contrast, all other transgenic callus samples, were found to contain enhanced levels of β-carotene ranging from 4.6 fold (UAS 7.2; 0.008 µg/mg dwt) to 72 fold (UAS 5.6; 0.127 µg/mg dwt) (Fig 3.11), with the exception of the creamy-yellow callus of line UAS 10.4 for which no pVA was detected. A direct correlation between intensity of colour and levels of β-carotene was observed with yellow-orange callus containing the highest β-carotene content. In addition, the height and area under the peak for their respective HPLC chromatograms was higher for orange callus compared to yellow and creamy-yellow callus (Fig 3.12A and B).

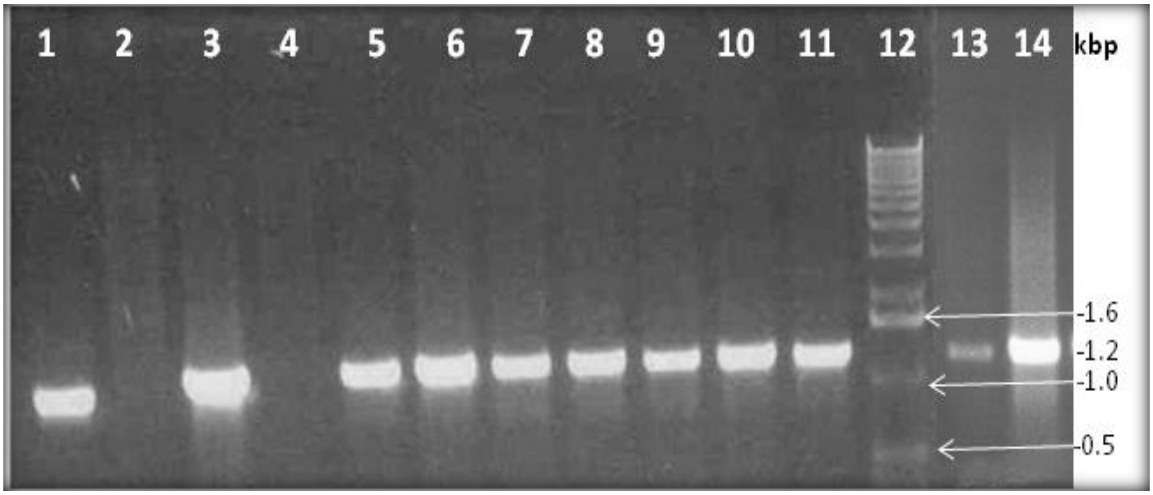


Figure 3.10 Representative agarose gel showing the PCR detection of APsy2a in T1 callus using gene-specific primers. Lane **1** (NT callus+ β-actin primers), lane **2** (NT callus + *APsy*2a primers) and lane **14** (pBIN-ZmUbi-*APsy*2a + *APsy*2a primers) are controls; while the remaining lanes are as follows; **3**. UAS 10.4 (antibiotic resistant yellow callus); **4**. UAS 8.1 (repeated in lane 13); **5.** UAS 2.4; **6**. UAS 2.5; **7**. UAS 5.2; **8.** UAS 5.3; **9**. UAS 5.5; **10.** UAS 5.6; **11.** UAS 5.7; **12**. Marker X (Roche); **13.** UAS 8.1.

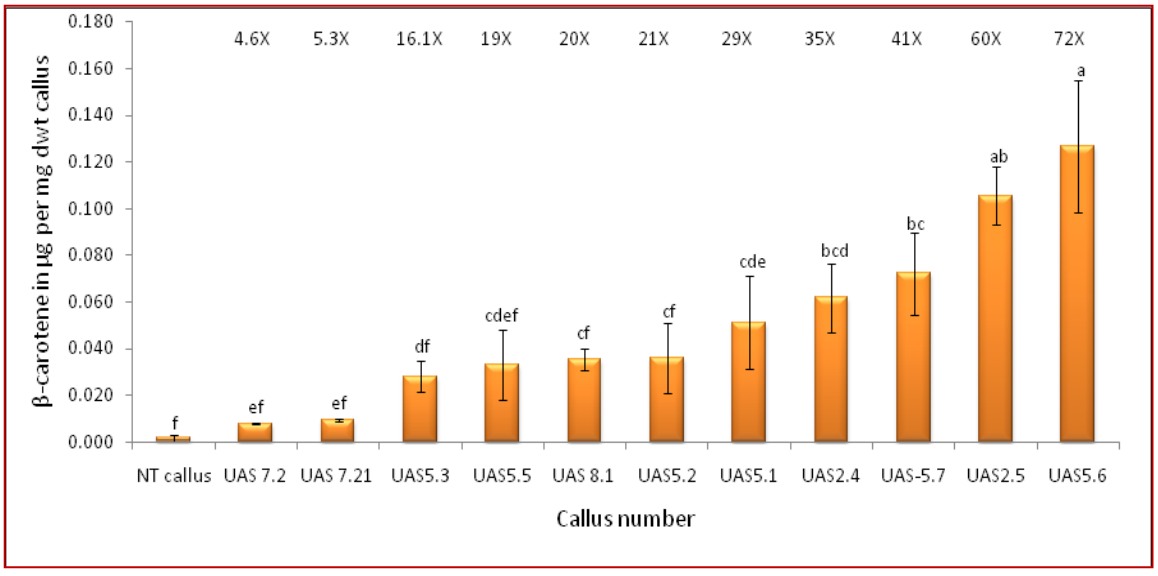


Figure 3.11 HPLC for estimation of β-carotene content in pBIN-ZmUbi-APsy2a-transgenic T1 callus. Bars represent mean β-carotene content in µg/mg dwt of rice callus. Error bars show standard error (n=3).

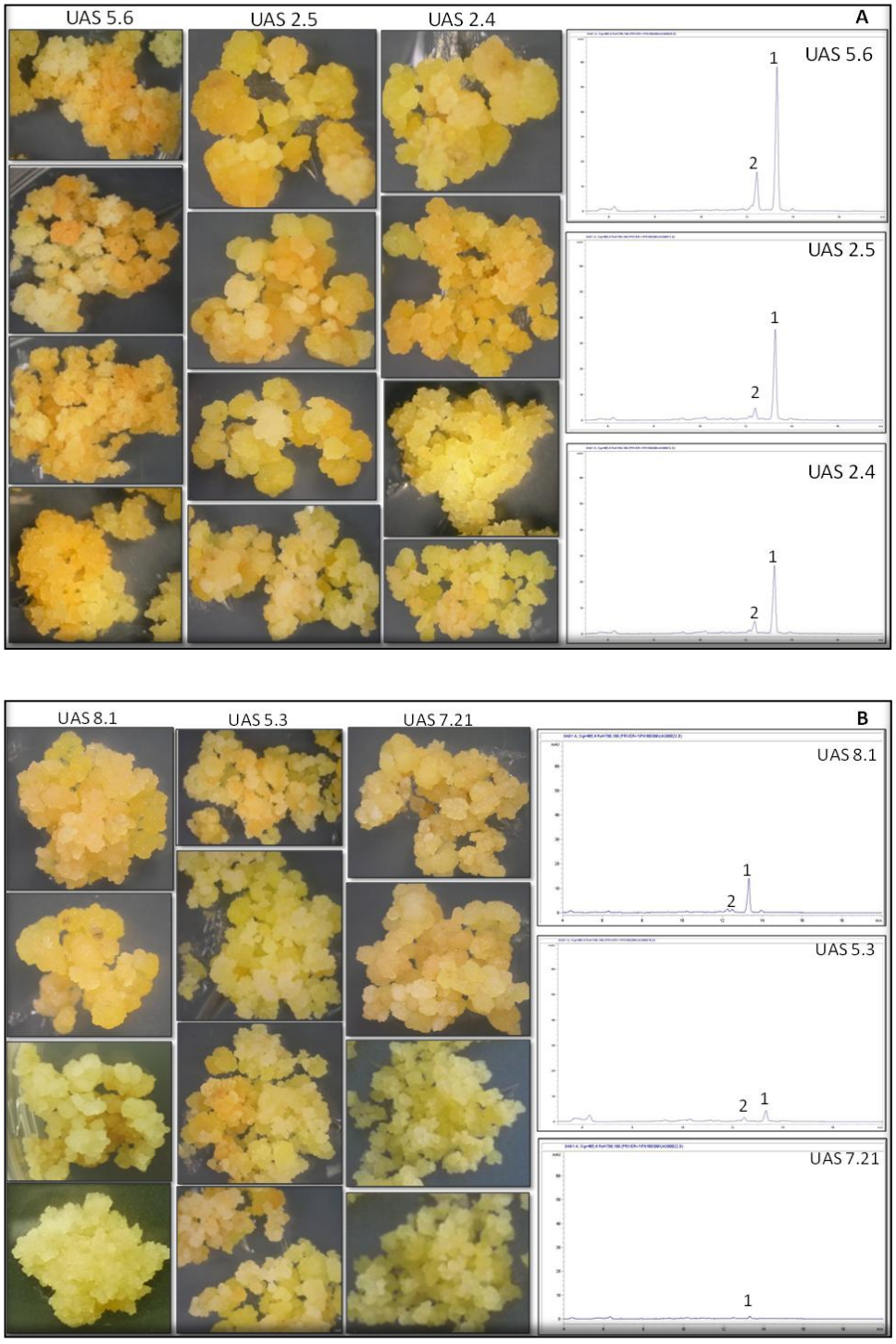


Figure 3.12 Comparison of the relationship between the colour of T1 callus and the height of carotenoid peaks observed on HPLC chromatograms. Left hand panels show representative T1 coloured callus clusters from which carotenoid was extracted for HPLC analysis. Callus line UAS 5.6 gave the highest peak and therefore the highest β-carotene content. The chromatograms illustrate the position and height of two peaks **1**. β-carotene and **2**. α-carotene whose decreasing height correspond to decreasing intensity of colour in representative samples UAS 5.6, UAS 2.5, UAS 2.4 (in Panel A) and UAS 8.1, UAS 5.3 and UAS 7.21 (in Panel B) respectively.

3.3.2 Seed-specific expression of *Psy* under the control of the rice glutelin1 promoter

3.3.2.1 Generation of transgenic plants

A total of 712 and 518 clumps of rice callus were each co-transformed with pCA-Gt1-*APsy*2a/pCA-Gt1-*CrtI* and pCA-Gt1-*ZmPsy*1/pCA-Gt1-*CrtI*, respectively, while 387 callus clumps were transformed with pCA-Gt1-GUS (Table 3.6). As a selection control, 105 non-transformed callus clusters were included. Hygromycin-resistant callus clusters were selected by culturing on 2N6-S media containing 50 mg L^{-1} hygromycin and 200 mg L^{-1} timentin. After 4-6 wks on selection media, none of the non-transformed control callus had proliferated; these eventually became necrotic and died with continued culture on antibiotic selection. In contrast, up to 11% of the putatively transgenic callus clusters derived from each transformation appeared healthy and were proliferating on antibiotic selection (Fig 3.13; Table 3.6). Each resistant callus cluster was treated as a putatively transformed line and clumps from each line were placed on multiplication media with selection. In order to regenerate putative transgenic plants, clumps from each line were transferred to regeneration media with selection. Of the eight, ten and eight germinating callus lines that were putatively transgenic for *APsy2a*/*CrtI*, *ZmPsy*1/*CrtI* and GUS respectively, plants were only regenerated from four, seven and three of these respective lines (Table 3.6). From these, the number of plants acclimatised in the glasshouse for further analysis was 38 from the four *APsy2a*/*CrtI*-transformed lines, 32 plants from the seven *ZmPsy*1/*CrtI*-transformed lines and 13 plants from the three GUS-transformed lines (Table 3.7)

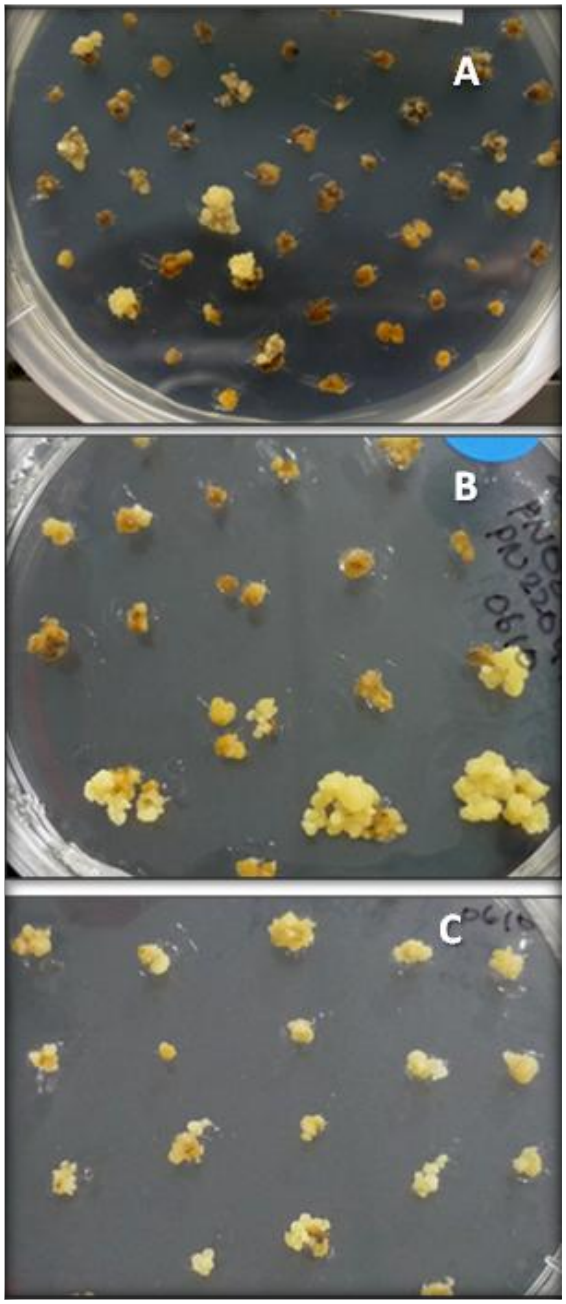


Figure 3.13 Selection of rice callus co-transformed with either pCA-Gt1-APsy2a or pCA-Gt1-ZmPsy1 and pCA-Gt1-CrtI on 2N6-S media. An example of proliferating antibiotic resistant callus clusters, 6 wks after transformation shows **A**. pCA-Gt1-*APsy*2a +pCA-Gt1-*CrtI* and **B**. pCA-Gt1-GUS. Plate **C** shows the non-transformed selection control callus with no proliferating clusters. Proliferating callus clusters were transferred to fresh selection media and multiplied as putatively transformed lines.

Table 3.6 Rice embryogenic callus transformed with the pVA biosynthesis genes under the control of glutelin1 promoter

Transformation vector	Av. no. of callus clumps transformed	No. of hygromycin resistant callus clusters	% putatively transgenic callus lines	No. of callus lines germinating	No. of callus lines taken to glasshouse
pCA-Gt1-*APsy*2a/pCA-Gt1-*CrtI*	712	46	6.5	8	4
pCA-Gt1-*ZmPsy*1/pCA-Gt1-*CrtI*	518	21	4.1	10	7
pCA-Gt1-GUS	387	41	10.6	8	3
NT-selection control	105	0	0.0	0	0

Table 3.7 Transgenic plants regenerated from rice callus transformed with pCA-Gt1 for the expression of pVA carotenoid synthesis genes

Treatment (vector(s)	Callus line ID	Number of plants per line acclimatised at glasshouse	PCR analysis for transgene			
			Positive for *APsy*2a	Positive for *ZmPsy*1	Positive for *CrtI*	Escapes
pCA-Gt1-*APsy*2a/pCA-Gt1-*CrtI*	2	5	5	NA	0	0
	3	16	0	NA	16	0
	12	13	10	NA	0	3
	14	4	0	NA	4	0
pCA-Gt1-*ZmPsy*1/pCA-Gt1-*CrtI*	2	7	NA	1	0	6
	3	10	NA	0	10	0
	4	1	NA	1	0	0
	5	4	NA	0	4	0
	8	8	NA	6	2	0
	9	1	NA	1	0	0
	11	1	NA	0	1	0
Total number of plants		**(70)**	**(15)**	**(9)**	**(37)**	**(9)**
pCA-Gt1- GUS	3	5	Seeds from these plants were harvested and stained for GUS activity			
	7	5				
	8	3				
Total of control plants		**(13)**				

3.3.2.2 PCR analysis to detect the presence of transgenes

To confirm the presence of the respective transgenes, plant gDNA was extracted from each of the 70 putatively transformed plants and analysed by PCR using primers specific for either *APsy*2a (1200 bp), *ZmPsy*1 (685 bp) or *CrtI* (1500 bp) (Table 3.2). Prior to this, the quality of gDNA was first screened by PCR using primers designed to amplify part of the house-keeping gene, β-actin (1100 bp) (Fig 3.14B). Further, in order to eliminate false positives due to residual *Agrobacterium* in plant cells, gDNA was also screened using primers specific for the VirC operon sequence (located in the Ti plasmid (pTiBo542) of *Agrobacterium*) (Fig. 3.14C). A negative result indicated that gDNA was clean from *Agrobacterium* contamination. A summary of these molecular characterisation results is shown in Table 3.7, while representative agarose gels showing transgene detection using gene-specific primers are shown in Figs 3.15, 3.16 and 3.17. In summary, all plants contained either the *APsy*2a, *ZmPsy*1 or *CrtI* transgene, but none of the plants was found to be co-transformed.

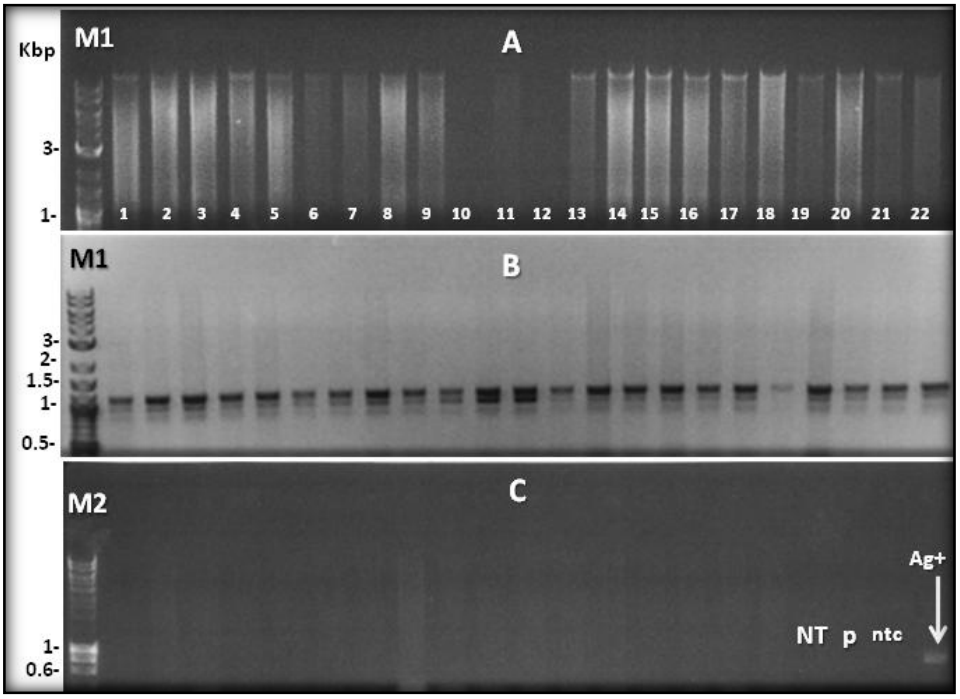


Figure 3.14 PCR analysis of putative (Gt1-ZmPsy1/Gt1-CrtI) transgenic rice plants for detection of residual Agrobacterium. Representative 1% agarose gels showing **A.** gDNA extracted by quick CTAB method, **B.** PCR with β-actin primers and **C.** screening gDNA for residual *Agrobacterium* using VirC primers, **NT.** Non-transformed control, **p.** negative plasmid DNA (pCA-Gt1-*ZmPsy*1), **ntc.** no template control, **Ag+** = *Agrobacterium* gDNA control (~738bp), **M1.** 2-log DNA ladder (Biolabs), **M2.** HyperLadder (Bioline).

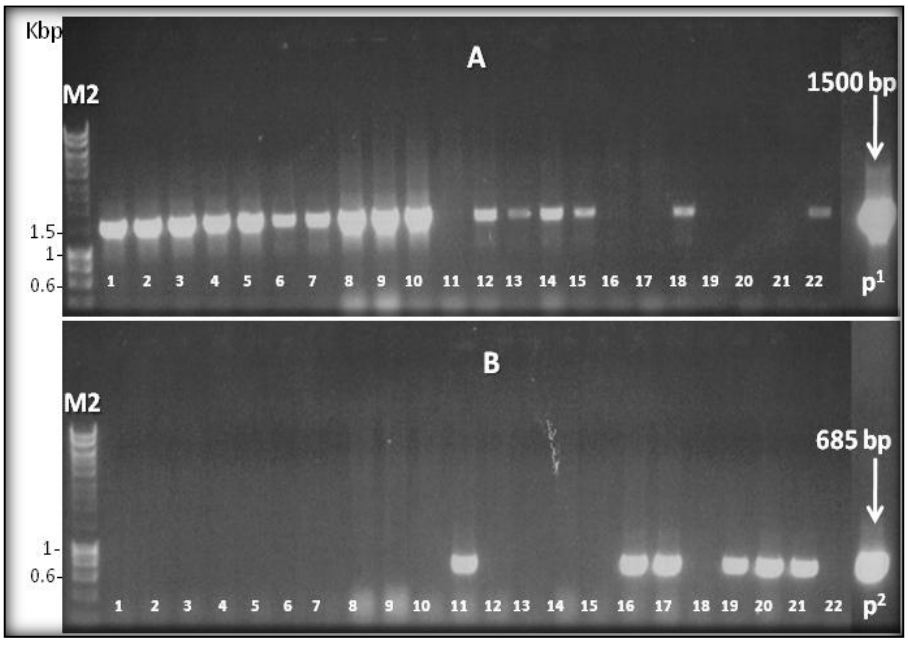


Figure 3.15 PCR analysis of putative (Gt1-ZmPsy1/Gt1-CrtI) transgenic rice plants for transgenes. Representative agarose gels show PCR products for 22 transgenic plants analysed for **A**. *CrtI* (1500 bp) and **B.** *ZmPsy*1 (685 bp) **p^1.** plasmid DNA positive control being pCA-Gt1-*CrtI* and **p^2**. pCA-Gt1-*ZmPsy*1). **M2.** HyperLadder (Bioline).

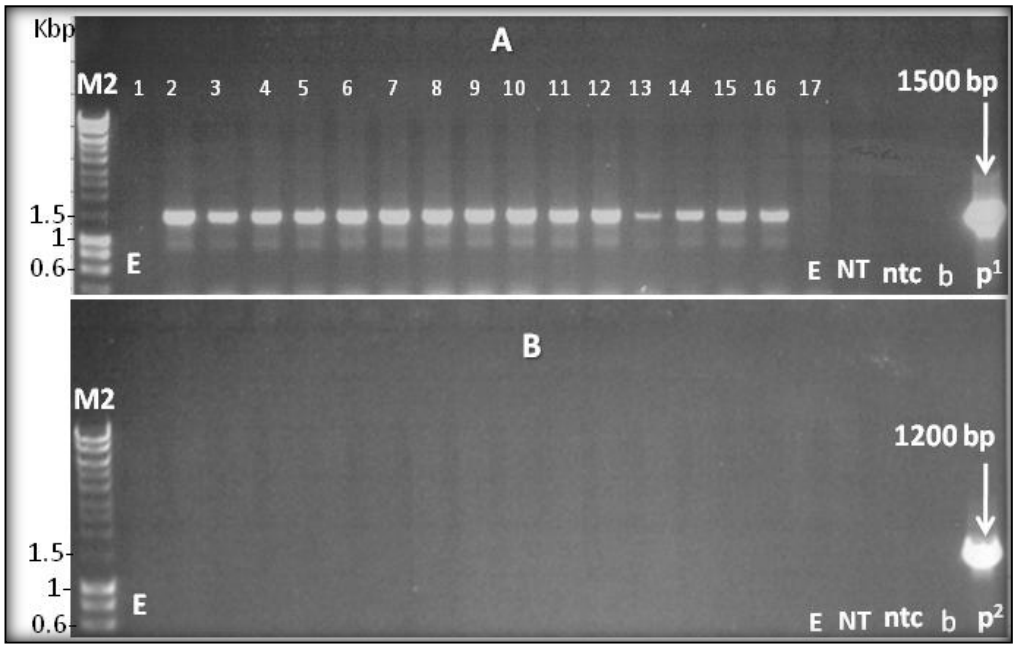


Figure 3.16 PCR analysis of putative (pCA-Gt1-APsy2a/pCA-Gt1-CrtI) transgenic rice plants for transgene. Representative agarose gels show PCR products for 17 plants analysed for **A**. *CrtI* (1500 bp) and **B.** *APsy*2a (1200 bp), **E.** escapes (plants 1 and 17), **NT.** non transformed control, **ntc.** no template control, **b.** blank well, **M2.** HyperLadder, **p^1.** plasmid DNA positive control being pCA-Gt1-*CrtI* and **p^2**. pCA-Gt1-*APsy*2a).

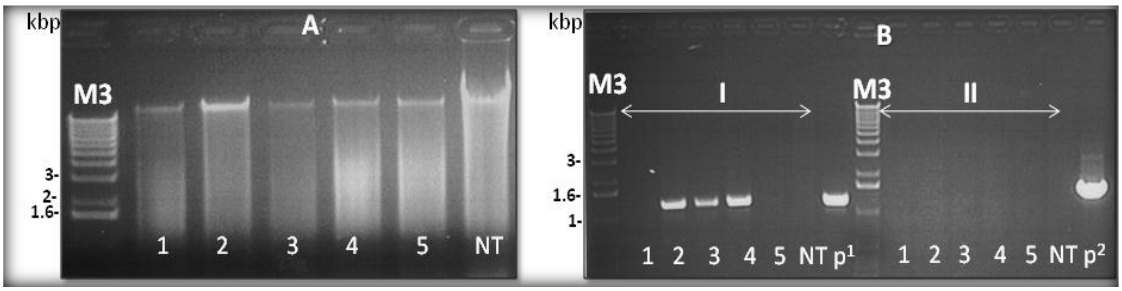


Figure 3.17 PCR analysis of putative (pCA-Gt1-APsy2a/pCA-Gt1-CrtI) transgenic rice plants for transgene. Agarose gel electrophoresis shows **A.** rice genomic DNA from 5 putative rice plants and **B.** PCR products for **I.** *APsy*2a and **II.** *CrtI* primers; **NT** = non transformed negative control; **p^1** = plasmid (pCA-Gt1-*APsy*2a) positive controls showing *APsy*2a (1200 bp); **p^2** = plasmid (pCA-Gt1-*CrtI*) showing *CrtI* (1500 bp); **M3** = Marker X (Roche).

3.3.2.3 Transplanting of Gt1-*Psy*-transgenic plants and production of T1 seed

As indicated in 3.3.2.1, regenerated transgenic rice plants were acclimatised and subsequently grown to maturity in the glasshouse as previously described (Chapter 2.5.4, Fig 3.18). Panicles (inflorescences) began to develop on the plants between 6-8 wks after potting and the seed was considered ready for harvest when the seed husks turned brown and dry (4-5 months after potting). Due to difficulties with acclimatisation conditions, all non-transformed control plants died. Although not a strictly ideal control, substitute control plants were raised by germinating rice seed directly in the soil at the glasshouse and used for comparative purposes. All transgenic plants displayed a normal phenotype for tillering, leaf colour and flowering when compared to wild-type controls (Fig 3.19). Furthermore, the extent of seed filling of all transgenic plants was similar to that of the wild-type plants. Although the size of panicles that developed on the different tillers of the same transgenic plant was found to vary, most of these were the same size as those that developed on wild-type plants (Fig 3.20). Additionally, most of the seeds harvested from the transgenic plants were visually indistinguishable from wild-type seeds, with some transgenic seeds showing a brown hue. The T1 seeds were subsequently subjected to further analysis by Raman spectroscopy for *in-situ* detection of pVA carotenoids.

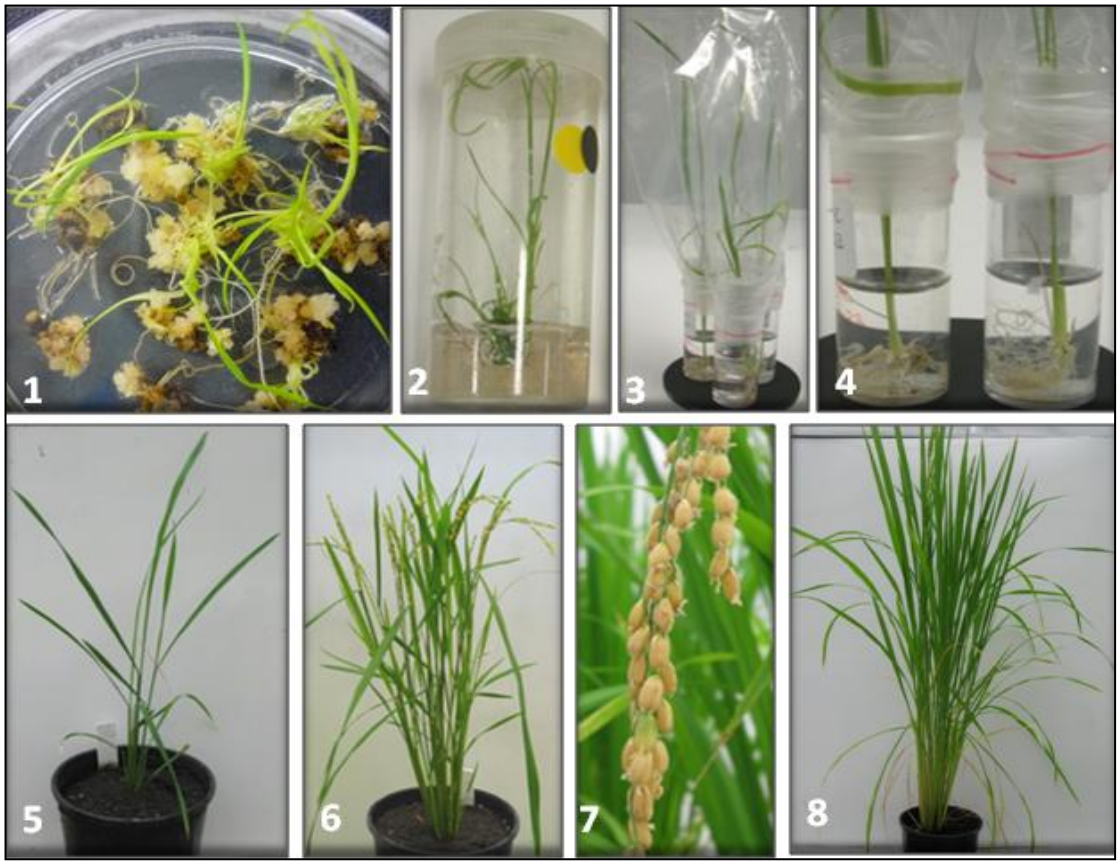


Figure 3.18 Regeneration and acclimatisation of putative transgenic rice plants derived from callus co-transformed with APsy2a or ZmPsy1 with CrtI each under the control of the Gt1 promoter. Plate **1** shows *in-vitro* regeneration of putative plants from callus co-transformed with pCA-Gt1-*APsy2a*/pCA-Gt1-*CrtI;* **2.** rooting of shoots to a sufficient root mass; **3** and **4**. pre-acclimatisation of rice shoots in water before transfer to soil; **5.** a putative transgenic rice plant 3 wks after potting and acclimatised under glasshouse conditions; **6.** mature transgenic rice plant; **7**. a panicle of rice showing seed formation in a transgenic plant; **8**. wild-type rice plant at flowering stage.

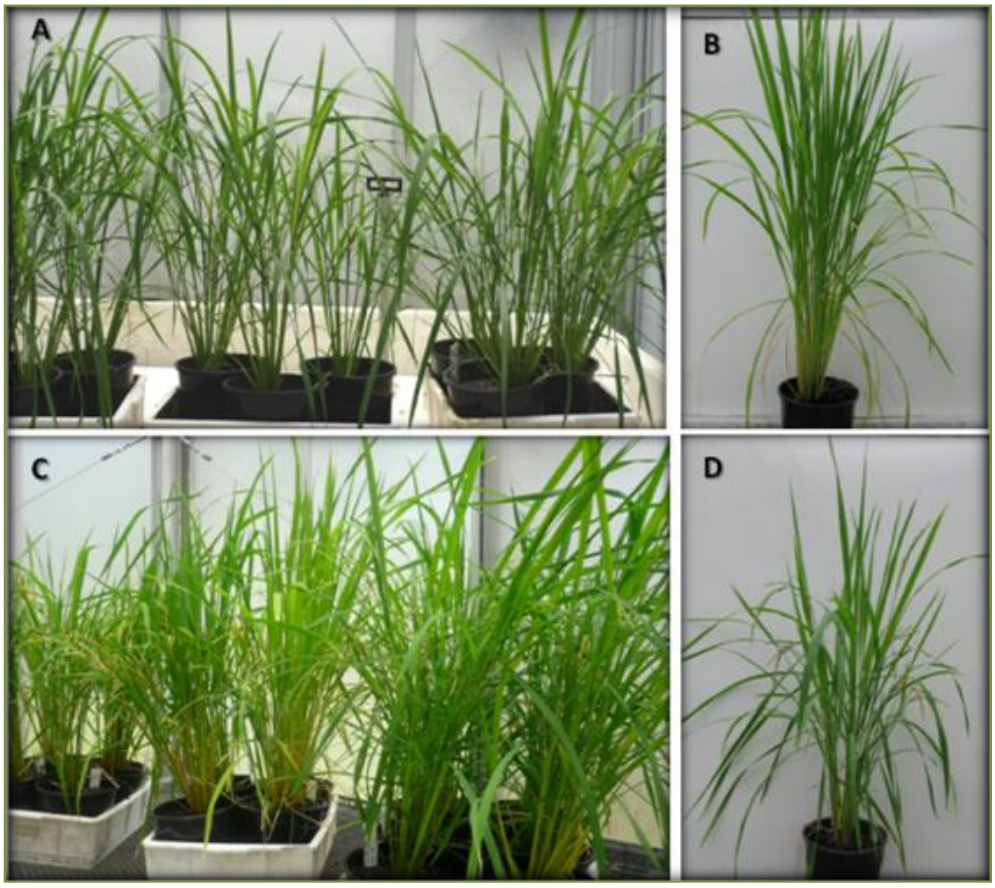


Figure 3.19 Phenotype of wild-type and transgenic rice plants growing at the glasshouse.

A = wild-type rice plants, **B** = close-up of wild-type rice plant, **C** = Gt1-*APsy*2a plants; **D** = Close-up of a Gt1-*APsy*2a plant.

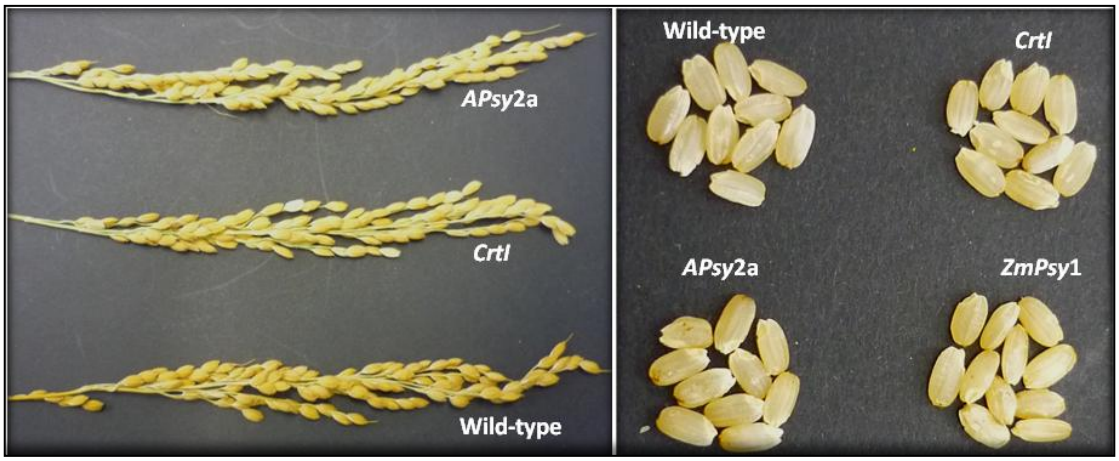


Figure 3.20 T1 rice seed generated from plants transformed with seed-specific-expressed pVA genes.

3.3.2.4 Raman spectroscopy for pVA carotenoids in T1 rice seed derived from Gt1-*Psy* plants

Raman spectroscopy was used to determine the *in-situ* presence of pVA carotenoids (in particular β-carotene) in rice seeds harvested from plants transgenic for Gt1-*APsy*2a, Gt1-*ZmPsy*1 or Gt1-*CrtI*. As a control, seed derived from wild-type plants were included in the analysis. Following exposure to a laser beam, the excitation of molecules in the wild-type rice seed produced Raman peaks characteristic of starch (Kim *et al.,* 2009) and no peaks for β-carotene were detected. Representative transgenic seed for Gt1-*APsy*2a, Gt1-*ZmPsy*1 or Gt1-*CrtI* gave Raman spectra identical to that observed from wild-type seed with no peaks corresponding to any carotenoid compounds (Fig 3.21 and 3.22).

3.3.3 Histochemical assay for reporter gene controls

As a transformation control, embryogenic rice callus was transformed with the *uidA* (GUS) gene under the control of either the maize poly-ubiquitin or glutelin1 promoter and transgenic plants were regenerated. When randomly selected callus clumps transformed with ZmUbi-GUS were histochemically assayed for GUS expression three days post-transformation, strong GUS expression was observed in all clumps tested (Fig 3.23 A). In addition, strong GUS expression was observed throughout the entire leaf taken from each of the four stably transformed ZmUbi-GUS-plants (Fig 3.23 B). It was not possible to assess constitutive GUS expression in rice seeds since no seeds were produced from any pBIN-ZmUbi-GUS-transgenic plants generated.

No GUS expression was observed in randomly selected clumps of pCA-Gt1-GUS-transformed rice callus after 3 days post-transformation. However, strong GUS expression was observed in the seeds obtained from 5/5, 2/5 and 3/3 pCA-Gt1-GUS-transgenic plants derived from lines 3, 7 and 8, respectively (Table 3.7), thereby confirming expression of the glutelin1 promoter in rice seed (Fig 3.23 C).

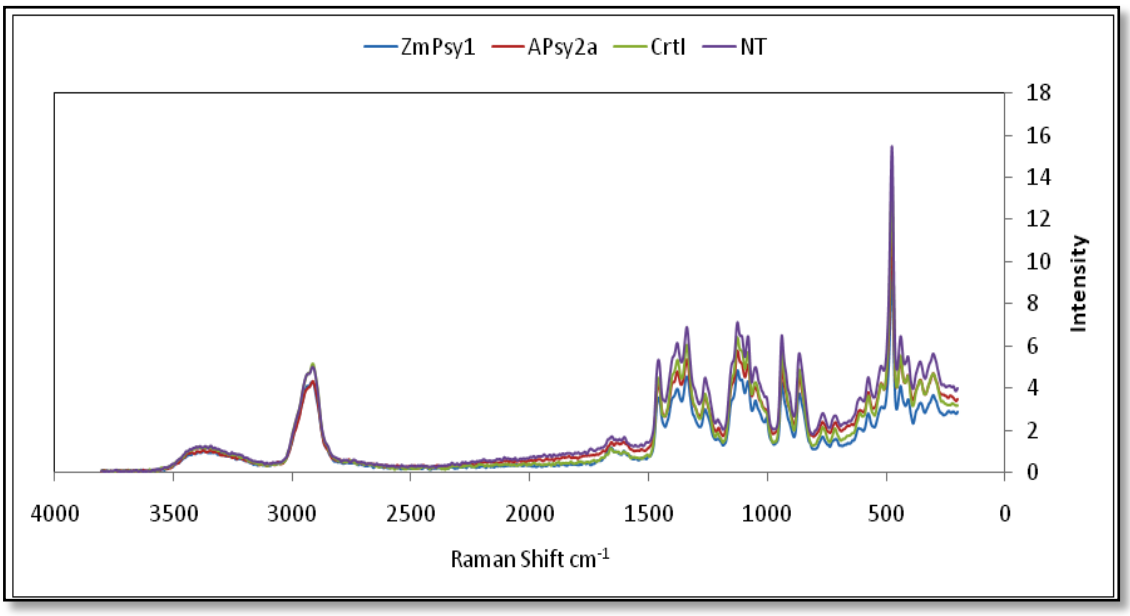


Figure 3.21 Full Raman spectra for representative rice seed for *in-situ* detection of pVA carotenoids

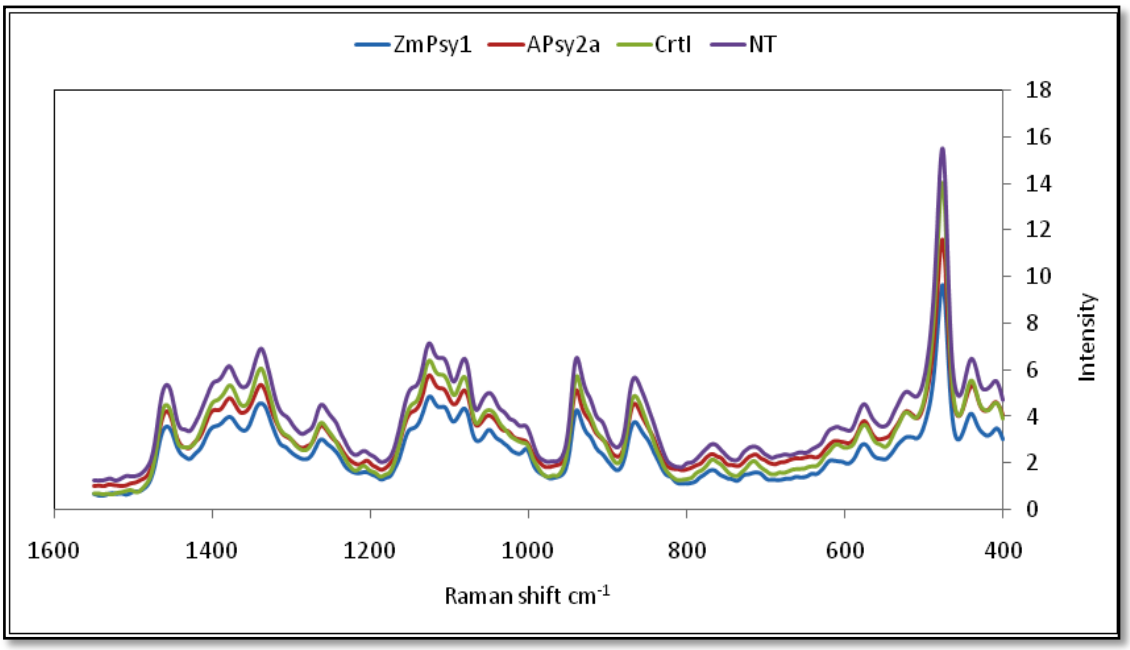


Figure 3.22 Close up of Raman spectra in the range 400-1500 cm^{-1} for rice seed. The Raman shifts observed show characteristic peaks for starch. No peaks corresponding to β-carotene were observed in transgenic rice seed.

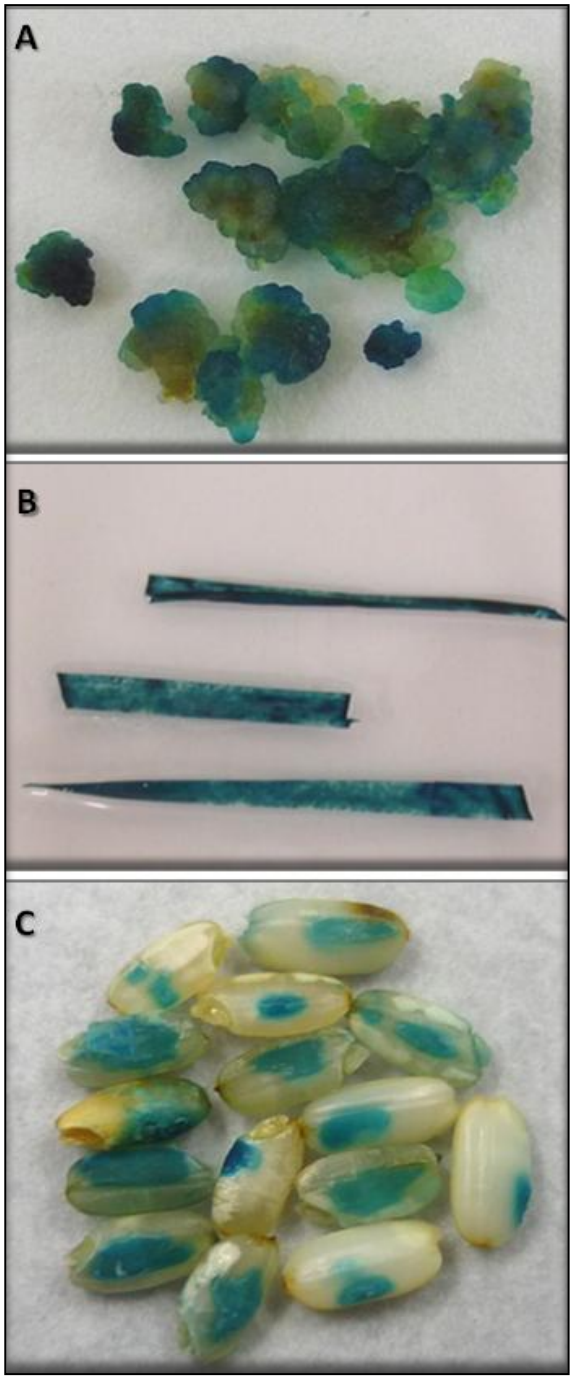


Figure 3.23 Histochemical assay for GUS activity in transgenic rice tissue. Constitutive GUS expression is shown in rice callus (**A)** and leaf (**B)** under the control of ZmUbi promoter, while GUS expression in seed is shown in seed (**C**) under the control of glutelin1 promoter.

3.4 Discussion

The results from this research have shown that the phytoene synthase gene (*APsy*2a) derived from the F'ei banana, Asupina, is functional in rice callus since constitutive expression of *APsy*2a resulted in increased levels of pVA. Preliminary evidence for the increase in pVA carotenoids was provided by the development of yellow/orange colour, characteristic of high β-carotene levels, in both T0 and T1 rice callus transformed with *APsy*2a. The control rice callus transformed with constitutively expressed, maize-derived *Psy*1 alone also developed an orange colouration which was generally more intense than that observed with *APsy*2a. In contrast, callus transformed with constitutively expressed *CrtI* alone, showed no colour changes suggesting no increase in pVA carotenoids. This latter observation suggests that *CrtI* alone requires phytoene for its function in rice callus. The development of yellow-orange colour in transformed callus constitutively expressing phytoene synthase, has been previously reported in maize (Paine *et al.,* 2005) where the intensity and amount of yellow-orange colour in transformed callus was shown to be indicative of the potential efficiency of the transgene for enhancement of pVA carotenoids. Based on this correlation, the differences observed in the intensity and amount of yellow-orange colour between callus transformed with ZmUbi-*APsy*2a or ZmUbi-*ZmPsy*1 suggest that the *Psy* from maize, which belongs to the same family as rice i.e. *Poaceae*, is more active than the banana-derived *APsy*2a in rice callus.

The *in-situ* increased accumulation of carotenoids in yellow/orange transgenic callus constitutively expressing *APsy*2a or *ZmPsy*1 was confirmed by Raman spectroscopy. The presence of higher peaks mainly in ZmUbi-*ZmPsy*1 transgenic callus compared to ZmUbi-*APsy*2a transgenic callus correlated with increases in colour intensity, suggesting a higher carotenoid content in the more intensely coloured callus. The Raman peaks for β-carotene were observed in the same range as that previously reported at 1520, 1156 and 1007 cm^{-1} (Schulz *et al.,* 2005). The strong peaks in the 1500-1550 cm^{-1} and 1150-1170 cm^{-1} range have been previously reported as a result of the in-phase C=C double bond and C-C single bond, respectively, stretching vibrations of the polyene backbone of the carotenoid molecules. An additional peak of medium intensity, observed in the range 1000-1020 cm^{-1}, corresponds to the in-plane motion of the methyl group (CH_3)

(Li-Chan 1996; Schulz *et al.,* 2005; Davey *et al.,* 2007). Raman scattering depends on changes in the polarizability of the functional groups as the atoms vibrate such that non-polar groups (C=C or C-C) have intense Raman shifts compared to polar groups (C=O or O-H). In addition, stretching vibrations for multiple bonds are enhanced such that C=C vibrations are stronger than those from C-C (Ram *et al.,* 2003). Although slight shifts in the wave positions were observed between the pure β-carotene standard compound and those from *in-situ* measurements in rice callus, these are considered normal and are thought to arise from the effect of interactions with other molecules within the plant tissue (Schulz *et al.,* 2005).

Regeneration of rice plants from coloured callus transformed with *ZmPsy*1 under the control of a constitutive promoter proved very difficult. In general, the ability to regenerate plants decreased with increasing colour intensity. With orange callus, for example, no normal shoots were obtained. Instead, stunted shoot-like structures with thick leaves were observed and the callus clumps turned brown and eventually died. Such phenotypic abnormalities, in particular dwarfism, have been observed in other crops such as tobacco and tomato when *Psy* was constitutively over-expressed (Fray *et al.,* 1995; Busch *et al.,* 2002). Further, in related studies at QUT, regeneration of banana embryos expressing *ZmPsy1 or APsy*2a under the control of a constitutive promoter was inhibited (Khanna personal communication). The difficulties reported with regeneration of transgenic plants expressing pVA genes has been attributed to interactions between different pathways in plants which share a common precursor, namely geranyl geranyl di-phosphate (GGPP). In addition to production of *Psy*, GGPP is a precursor for other compounds such as gibberellins, phytol, tocopherol, phylloquinone and plastoquinone which are essential for plant growth. Therefore, it is believed that over-expression of *Psy* re-directs GGPP from other pathways, thereby creating an imbalance that leads to abnormalities in plant regeneration and growth (Fray *et al.,* 1995; Shewmaker *et al.,* 1999; Busch *et al.,* 2002). In addition**,** enhanced production of β-carotene also leads to an increased production of zeaxanthin, a derivative of β-carotene (Refer to Fig 1.1). Zeaxanthin is the first committed step for the production of abscisic acid which is a known growth inhibitor that has been implicated in initiation and maintenance of seed dormancy (Finkelsteina and Rock

2002; Gulden *et al.,* 2004.). Enhanced levels of β-carotene therefore create a negative feedback where its by-products deter plant regeneration/growth.

The transgenic plants regenerated from callus constitutively expressing *APsy*2a appeared phenotypically similar to wild-type plants. Apart from a brown hue in some seeds, the seeds produced from transgenic plants, were of a similar colour to those of wild-type plants. In the scope and time-frame of this study, it was not possible to generate homozygous rice plants. In order to permit analyses for pVA carotenoids, callus from T1 seed was induced and analysed for β-carotene using HPLC. Callus generated from the transgenic T1 seed produced a range of colours from yellow to orange. When the carotenoid content (β-carotene) of the *APsy*2a-transgenic callus was quantified by HPLC and compared to that of non-transgenic control callus, the highest level of β-carotene content was obtained from orange callus with a corresponding decrease in the amount of β-carotene with decreasing intensity in colour. The correlation between colour and pVA carotenoid content has previously been reported in banana fruit (Englberger *et al.,* 2003c) and carotenogenic maize callus transformed with constitutively expressed *Psy* genes (Paine *et al.,* 2005). The HPLC analyses supported the results obtained by Raman spectroscopy in which the highest Raman peaks were mainly observed from the most intensely coloured T0 callus. The pVA carotenoid peaks detected in rice callus by HPLC were mainly β-carotene, α-carotene and lutein. Although α-carotene and lutein were not quantified, their identity was determined from their retention time in combination with their spectral characteristics as detected by a photodiode array detector.

The constitutive over-expression of *APsy*2a led to enhanced levels of β-carotene in T1 seed of more than 72 times that observed in wild-type callus which contained only a small amount of β-carotene (0.002 μg/mg dwt). The variation observed in seeds from transgenic plants which were originally derived from the same transformation event may be due to homozygosity or heterozygosity. In addition, the variation observed may be a due to difference in transgene copy number in each plant. However, this study focused on establishing gene function and no Southern hybridisation data was generated in these experiments. The presence of a small amount of pVA carotenoids in

non-transformed rice callus suggests that the endogenous pVA biosynthetic pathway in wild-type rice callus is functional, but that the native phytoene synthase and desaturase genes are probably not up-regulated. In addition, the increase in pVA carotenoids, as detected *in-situ* in T0 callus and as quantified in T1 callus constitutively expressing *APsy*2a alone shows that the native desaturase activity in rice callus is sufficient to process enhanced levels of phytoene. In contrast, on its own, constitutively expressed *CrtI* did not increase the amount of pVA coloured carotenoids in rice callus suggesting that the desaturase gene needs to be supplemented with *Psy.* Again, as previously shown in maize callus (Paine *et al.,* 2005) where a range of *Psy* genes were tested in the development of Golden Rice, the proportion of highly coloured callus and the total amount of carotenoids were used as indicators of the efficacy of the transgene. In subsequent research to further improve the carotenoid content of Golden Rice, the same two *Psy* genes derived from maize and rice which produced the most intense colour and high levels of carotenoid content in transformed maize callus, were found to provide the highest levels of pVA carotenoids in "Golden Rice 2" (Paine *et al.,* 2005). Therefore, the observation of dark orange coloured T0 and T1 callus generated using *APsy*2a in this study indicates that *APsy*2a is a functional gene, which could potentially be used to enhance the levels of pVA carotenoids in transgenic bananas.

In contrast to the results obtained using the constitutive poly-ubiquitin promoter, seed-specific expression of *APsy*2a or *ZmPsy*1 alone in rice seed did not result in an increase in pVA carotenoids. In fact, using Raman spectroscopy, no β-carotene was detected in transgenic rice seed derived from plants expressing *APsy*2a, *ZmPsy*1 or *CrtI* alone under the control of the rice glutelin1 promoter. These observations are consistent with previous studies which have shown that the expression of both *Psy* and *CrtI* genes together is required in order to achieve complete carotenoid biosynthesis in rice seed (Ye *et al.,* 2000; Paine *et al.,* 2005). Studies in rice have also shown that *Psy* is the limiting step for carotenoid biosynthesis. This was based on the detection of mRNA transcripts for all relevant carotenoid biosynthesis genes, except *Psy,* in the endosperm of wild-type rice seed (Schaub *et al.,* 2005). Further, it has been shown that enhancement of pVA carotenoids in rice seed is not due to up-regulation of the

endogenous carotenoid synthesis pathway in response to a transgene. Rather, the need to supplement *Psy* with a desaturase-encoding gene such as *CrtI* has been attributed to the insufficient activity of the endogenous desaturases in the rice endosperm. It is for this reason that enhanced pVA carotenoid levels in rice seed have only been achieved by combined expression of *Psy* and *CrtI*. In the current study, out of the transgenic plants generated from callus co-transformed with pCA-Gt1-*APsy2*a or pCA-Gt1-*ZmPsy*1 with pCA-Gt1-*CrtI,* none contained both transgenes. Therefore, it was not possible to evaluate the ability of *APsy*2a to enhance pVA carotenoids in transgenic rice seed. The generation of transgenic plants consisting co-integrated genes was probably limited by the use of single-gene constructs during the co-transformation experiments. Importantly, the successful expression of GUS in rice seed produced from plants transformed with pCA-Gt1-GUS demonstrated the functionality of the rice glutelin1 promoter and the expression vectors used in these transformation experiments.

Unlike rice seed, a banana fruit is known to produce pVA carotenoids indicating the presence of a functional pVA-biosynthesis pathway. Enhanced levels of pVA carotenoids in tissues with a functional carotenogenic pathway have been reported in canola (*Brassica napus)* where seed-specific expression of a bacterial phytoene synthase (*Crt*B) resulted in a 50-fold increase in pVA carotenoids in seed. It was suggested that phytoene synthase was possibly the rate-limiting enzyme in the carotogenesis pathway and that the transgenic expression of *Crt*B overcame this bottleneck resulting in increased carotenoid levels (Shewmaker *et al.,* 1999; Hirschberg 2001). In the current study, an increase in pVA carotenoid content was achieved in rice callus by expressing *Psy* alone. As such, it is likely that, in bananas, *Psy* alone may be able to up-regulate the synthesis of pVA carotenoids. In complementary research being undertaken for banana biofortification at QUT, it has been shown that the constitutive expression of *Psy* in banana cell suspensions results in the production of yellow-orange embryos. In addition, stably transformed banana plants expressing *Psy* in the glasshouse and field showed a characteristic “golden leaf” phenotype. These observations show that over-expression of *Psy* alone in banana up-regulates the production of coloured carotenoids *in-vitro* and in vegetative tissue. These

observations augur well for using *Psy* to enhance the carotenoid content of banana fruit but ultimately, this will need to be confirmed once the fruit has been obtained and analysed.

A comparison of the *APsy*2a nucleotide sequence used in this study and that of rice *Psy* (*AJ715785)* showed 75% similarity. In studies comparing the effects of *Psy* genes from different plants on carotenoid enhancement in rice, the highest carotenoid levels were achieved using maize *Psy*, the sequence of which showed the highest similarity to rice *Psy* (89%). It was suggested that optimal functionality of maize *Psy* in rice could be attributed, in part, to the close evolutionary relationship of rice and maize (Paine *et al.,* 2005). The result could also be due to the fact that the endosperm of maize and rice is similar and maize *Psy* is known to be involved in carotenoid accumulation in maize endosperm plastids. In recent studies at QUT, banana *Psy* genes have been isolated from Cavendish and F'ei banana cv Asupina (Mlalazi 2010). A comparison of the nucleotide sequences revealed similarities between *APsy*2a and the Cavendish *Psy* genes (C*Psy*1, C*Psy*2a, C*Psy*2b and C*Psy*2c) of 74%, 98%, 85% and 86%, respectively. In addition, Mlalazi (2010) reported that the expression of *APsy*2a was predominantly found in fruit. By analogy with the results obtained in rice, (Paine *et al.,* 2005) it is probable that banana-derived *APsy*2a will be functional in the fruit of banana varieties such as Cavendish, and that the transgenic expression of this gene could be used to enhance the normally low pVA carotenoid content.

In summary, results in this chapter demonstrated that *APsy*2a is a functional gene whose constitutive over-expression in rice callus resulted into a yellow-orange phenotype that is characteristic of coloured carotenoids. A combined analysis using Raman spectroscopy and HPLC methods confirmed enhanced levels of β-carotene as a result of *APsy*2a transgene expression.

Chapter 4 Characterisation of promoters for the biofortification of banana

4.1 Introduction

Cooking bananas such as the East African Highland bananas (EAHB) constitute the staple diet of millions of people in the East African region. However, EAHB cooking varieties have low levels of micronutrients particularly pro-vitamin A (pVA) carotenoids and iron. The transgenic biofortification of such bananas for increased levels of these, and other, micronutrients requires the use of fruit-preferred promoters to target transgene expression in the fruit. Unfortunately, despite the availability of reliable transformation protocols for banana using either particle bombardment or *Agrobacterium*-mediated approaches, there is limited information regarding banana fruit promoters. Although several different promoters have been used in banana transformation studies, the constitutive cauliflower mosaic virus (CaMV 35S) promoter has been the most commonly used (Sagi *et al.,* 1995; Becker *et al.,* 2000; Khanna *et al.,* 2004; Ghosh *et al.,* 2009).

In an effort to identify promoters for banana biofortification, QUT scientists have isolated a number of promoter sequences from banana, some of which have been shown to regulate genes associated with fruit development and ripening and which could have fruit-preferred expression. Further characterisation of the strength and tissue-specificity of these promoters was required to assess their suitability for use in banana biofortification. Promoter activity is commonly characterised by fusing a reporter gene to the candidate promoter and then analysing reporter gene expression either transiently in plant tissues or preferably in stably transformed plants. Transient assays, most commonly done following either particle bombardment (Dugdale *et al.,* 2001; Agius *et al.,* 2005) or *Agrobacterium*-mediated infiltration (Spolaore *et al.,* 2001; Wroblewski *et al.,* 2005; Orzaez *et al.,* 2006; Hasan *et al.,* 2008) of target tissue, provide quicker results than the analysis of stably transformed plants since the procedures do not involve the lengthy steps of transformation and the subsequent regeneration of transgenic plants. Despite transient assays providing a rapid, preliminary assessment of promoter activity, conclusive quantitative analyses in stably transformed plants are

ultimately required since the results from transient analyses do not always correlate with those obtained in transgenic plants (Holtorf *et al.,* 1995; Hamilton *et al.,* 2000).

The strategy that the QUT research team is using to generate bananas with enhanced pVA carotenoids involves the expression of a phytoene synthase (*Psy*) gene derived from either maize (Paine *et al.,* 2005) or high β-carotene banana (F'ei cv Asupina, (Mlalazi 2010) with or without the bacterial-derived carotene desaturase gene (Misawa *et al.,* 1990). The use of a fruit-active promoter to drive transgene expression is of paramount importance to the success of this strategy since similar studies in other plants has shown that constitutive expression of *Psy* leads to severe *in-vitro* regeneration difficulties and phenotypic abnormalities (Fray *et al.,* 1995; Busch *et al.,* 2002). Further, as reported in the previous chapter, the regeneration of plantlets from rice callus constitutively expressing *Psy* was difficult while in related studies at QUT, regeneration of banana embryos constitutively expressing phytoene synthase genes was inhibited (Khanna, personal communication). Clearly, the expression of *Psy* with or without *CrtI* as target genes for enhancement of pVA biosynthesis in banana fruit will require the use of a fruit-specific promoter to (i) enable the successful regeneration of phenotypically normal, transgenic plants and (ii) direct carotenoid biosynthesis and accumulation to the fruit.

The aim of the research in this chapter was to assess the activity of different banana-derived promoters in banana fruit using transient assays, and to analyse their activity/specificity in non-target tissue in stably transformed banana plants. The specific objectives of this study were to: (i) investigate the potential of *Agrobacterium*-mediated infiltration as a procedure for transient analysis of promoter activity in banana fruit, (ii) assess and compare the transient activity of different promoters in banana fruit using particle bombardment and *Agrobacterium*-mediated infiltration method, (iii) assess the enhancement of pVA carotenoid content in banana fruit following transient expression of the banana-derived phytoene synthase gene (*APsy*2a) and (iv) assess the activity of different promoters in the leaves of stably transformed banana plants growing in the glasshouse and the field.

4.2 Materials and Methods

4.2.1 Transformation vectors

Ten expression vectors in pGEM4Z or pBINPLUS (binary) plasmid, each containing a different promoter controlling the expression of the *uidA* reporter gene encoding β-glucuronidase (GUS) were used. The promoters tested included Expansin1 (MaExp1), Expansin1 containing the rice actin intron (MaExp1a), Expansin4 (MaExp4), Extensin (MaExt), 1-aminocyclopropane-1-carboxylic acid synthase (MaACS), 1-aminocyclopropane-1-carboxylate oxidase (MaACO) and Metallothionein (MaMT2a), all isolated from Cavendish banana, and the phytoene synthase (APsy2a) promoter isolated from F'ei type banana cv Asupina. In addition, the constitutive maize poly-ubiquitin promoter (ZmUbi) (Christensen *et al.,* 1992; Christensen and Quail 1996) and BT4 promoter derived from *Banana bunchy top virus* (BBTV) (Dugdale *et al.,* 2000) were included as positive controls. Three additional binary expression vectors in pBINPLUS were also used, each containing the maize poly-ubiquitin promoter (ZmUbi) controlling the expression of either the banana phytoene synthase (*APsy*2a), maize phytoene synthase (*ZmPsy*1) or carotene desaturase (*CrtI*) from *Erwinia uredovora*. All expression vectors used in this chapter (Table 4.1) were cloned and kindly provided by Dr. Jason Geijskes, Mr. Don Catchpoole and Dr. Ella Whitelaw either in pGEM4Z (for particle bombardment) or in the pBINPLUS binary vector hosted in *Agrobacterium tumefaciens* (strain AGL1) for *Agrobacterium*-mediated transformation (Fig 4.1).

4.2.2 Transformation of green banana fruit using particle bombardment or *Agrobacterium*-mediated infiltration

For particle bombardment, banana fruit slices were prepared and transformed as described (Chapter 2.6.1 and 2.6.2). Briefly, 1 µg of plasmid DNA was coated onto gold particles and was bombarded into the fruit tissue at a controlled helium pressure of 550 kPa and chamber vacuum of –84 kPa (25 mmHg). Transformed fruit slices were incubated in the dark at 25°C for 3 days before histochemical assay. The surface area of the transformed fruit slice was determined against a calibrated grid (in cm^2) (Fig 4.2) and GUS activity was expressed as the number of blue foci counted per unit area transformed.

Table 4.1 Expression vectors for particle bombardment and *Agrobacterium*-mediated transformation

Vector ID	Expression vector detail*	
	Binary expression vector in AGL1	**Expression vector for particle bombardment**
pZmUbi-G	pBIN-ZmUbi-GUS-nos[1]	pGEM4Z-ZmUbi-GUS-nos[1]
pBT4-G	pBIN-BT4-GUS-nos[1]	pGEM4Z-BT4-GUS-nos[1]
pMaExp1-G	pBIN-MaExp1-GUS-nos[1]	pGEM4Z-MaExp1-GUS-nos[1]
pMaExp1a-G	pBIN-MaExp1a-GUS-nos[1]	pGEM4Z-MaExp1a-GUS-nos[1]
pMaExp4-G	pBIN-MaExp4-GUS-nos[2]	pGEM4Z-MaExp4-GUS-nos[2]
pMaExt-G	pBIN-MaExt-GUS-nos[2]	pGEM4Z-MaExt-GUS-nos[2]
pMaACS-G	pBIN-MaACS-GUS-nos[1]	pGEM4Z-MaACS-GUS-nos[1]
pMaACO-G	pBIN-MaACO-GUS-nos[1]	pGEM4Z-MaACO-GUS-nos[1]
pMaMT2a-G	pBIN-MaMT2a-GUS-nos[2]	pGEM4Z-MaMT2a-GUS-nos[3]
pAPsy2a-G	pBIN-APsy2a-GUS-nos[1]	pGEM4Z-APsy2a-GUS-nos[1]
pZmUbi-*ZmPsy*1	pBIN-ZmUbi-*ZmPsy*1-nos[1]	
pZmUbi-*APsy*2a	pBIN-ZmUbi-*APsy*2a-nos[1]	
pZmUbi-*CrtI*	pBIN-ZmUbi-*CrtI*-nos[1]	

*vector backbone-promoter-coding sequence-terminator. All binary expression vectors contained the *nptII* selection marker gene under the control of the nos promoter and nos terminator.

Vectors marked with 1 were provided by Dr. Jason Geijskes, 2 by Mr. Don Catchpoole, 3 by Dr. Ella Whitelaw.

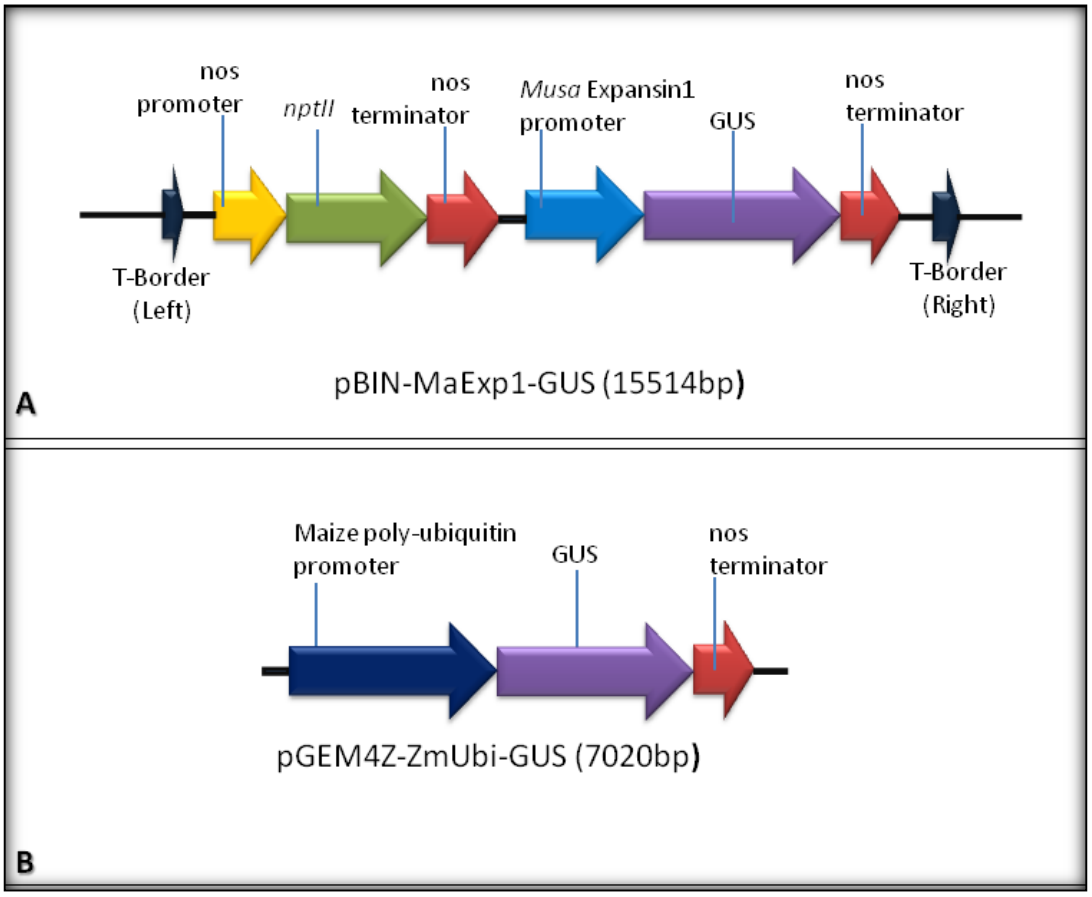


Figure 4.1 Schematic representation of expression vectors for transformation of banana fruit. Panel **A** shows a binary vector pBIN-MaExp1-GUS with the T-DNA between the left and right borders; Panel **B** shows the pGEM4Z-ZmUbi-GUS vector.

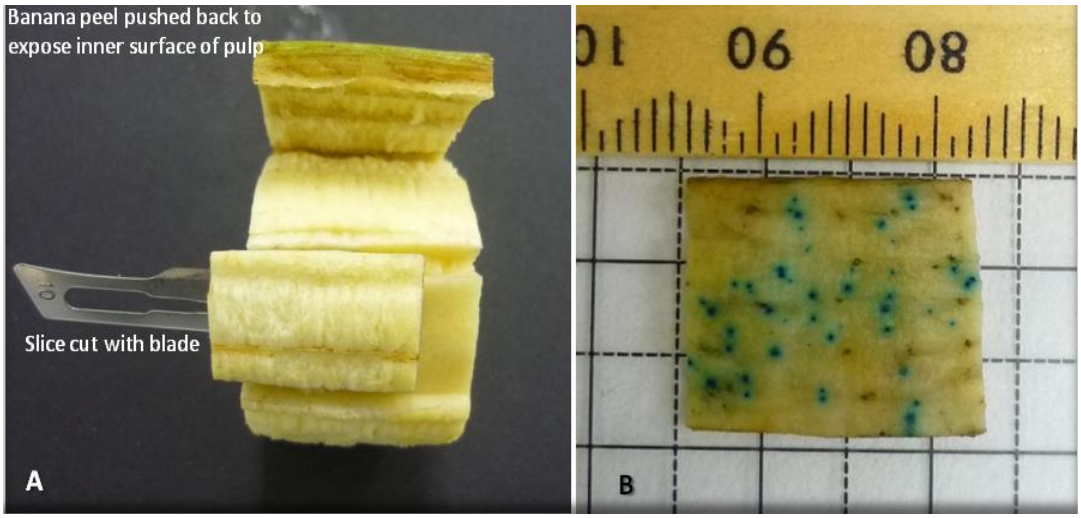


Figure 4.2 Preparation of banana fruit slices for particle bombardment.

Plate **A** Illustrates how the fruit slices were cut from the surface of the fruit pulp and the grid in **B** shows how the surface area of bombarded fruit slice was estimated.

Mature green banana fruit was transformed by *Agrobacterium*-mediated infiltration as described (Chapter 2.6.3) using a bacterial culture adjusted to OD_{600} = 0.8 and introduced into fruit using disposable surgical needles (size 21G; 0.8 mm X 38 mm). Transformed fruit was incubated in the dark at 23°C for 4 days post-infiltration (dpi) before GUS assay or further analysis for pVA carotenoid content. Transformed banana fruit were sequentially sliced into transverse sections before histochemical assay and stained fruit was photographed. The mean percentage pulp area stained was calculated from photographs of replicate transverse fruit sections using MatLab software (Kindly provided by QUT- High Performance Computing and Research Support).

4.2.3 GUS assay in banana fruit or leaf tissue – histochemical and fluorometric assay

All the transgenic plants from the glasshouse and the field were generated by Dr. H. Khanna and Ms. Fiona Banks while verification of transgene sequences was done by Dr. Jean-Yves Paul as part of the Bill and Melinda Gates-funded Banana biofortification project activities at QUT. Samples from the glasshouse-grown plants were obtained from the Queensland Crop Development Facility glasshouse at Cleveland, S.E Queensland, while field-grown plant samples were kindly collected and delivered to the laboratory by Dr. Doug Becker, Assoc. Prof. Rob Harding and D.Prof James Dale while visiting the QUT banana field trials located at the Department for Primary Industries (DPI), Centre for Wet Tropics Agriculture, South Johnstone, Innisfail, North Queensland. Mature green fruit (*Musa* spp. Cavendish) were obtained from Mr. Kurt Lindsay, a farmer from Rocksburg, S.E Queensland.

4.2.3.1 Histochemical assay for transient GUS expression in banana fruit or leaf

GUS expression in banana fruit or leaf tissue was assessed by histochemical assay using X-gluc at 37°C overnight as described (Chapter 2.8.2). After staining, chlorophyll in the banana leaf tissue was cleared by incubating the stained leaf sections in a solution of ethanol: acetic acid (3:1 v/v) overnight at room temperature. Stained banana tissue was stored in 70% ethanol.

4.2.3.2 Fluorometric assay for GUS expression in banana leaf tissue

Soluble protein was extracted from four freeze-dried banana leaf discs (approx. 2.8 mg dry weight) in 180 µL of GUS extraction buffer supplemented with 27.2 mg mL^{-1} (dry weight) Dowex 1X2-400 resin and 500 nM DTT as described (Chapter 2.8.1). Aliquots (10 µL each) were taken for protein assay or 4-methylumbelliferone (4-MU) assay. Protein concentration was determined from a 10 µL sub-sample using the BCA™ Protein Assay Kit (Thermo Scientific) containing BCA™ reagent A and B as well as a 2 mg mL^{-1} bovine serum albumin (BSA) following the manufacturer's instructions. After incubation for 30 min at 37°C, absorbance was determined at 562 nm on a plate reader (AD 200C, Beckman Coulter with ADLD analysis software version 1.6). The amount of protein measured was calculated in mg/10 µL of sample against a BSA standard curve.

In addition, fluorometric assays were carried out from a 10 µL sample in a 200 µL reaction volume containing 20 µL extraction buffer, 30 µL of pre-warmed 4-MU substrate (2 mM) and 200 µL of stop buffer in 96-well microtitre plates. Fluorescence was determined using a Luminescence spectrometer [PerkinElmer LS50B] at excitation wavelength of 365 nm and emission wavelength of 455 nm with a slit width of 5 nm. Enzymatic activity was calculated from fluorescence values against a standard curve of 4-MU. The final GUS activity was determined and expressed as ρmol of 4-MU/mg of protein produced/min.

4.2.4 Quantification of carotenoid content in banana fruit by HPLC

Six pre-weighed infiltrated fruit slices were placed into 50 mL Falcon tubes and freeze-dried. For analysis, the freeze-dried samples were ground into a fine powder using a mortar and pestle. A 500 mg sub-sample of the powder was weighed into a 15 mL Falcon tube from which the total carotenoids were extracted using 2 mL of acetone and separated using 2 mL of PE: DE (2:1; v/v) consisting of petroleum ether (40-60°C fraction) and diethyl ether as described (Chapter 2.7.1). Detection and quantification of carotenoids (β-carotene) was carried out by RP-HPLC as described (Chapter 2.7.1 and Appendix 3). Alpha tocopherol acetate was used as an internal standard in carotenoid extraction and quantification. The amount of β-carotene in test samples was estimated based on the area under the curve against a calibration curve equation for a pure β-

carotene compound (Sigma) (Chapter 2.7.1.4). The final amount of β-carotene quantified was expressed in µg/100 g of raw fresh weight.

4.3 Results

4.3.1 Assessing promoter activity in banana fruit by transient expression

The function of the Expansin1 (MaExp1), Expansin1 containing the rice actin intron (MaExp1a), Expansin4 (MaExp4), Extensin (MaExt), ACS (MaACS), ACO (MaACO), Metallothionein (MaMT2a) and phytoene synthase (APsy2a) promoters (Table 4.1) in intact banana fruit was analysed using two different transformation methods, particle bombardment and *Agrobacterium*-mediated infiltration. As controls, the constitutive maize poly-ubiquitin (ZmUbi) and BT4 promoters were also included.

4.3.1.1 Transient promoter activity in banana fruit transformed by particle bombardment

When fruit slices separately transformed with each of the ten different promoter constructs were histochemically assayed for transient GUS activity three days post-bombardment, blue foci were observed on the surface of all bombarded fruit slices (Fig 4.3). No blue foci were observed on any non-transformed control fruit slices. To enable a comparison of the relative strengths of each promoter, GUS activity was determined and expressed as the mean number of blue foci per unit area transformed. For many of the promoters tested, a considerable amount of variation was observed in the number of blue foci observed both within replicates from a single experiment (results not shown) and between the three independent transformation experiments (R1, R2 and R3; Fig. 4.4). For example, in the first transformation experiment (R1), the transient activity of the MaMT2a promoter was found to be significantly higher than all others tested including the constitutive ZmUbi control (Fig 4.4 R1). However, in repeat experiments two and three (R2, R3), the activity of MaMT2a decreased approximately 10-fold and 1.3-fold, respectively, and was significantly lower than the ZmUbi control and several of the other promoters tested (Fig 4.4 R2, R3). In general, however, the promoters which consistently showed the highest activities were ZmUbi, BT4, MaExp1,

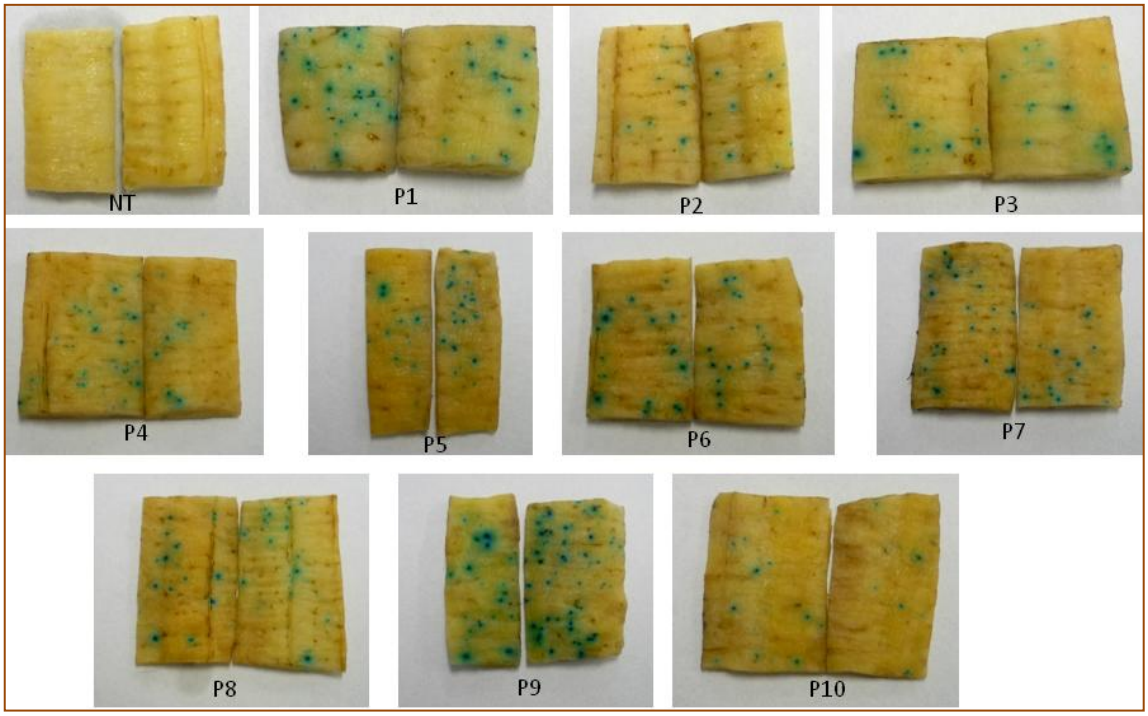
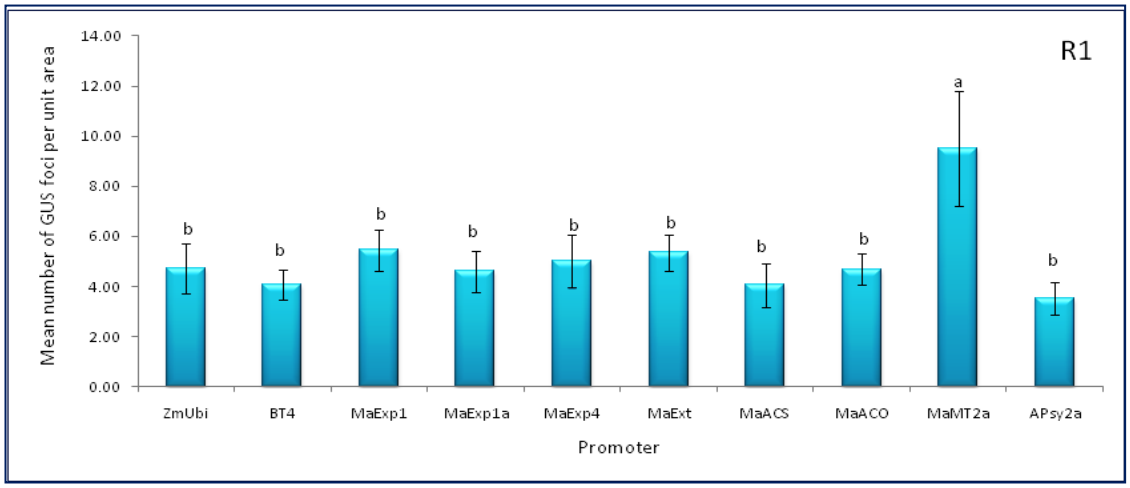
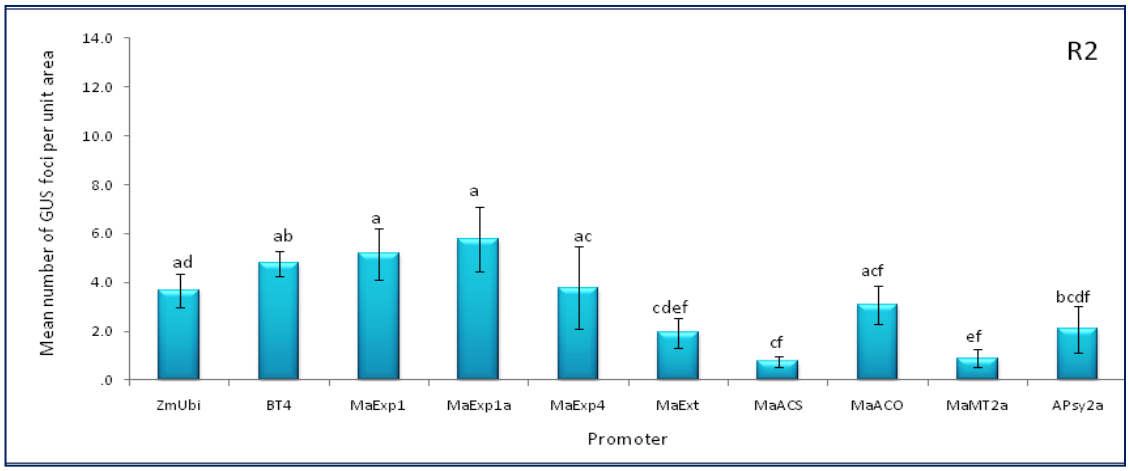


Figure 4.3 Histochemical assay for transient GUS expression in banana fruit slices transformed via particle bombardment. **NT** shows non-bombarded slices. The blue foci indicate the sites of GUS activity in fruit slices bombarded with GUS under the control of the **P1**. ZmUbi; **P2**. BT4; **P3**. MaExp1; **P4.** MaExp1a; **P5**. MaExp4; **P6**. MaExt; **P7.** MaACS; **P8**. MaACO; **P9.** MaMT2a; and **P10**. APsy2a promoters.





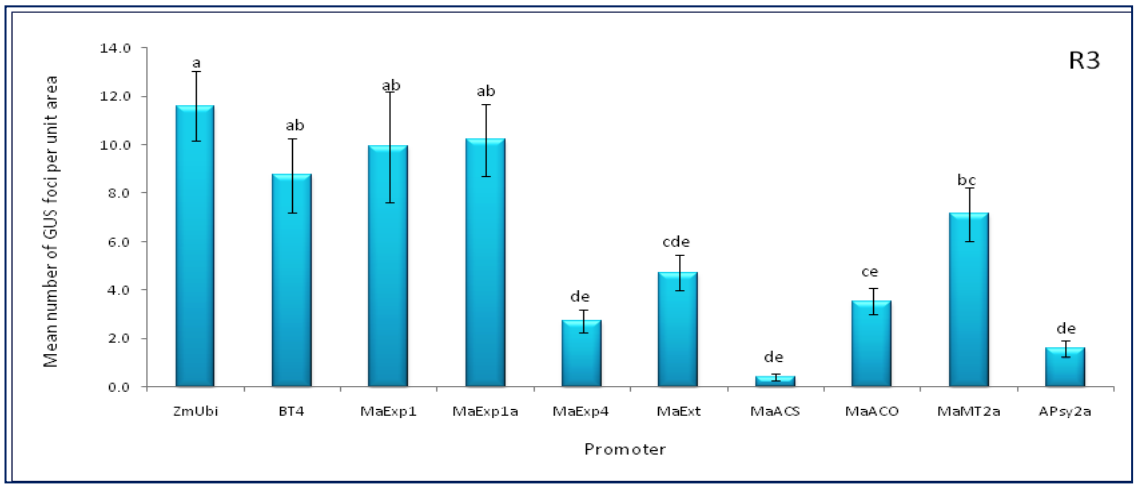


Figure 4.4 Promoter activities in banana fruit transformed by particle bombardment. Univariate ANOVA was used to determine differences between means. Bar graphs represent means ± standard error (n=14) for each of the ten promoters driving the GUS gene in transformation replicates R1, R2 and R3. Promoters marked with the same letter were not significantly different ($p \leq 0.05$).

MaExp1a and MaMT2a. Interestingly, the inclusion of the rice actin intron to MaExp1 did not result in any significant difference ($p \leq 0.05$) to its promoter activity.

When the results from all three experiments were pooled and analysed (Fig 4.5), the same general trends in promoter activity noted in the individual experiments was observed. Overall, the MaExp1 and MaExp1a promoters showed the highest activities which were very similar to, and not significantly different from, that of ZmUbi. The BT4 and MaMT2a promoters showed the next highest activities while the activity of the MaExp4, MaExt and MaACO promoters was about two thirds that of MaExp1 and MaExp1a. The MaACS and APsy2a promoters gave the lowest levels of transient GUS activity which were about one third that of MaExp1 and MaExp1a.

4.3.1.2 Assessment of *Agrobacterium*-mediated infiltration as a method to analyse transient promoter activity in banana fruit

Since an agro-infiltration procedure had not been previously reported for transformation of banana fruit, the utility of this approach for analysing transient promoter activity in banana fruit was first assessed using the constitutive ZmUbi promoter. Using a 1 mL size syringe and wide bore needle (0.8 mm X 38 mm), a 1 mL suspension of *Agrobacterium* (strain AGL1 at OD_{600} = 0.5) containing the pBINZmUbi-GUS vector was injected into fruit (Fig 4.6) obtained from a commercial outlet. This fruit is normally gassed with ethylene to initiate ripening prior to sale. When infiltrated fruit were sliced and analysed by histochemical assay at 3 days post injection (dpi), no GUS expression was observed. Therefore, an identical experiment was done to compare transient GUS activity in two gassed fruit and two un-gassed fruit following agro-infiltration with pBIN-ZmUbi-GUS. Whereas GUS expression was observed in the two un-gassed fruit, no GUS activity was again observed in gassed fruit (Fig 4.7). Furthermore, the distribution of *Agrobacterium* within the fruit pulp and subsequent GUS expression was observed in the fruit centre and often restricted to the fruit locules (Fig 4.8) indicating that the procedure was targeting *Agrobacterium* to the appropriate location within the fruit. Based on these results, freshly harvested and mature green banana fruit was used in all subsequent experiments.

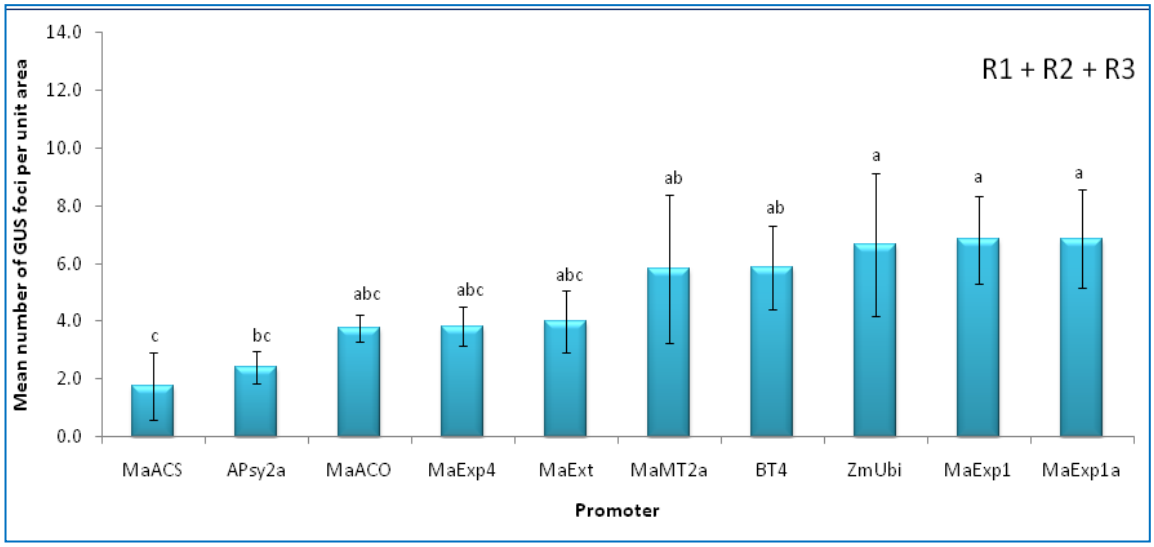


Figure 4.5 Summary of the transient promoter activity in banana fruit slices transformed by particle bombardment. Univariate ANOVA was used to determine the difference between means. Bars represent the overall mean number of GUS foci per unit area of fruit transformed per promoter in the three separate transformation experiments in Figure 4.4. Error bars represent standard error (n=3) and promoters whose bars are marked with the same letter were not significantly different ($p \leq 0.05$).

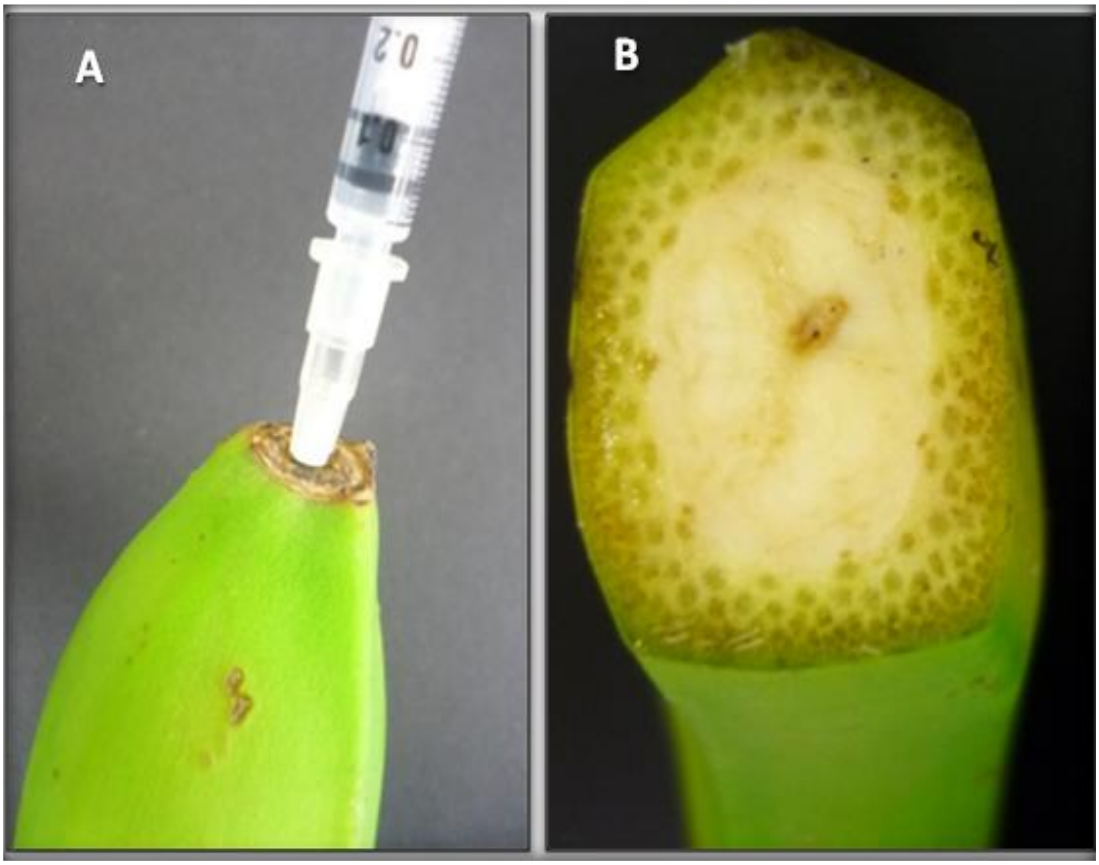


Figure 4.6 The method used for agro-infiltration of banana fruit. Injection was carried out through the distal end (**A);** with the needle targeted into the centre of the fruit pulp (**B**) to enable proper distribution of *Agrobacterium*.

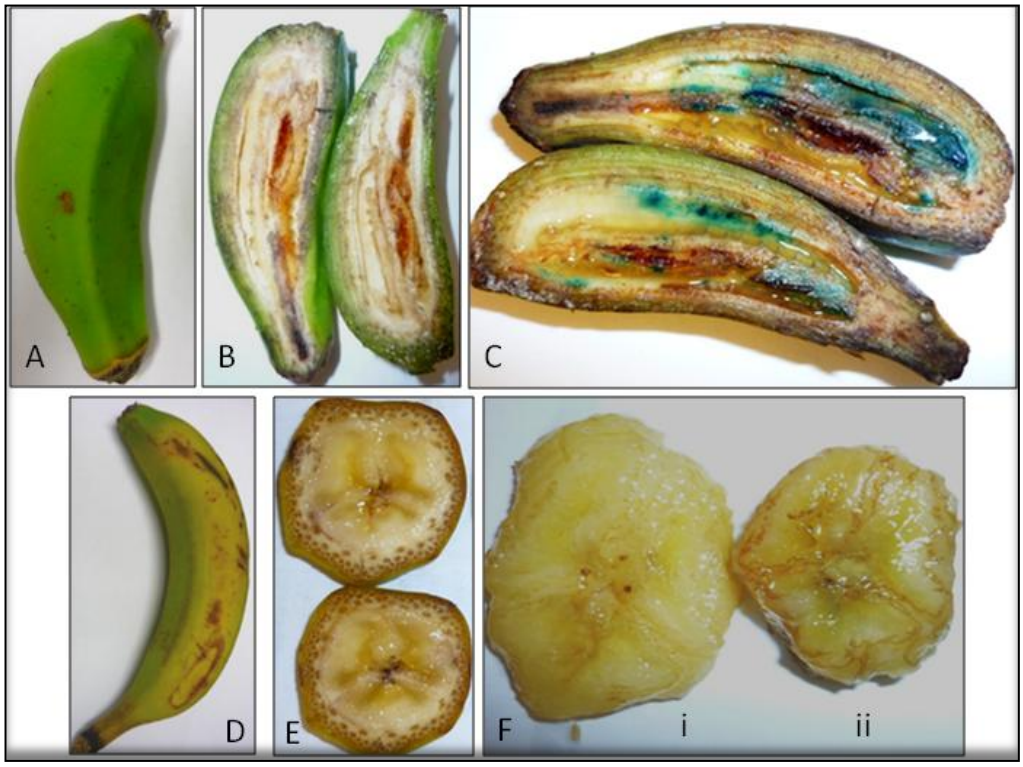


Figure 4.7 Transient GUS expression in gassed and un-gassed banana fruit. Preliminary expression of GUS under the control of the ZmUbi promoter in **A.** un-gassed green fruit; longitudinal section of un-gassed fruit before (**B)** and after (**C)** histochemical assay; **D**. gassed fruit; cross sections of gassed fruit before (**E)** and after (**F)** histochemical assay showing no GUS expression in **(i)** ripe and **(ii)** raw fruit.

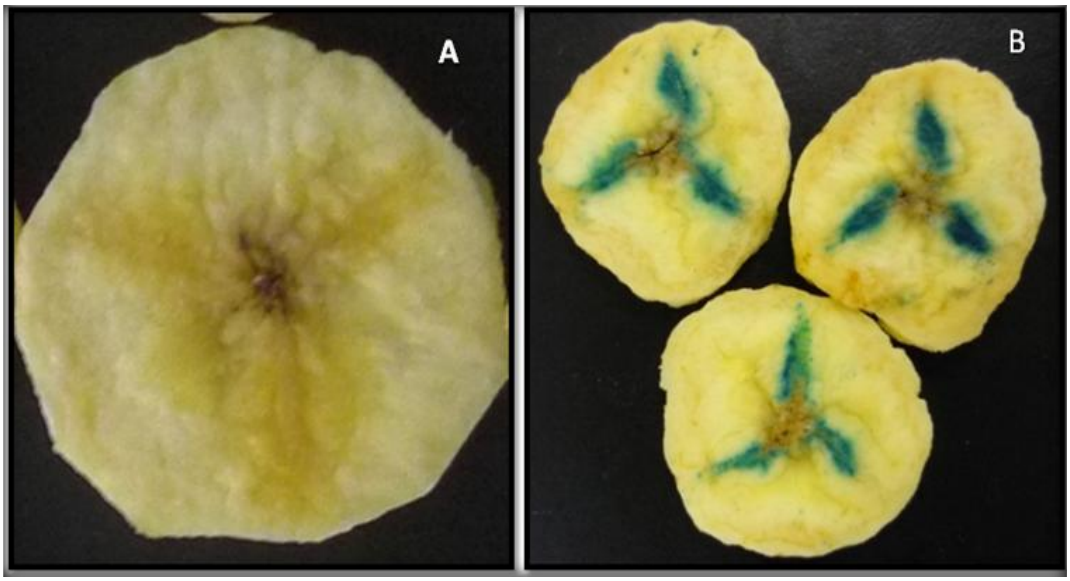


Figure 4.8 Illustration of the distribution of Agrobacterium in infiltrated banana fruit. Plate (A) shows the sites of *Agrobacterium* distribution while (B) shows GUS expression localised in the locules of the fruit pulp.

In an attempt to optimise the assay protocol, different parameters, including the volume (1 or 2 mL) and optical density (OD_{600} = 0.5 or 0.8) of *Agrobacterium* suspension used for infiltration and the incubation time post-infiltration before assaying (3 or 4 days), were also examined. In these studies, the highest transient GUS expression levels were consistently obtained in fruit infiltrated with 2 mL of bacterial suspension at an OD_{600} = 0.8 and incubated for 4 days prior to histochemical assay. The effect of *Agrobacterium* concentration on GUS expression from fruit infiltrated with a 2 mL volume and assayed at 4 dpi is shown in Fig. 4.9. Based on these results, all subsequent fruit infiltrations were done using 2 mL of *Agrobacterium* suspension at OD_{600} = 0.8 and incubated for 4 dpi before further analysis.

Finally, to examine whether GUS activity varied along the length of the fruit pulp, three infiltrated fruit were sliced into approximately 4 mm cross sections sequentially from the point of infiltration and stained by histochemical assay. The intensity of GUS expression was found to increase from slice number 3 and attained a maximum between fruit slices 4-8 before gradually decreasing along the length of the fruit (Fig 4.10 A and B). Therefore, fruit slices 3-8 were selected for use in all subsequent assays.

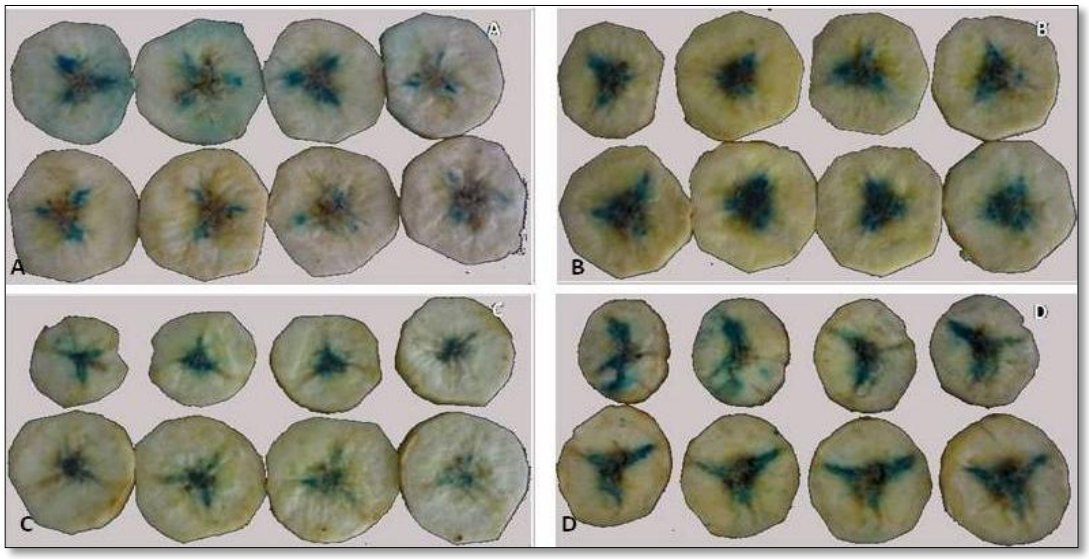
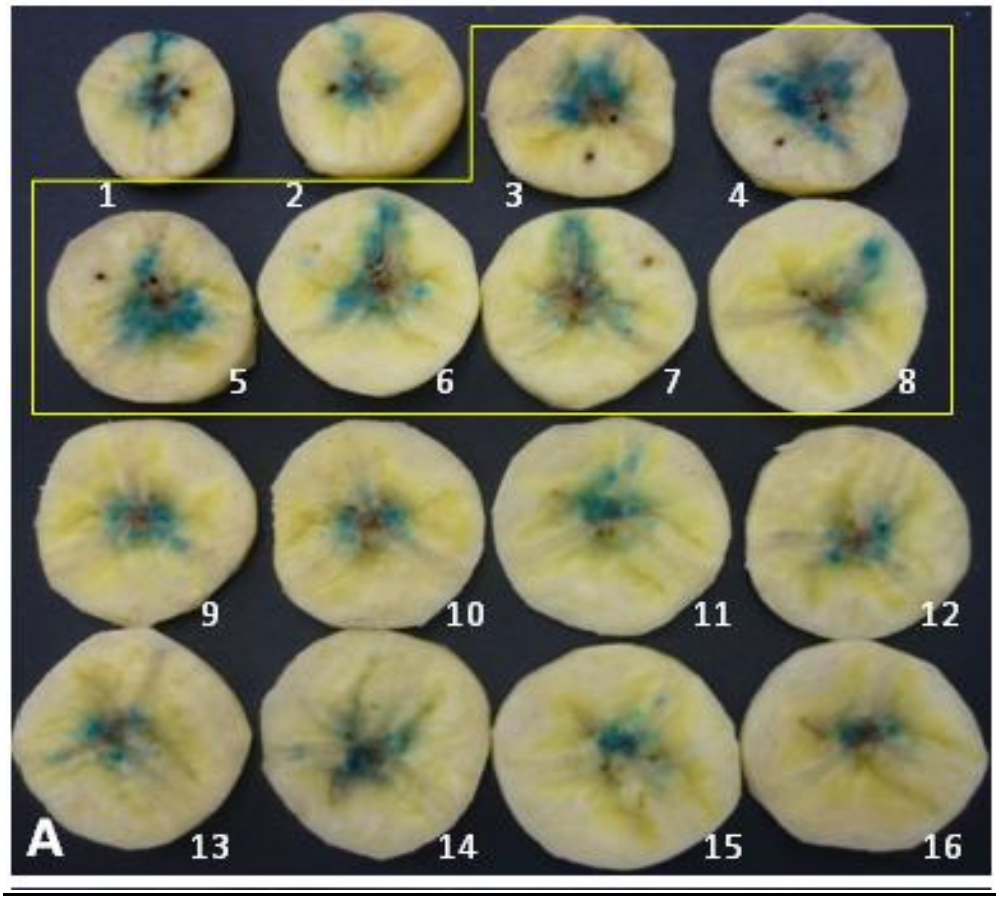


Figure 4.9 Effect of the concentration of Agrobacterium on the intensity of GUS expression in infiltrated banana fruit. Banana fruit were infiltrated with 2 mL of *Agrobacterium* suspension containing GUS under the control of **A.** ZmUbi at OD_{600} = 0.5; **B**. ZmUbi at OD_{600} = 0.8, **C.** MaExp1 at OD_{600} = 0.5 and **D**. MaExp1 at OD_{600} = 0.8. All fruit were stained 4 days after infiltration.



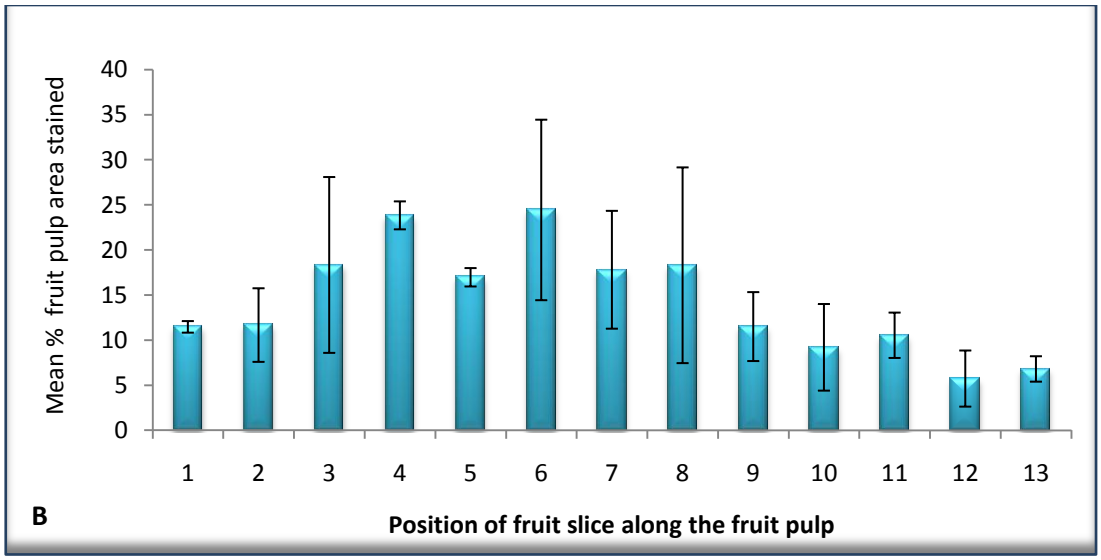


Figure 4.10 Representative histochemical assay showing positions of maximum GUS activity in agro-infiltrated banana fruit. **A.** The intensity of GUS signal at sites of histochemical staining along the pulp of banana fruit infiltrated with GUS under the control of ZmUbi, **B.** Bars show the mean pulp area stained, with maximum GUS activity between slice numbers 3 to 8. Error bars represent standard deviation for 3 fruit.

4.3.1.3 Assessing transient promoter activity in banana fruit via *Agrobacterium*-mediated infiltration

In order to assess the activity of the different promoters in fruit, the ten binary expression vectors in pBINPLUS (Table 4.1), each with a different promoter driving the GUS gene, were agro-infiltrated into intact banana fruit. In two separate experiments, three and two fruit were each agro-infiltrated with one of the ten binary expression vectors. As controls, two fruit were each infiltrated with *Agrobacterium* suspension or bacterial re-suspension media (BRM) alone while one fruit was left as an untransformed control. When the fruit were histochemically assayed at 4 dpi, no detectable GUS expression was observed in the non-transformed control or fruit infiltrated with either *Agrobacterium* only or bacterial re-suspension media (Fig 4.11). In contrast, various levels of GUS activity were observed from the different promoters tested as illustrated in Fig. 4.11 which shows three representative fruit slices taken from between positions 3 to 8 from one experiment**.** To provide more quantitative data for each promoter, the mean percentage pulp area stained from the region of maximum GUS activity (i.e. slices 3 to 8) was determined using MatLab 7.10 software. The constitutive ZmUbi and BT4 promoters, as well as MaExp1 and MaExp1a, directed the highest levels of GUS activity of all promoters tested with between 16 to 26% of transformed fruit pulp staining positive for GUS (Fig 4.12). The level of GUS activity observed from the MaACO and MaExt promoters was lower than MaExp1 and MaExp1a but not significantly different ($p \leq 0.05$) from the BT4 promoter. The MaExp4 and MaMT2a promoters directed minimal transient GUS activity which covered less than 5% of the fruit pulp. No detectable GUS expression was obtained from the MaACS and APsy2a promoters **(**Fig 4.12).

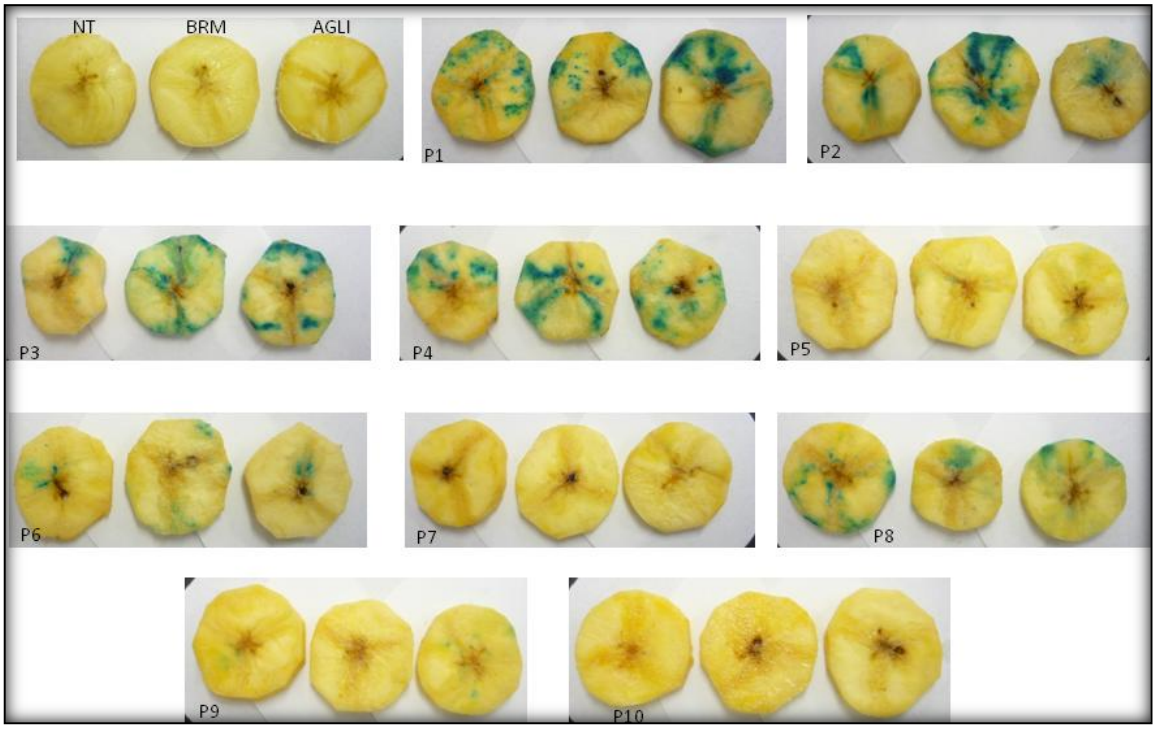


Figure 4.11 Histochemical assays for transient GUS expression from different promoters in banana fruit transformed by Agrobacterium-mediated infiltration. Representative fruit slices show non-transformed fruit (**NT)** and fruit infiltrated with bacterial re-suspension media (**BRM)** or *Agrobacterium* suspension only (**AGL1).** The blue colour indicates GUS activity at the sites of histochemical staining in fruit slices agro-infiltrated with GUS under the control of **P1.** ZmUbi, **P2.** BT4, **P3.** MaExp1, **P4.** MaExp1a, **P5.** MaExp4, **P6.** MaExt, **P7.** MaACS, P8**.** MaACO, **P9.** MaMT2a and **P10.** APsy2a promoters.

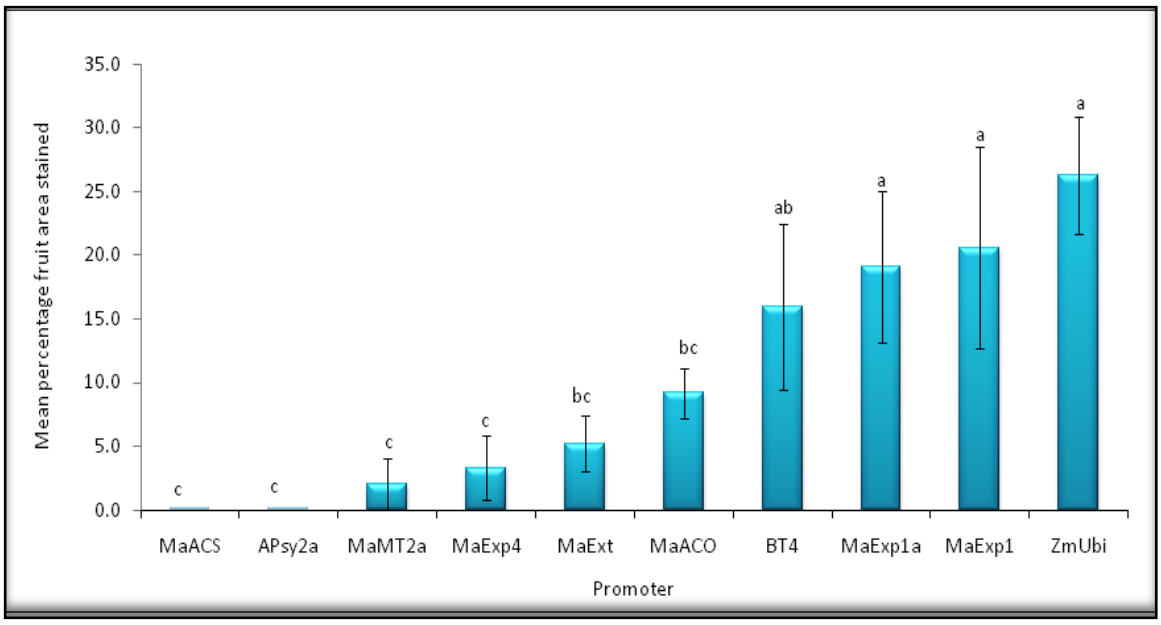


Figure 4.12 Transient promoter activity in banana fruit transformed by Agrobacterium-mediated infiltration. Univariate ANOVA was used to compare the mean percentage fruit pulp area stained for each promoter driving GUS expression in banana fruit in two experiments. Bars represent mean ± standard error (n=5). Promoters whose bars are marked with the same letter were not significantly different ($p \leq 0.05$).

4.3.2 Constitutive transient expression of phytoene synthase (*Psy)* in banana fruit

4.3.2.1 Transformation of banana fruit with transiently expressed pVA genes

Agrobacterium-mediated infiltration was used to assess transient expression of genes involved in pVA biosynthesis in mature green banana fruit. Prior to the commencement of these studies, validation of the HPLC method for identification and quantification of pVA carotenoids in Cavendish banana was done. For this purpose, a chromatogram for a pure commercial β-carotene standard compound was generated as a reference against which the pVA carotenoids in banana extracts were identified (Fig 4.13). Total carotenoids were then extracted from six wild-type non-transformed banana (Cavendish) fruit (obtained from one farm) and analysed by HPLC. In all extracts examined, chromatographic separation produced three distinct peaks (Fig 4.14) corresponding to the common carotenoids reported in banana fruit, namely lutein, α-carotene and β-carotene. The identity of lutein, α-carotene and β-carotene was determined based on their retention times (at 6.4, 12.5 and 13.4 min, respectively) and was further verified by analysing the shape of their spectrum (i.e. the fine spectral characteristics of their individual peaks) at wavelengths of maximum absorbance (λ_{max}) when separated on a C30 reverse phase (RP)-HPLC system using a methanol-tert-butyl methyl ether solvent system and a photodiode array detector. Whereas β-carotene showed two peaks at 451.2 and 477.9 nm with a shoulder, lutein and α-carotene each showed 3 peaks at 420.6, 444.1, 472.4 and 423.2, 445.8, 473.9, respectively. A detailed analysis of their spectral characteristics is shown in Fig 4.15**.** Based on the spectral analyses and area under the peak, the β-carotene content of fruit of Cavendish was estimated at 23 µg/100 g raw fresh weight. These experiments indicated that HPLC was a reliable method for the identification and quantification of β-carotene in banana fruit.

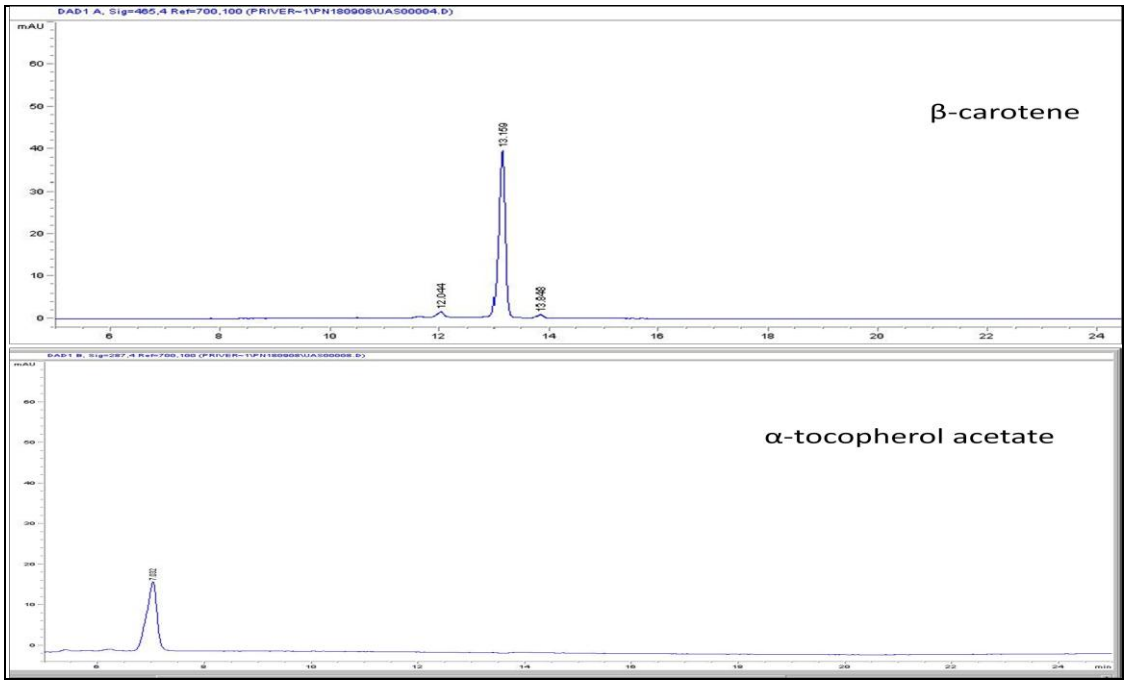


Figure 4.13 HPLC chromatogram for the pure standard compounds β-carotene and α-tocopherol acetate. The signal for β-carotene was detected at λ_{max} 465 nm after 13.1 min while α-tocopherol was detected at λ_{max} 287 nm after 7 min retention time.

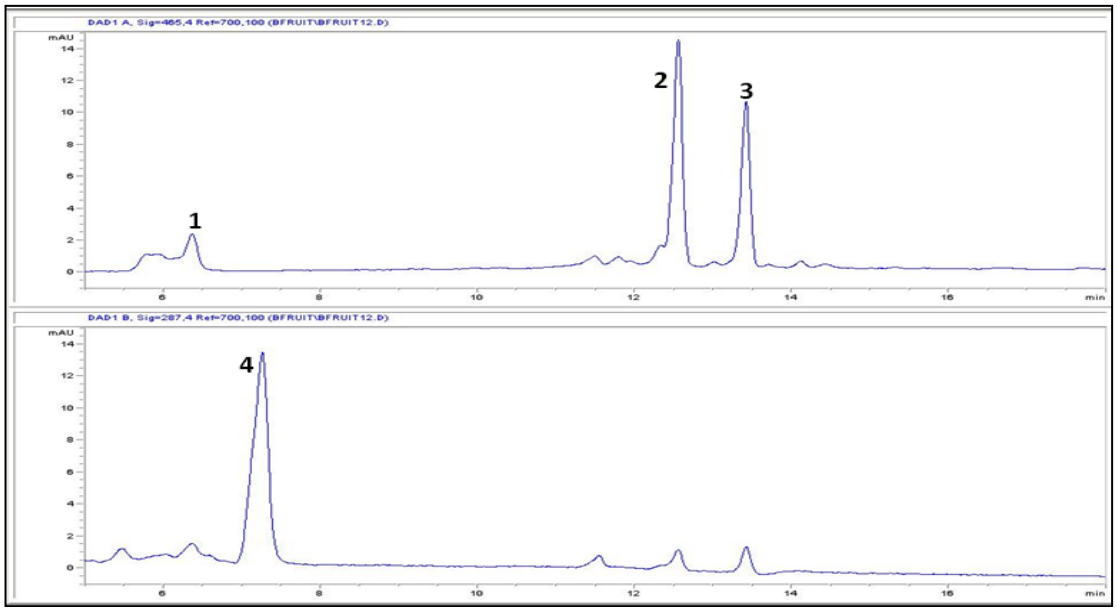
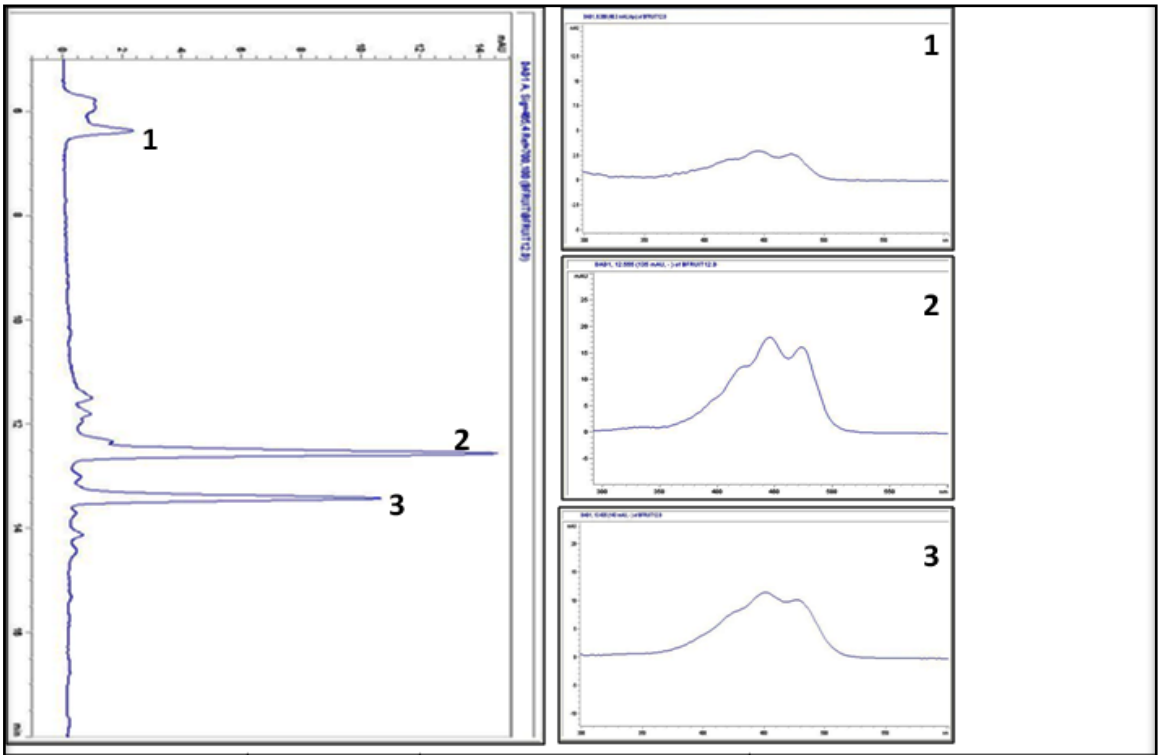


Figure 4.14 Representative HPLC chromatogram for banana fruit extract. Carotenoid peaks detected by photodiode array detector at λ_{max} 456 nm show **1**. lutein, **2**. α-carotene and **3.** β-carotene. Peak **4** represents α-tocopherol acetate (internal standard) detected at λ_{max} 278 nm.



Peak/Compound	Retention time (min)	Peak spectral λ (nm) observed	Reported spectral λ (nm) (Fraser *et al.*, 2000)
1. Lutein	6.4	420.6, 444.1, 472.4 nm	421.0, 443.9, 477.8 nm
2. α-carotene	12.5	423.2, 445.8, 473.98 nm	423.0, 446.3, 474.1 nm
3. β-carotene	13.4	-----, 451.2, 477.9 nm	----, 453.2, 478.0 nm

Figure 4.15 Spectral characteristics of β-carotene, α-carotene and lutein.

Each compound was separated using the reverse-phase HPLC system with a C30 column and a methanol-tert-butyl methyl ether-based mobile phase. Spectra shown were identified using a photodiode array detector.

Banana fruit was subsequently agro-infiltrated with binary expression vectors in which the constitutive maize poly-ubiquitin (ZmUbi) promoter controlled the expression of either the banana phytoene synthase (*APsy*2a), carotene desaturase *(CrtI*) gene from *Erwinia uredovora* or the maize phytoene synthase gene (*ZmPsy*1) as a control. The ZmUbi promoter was used to ensure the highest levels of pVA carotenoids were expressed. As additional controls, fruit were infiltrated with *Agrobacterium*, bacterial re-suspension media, or pBIN-ZmUbi-GUS vector while some fruit were left as untransformed controls.

In an initial experiment, two fruit were each agro-infiltrated with either pBIN-ZmUbi-*CrtI,* pBIN-ZmUbi-*ZmPsy*1 +/- pBIN-ZmUbi-*CrtI* or a pBIN-ZmUbi-GUS. The efficacy of the agro-infiltration was verified by the GUS expression (blue colour) observed when one of the two fruit infiltrated with pBIN-ZmUbi-GUS was analysed by histochemical assay (result not shown). When sections of fruit agro-infiltrated with constitutively expressed *CrtI* or *ZmPsy*1 alone or *ZmPsy*1/*CrtI* were visually observed at 4 dpi, a slight increase in the intensity of yellow pulp colour was observed compared to non-transformed fruit or those infiltrated with *Agrobacterium* or BRM (Fig 4.16A) suggesting a possible enhancement of coloured carotenoids. To quantify the pVA content, total carotenoids were extracted from freeze-dried slices (position 3-8) of one fruit per treatment and each extract was analysed by HPLC. Up to 20% more β-carotene was present in fruit infiltrated with constitutively expressed *CrtI, ZmPsy*1 or *ZmPsy*1/*CrtI* compared to the non-transformed controls at 60 µg/100 g raw fresh weight (Fig 4.16B).

Based on these promising results, additional experiments were carried out in which three fruit were each agro-infiltrated with the *CrtI, ZmPsy*1 +/- *CrtI* or *APsy*2a +/- *CrtI* each under the control of Ubiquitin promoter. Despite repeating the experiment at least six times, inconsistencies and considerable variation in the colour of infiltrated fruit, both within and between experiments, was observed. For example, whereas an increase in the intensity of yellow colour was observed in fruit infiltrated with pBIN-ZmUbi-*CrtI* in the preliminary studies, no distinct change in pulp colour was observed in any fruit infiltrated with the *CrtI* vector when the experiment was repeated. Instead, a characteristic brown colouration, similar to that observed in fruit infiltrated with

Agrobacterium only, was mostly observed. Although slight changes in pulp colour were observed in banana fruit agro-infiltrated with *APsy*2a +/- *CrtI* or *ZmPsy*1 +/- *CrtI,* each under the control of ZmUbi promoter, the change was not consistent in repeated individual treatments, neither between fruit in one experiment nor between experiments. Fig 4.17 shows the results of one experiment and illustrates the colouration in fruit slices (#5 and 6) taken from 1 out of 3 fruit agro-infiltrated with *CrtI, APsy*2a +/- *CrtI* or *ZmPsy*1 +/- *CrtI* vectors compared to non-transformed control fruit. A slight change in the yellow pulp-colour was observed in fruit agro-infiltrated with *APsy*2a or *ZmPsy*1 while a deeper yellow colouration was observed in fruit infiltrated with *APsy*2a/*CrtI* but not *ZmPsy*1/*CrtI* vectors.

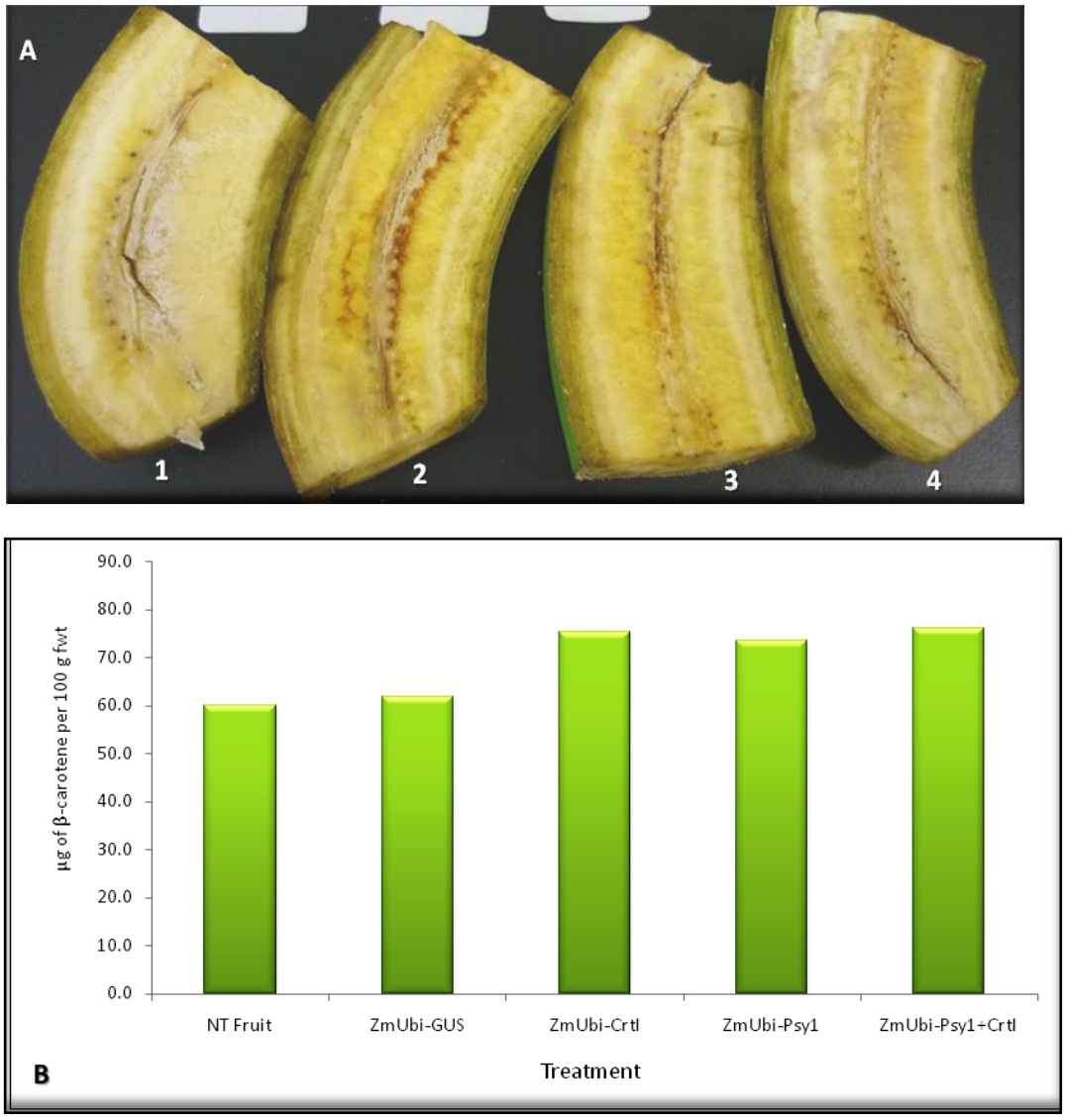


Figure 4.16 Preliminary quantification of β-carotene content in banana fruit agro-infiltrated with genes for pVA biosynthesis. Panel **A** shows longitudinal sections at 4 dpi for fruit infiltrated with **1.** BRM transformation media (NT control), **2**. pBIN-ZmUbi-*CrtI*, **3**. pBIN-ZmUbi-*Psy*1, and **4**. pBIN-ZmUbi-*Psy*1/ZmUbi-*CrtI.* Bars in graph **B**, show the amount of β-carotene in µg per 100 g raw fresh weight quantified from single HPLC injections/carotenoid extract/fruit.

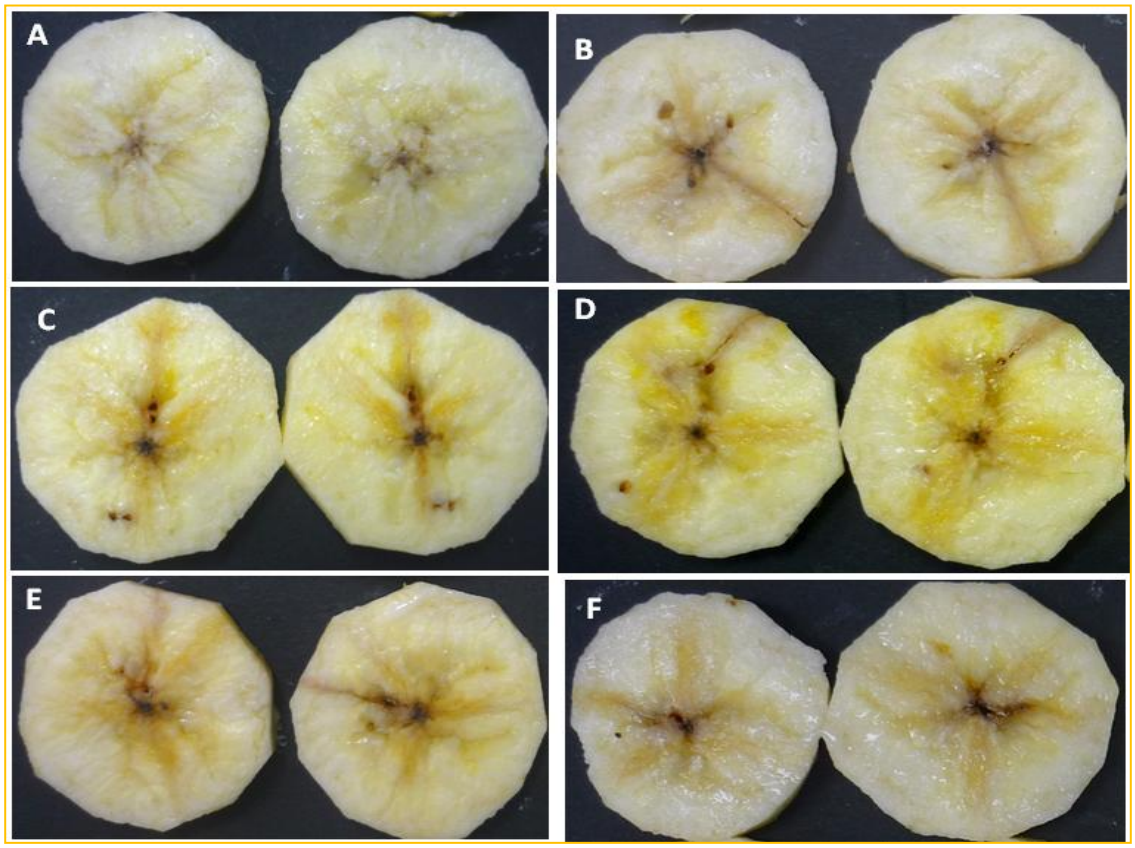


Figure 4.17 Distinct yellow colour in transverse sections of banana fruit agro-infiltrated with constitutively expressed pVA genes. Fruit slices #5 and 6 taken from the region of maximum expression are shown for **A**. non-transformed fruit and fruit infiltrated with **B.** *CrtI*, **C.** *APsy*2a, **D.** *APsy*2a/*CrtI*, **E**. *ZmPsy*1 and **F**. *ZmPsy*1/*CrtI* each under the control of the ZmUbi promoter.

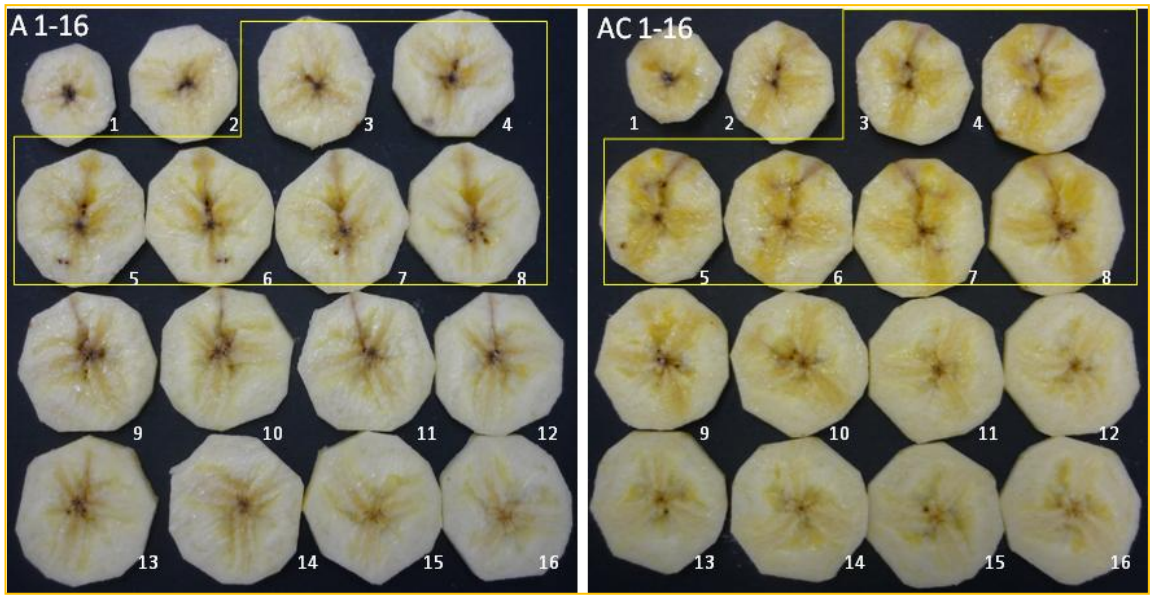


Figure 4.18 Transverse sections of banana fruit agro-infiltrated with APsy2a +/- CrtI under the control of ZmUbi promoter. The 16 fruit sections of fruit infiltrated with *APsy*2a (**A 1-16**); and *APsy*2a/*CrtI* (**AC 1-16)** are shown. Marked fruit slices (3-8) were sampled for carotenoid extraction.

4.3.2.2 Quantification of β-carotene in agro-infiltrated banana fruit

To analyse carotenoid content in the agro-infiltrated fruit, total carotenoids were extracted from representative fruit sections (#3 to 8, Fig 4.18) taken from each of three fruit infiltrated with GUS, *CrtI, APsy*2a +/- *CrtI* or *ZmPsy*1 +/- *CrtI* each under the control of ZmUbi promoter. Extracts were also prepared from three non-transformed fruit. Despite repeating the experiment at least six times, a considerable amount of within- and between-treatment variation was observed in the amount of β-carotene quantified (Fig 4.19). For example, in the results of one experiment shown in Fig. 4.18, the amount of β-carotene found in the three fruit independently agro-infiltrated with pBIN-ZmUbi-*ZmPsy1* alone ranged from less than 10 to more than 60 μg per 100 g fresh weight. There was also considerable variation in the β-carotene levels between treatments. Similar variation was observed between the replicate experiments (data not shown). In one experiment, for example, the amount of β-carotene estimated from some of the fruit transformed with pBIN-ZmUbi-*APsy2a*/*CrtI* or pBIN-ZmUbi-*ZmPsy*1/*CrtI* was lower than that obtained in the non-transformed controls (Fig 4.19) whereas in other treatments it was higher. In addition, no consistent correlation was observed between the colour of the infiltrated fruit and the amount of β-carotene quantified. For example, the β-carotene content of fruit #3 agro-infiltrated with pBIN-ZmUbi-*APsy*2a/*CrtI*, whose pulp showed a deep yellow pigmentation (Fig 4.17D), was found to be lower than that of the fruit agro-infiltrated with pBIN-ZmUbi-*APsy2a,* pBIN-ZmUbi-*ZmPsy*1 or pBIN-ZmUbi-GUS alone which exhibited a less intense yellow colouration. As a result, the change in colour of the fruit pulp and the β-carotene content could not be attributed to a specific set of treatments and therefore it was not possible to ascertain a defined transient expression pattern for pVA genes in green banana fruit.

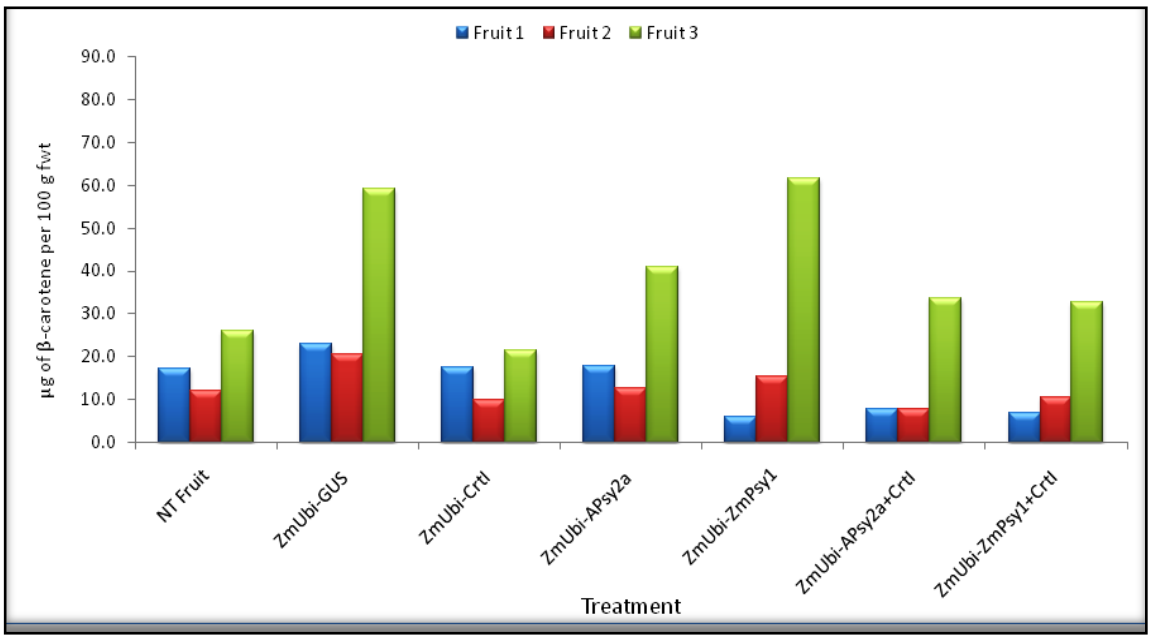


Figure 4.19 Estimation of β-carotene content from banana fruit agro-infiltrated with genes for pVA biosynthesis. Bars show the amount of β-carotene in µg/100 g raw fresh weight estimated from single injections/carotenoid extract/fruit.

4.3.3 Assessing promoter activity in leaves of stably transformed banana plants

4.3.3.1 Glasshouse-grown banana plants

A total of 33 three month old transgenic glasshouse-grown banana plants (cv Cavendish and Lady Finger), stably transformed with the *uidA* (GUS) gene under the control of either the constitutive ZmUbi or BT4 promoters or the fruit-active MaExp1, MaACO and APsy2a promoters (Table 4.1) were used for promoter analysis along with two somatic embryo-derived wild-type banana plants (cv Cavendish) as controls. Leaf discs were taken from the youngest, fully expanded leaf of each of the independently transformed plants (Fig 4.20) and were analysed for GUS expression using histochemical and fluorometric assays.

For histochemical assays, six leaf discs from each plant were examined and the intensity of blue staining was rated as weak (+), medium (++) or strong (+++). No blue signal was observed in any of the leaf discs taken from the wild-type control plants. In contrast, all of the nine, two and two plants stably transformed with the GUS gene under the control of the ZmUbi, BT4 and APsy2a promoter, respectively, gave a medium or strong GUS signal (Table 4.2, Fig 4.21 and Fig 4.22). A weak GUS signal was observed from one leaf out of the 10 MaExp1-GUS plants while of the 10 MaACO-GUS plants examined, two gave a weak GUS signal while two gave a medium GUS signal (Table 4.2, Fig 4.23).

To quantify GUS activity in the leaves of the glasshouse-grown transgenic plants, eight leaf discs taken from the youngest, fully expanded leaf of each plant were freeze-dried and pooled into 2 x 4 disc samples. Protein was subsequently extracted from the replicate samples on two separate days and the GUS activity was quantified by fluorometric assay (Table 4.2). An average of approximately 21 ρmol 4-MU/mg protein/min was detected in the wild-type negative controls (cv Cavendish). Consistent with the results of the histochemical assays, the highest levels of GUS activity were detected in plants stably transformed with GUS under the control of constitutive ZmUbi promoter.

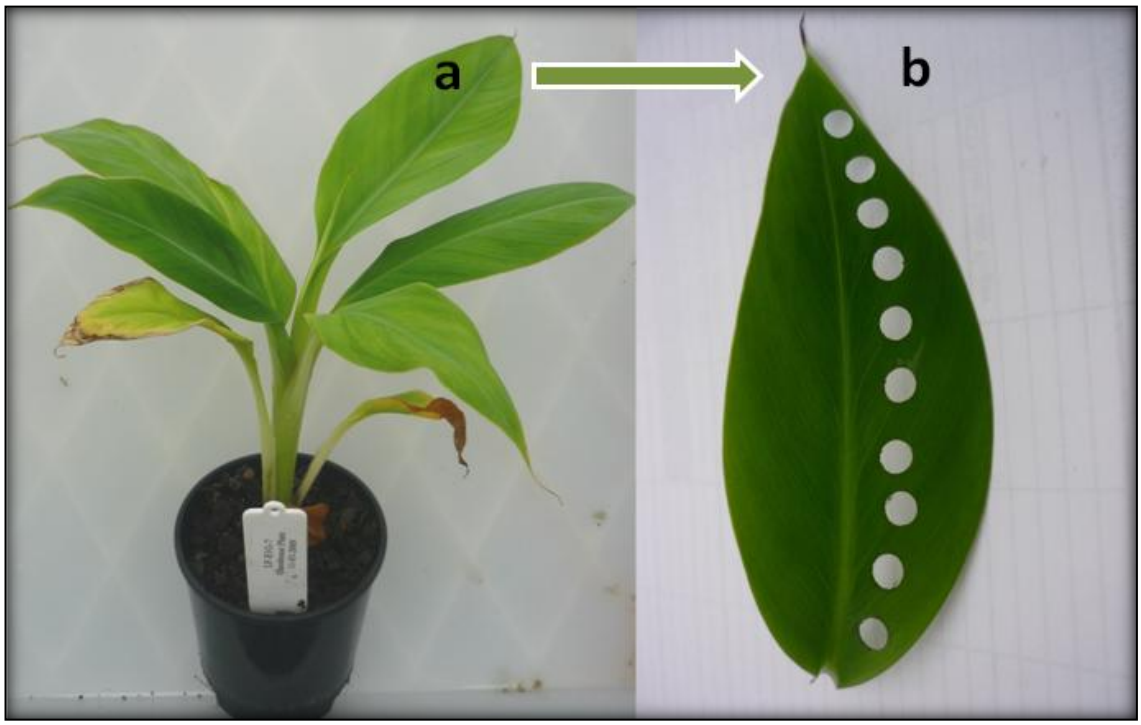


Figure 4.20 Sampling of leaf discs from a glasshouse-grown transgenic banana plant. The youngest fully expanded leaf **(a**) and the positions of the leaf blade sampled (**b)** for GUS assays are shown.

Table 4.2 Comparing histochemical and fluorometric assay in leaves of stably transformed glasshouse-grown banana plants

Plant ID*	Sample number	4-MU Rep 1**	4-MU Rep 2**	Mean GUS activity (pmol 4-MU/mg protein/min)	STDev	Histochemical assay***
WT 1	NT 1	25.8	15.2	20.5	7.5	-
WT 2	NT2	27.1	15.6	21.4	8.1	-
LF E1G 5	7	42.5	47.5	45.0	3.5	-
LF E1G 2	4	45.2	48.4	46.8	2.2	-
LF E1G 27	13	52.8	42.0	47.4	7.6	-
LF E1G 16	11	58.7	59.2	59.0	0.4	-
LF E1G 17	12	62.7	55.5	59.1	5.1	-
LF E1G 4	6	61.7	59.5	60.6	1.5	+
LF E1G 7	8	49.8	74.9	62.4	17.7	-
LF E1G 14	10	97.7	35.4	66.5	44.1	-
LF E1G 11	9	67.3	80.5	73.9	9.3	-
LF E1G 3	5	75.2	80.3	77.8	3.6	-
W ACOG 3	33	14.4	48.4	31.4	24.0	-
LF ACOG 3	29	11.4	61.3	36.4	35.2	-
LF ACOG 10	31	17.2	60.4	38.8	30.6	++
W ACOG 13	36	28.5	53.7	41.1	17.8	+
W ACOG 5	34	38.4	55.1	46.8	11.8	+
W ACOG 8	35	42.5	51.5	47.0	6.4	-
LF ACOG 1	28	14.5	89.0	51.8	52.6	-
LF ACOG 8	30	31.3	74.8	53.0	30.8	++
LF ACOG 13	32	43.5	65.5	54.5	15.6	-
W ACOG 15	37	38.6	73.2	55.9	24.5	-
W APG 1	38	30.1	337.1	183.6	217.1	+++
W APG 2	39	58.7	490.0	274.3	305.0	+++
LF BT4G 2	17	131.9	154.2	143.0	15.7	+++
LF BT4G 14	18	221.3	173.7	197.5	33.7	+++
LF UbiG 13	21	58.2	93.3	75.7	24.8	++
LF UbiG16	23	64.2	97.2	80.7	23.4	+++
LF UbiG 3	19	1896.9	600.1	1248.5	917.0	+++
LF UbiG 15	22	2318.2	938.0	1628.1	975.9	+++
LF UbiG 12	20	1403.3	2431.5	1917.4	727.1	+++
LF UbiG 33	24	3532.2	1838.7	2685.4	1197.5	+++
W UbiG 1	25	10975.0	2977.9	6976.5	5654.8	+++
W UbiG 3	26	20376.5	22179.5	21278.0	1274.9	+++
W UbiG 5	27	28654.7	30686.8	29670.7	1436.9	+++

*ID = identity of the sample plant representing (cultivar-promoter/gene-plant number); WT = wild-type; W = Cavendish; LF = Lady Finger; E1 = MaExp1; ACO = MaACO; AP = APsy2a; Ubi = ZmUbi; BT = Bunchy top; G = GUS; the number (in front of G) represents the glasshouse identification number for each plant.

** Rep = Replicate analyses 1 and 2 for GUS activity (pmol 4-MU/mg protein/min)

*** + = weak positive; ++ = medium positive; +++ = strong positive; - = no detectable blue colour

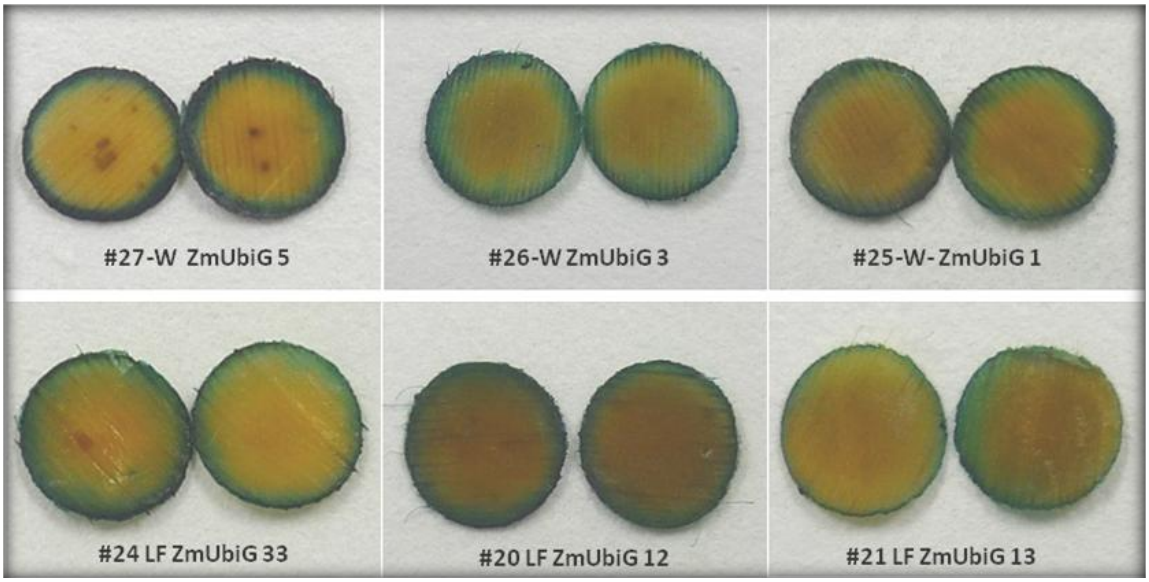


Figure 4.21 Histochemical assay for GUS expression in leaves of glasshouse-grown banana plants stably transformed with the GUS gene under the control of the ZmUbi promoter.

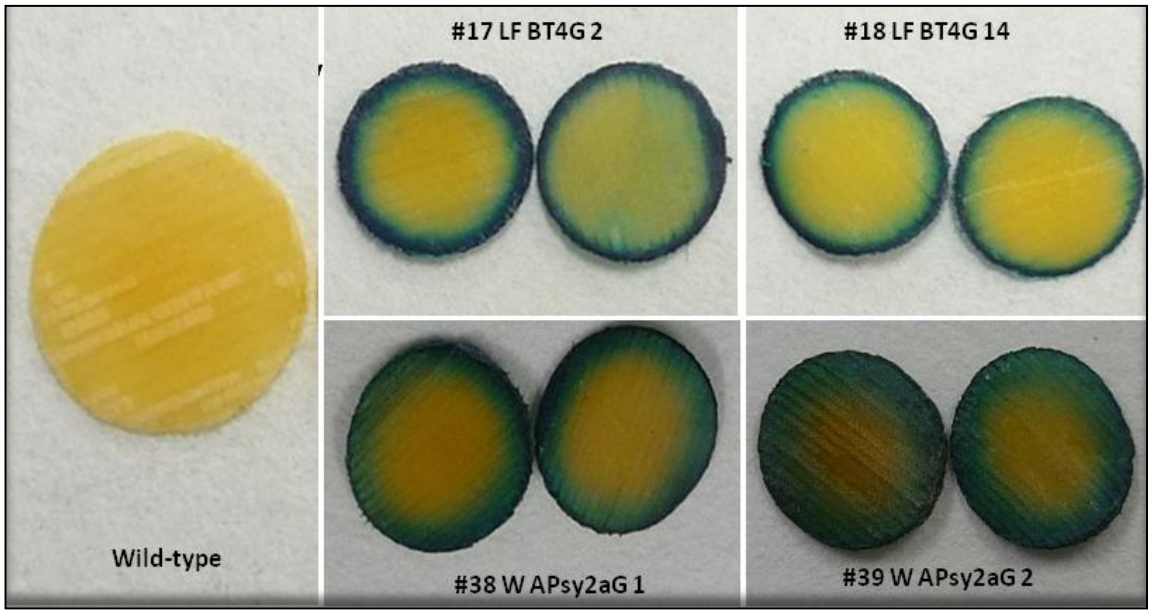


Figure 4.22 Histochemical assay for GUS expression in leaves of glasshouse-grown banana plants stably transformed with the GUS gene under the control of the BT4 and APsy2a promoters.

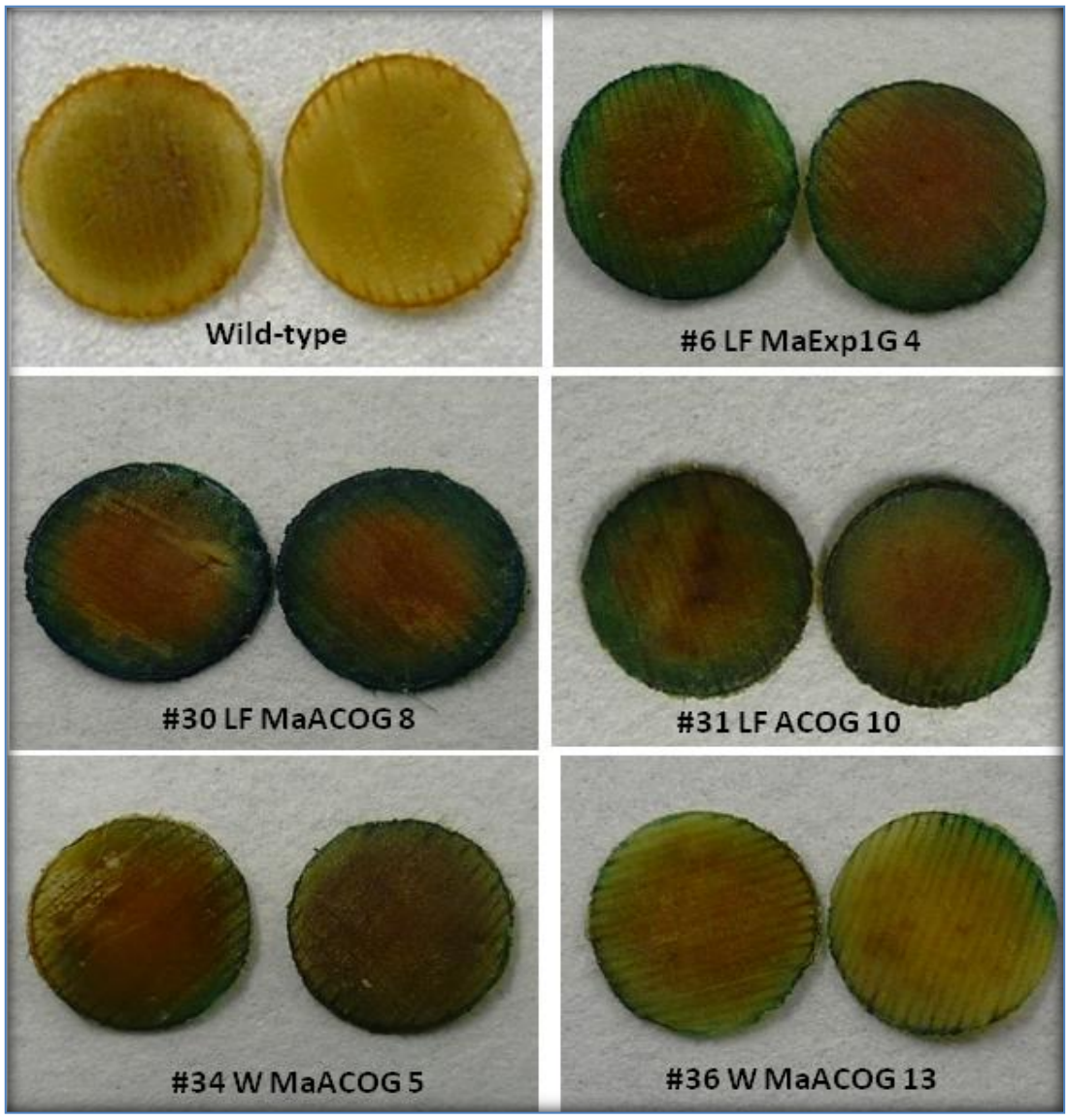


Figure 4.23 Histochemical assay for GUS expression in leaves of glasshouse-grown banana plants stably transformed with the GUS gene under the control of the MaExp1 and MaACO promoters.

Interestingly, there appeared to be a cultivar-dependent effect, with the highest mean GUS activity in leaves from the ZmUbi-transgenic Cavendish cultivar (29,670.7 pmol 4-MU/mg protein/min) more than 10-fold higher than that found in leaves from a transgenic Lady Finger cultivar (2,685.4 pmol 4-MU/mg protein/min). The GUS activities in leaves from both BT4-GUS transgenic plants (cv Lady Finger) (143.0 and 197.5 pmol 4-MU/mg protein/min) was considerably lower than the GUS activities in leaves taken from the ZmUbi-transgenic plants.

Although the mean GUS activities in leaves from the two APsy2a-GUS plants (cv Cavendish) were similar to those obtained from the BT4-GUS transgenic Lady Finger plants, considerable variation, up to 11-fold, was observed in the replicate assays (Table 4.2). Compared to the constitutive ZmUbi and BT4 promoters, the MaACO and MaExp1 promoters directed low levels of GUS activity, up to ~650 fold less, with the activity of MaExp1 slightly higher at 45.0-77.8 pmol 4-MU/mg protein/min than the MaACO promoter at 31.4-55.9 pmol 4-MU/mg protein/min. Again, variable GUS activity was obtained in some replicate assays with at least two MaExp1-GUS plants, E1G 7 and E1G 14, giving very high standard deviations (Table 4.2). Interestingly, there was no direct correlation between the results of the histochemical and fluorometric assays in plants transformed with MaACO-GUS or MaExp1-GUS. For example, whereas leaf discs from MaACO-GUS transgenic plant #10, with a mean GUS activity of 38.8 pmol 4-MU/mg protein/min, gave a medium intensity histochemical staining reaction, six out of seven MaACO-GUS plants with higher mean GUS activities, such as plant WACOG #13 and 15, gave either a weak positive or a negative result in histochemical assay (Table 4.2).

The overall comparison of GUS activity controlled by the different promoters analysed by fluorometric assay is presented in Fig 4.24. In summary, these results showed that the constitutive ZmUbi and BT4 promoters direct high levels of GUS activity in leaves of stably transformed banana plants compared to the fruit-active promoters analysed. In addition, histochemical assay alone appeared to be an unreliable indicator of promoter activity.

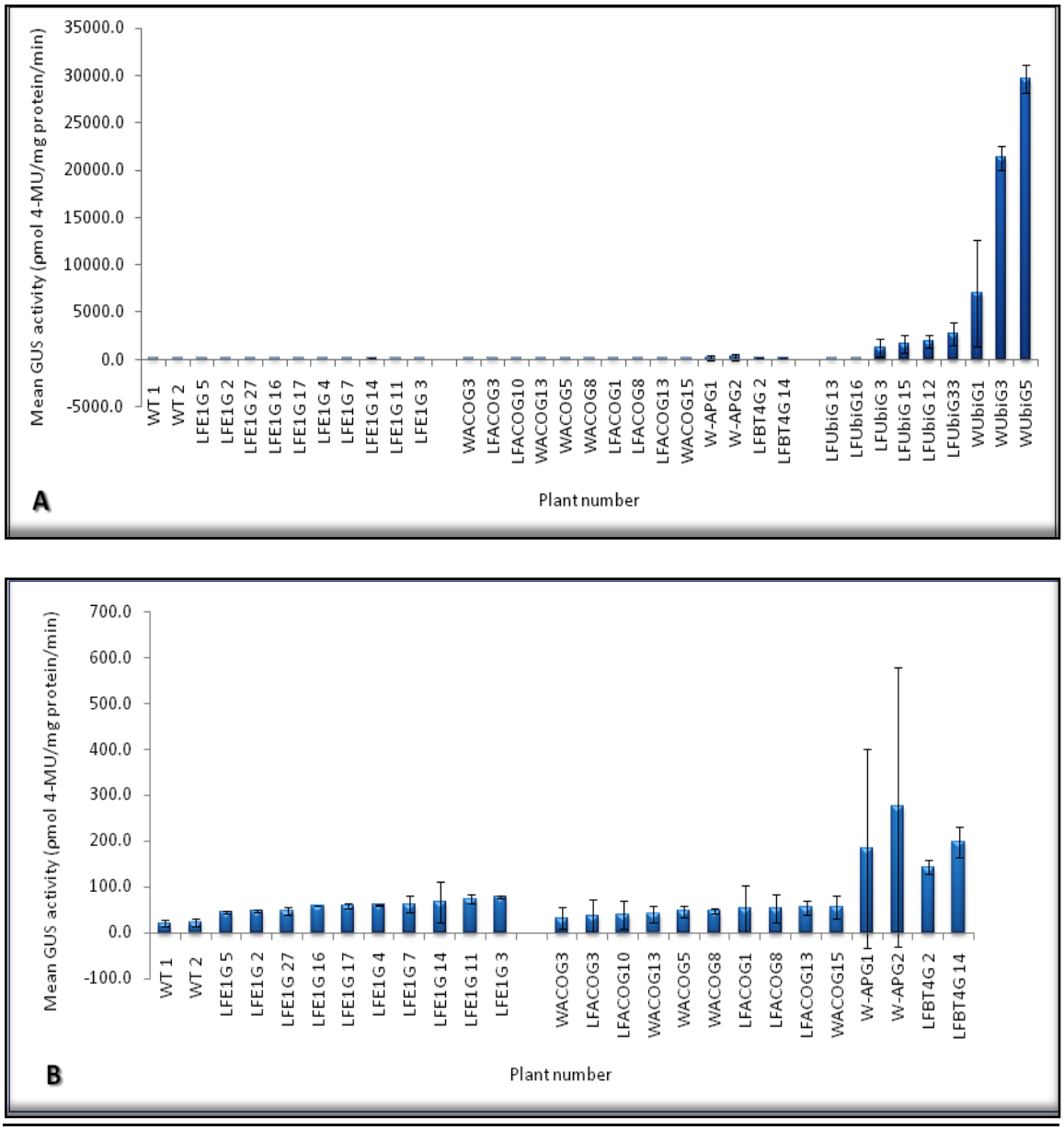


Figure 4.24 Fluorometric assay for promoter activity in the leaves of glasshouse-grown transgenic banana plants. Graph **A** compares the activity of all promoters analysed, while graph **B** shows all except the ZmUbi promoter to highlight differences within and between promoter activities. Bars represent mean GUS activity (pmol 4-MU/mg protein/min) ± standard deviation for two replicate analyses.
WT = wild-type; W = Williams; LF = Lady Finger; E1 = MaExp1; ACO = MaACO; AP = APsy2a; BT = Bunchy top; G = GUS

4.3.3.2 Field-grown transgenic banana plants

To examine promoter activity in field-grown plants, leaf samples were taken from two and 31 four-month old transgenic banana plants (cv Cavendish) stably transformed with the *uidA* (GUS) gene under the control of the constitutive BT4 and putative fruit-active MaExp1 promoters, respectively. As controls, six Cavendish banana plants derived from somatic embryos in the QUT plant tissue culture laboratory and three shoot-tip-cultured plants derived from an external laboratory were used. All the plants were growing in the field at the DPI, Centre for Wet Tropics Agriculture, South Johnstone, North Queensland. For each plant, two leaf samples (hereafter referred to as "leaf 1" and "leaf 2") were collected at a three week interval from the youngest fully-expanded leaf and analysed for GUS activity using both histochemical and fluorometric assays. For the second leaf sample, only three control plants were included in the analysis.

When the "leaf 1" samples were analysed by histochemical assay for GUS expression, no blue signal was observed in any of the six leaf discs taken from the nine wild-type control plants. In contrast, discs taken from the leaves of the two BT4-GUS transgenic plants gave a strong GUS signal (Table 4.3; Fig 4.25). Further, discs taken from the leaves of one (E1G 281), seven and six of the 31 MaExp1-GUS transgenic plants gave strong, medium and weak GUS reactions, respectively (Table 4.3; Fig. 4.25). To quantify GUS activity in the "leaf 1" samples, discs were taken from the youngest, fully expanded leaf of each plant, freeze-dried and pooled into 3 x 4 disc samples. Protein was subsequently extracted from the three replicate samples on separate days and the GUS activity was quantified by fluorometric assay (Table 4.3; Fig. 4.26). On average, the levels of GUS activity detected in the nine wild-type controls ranged from 12.13-24.02 pmol 4-MU/mg protein/min. Consistent with the results of the histochemical assays, the highest levels of GUS activity detected in the "leaf 1" samples were from the two BT4-GUS transgenic plants and the MaExp1-GUS transgenic plant #281 (Table 4.3; Fig. 4.26). With few exceptions such as MaExp1-GUS transgenic plants #284, 263 and 266, the results of the histochemical and fluorometric assays were consistent across the "leaf 1" samples.

Table 4.3 Fluorometric and histochemical assay for the leaf tissue of field-grown banana plants – Leaf 1

Plant ID	4-MU (pmol/mg protein/min)			Mean GUS activity (4-MU pmol/mg protein/min)	STDev	Histochemical assay
	Rep 1	Rep 2	Rep 3			
E1G 275	6.28	-*	4.56	5.42	1.2	-
E1G 274	6.44	3.63	6.20	5.42	1.6	-
E1G 276	10.78	4.52	10.91	8.74	3.7	-
E1G 277	10.61	8.28	12.42	10.44	2.1	-
E1G 260	13.94	-*	8.93	11.44	3.5	-
E1G 279	21.56	5.20	8.03	11.60	8.7	-
E1G 284	20.68	8.67	6.08	11.81	7.8	+
157- WT	10.45	13.34	12.59	12.13	1.5	-
E1G 285	7.34	9.72	20.51	12.52	7.0	-
Grid 4	5.87	18.46	15.34	13.22	6.6	-
156-WT	13.81	13.93	12.34	13.36	0.9	-
E1G 261	18.76	-*	10.84	14.80	5.6	-
E1G 278	22.19	10.19	12.39	14.92	6.4	-
E1G 262	24.92	4.46	15.86	15.08	10.3	-
170-WT	8.57	20.21	17.92	15.56	6.2	-
E1G 255	14.51	18.08	14.64	15.74	2.0	-
Grid 3	5.83	21.28	22.39	16.50	9.3	-
E1G 263	20.66	13.15	16.13	16.65	3.8	+
E1G 259	18.93	18.94	16.96	18.28	1.1	-
E1G 283	18.08	17.99	19.99	18.68	1.1	-
E1G 269	25.29	14.04	16.81	18.71	5.9	-
E1G 254	19.06	21.04	18.69	19.60	1.3	-
E1G 264	31.85	12.08	18.11	20.68	10.1	-
Grid 5	18.32	24.31	21.48	21.37	3.0	-
171- WT	16.15	26.76	23.38	22.10	5.4	-
158-WT	35.45	15.82	17.13	22.80	11.0	-
E1G 266	41.57	14.35	15.52	23.81	15.4	+
172-WT	13.56	26.94	31.56	24.02	9.4	-
E1G 265	33.07	19.52	23.59	25.39	7.0	-
E1G 257	28.67	29.60	24.64	27.64	2.6	+
E1G 268	63.13	16.88	15.90	31.97	27.0	+
E1G 271	60.80	15.71	20.72	32.41	24.7	++
E1G 270	57.44	25.40	22.74	35.19	19.3	++
E1G 253	50.84	28.45	27.33	35.54	13.3	++
E1G 282	52.17	30.01	26.48	36.22	13.9	+
E1G 267	81.29	21.79	24.60	42.56	33.6	++
E1G 272	150.98	18.62	23.12	64.24	75.2	++
E1G 273	162.59	19.35	25.46	69.13	81.0	++
E1G 280	310.31	29.71	24.59	121.54	163.5	++
E1G 281	477.81	71.44	33.66	194.30	246.2	+++
BT4G 252	261.56	91.29	191.84	181.56	85.6	+++
BT4G 251	1822.00	793.55	735.32	1116.96	611.3	+++

*absorbance readings from Rep 2 were excluded from this analysis since they were <0 and therefore regarded as spurious. WT = wild-type; E1 = MaExp1; BT = Bunchy top; G = GUS

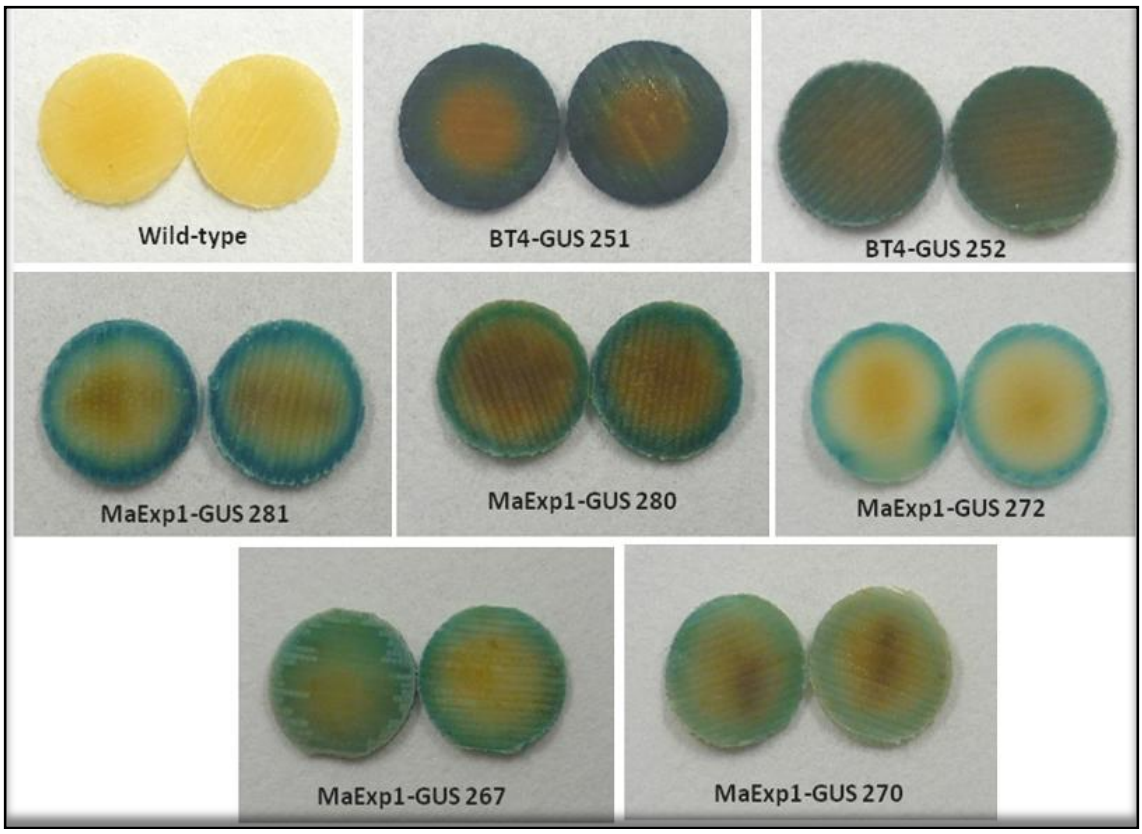


Figure 4.25 Representative histochemical assays of leaf samples taken from field-grown banana plants stably transformed with the GUS gene under the control of the BT4 or MaExp1 promoters. Plants with the strongest histochemical signal in "leaf 1" gave the highest GUS activity determined by fluorometric assay.

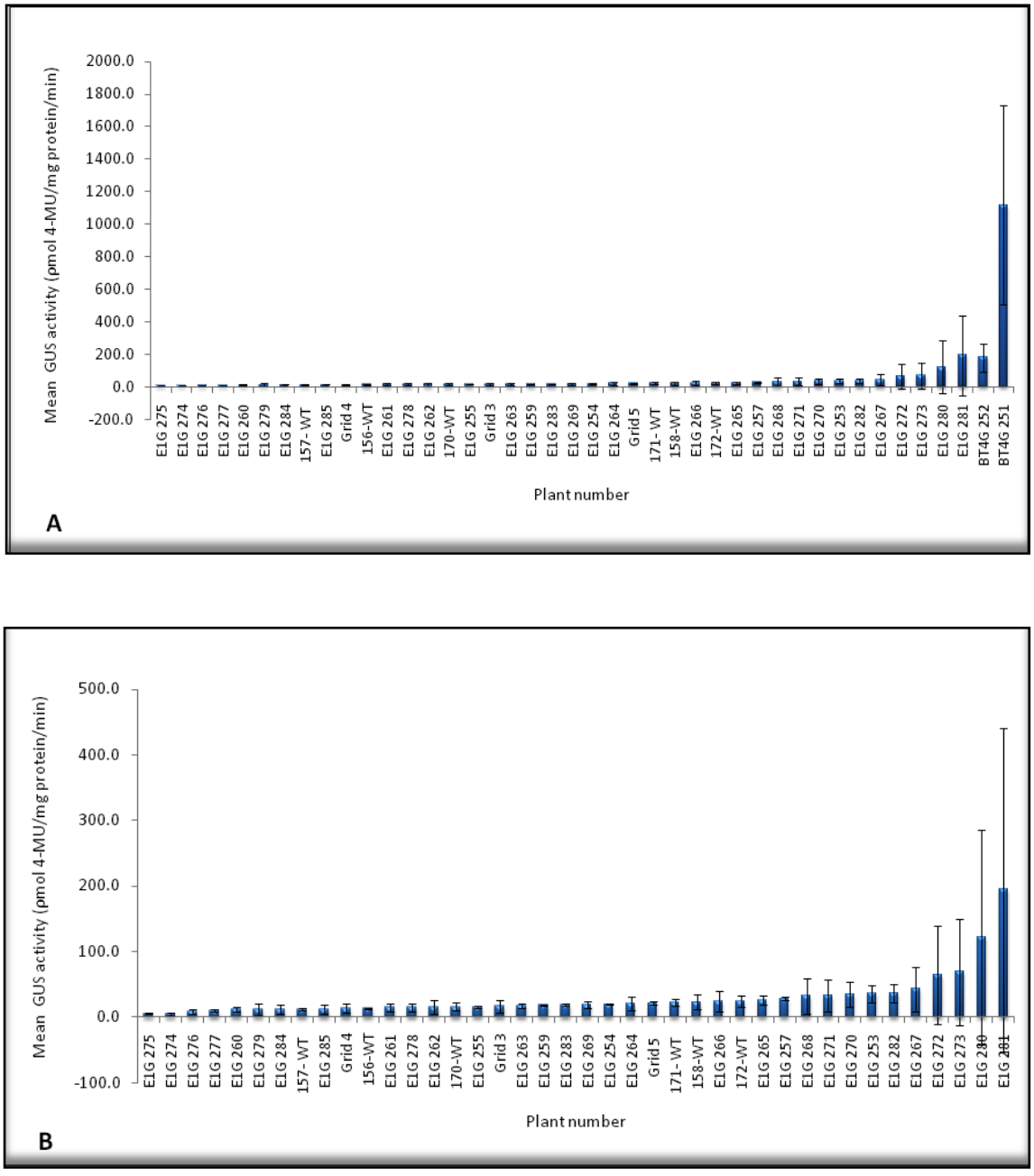


Figure 4.26 Fluorometric assay for promoter activity in leaves of field-grown transgenic banana plants (Leaf 1). Graphs show a comparison of promoter activity in leaves of banana plants stably transformed with the GUS gene under the control of the constitutive BT4 or putative fruit-active MaExp1 promoters. Graph **A** compares the activity of BT4 and MaExp1. Graph **B** compares the variation between the MaExp1-GUS plants. Bars represent mean GUS activity determined as 4-MU pmol/mg protein/min ± standard deviation. E1G = MaExp1-GUS; WT = wild-type plants generated through somatic embryogenesis; Grid = wild-type plants generated through shoot-tip culture.

To investigate possible temporal effects on promoter activity, the subsequently developed fully-expanded leaf from the same field-grown plants was sampled three weeks after the first samples were taken. As controls, only three wild-type plants were re-sampled for analysis. Histochemical assays were only done on selected samples while fluorometric assays were done once on a pooled and freeze-dried four-disc sample for every plant. Similar to the results obtained with the "leaf 1" samples, none of the discs taken from the three wild-type control plants tested positive for GUS expression following histochemical staining (Table 4.4). Of the samples taken from the two BT4-GUS transgenic plants, one (#251) gave a strong positive reaction similar to that obtained in the "leaf 1" sample while the second sample (#252) only gave a weak positive reaction (Table 4.4). Out of the six samples taken from the MaExp1-GUS transgenic plants, only one (#257) gave an identical result to that obtained using the "leaf 1" sample. In the other five MaExp1-GUS "leaf 2" samples, the intensities of GUS expression were lower than that observed from the "leaf 1" samples (Table 4.4). Unlike the "leaf 1" analyses, inconsistencies were seen in the results of the "leaf 2" histochemical and fluorometric assays. For example, whereas weak histochemical reactions were correlated with GUS activities ranging from 3.82-50.39 pmol 4-MU/mg protein/min in "leaf 2" analyses, weak histochemical reactions in "leaf 1" correlated to activities of 11.81–36.22 pmol 4-MU/mg protein/min. Despite the inconsistencies, in general, similar trends were observed from both leaf samples, with the BT4-GUS transgenic leaves showing the highest GUS activities while the MaExp1-GUS transgenic leaves showed lower activities (Fig. 4.27).

An unexpected observation from both leaf sample analyses was the relatively high GUS activities in many of the wild-type control samples compared to the MaExp1-GUS samples. For example, the mean GUS activity in the "leaf 1" sample from control plant 172-WT was 24.02 pmol 4-MU/mg protein/min which was higher than the mean GUS activities obtained from 19 out of 31 MaExp1-GUS-plants in the "leaf 1" samples (Table 4.3). A considerable amount of variation was also observed in the three fluorometric assay replicate results for the "leaf 1" samples resulting into high standard deviations (Table 4.3).

Table 4.4 Fluorometric and histochemical assay for leaf tissue of field-grown banana plants – Leaf 2

Plant ID	Mean GUS activity (pmol 4-MU /mg protein/min)	STDev*	Histochemical assay
E1G 276	1.79	0.29	
Grid 4	2.73	0.83	-
E1G 266	2.73	0.21	
E1G 264	3.34	0.57	
158-WT	3.80	0.13	-
E1G 253	3.82	0.34	+
E1G 255	4.55	0.22	
E1G 262	5.27	0.15	
172-WT	5.29	0.40	-
E1G 269	5.48	1.16	
E1G 283	5.58	0.94	
E1G 273	5.83	0.46	
E1G 272	5.86	1.56	-
E1G 260	6.58	0.43	
E1G 270	7.21	0.86	
E1G 282	7.28	0.79	
E1G 259	7.47	1.02	
E1G 254	7.62	0.29	
E1G 271	7.97	2.14	
E1G 279	9.38	0.53	
E1G 257	9.70	0.35	+
E1G 275	12.09	0.40	
E1G 261	12.99	0.96	
E1G 278	13.22	1.29	
E1G 265	13.54	0.89	
E1G 277	13.60	0.65	
E1G 267	14.23	0.47	
E1G 280	14.32	0.31	+
E1G 268	14.34	1.11	
E1G 263	15.78	0.77	+
E1G 281	19.90	1.32	+
E1G 284	22.36	0.99	
E1G 274	23.04	1.67	
E1G 285	28.23	3.19	
BT4G 252	50.39	1.97	+
BT4G 251	955.13	78.51	+++

*standard deviation represents three 4-MU reactions all taken from one protein extract
E1 = MaExp1; BT = Bunchy top; G = GUS

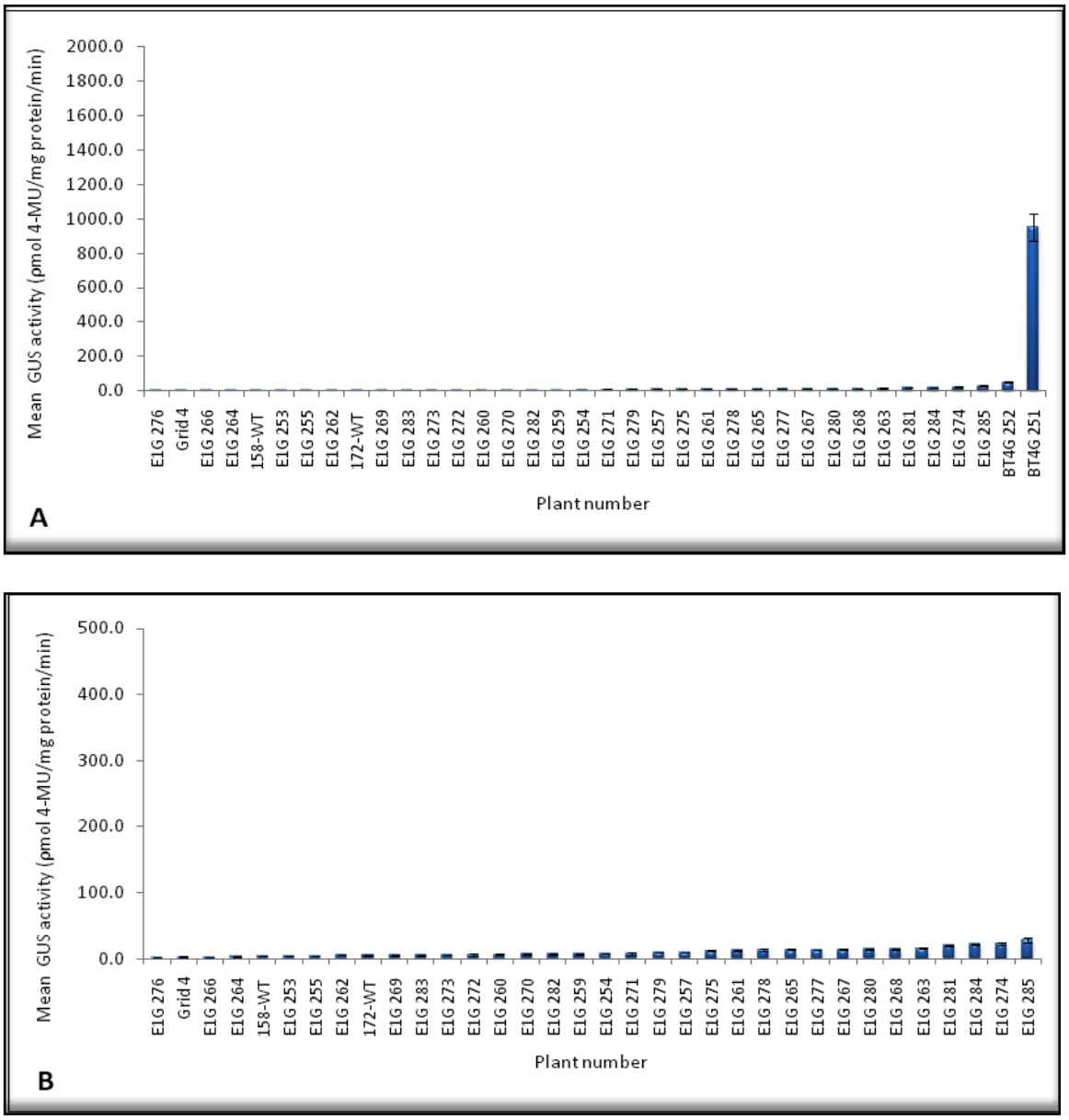


Figure 4.27 Fluorometric assay for promoter activity in leaves of field-grown transgenic plants (Leaf 2). Graphs show a comparison of promoter activity in leaves of banana plants stably transformed with the GUS gene under the control of the constitutive BT4 or putative fruit-active MaExp1 promoters. Graph **A** compares the activity of BT4 and MaExp1. Graph **B** compares the variation between the MaExp1-GUS plants. Bars represent mean GUS activity determined as 4-MU pmol/mg protein/min ± standard deviation. E1G = MaExp1-GUS; WT = wild-type plants generated through somatic embryogenesis; Grid = wild-type plants generated through conventional shoot-tip culture.

4.4 Discussion

The initial component of the research described in this chapter involved assessing and comparing particle bombardment and *Agrobacterium*-mediated infiltration as methods to analyse transient promoter activity in banana fruit. The Expansin1 (MaExp1), Expansin4 (MaExp4), Extensin (MaExt), 1-aminocyclopropane-1-carboxylic acid synthase (MaACS), 1-aminocyclopropane-1-carboxylate oxidase (MaACO) and Metallothionein (MaMT2a) promoters that were used in this study had been isolated from Cavendish while the phytoene synthase (APsy2a) promoter had been isolated from F'ei type banana cv Asupina. These promoters were chosen because they are associated with genes that are involved in fruit development and ripening (Holdsworth M J *et al.,* 1987; Clendennen and May 1997; Trivedi and Nath 2004; Asha *et al.,* 2007; Mlalazi 2010) and were therefore likely to be fruit-active. In addition, homologues of some of the selected promoters such as the ACO have been identified in other fruits such as peach (Ruperti *et al.,* 2001; Rasori *et al.,* 2003) apple (Atkinson *et al.,* 1998) and tomato (Blume and Grierson 1997) and have also been shown to be active during fruit ripening.

Using both particle bombardment and *Agrobacterium*-mediated infiltration methods, several of the banana-derived promoters were identified which could potentially be used to drive transgene expression in banana fruit. With the exception of the metallothionein promoter (MaMT2a), similar trends in promoter activity were observed using both transformation methods. The constitutive ZmUbi and BT4 promoters used as controls were shown to direct high levels of GUS expression in fruit using both methods, with similar high expression levels obtained using the MaExp1 and MaExp1a promoters. Lower levels of promoter activity were obtained in both methods using the MaACO and MaExt promoters although MaACO activity was not significantly different from that of the BT4 promoter, while the MaExp4, MaACS, and APsy2a promoters directed the lowest GUS activity in banana fruit. Although the activity of the MaMT2a promoter was found to be high in bombarded fruit, its activity in infiltrated fruit was weak. Based on the results of the transient assays, the MaACO and MaExp1 promoters represent potentially useful promoters for the biofortification of banana fruit since they were capable of directing high levels of transgene expression which

were equivalent to, or higher than, those of the constitutive ZmUbi and BT4 promoters. Interestingly, the inclusion of the rice actin intron in the MaExp1 promoter did not result in a significant difference to its activity. The inclusion of an intron to enhance promoter activity (known as intron-mediated enhancement (IME)) has been well documented and is commonly used to increase the activity of monocot-derived promoters such as the maize poly-ubiquitin promoter (Christensen and Quail 1996; Dugdale *et al.,* 2001). Further, using banana embryogenic cell suspensions, the inclusion of the rice actin intron to the *Banana bunchy top virus*-derived BT6.1 promoter was found to enhance reporter gene expression levels by 300-fold (Dugdale *et al.,* 2001). Although IME of promoter activity/transgene expression has been demonstrated in plants such as barley (Bartlett *et al.,* 2009), rice (Lu *et al.,* 2008) and *Arabidopsis* (Rose 2004), the phenomenon is reported to be dependent on tissue type, position of the intron in the expression vector and the promoter used.

Despite both the particle bombardment and agro-infiltration transformation methods yielding similar results, a considerable amount of variation in promoter activity was nonetheless observed both within and between experiments. For example, the transient GUS activity directed by the MaMT2a promoter differed by 10-fold between two particle bombardment experiments. Such variation in GUS expression has been reported in transient assays in fruit of several different crops (Wroblewski *et al.,* 2005) and has been recently reported in experiments with agro-infiltrated immature banana fruit (Matsumoto *et al.,* 2009). The observed variability in the results of this, and other studies, is most likely a reflection of the potential strengths and weaknesses of the transformation methods used. The success of agro-infiltration as a transient expression system, for example, is influenced by the effectiveness of *Agrobacterium* to infect plant tissues. The ability of *Agrobacterium* to infect plant tissue has previously been shown to be dependent on the physiological status of the target tissue at the time of transformation. As a result, variable transgene expression can occur in identical target tissue which has been sampled at different times (Wroblewski *et al.,* 2005). This may explain the differences in transient GUS expression observed in un-gassed agro-infiltrated banana fruit compared to gassed fruit (from the supermarket) which suggested an inhibitory effect resulting from exposure to ethylene gas. Further,

although a seasonal effect was not deliberately investigated in this study, fruit harvested in summer appeared to be more responsive to agro-infection than fruit harvested during winter (results not shown). In view of these observations, agro-infiltration is recommended for determining transient expression in intact fruits where particle bombardment may not be easily applicable. However, agro-infiltration should be utilised with tissues whose physiological status is relatively uniform such as those derived from plants growing in similar environmental conditions and/or subjected to similar post-harvest treatments. Furthermore, for both agro-infiltration and particle bombardment, a large number of samples must be analysed in order to capture the expected variation. Once this is done, reliable inferences can be made despite variations within and between experiments.

Following the successful demonstration of agro-infection as a method to analyse transient reporter gene expression in banana fruit, the protocol was subsequently used to assess the expression of pVA biosynthesis genes in this tissue. For this purpose, banana fruit was infiltrated with constructs in which the constitutive maize poly-ubiquitin promoter controlled the expression of the banana-derived phytoene synthase gene (*APsy*2a), with and without the bacterial carotene desaturase (*CrtI*), and with the maize phytoene synthase gene (*ZmPsy*1) included as a control. Since coloured carotenoids are responsible for the characteristic bright colours usually seen in many fruits and flowers (Bartley and Scolnik 1995; Vershinin 1999) and the intensity of banana pulp colour has been shown to correspond to the carotenoid content (Englberger *et al.,* 2003b; Englberger *et al.,* 2003c), agro-infiltrated fruit were assessed for increased pVA synthesis both qualitatively, by visual examination for increased colouration, and quantitatively using HPLC.

Prior to the commencement of these analyses, the use of HPLC as a protocol to identify and quantify carotenoids in Cavendish banana fruit was examined. Most carotenoids are known to absorb ultraviolet/visible light maximally at three wavelengths resulting in a three peak spectrum, the wave numbers of which shift slightly depending on the stationary and the mobile phase in use. Using a C30 column and a methanol-tert-butyl methyl ether solvent system for carotenoid separation in this study, three well-defined peaks were obtained for lutein and α-carotene, while two peaks and a shoulder were

obtained for β-carotene. This was consistent with the spectral characteristics and wavelengths of carotenoids reported under similar chromatographic separation conditions (Fraser *et al.,* 2000; Rodriguez-Amaya and Kimura 2004). Further, similar to other studies (Englberger *et al.,* 2003b; Davey *et al.,* 2006; Davey *et al.,* 2009) more than 90% of the carotenoids detected in banana consisted of α- and β-carotene with minor amounts of lutein. Using HPLC, the average β-carotene content estimated from fruit of non-transgenic Cavendish from two different farms ranged from 23-60.0 µg/100 g raw fresh weight. These amounts were comparable to those reported elsewhere (Holden *et al.,* 1999; USDA 2005).

In preliminary analyses, transient expression of *ZmPsy*1 +/- *CrtI* resulted in slight colour changes in the pulp of agro-infiltrated banana fruit which was presumed to be the result of enhanced levels of coloured carotenoids. In subsequent experiments, however, a considerable amount of variation and inconsistency was observed in colour changes in individual treatments within and between agro-infiltration experiments. Moreover, in repeated experiments, the observed colour changes in the banana fruit pulp did not correspond to enhanced levels of carotenoids. In summary, the amount of β-carotene quantified by HPLC (above the basal content in the fruit) did not reflect any consistent patterns as a consequence of transient expression of pVA biosynthesis genes in agro-infiltrated fruit. There is no clear-cut explanation for these results since the transient GUS assays showed that the ZmUbi promoter as well as the agro-infiltration method works in banana fruit. However, it is possible that a range of unpredictable physiological interactions could be taking place between *Agrobacterium* and the fruit in the presence of pVA biosynthesis genes which appear to interfere with the transient production and/or stability of carotenoids in the fruit.

The final component of the research described in this chapter was an assessment of the activity of selected banana-derived fruit-active promoters in stably transformed glasshouse and/or field-grown banana plants. As controls, the activities of the constitutive ZmUbi and BT4 promoters were also assessed. In the previous section, these promoters showed various levels of fruit activity using transient assays in banana fruit. Although transient assays provide useful preliminary results, analysis of stably

transformed whole plants is ultimately needed to provide a conclusive assessment of the fruit-activity of the promoter since the ultimate goal is to enhance the nutrient quality of the banana fruit. At the time of this study, banana plants stably transformed with a range of different promoters driving the expression of the banana phytoene synthase (*APsy*2a), maize phytoene synthase *(ZmPsy*1) or GUS gene were growing in the glasshouse. Further, a selection of these transgenic plants had already been planted in the field in North Queensland as part of the related studies at QUT towards the biofortification of banana. Although these plants had not yet produced fruit, they nonetheless provided an opportunity to examine the level of promoter activity/transgene expression in, and the effects of transgene expression on, non-target tissue such as leaves.

The constitutive ZmUbi promoter directed the highest levels of GUS expression in the leaves of glasshouse-grown transgenic plants. Interestingly, the levels of expression from the ZmUbi promoter in leaves taken from the transgenic Cavendish cultivar were up to 10-fold higher than those found in leaves taken from the transgenic Lady Finger cultivar. This seemingly cultivar-dependent effect on transgene expression was only observed using the ZmUbi promoter, however, so the significance of this observation, if any, requires further investigation. Although generally lower than ZmUbi, the levels of expression directed by the APsy2a and the constitutive BT4 promoters were similar. However, a 10-fold variation in the results of the replicate fluorometric assays for the APsy2a promoter casts some doubt on the validity of this result which is most likely attributed to an experimental error. When compared to constitutive controls, the activities of the fruit-active MaExp1 and MaACO promoters were very low in leaves of stably transformed glasshouse-grown plants, demonstrating that the promoters are only weakly expressed in leaf tissue. Interestingly, many of the banana plants transformed with ZmUbi-*ZmPsy*1 displayed a yellow (“golden leaf”) phenotype (results not shown) and did not thrive whereas plants transformed with other promoters appeared phenotypically normal. Although the golden leaf phenotype was indicative of enhanced carotenoid production in banana and thus very encouraging, the poor growth of these plants clearly indicated that constitutive expression of the *Psy* gene

was not ideal and supported the need for a promoter with low constitutive, but strong fruit activity.

As part of the related banana biofortification project, a number of transgenic plants expressing the banana phytoene synthase (*APsy*2a) gene and the maize phytoene synthase *(ZmPsy*1) gene under the control of the fruit-active MaExp1 promoter were growing in a field trial in North Queensland. Included in this trial were several banana plants which were stably transformed with the GUS gene under the control of the fruit-active MaExp1 or constitutive BT4 promoters. Although the transgenic plants were only 3 months old, they again provided an opportunity to analyse promoter activity in field-grown plants. Overall, similar trends in activity were seen in the glasshouse and field-grown plants with the activity of the BT4 promoter higher than that of the MaExp1 promoter. Further, the overall activity of the promoters in field-grown plants was generally lower than that observed in glasshouse-grown plants. Whether this effect was real or simply due to the variability that was observed with the fluorometric assay replicates will require further investigation. However, the use of histochemical assays alone to assess GUS expression was not found to be reliable. For example, using glasshouse-grown MaACO-GUS or MaExp1-GUS transgenic plants, no direct correlation was observed between the results of histochemical and fluorometric assay. Similar inconsistencies were observed in replicate samples taken from the same leaf of field-grown MaExp1-GUS transgenic plants. The differences in GUS expression with-in leaf and between the “leaf 1” and “leaf 2” samples from the same plant may be due to temporal promoter activity between cells at different developmental stages or environmental effects (in the case for field plants) or variability in the GUS assay (Serres *et al.,* 1997; Côté and Rutledge 2003). In addition, inconsistencies in the results of GUS activity could also be due to endogenous GUS activity (Sudan *et al.,* 2006) and this was the case in one MaExp1-GUS field-grown plant where the variation observed between fluorometric and histochemical assay was a result of endogenous GUS expression. Overall these results affirm that the fruit-active MaExp1 promoter directs very low levels of transgene activity in leaves of stably transformed banana plants and may prove to be a suitable promoter for banana biofortification.

Similar to that observed in glasshouse-grown plants, the vast majority of field-grown plants expressing *Psy* genes under the control of the constitutive ZmUbi or BT4 promoter showed severe dwarfism and/or the characteristic "golden leaf" phenotype (Fig 4.28), presumably as a result of the over-expression of pVA biosynthesis genes. In contrast, the majority of field-grown banana plants stably transformed with *APsy*2a and *ZmPsy*1 genes under the control of the fruit-active MaExp1 promoter showed normal phenotypic characteristics (including suckering and leaf colour/shape) similar to those of wild-type plants (Fig 4.29). However, a small number of plants did exhibit dwarfism and the "gold leaf" phenotype (Fig 4.29) similar to, but not as severe as, that seen in plants transformed with constitutively expressed *Psy* genes. Considering that the MaExp1 promoter controls the expression of genes associated with cell expansion and growth (Lee *et al.,* 2001) it was not surprising that the "golden leaf" phenotype was observed in the young expanding leaves mainly in the lower half of the leaf (Fig 4.29). With most of the MaExp1-*Psy*-banana plants, the golden leaf phenotype reverted to a normal green leaf once it had expanded fully and continued to grow normally (Khanna personal communication), further confirming temporal promoter activity in newly expanding tissue.

In conclusion, results in this chapter showed comparable promoter activity in banana fruit transformed by particle bombardment or *Agrobacterium*-mediated infiltration methods. The constitutive BT4 and ZmUbi promoters showed the highest activities in banana fruit for both assays and also in leaves of stably transformed glasshouse- and field-grown plants. Two banana-derived promoters, MaExp1 and MaACO, directed high levels of transient GUS activity that was comparable to, or even higher than, that of the constitutive promoters in mature green fruit while their overall activity in leaf tissue of stably transformed banana plants was very low. This research demonstrated that MaExp1 and MaACO promoters are fruit-active and therefore are candidates for directing targeted fruit-preferred expression of genes of interest, in banana, whose products are preferentially required to enhance the nutritional quality of the fruit. Ultimately, however, analysis of fruit from the field-grown transgenic plants will be required to fully evaluate the suitability of the promoters for fruit biofortification.

Figure 4.28 Phenotype of field-grown banana plants expressing the ZmPsy1 or APsy2a gene under the control of the constitutive ZmUbi or BT4 promoters. Representatives of **(A)** ZmUbi-*APsy*2a, **(B)** BT4-*ZmPsy*1 and **(C)** BT4-*ZmPsy*1 transgenic plants displayed various degrees of stunting with the characteristic "golden leaf" phenotype (arrowed). Plate **(D)** shows a ZmUbi-*APsy*2a-transgenic plant with a normal phenotype while a wild-type cell culture-derived control plant is shown in plate **(E)**.

Figure 4.29 Phenotype of field-grown banana plants expressing the ZmPsy1 or APsy2a gene under the control of the MaExp1 promoter. Normal phenotype shown by **(A)** MaExp1-*APsy*2a and **(B)** MaExp1-*ZmPsy*1 transgenic plants similar to that observed in the wild-type control plants. Plate **(C)** MaExp1-*APsy*2a and **(D)** MaExp1-*ZmPsy*1 transgenic plants show mild stunting with the "golden leaf" phenotype (arrowed).

Chapter 5 Functional evaluation of the banana ferritin (*BanFer*1) gene for enhanced accumulation of iron using rice as a model

5.1 Introduction

Iron deficiency is the leading nutritional disorder worldwide, affecting both the developed and developing countries, with at least 30% of the world's population suffering from iron deficiency anaemia (Clugston and Smith 2002; WHO 2003). Food crops are a major source of dietary iron. Although dietary iron is only required in small amounts i.e. 10 – 18 mg per day (National Academy of Sciences 2004), a daily diet that is mainly composed of a single food crop usually does not provide sufficient iron to meet recommended daily requirements. In effect, populations who primarily depend on staple food crops with limited or no diet diversification are highly susceptible to iron deficiency anaemia.

Previous efforts to overcome iron deficiency have focused largely on food fortification and the use of iron supplements (Goto and Yoshihara 2001; Nestel *et al.,* 2006). Although these interventions have led to positive health improvements, their success remains limited to developed countries. In developing countries, supplementation programs are often supported by short-term, grant-based projects which are limited in their scope, population coverage and continuity of funding and are therefore unsustainable (Bouis *et al.,* 2003b; Zhu *et al.,* 2007). In addition, food fortification and diet diversification are neither accessible nor affordable in rural and often poor communities who depend on staple food crops for their daily diet. Therefore, improving the iron content of a staple food crop is an important step towards sustainable improvement of the quality of life for such people. Improvement of micronutrients in crops can be achieved through biofortification by over-expression of novel genes through transgenic approaches (Goto and Yoshihara 2001; Poletti and Sautter 2005; Ye *et al.,* 2008; Djennane *et al.,* 2010; Gómez-Galera *et al.,* 2010; Lin *et al.,* 2010). East African highland bananas (EAHB) are a staple food for communities in East Africa but the amount of iron in fruits of the cultivated varieties is not sufficient to meet the recommended daily allowances. In addition, there are no known banana

varieties that accumulate high levels of iron. However, with recent advances in transformation and transgene expression in bananas, genetic engineering is now a viable approach to improve cultivated varieties through over-expression of genes that have the potential to improve the plants ability for iron uptake and storage in the edible fruit. Improving varieties that are already selected for agronomically important traits offers a feasible and sustainable solution that would not change cropping systems or impose re-current costs to farmers.

Improving the ability of plants to accumulate enhanced levels of iron has been demonstrated in rice by over-expression of the ferritin gene derived from soybean (Goto *et al.,* 1999; Drakakaki *et al.,* 2000; Vert *et al.,* 2002; Van Lieshout *et al.,* 2003; Vasconcelos *et al.,* 2003). In an effort to identify banana genes related to iron uptake and storage, scientists at QUT have isolated a new ferritin gene (*BanFer*1) from *Musa* spp. (cv Cavendish). However, due to the lengthy time from banana transformation to transgenic fruit production, at least 2-3 years is required to characterise the function of this gene in banana fruit. Rice has previously been used as a model plant to study gene regulation of monocotyledonous plants (Tyagi *et al.,* 1999), and an efficient transformation system is available (Hiei *et al.,* 1994; Nishimura *et al.,* 2006). Therefore**,** rice was considered a suitable alternative plant to analyse the function of banana ferritin. The aim of this study was to evaluate the function of the *BanFer*1 gene and its potential for increasing iron content in plant tissue using rice as a model system and the soybean ferritin gene as a control. The specific objectives of this study were to: (i) transform embryogenic rice callus with *BanFer*1 under the control of the constitutive maize poly-ubiquitin promoter, (ii) select and multiply transformed/antibiotic resistant callus lines and analyse for transgene expression and (iii) quantify the amount of iron in transgenic rice callus by FAAS.

5.2 Materials and methods

5.2.1 Target genes

The banana ferritin gene (*BanFer*1), isolated from Cavendish, was kindly provided by Ms. Ada Rosier (QUT). The soybean ferritin gene (*SoyFer*) used for comparative purposes, and the *iudA* reporter gene encoding β-glucuronidase gene (GUS) used as a transformation control, were kindly provided by Dr. Jason Geijskes and Mr. Don Catchpoole-CTCB-QUT.

5.2.2 Construction of binary transformation vectors

Transformation vectors for the *BanFer1, SoyFer* and GUS genes, under the control of maize poly-ubiquitin (ZmUbi) promoter, were constructed into the binary pCAMBIA 2300 vector. Template DNA (cds and terminator) for *BanFer*1, *SoyFer* and GUS genes were derived from three parent vectors pCAM-35S-*BanFer1*-nos-35S-*nptII*-35S, pBIN-ZmUbi-*SoyFer*-nos-nos-*nptII*-nos and pBIN-ZmUbi-GUS-nos-nos-*nptII*-nos. Template DNA for the base vector was derived from pCAM-ZmUbi-Vte1-nos-35S-*nptII*-35S**.** All parent vectors (provided by CTCB-QUT scientists) had the restriction site *Bam*H1 upstream of the gene cds and *Eco*R1 downstream of the nos terminator (Table 5.1).

Template DNA fragments (Table 5.1) were excised from parent vectors by double digestion with *Bam*H1-*Eco*R1 in restriction buffer B (Chapter 2.6.2). DNA fragments were separated on a 1% (w/v) agarose gel, the appropriately sized DNA bands were excised and DNA was recovered by the freeze and squeeze method (Chapter 2.6.4). Fragments for *BanFer*1-nos (810 bp), *SoyFer*-nos (1028 bp) and GUS-nos (2322 bp) were ligated (Chapter 2.6.5) into the corresponding *Bam*H1-*Eco*R1 site of the 10695 bp pCAM-ZmUbi--35S-*nptII*-35S fragment to generate pCAM-ZmUbi-*BanFer*1-nos-35S-*nptII*-35S, pCAM-ZmUbi-*SoyFer*-nos-35S-*nptII*-35S and pCAM-ZmUbi-GUS-nos-35S-*nptII*-35S, respectively.

Subsequently, each ligation mixture was transformed into competent *E.coli* cells (XL1-Blue) using the heat-shock method (Chapter 2.4.2 and 2.4.4) and selected on LB media containing 100 mgL^{-1} kanamycin. A bacterial suspension was cultured from selected resistant *E.coli* colonies (Chapter 2.5.1) from which plasmid DNA was extracted by

alkaline lysis (Chapter 2.6.1). In order to confirm the presence of the cloned fragments, pDNA from each vector was digested by *Bam*H1-*Eco*R1 in buffer B and the products were separated by agarose gel electrophoresis to identify inserts of the expected size. In addition, the sequence integrity of cloned fragments and their orientation in relation to the ZmUbi promoter was confirmed by capillary electrophoresis (Chapter 2.6.6). Binary vectors were sequenced using primers listed in Table 5.2. The position and integrity of the promoter junction in relation to the vector backbone was analysed using the universal M13 primer. In addition, the orientation of the start codon at the promoter-gene junction was analysed using the Ubi-Int sequencing primer (designed 200 bp into the end of the ZmUbi intron of the ZmUbi promoter). The orientation of the ferritin coding sequence in relation to the nos terminator was verified using the M13-Rev (pCAM) reverse primer.

5.2.3 Transformation of *Agrobacterium* with vector DNA

Plasmid DNA from one clone selected by restriction digestion and sequencing was transformed into *Agrobacterium* (strain AGL1) by electroporation (Chapter 2.4.3).

5.2.4 Transformation, selection and multiplication of rice callus

Rice embryogenic callus was induced from mature rice seed (*Oryza sativa* cv *japonica* var Nipponbare) on 2N6 callus induction media and transformed by *Agrobacterium*-mediated transformation (Chapter 2.5.1, 2.5.2 and Appendix 1). Transformed callus was selected on 2N6S media containing 50 mgL^{-1} geneticin and 200 mgL^{-1} timentin as described (Chapter 2.5.2). Individual antibiotic-resistant callus clusters which proliferated on antibiotic selection (i.e. representing putatively transformed callus lines) were transferred onto multiplication media and further multiplied separately for the analysis of iron content by flame atomic absorption spectrometry (FAAS) (Chapter 2.7.2 and Appendix 3).

Table 5.1 Template DNA fragments for the cloning of ferritin transformation vectors

Parent vectors	***Template fragments (bp)**	**Other fragments (bp)**
pBIN-ZmUbi(*Bam*H1)**GUS-nos**(*Eco*R1)nos-*nptII*-nos	2322	14348
pBIN-ZmUbi(*Bam*H1)***SoyFer*-nos(***Eco*R1)nos-*nptII*-nos	1028	14348
pCAM-35S(*Bam*H1)***BanFer*1-nos(***Eco*R1)-35S-*nptII*-35S	1056	9247
pCAM-ZmUbi(*Bam*H1)*Vte*1-nos(*Eco*R1)**35S-*nptII*-35S**	10695	1735

*the components of the parent vector corresponding to the required template for cloning purposes are highlighted in bold in the left hand column

Table 5.2 Primer sequences for cloning and PCR

Primer #	**Purpose/transgene**	**Primer name**	**Primer sequence (5'-3')**[a]	**Product size (bp)**
1	PCR/*BanFer*1	bFerATG*Bam*H1-Fwd	GGATCCATGCTTCTCAAGGCCTCTGCTTCT	810
2	PCR/*BanFer*1	bFerTAA*Xba*1-Rev	TCTAGATTATGCAGCATCTCCCTGAAG	
3	sequencing	M13- Rev	CACACAGGAAACAGCTATGACCATG	
4	sequencing	M13 universal	GTAAAACGACGGCCAGT	
5	sequencing	M13-Fwd	GGTTT CCCAGTCACGAC	
6	sequencing	M13-Rev (pCAM)	CACAC*C*GGAAACAGCTATGACC	
7	sequencing	Ubi-Int	GATTTTTTTAGCCCTGCCTTC	
8	cDNA synthesis	OligodT	GACTCGAGTCGACATCGTTTTTTTTTTTTTTT	
9	PCR/*SoyFer*	*SoyFer*Int-Fwd	GCGAGCTTGCTGTTCCAACTGC	562
10	PCR/*SoyFer*	*SoyFer*-Rev	GCAAGATCTTCCTAATCAAGAAGTC	
11	PCR	β-actin-Fwd	GGTGTYATGGTWGGKATGGG	1100
12	PCR	β-actin-Rev	CCTCCAATCCAGACACTGTAC	

[a] Restriction sites within the primer sequence are underlined

5.2.5 Reverse transcriptase (RT)-PCR

Total RNA was extracted from rice callus tissue as previously described (Chapter 2.7.3). Total RNA was treated with Turbo DNA-free™ (Ambion) according to the manufacturer's instructions to remove contaminating DNA. Transgene expression in antibiotic resistant callus was analysed by two-step RT-PCR using the ImProm-II™ reverse transcription system (Promega). Synthesis of cDNA was carried out using anchored oligo (dT) mega primers (Table 5.2) and reverse transcriptase (ImProm-II™, Promega) in a 20 µL reaction (Chapter 2.3.7.2). Subsequently, 2 µL of cDNA was subjected to PCR analysis using gene-specific primers for *BanFer*1 or *SoyFer* (#1 and 2 or #9 and 10, respectively; Table 5.2). PCR mixes were prepared using GoTaq green master mix (Promega) as described (Chapter 2.3.7.1), and amplicons were separated and visualised by agarose gel electrophoresis (Chapter 2.6.3).

5.2.6 Quantification of iron content in transgenic rice callus

Proliferating rice callus from each transformed callus line was harvested, washed in sterile MilliQ water before freeze drying (2.7.2.1). Iron was extracted from 500 mg of grounded dry callus using 10 ml of 70% HNO_3 (TraceSELECT grade, Fluka,) by CEM microwave digestion as described (Chapter 2.7.2). Tomato leaf powder (containing 368±7 mg of Fe/kg of dwt; certified SRM # 1573a, certified by National Institute of Standards and Technology, US Department of Commerce) was included as a reference control for iron extraction. For comparison, non-transformed callus was included as a control. Each digested sample was diluted 1:5 in 10% nitric acid and analysed by FAAS using a Varian SpectrAA 220 FS spectrometer with a 10 mA hollow cathode lamp as described (Chapter 2.7.2 and Appendix 2). The amount of iron in test samples was estimated against a standard calibration curve for iron as described (Chapter 2.7.2.5). The percentage extraction efficiency was calculated from the amount of iron quantified from the reference tomato leaf sample with reference to the actual iron content (i.e. 368±7 mg Fe/kg dwt). The extraction efficiency was used to standardise the actual quantified iron content in test samples. The mean iron content in test samples was determined from two FAAS measurements of one iron extract per callus line. Univariate analysis of variance (ANOVA) was used to compare mean iron content

between individual callus lines/treatment. The final iron content in a transformed callus line was expressed as mg of Fe/kg dwt of tissue.

5.2.7 Histochemical staining of ferric iron

Transgenic and non-transgenic rice callus was stained with potassium ferrocyanide in acidic solution according to Mallory's Iron Prussian blue reaction (Mallory 2007). The staining solution was freshly prepared by mixing equal volumes of 5% potassium ferrocyanide and 5% hydrochloric acid, and incubated at room temperature for 30 min before use. Callus, pre-hydrated in Milli-Q water for at least 1 hr, was submerged in staining solution for 5–10 min and rinsed twice in Milli-Q water for 2 min.

5.3 Results

5.3.1 Expression vectors for transformation of rice

In order to construct expression vectors for transformation of rice, insert DNA fragments for *BanFer*1, *SoyFer*, GUS and the base vector (pCAMBIA 2300) were isolated from their parent vectors by restriction digestion (Fig 5.1). Following gel purification, the DNA fragments were cloned into a binary pCAMBIA 2300 vector under the control of the ubiquitin promoter to generate three expression vectors for (i) banana ferritin, (ii) soybean ferritin and (iii) GUS. The presence of the cloned inserts in each of the binary vectors was confirmed by restriction digestion (Fig 5.2) and their integrity was verified by sequencing. A schematic representation of the strategy for generating the expression vectors used for transformation of rice callus, using banana ferritin as an example, is shown in Fig 5.3.

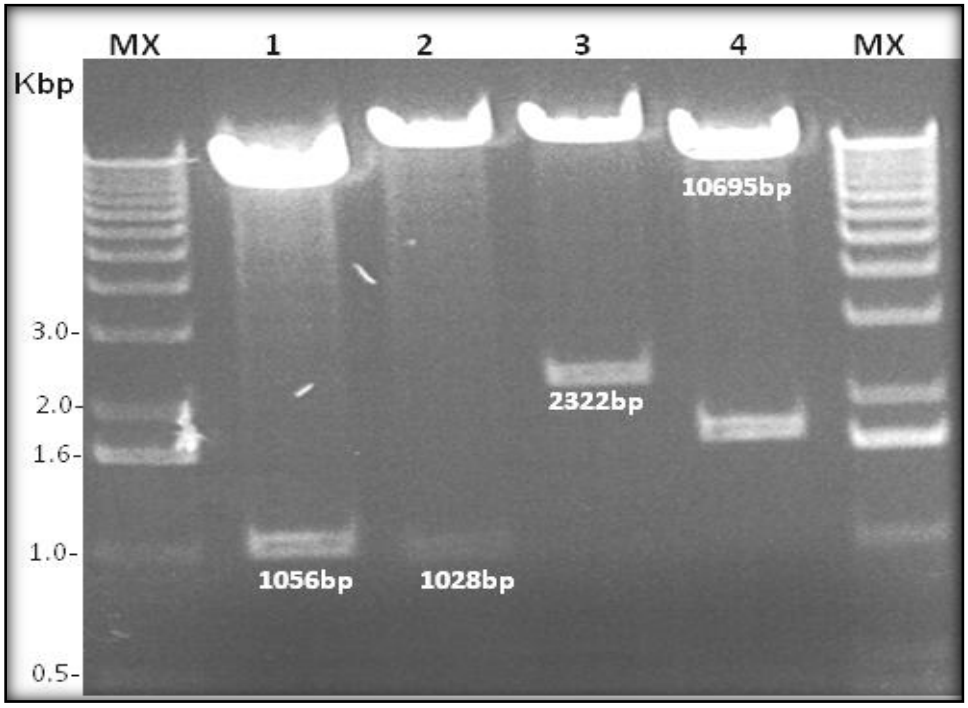


Figure 5.1 Agarose gel electrophoresis of restriction digests of parent vectors to separate insert DNA fragments. Lane **1**. *BanFer*1-nos (1056 bp), **2**. *SoyFer*-nos (1028 bp), **3**. GUS-nos (2322 bp), **4**. pCAM-ZmUbi--35S-*nptII*-35S (10695 bp). **MX**= Molecular weight marke**r (**Roche)

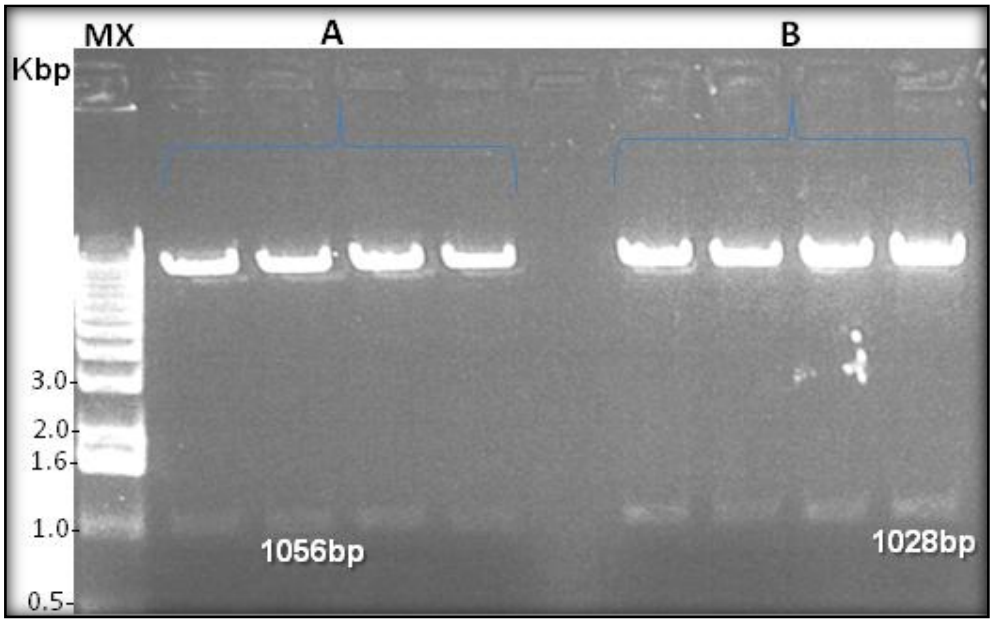


Figure 5.2 Confirmatory restriction digestion of pDNA of expression vectors to confirm the presence of cloned gene fragments. The agarose gel shows products of *Bam*H1-*Eco*R1 double digestion for **A.** pCAM-ZmUbi-*BanFer*1-nos and **B.** pCAM-ZmUbi-*SoyFer*-nos. The lower bands represent *BanFer*1-nos (1056 bp) and *SoyFer*-nos (1028 bp). **MX** = Molecular weight marker (Roche).

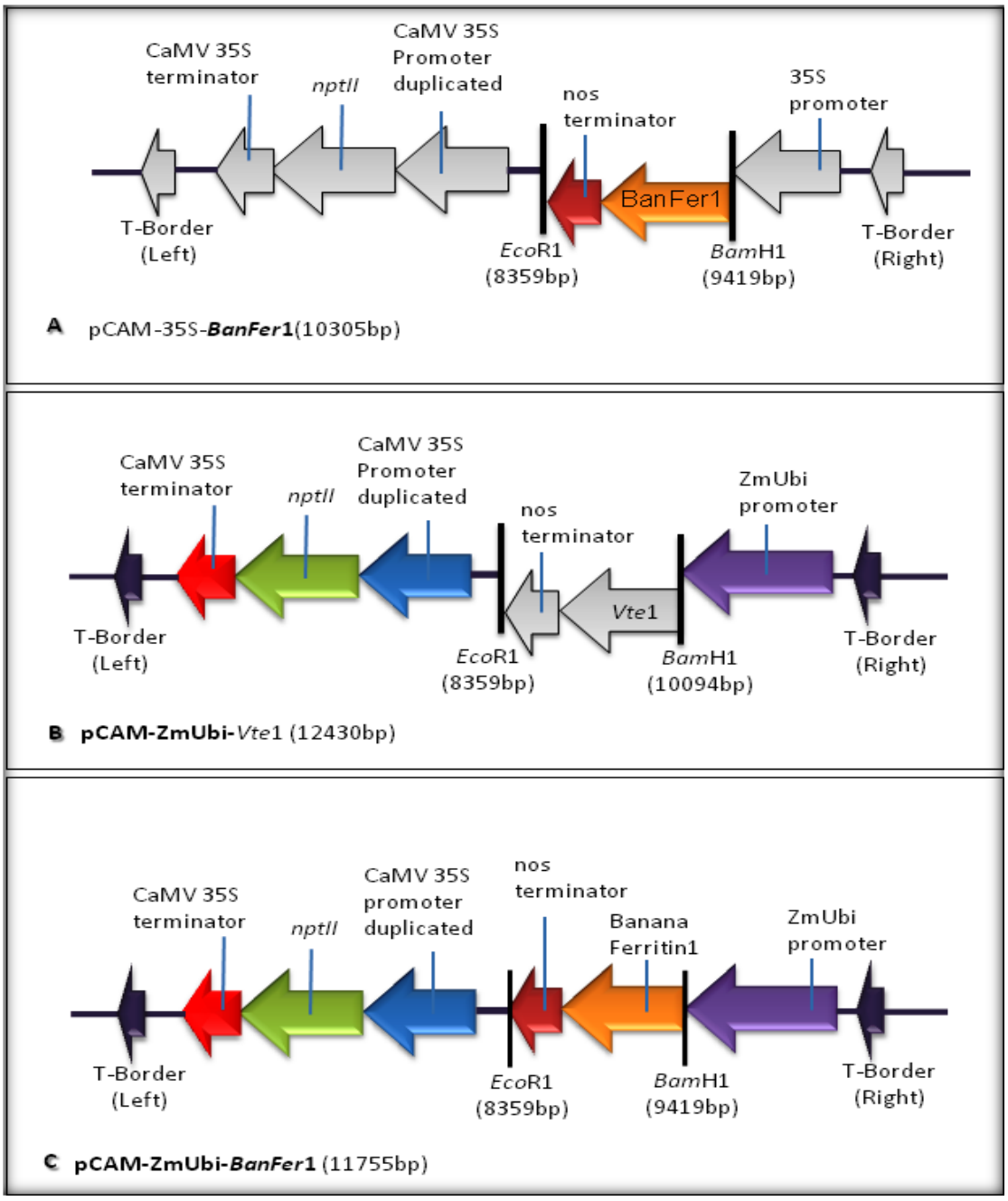


Figure 5.3 Schematic representation of the generation of the binary expression vector, pCAM-ZmUbi-BanFer1. The *BanFer*1-nos fragment was isolated from its parent vector (**A)** by *Bam*HI-*Eco*R1 double-digestion and ligated into the corresponding *Bam*HI-*Eco*R1 sites of the backbone vector (**B)** to generate the final expression vector (**C**) pCAM-ZmUbi-*BanFer*1 used to transform rice callus. The required fragments from parent vectors **A** and **B** are highlighted in colour. The T-DNA is located in between the left and right borders.

5.3.2 Selection and multiplication of rice callus

A total of 208 rice callus clusters were each transformed with pCAMZmUbi-*BanFer*1 and pCAMZmUbi-*SoyFer*, while 182 callus clusters were transformed with pCAMZmUbi-GUS as a transformation control. Additionally, 52 callus clusters were included as non-transformed selection controls while 48 non-transformed callus clusters were also included as regeneration controls. After 6 wks on selection media, none of the 52 non-transformed callus clusters showed any signs of proliferation (Fig 5.4). In contrast, 21 of the 48 (43.8%) non-transformed callus clusters placed on non-selective media as transformation controls enlarged in size and began proliferating, and these were pooled and multiplied together. The transformation efficiency of embryogenic rice callus using the *BanFer*1, *SoyFer* and GUS transgenes ranged from 8.2 to 17% (Table 5.3; Fig 5.4). An individual antibiotic resistant/proliferating callus cluster was treated as an independent putatively transformed callus line. Clumps in each cluster (line) were separated into smaller pieces, placed on fresh 2N6S media to (i) multiply and increase in quantity or (ii) select against any clumps that may have been escapes. To verify the success of the transformation procedure, rice callus transformed with the GUS reporter gene was subjected to histochemical GUS assays. All the proliferating, antibiotic-resistant callus clusters stained positive for GUS expression (Fig 5.5).

A selection of proliferating callus lines was chosen for further analysis including 12, 11 and 3 lines transformed with *BanFer*1, *SoyFer* and GUS, respectively. Clumps from each line were pooled and multiplied in liquid 2N6S media. Although all of these callus clusters were available for analysis of transgene expression, the proliferation rate of some callus lines declined over time such that the number of lines with sufficient amount of callus for iron analyses was only 9 for *BanFer*1, 11 for *SoyFer* and 2 for GUS.

Table 5.3 Rice embryogenic callus transformed with *Agrobacterium* and their transformation efficiency

Treatment	Number of callus clumps transformed	Number of callus clusters (lines) proliferating after 6 wks on selection media	Transformation efficiency (%)
pCAMZmUbi-*BanFer*1	208	17	8.2
pCAMZmUbi-*SoyFer*	208	33	15.9
pCAMZmUbi-GUS	182	31	17.0
NT selection control	52	0	0

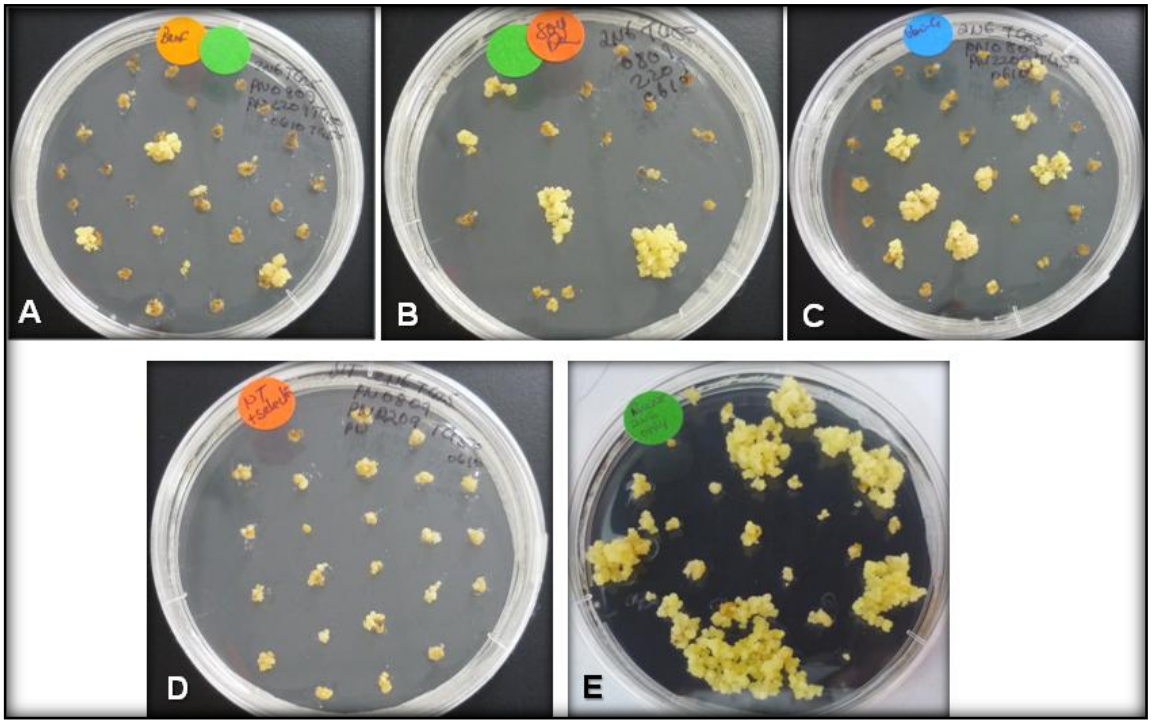


Figure 5.4 Selection of rice callus transformed with ferritin genes under the control of the maize poly-ubiquitin promoter. Plates show callus transformed with **A**. pCAM-ZmUbi-*BanFer*1, **B**. pCAM-ZmUbi-*SoyFer* **C.** pCAM-ZmUbi-GUS, **D**. non-transformed callus on 2N6S containing 50 mgL^{-1} geneticin, and **E.** non-transformed callus on 2N6 without selection after 6 wks.

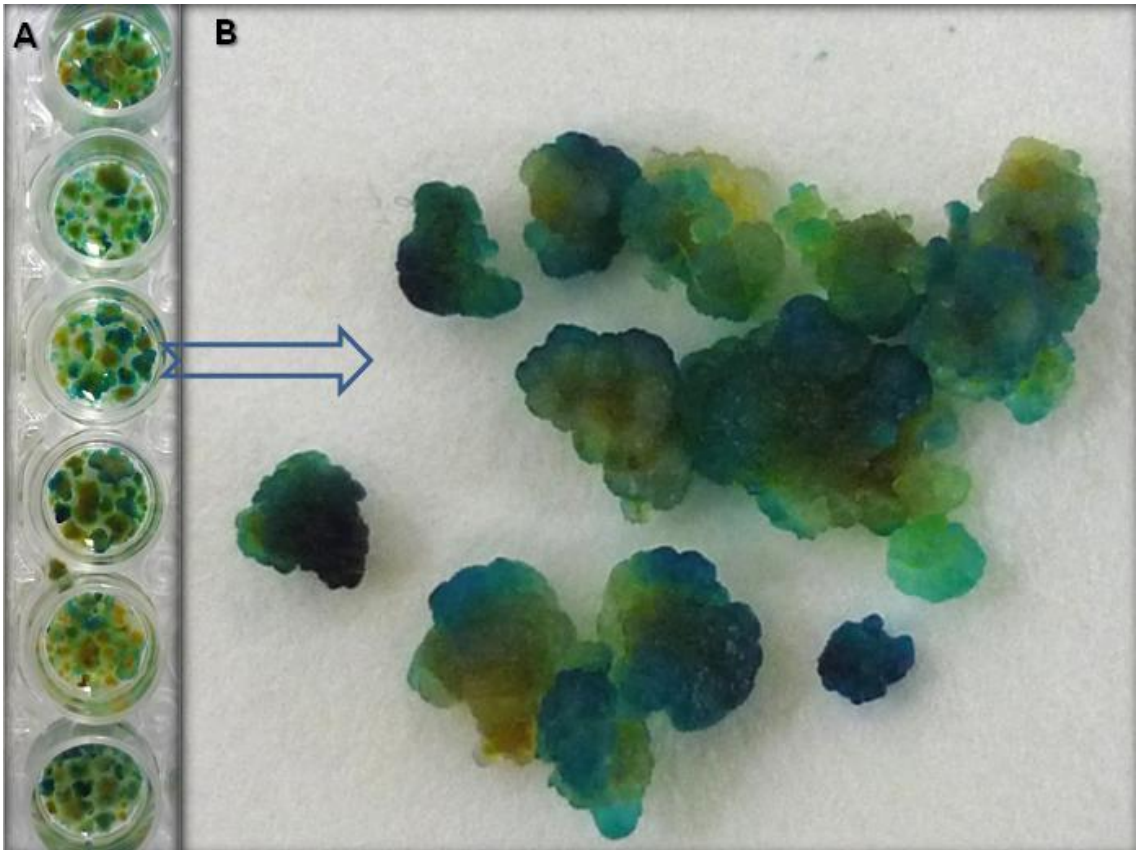


Figure 5.5 Histochemical assay of rice callus transformed with the GUS gene under the control of the maize poly-ubiquitin promoter. The left hand panel (**A**) shows representatives of transformed callus lines and **B.** shows a close up of stained callus.

5.3.4 Analysis of transgene expression

To determine whether the ferritin genes were transcribed in the proliferating callus, total RNA was extracted from each of the callus lines independently transformed with *BanFer*1 (12 lines) or *SoyFer* (11 lines) and was analysed by reverse transcriptase (RT)-PCR using transgene-specific primers (Table 5.2). Bands of the expected size of 810 bp and 562 bp were amplified from each of the callus lines transformed with *BanFer*1 and *SoyFer*, respectively (Fig 5.6 and 5.7). The absence of amplicons from DNase-treated RNA and RNA extracted from non-transformed callus, confirmed the specificity of the RT-PCR.

5.3.5 Pearl's Prussian blue staining for iron in the rice callus

Pearl's Prussian blue staining technique was used to demonstrate the presence of stored ferric iron (Fe^{3+}) in transgenic callus. When callus from four *SoyFer*- and six *BanFer*1-transgenic callus lines and one non-transformed line were stained, the intensity of blue colour was much stronger in all transgenic callus compared to non-transformed tissue illustrating that rice callus transformed with ferritin accumulated ferric iron (Fig 5.8). Further, the intensity of Prussian blue colour in rice callus transformed with the *BanFer*1 was stronger compared to *SoyFer,* suggesting that *BanFer*1 led to accumulation of more iron stores than *SoyFer*.

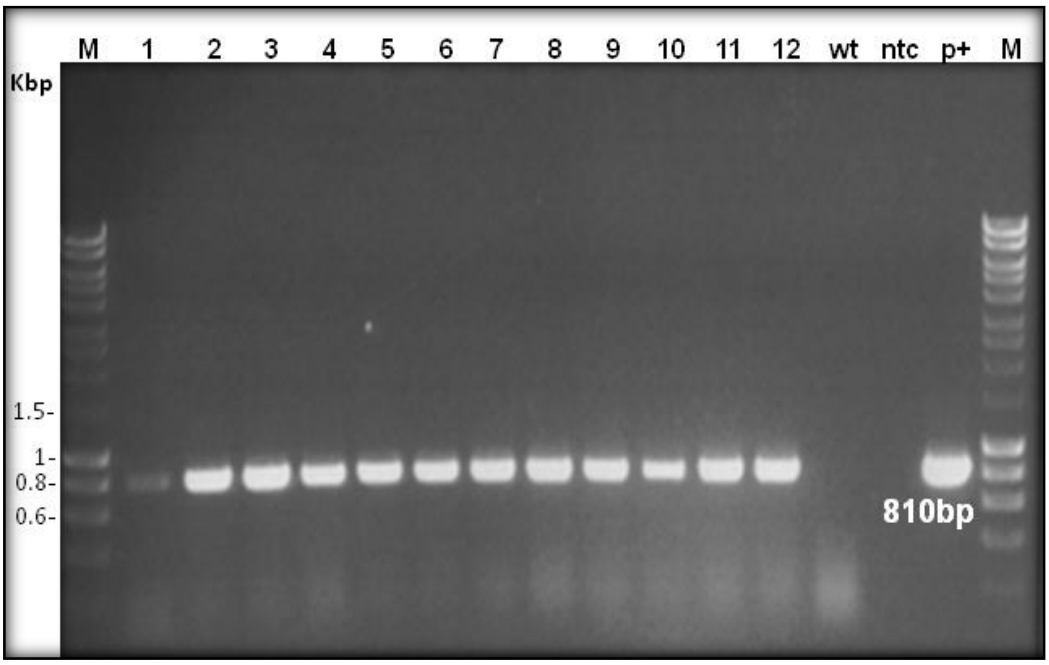


Figure 5.6 RT-PCR analysis for the expression of BanFer1 in transgenic rice callus. **1-12** represents the different callus lines transformed with pCAM-ZmUbi-*BanFer*1, **wt** = wild-type negative control, **ntc** = no template control, **p+** = plasmid DNA positive control (pCAM-ZmUbi-*BanFer*1), **M** = molecular weight marker (Hyperladder-Bioline).

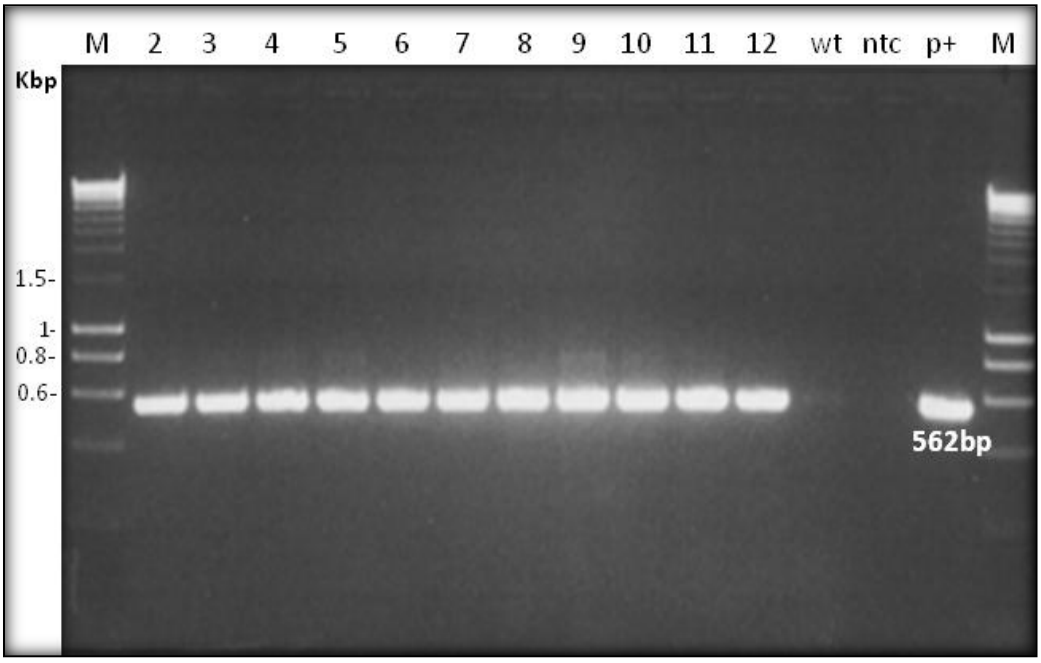


Figure 5.7 RT-PCR analysis for the expression of SoyFer in transgenic rice callus. 2-12 represents the different callus lines transformed with pCAM-ZmUbi-*SoyFer;* **wt** = wild-type negative control, **ntc** = no template control, **p+** = plasmid DNA positive control (pCAM-ZmUbi-*SoyFer*), **M** = molecular weight marker (Hyperladder-Bioline).

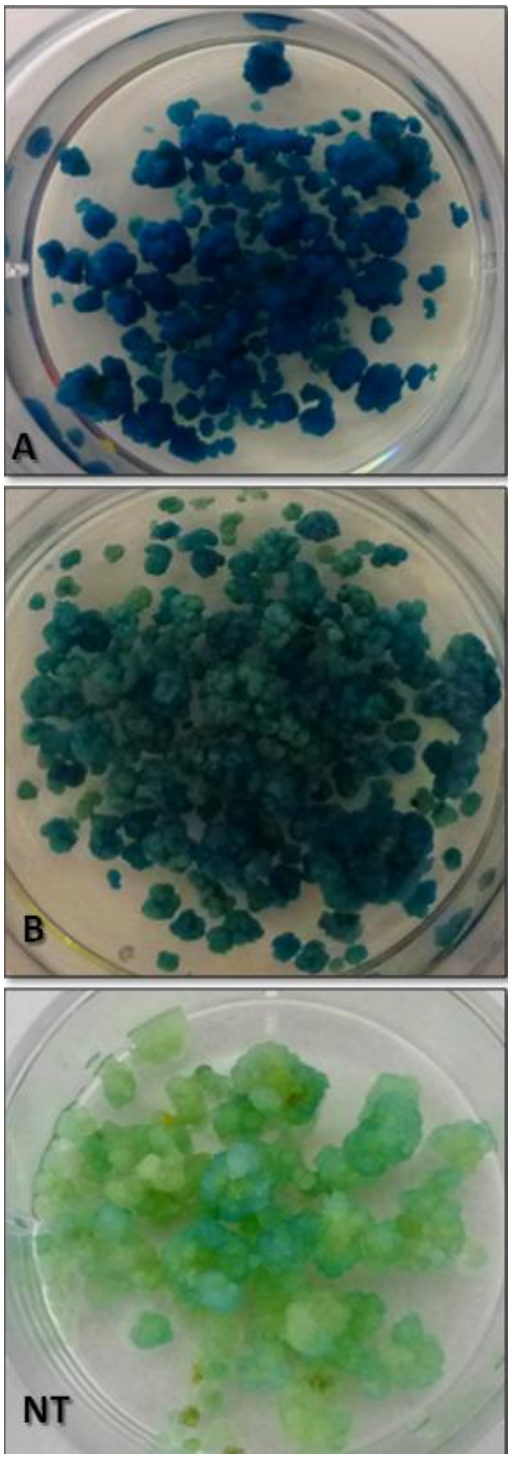


Figure 5.8 Pearl's Prussian blue staining of transgenic rice callus for ferric iron. The representative stained callus shows *BanFer*1- line 6 (**A**); *SoyFer*- line 21 (**B)** and the non-transformed control **(NT**).

5.3.5 Analysis of iron content in rice callus

To determine iron content in rice callus, extracts of iron from nine lines of callus expressing *BanFer*1, 11 lines expressing *SoyFer*, two lines expressing GUS and one line of non-transformed callus were analysed by FAAS. The mean iron content in test samples was determined from two FAAS measurements of one iron extract per callus line. The mean iron content in non-transformed callus was 39.3 mg Fe/kg dwt. In the nine transformed lines of rice callus expressing *BanFer*1, the mean iron content ranged from 77.9 to 1179.5 mg Fe/kg dwt of callus while in callus expressing *SoyFer*, iron content ranged from 40.5 to 555.9 mg Fe/kg dwt of callus (Fig 5.9). Using the *BanFer*1 transgene, the highest iron content obtained (line B1) was 30 times higher than that obtained from non-transformed callus and 2.1 times higher than that obtained using the *SoyFer* transgene. In contrast, the highest iron content obtained by over-expressing *SoyFer* was only 14.2 times higher (line S18) than that found in non-transformed callus. When the results were subjected to ANOVA, the amount of iron in 77.8% (seven out of nine) of samples expressing *BanFer*1 and 27.3 % (three out of eleven) of samples expressing *SoyFer* had a significantly higher ($p \leq 0.05$) iron content than the non-transformed controls, while the amount of iron in the GUS controls was not significantly different ($p \leq 0.05$) from that in the non-transformed controls. These observations were consistent with qualitative assays using Prussian blue staining where the intensity of the blue colour from callus transformed with *BanFer*1 was stronger than that observed with *SoyFer.*

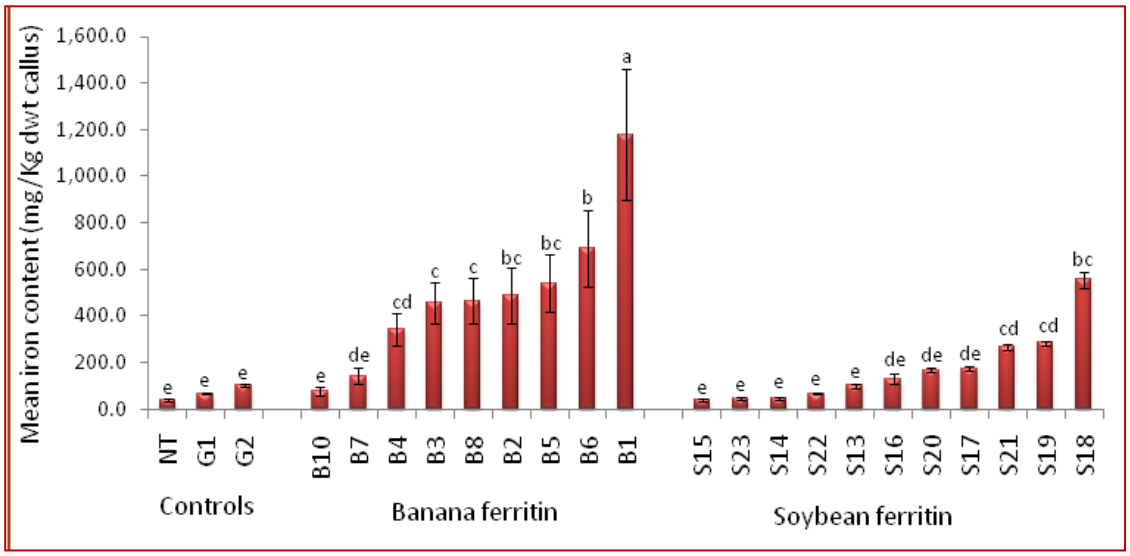


Figure 5.9 Comparison of iron content in transgenic rice callus expressing BanFer1 and SoyFer. Each bar represents the mean iron content in pooled callus which was multiplied from one transgenic callus line. Error bars represent standard error (n=2 or n=4 for NT) **NT** = non-transformed, **G** = GUS, **B** = *BanFer*1, **S** = *SoyFer*; letters and numbers on the X-axis represent the *in-vitro* ID of each callus line.

5.4 Discussion

In this study, the function of a banana ferritin (*BanFer*1) gene was demonstrated in transgenic rice callus by over-expressing *BanFer*1 under the control of the constitutive maize poly-ubiquitin promoter. The iron content of transgenic rice callus transformed with *BanFer*1 and the *SoyFer* was significantly higher than that observed in non-transformed callus, with up to 30-fold and 14-fold increases, respectively. In addition, these results showed that the constitutive expression of banana-derived ferritin led to an accumulation of high levels of iron content in rice callus and was significantly higher than that obtained from the expression of the soybean-derived ferritin. In previous reports, *SoyFer* has been constitutively expressed in rice (Drakakaki *et al.,* 2000) and in tobacco (Goto *et al.,* 1998) leading to a 2-fold and 1.3-fold increase in iron content in leaves of respective transgenic plants. *SoyFer* has also been over-expressed in rice seed under the control of a seed-specific promoter, leading to a 2-3-fold increase in iron content (Goto *et al.,* 1999; Vasconcelos *et al.,* 2003).

The detection of ferritin-specific transcripts in transgenic callus by reverse transcriptase (RT)-PCR suggested that the observed increase in iron accumulation was a result of ferritin synthesis. Unfortunately, ferritin antibodies were not available for use in this study which precluded the quantification of ferritin levels by serological assays. Consequently, it was not possible to directly measure ferritin protein levels and how they may have contributed to the observed increase in iron content. Due to the unavailability of ferritin antibodies, Prussian blue staining was used to qualitatively analyse the iron content in transgenic callus. Iron is stored in ferritin as Fe^{3+} in a crystalline molecule made of oxygen ions and a phosphate group, which is responsible for binding minerals to the residues on the inside of the ferritin protein shell. When treated with potassium ferrocyanide in acidic condition, any ferric ion present in cells stains a characteristic blue colour (also called Prussian blue or ferric ferrocyanide). In this study, all *BanFer*1- and *SoyFer*-transgenic rice callus lines stained blue after treatment with Prussian blue, while the non-transformed control only developed a light green/blue colour. Further, the intensity of the blue colour was much stronger in *BanFer*1-transgenic rice callus than that transformed with *SoyFer.* These observations were consistent with the quantitative iron analysis results obtained using FAAS thereby providing a correlation between the intensity of the blue colour and iron levels in

tissues. Therefore, Prussian blue staining has great potential as a screening tool useful for early monitoring of the expression of ferritin transgenes in transformed cells and embryos in future transgenic banana plant production. The observed differences in iron content suggest that tissues expressing *BanFer*1 accumulated more ferritin and, therefore, more iron stores. The reasons for this are unknown but may be due to differences between the respective iron-binding affinities of the *BanFer*1 or *SoyFer* genes in rice callus. It is also possible that, being a monocot-derived sequence, *BanFer*1 may be processed more efficiently than the dicot-derived *SoyFer* sequence in the monocot, rice. Further studies will be necessary to resolve these questions.

This study showed that the iron content in rice callus can be significantly increased by expressing *BanFer*1 under the control of the constitutive ZmUbi promoter. These results are consistent with previous reports which showed that increasing the capacity for iron storage in plants by over-expressing ferritin genes induces the activities of their iron uptake mechanism, resulting into enhanced iron content (Van Wuytswinkel *et al.,* 1998; Goto and Yoshihara 2001; Curie and Briat 2003). As such, *BanFer*1 represents a potentially useful gene for the enhancement of iron uptake and accumulation into staple food crops such as cooking bananas, which have low levels of iron. By increasing the capacity for iron storage in these crops, iron uptake mechanisms may also be activated or up-regulated which may enable the plant to extract more iron from the soil. Activation of the iron uptake/transport systems and subsequent accumulation of iron in leaves as a result of constitutive over-expression of ferritin was observed in tobacco, a dicotyledonous plant which utilises the strategy I mechanism for iron acquisition from the soil (Van Wuytswinkel *et al.,* 1998; Curie and Briat 2003). In a complementary study at QUT, it has been established through *in-vitro* assays that banana also utilises strategy I for the uptake of iron (Ms. Ada Rosier, PhD Thesis in preparation), and therefore may behave similarly if transformed to over-express ferritin genes. However, it is desirable that accumulation of iron occurs in the edible plant parts. Therefore, a suitable strategy would be the expression of banana ferritin under the control of a fruit-specific promoter to specifically increase iron stores in the edible fruit.

The generation of iron-enhanced, stably transformed cooking bananas using a banana-derived promoter, such as MaExp1 promoter reported in the previous Chapter, controlling the expression of a banana-derived transgene gene offers several advantages over using non-plant derived sequences. Firstly, it would contribute to the understanding of the mechanisms of iron transport, translocation and accumulation in banana plants. Moreover, plant-derived genes are desirable for production of transgenic food crops especially with regard to regulatory perspectives, public acceptance and also preserving the genetic background of selected varieties (Poletti and Sautter 2005). Therefore, introducing *BanFer*1 into low-iron cooking banana cultivar, preferably using a fruit-specific banana-derived promoter, may be a viable strategy for the production of transgenic banana plants with iron-enhanced fruit. The potential of iron-rich foods has been demonstrated for rice (Lucca *et al.,* 2002; Vasconcelos *et al.,* 2003) with potential economic benefits. Therefore, staple cooking banana varieties that are rich in iron content represent an economical target for iron fortification in a normal banana diet. One of the limiting factors for genetic engineering is the number and diversity of available genes for specific traits (Poletti and Sautter 2005). Therefore, the potential of *BanFer*1 demonstrated in this chapter is a contribution towards the improvement of iron content in staple banana varieties by increasing the number of known functional genes that can be utilised.

In summary, the function of the banana ferritin gene and its potential for increasing iron content was demonstrated in rice callus. Both qualitative (Prussian blue) and quantitative (FAAS) analysis showed increased iron stores in transgenic callus compared to non-transformed controls. Overall, this work provides preliminary evidence of the function of the banana ferritin gene. However, due to time constraints, iron accumulation was only analysed at the callus stage. Therefore, further work remains to be done to determine whether the iron accumulated in rice callus as a result of ferritin expression is maintained in stably transformed plants, particularly, in the edible plant part.

Chapter 6 General Discussion and Conclusions

A diet that contains sufficient essential macro- and micro-nutrients represents the primary contribution to human health and growth. Some of the essential micronutrients include vitamin A, E and folate, as well as mineral elements such as iodine, iron, zinc and selenium (Poletti and Sautter 2005; Gómez-Galera *et al.,* 2010). Micronutrients are, however, often not available in sufficient amounts in many foods and a diet that is solely based on a single staple food crop usually results in chronic dietary deficiencies. Two of the major deficiencies are VAD and IDA, which affect both developing and developed countries (WHO 2003). Unfortunately, the majority of people who suffer from the lack of essential vitamins and minerals in their diet are not aware that their diets lack such nutrients and, therefore, they do not associate diet deficiencies with frequent illnesses such as defective eye sight, impaired physical growth or learning capacity or high mortality. As a result, the problem of micronutrient malnutrition has been described as the hidden hunger (WHO 2003; Bouis *et al.,* 2003b) and remains a major cause of ill health worldwide.

For various reasons, including reliance on adequate/sustained funding, efficient health and distribution system, technical limitations, affordability and client compliance, micronutrient supplementation and fortification are not always appropriate strategies, especially in rural communities such as those where farmers depend on a staple crop (Bouis *et al.,* 2003b; Zhu *et al.,* 2007; Zimmermann and Hurrell 2007). Conventional breeding to improve the qualities of staple crops, such as the East African highland bananas, is often difficult due to low fertility and low embryo germination levels (Ssebuliba *et al.,* 2006). In addition, bananas have a long cropping cycle, are parthenocarpic and vegetatively propagated, characteristics that make the most popular banana cultivars difficult to cross breed. In many cases, biotechnology has the potential to overcome such breeding limitations, making it possible to isolate genes from species where the conventional breeding approach is not possible and use them to improve specific traits through genetic engineering. Biofortification through gene manipulation is one such strategy that can be used to directly improve the

micronutrient content of crop plants (Zhu *et al.,* 2007), and can potentially provide cost effective and sustainable benefits (Meenakshi *et al.,* 2010). Therefore, biofortification of a staple food crop would ensure that the much needed micronutrients are available for consumption by vulnerable populations. An example of the successful enhancement of micronutrient content in a staple crop through genetic engineering is the development of the Golden Rice. In this case, rice seed, which ordinarily produces no carotenoids, was genetically modified to synthesise enhanced levels of pVA carotenoids (Ye *et al.,* 2000; Paine *et al.,* 2005). Existing simulation studies investigating the health and economic benefits of Golden Rice in women and children have projected that biofortified rice has the potential to alleviate VAD (Zimmermann and Qaim 2004; Stein *et al.,* 2008). These findings provide promise, particularly to the people in South East Asia who depend on rice for a staple diet. The availability of well characterised and suitable genes, tissue specific promoters and transformation technologies played a vital role in the successful development of the Golden Rice.

The banana biofortification project supported by the Bill and Melinda Gates Foundation under the Grand Challenges in Global Health Program (GCGH) was initiated with the ultimate aim to address the chronic micronutrient malnutrition in Uganda through transgenic biofortification of the major staple food, East African Highland bananas. This project is being implemented as collaboration between Queensland University of Technology (QUT), Australia, and the National Agricultural Research Organization (NARO), Uganda, and specifically aims at improving the nutritional status in Uganda and surrounding countries through the generation of farmer- and consumer-acceptable edible banana cultivars with significantly increased levels of pro-vitamin A and iron in the fruit. However, one of the limitations to the application of genetic engineering in food crops is the number of suitable genes available for a specific trait (Poletti and Sautter 2005). To address this gap, one of the activities of the banana biofortification project at QUT has been the isolation and testing of a range of genes and promoters. The research undertaken in this study focussed on evaluating the function of selected banana-derived genes and promoters towards the biofortification of cooking bananas for enhanced pVA carotenoids and iron content. The use of native genes and promoters is a desirable strategy to ensure transgene compatibility with the crop's

genetic makeup and therefore preserving the genetic integrity of transformed cultivars. Furthermore, due to stringent regulations governing the screening and use of GMOs, and issues associated with public acceptance of GM crops worldwide (Myhr and Traavik 2002; Van den Belt and Gremmen 2002; Varzakas *et al.,* 2007), the use of plant-derived genes to improve traits in food crops would be less controversial and more ethically acceptable than the use of genes derived from non-plant sources.

One important initial outcome of this research was the demonstration that the banana phytoene synthase gene (*APsy*2a) was functional, with the constitutive over-expression of this transgene leading to increased levels of pVA carotenoids in transgenic rice callus (Chapter 3). By over-expressing *APsy*2a alone, very high levels of β-carotene were obtained in rice callus tissue that otherwise contains negligible carotenoid content. It was therefore postulated that, in tissues where the pVA biosynthetic pathway is functionally complete and carotenoids are accumulated such as the banana fruit, the over-expression of *Psy* alone may be sufficient to boost pVA carotenoid content. Biofortification of banana fruit will, however, require the use of a fruit-specific promoter since constitutive over-expression of both the banana and maize-derived phytoene synthase genes in rice callus led to phenotypic abnormalities, similar to those reported in tobacco (Busch *et al.,* 2002) and tomato (Fray *et al.,* 1995). The use of fruit-specific promoters for the expression of *Psy* in banana will be required to allow normal growth of transgenic plants, while targeting transgene expression in the fruit where the accumulation of pVA carotenoid is needed. Although the banana fruit is known to accumulate pVA carotenoids, (Holden *et al.,* 1999; USDA 2005; Wall 2006; Davey *et al.,* 2009), the content in most cultivated banana cultivars is too low to meet the RDA in a normal diet. For the banana biofortification project, the target pVA content for the EAHB is a 4-fold increase which is expected to provide up to 50% of the vitamin A RDA for children. Based on the results obtained from Chapter 3, it is feasible that, using an appropriate fruit-specific promoter, pVA biosynthesis in banana cultivars that have low levels of pVA carotenoids could be improved by over-expressing the *Psy* gene alone.

The activity of several banana-derived promoters in mature green banana fruit was also characterised in this study, with the MaExp1, MaExp1a and MaACO promoters showing fruit-activity and directing high levels of GUS expression (Chapter 4). Therefore, the

MaExp1 and MaACO promoters appear to be potentially useful as "fruit-specific" promoters for transgene expression in banana. Fruit-specific expression of the *Psy* gene is required, not only to direct synthesis of pVA carotenoids in fruit, but also to enable the regeneration and growth of phenotypically normal plants. When the activity of the fruit-active MaExp1 and MaACO promoters was analysed in leaves of stably transformed banana plants, results showed that their activity was very low in leaf tissue and these characteristics makes these promoters attractive for transgene expression in banana fruits. Therefore, future work for the generation of banana transgenic plants for enhanced fruit quality should utilise MaExp1 and MaACO promoters driving the target transgenes. In transgenic banana expressing Psy, a complete analysis for phenotypic characteristics during the vegetative stage and the quantification of pVA carotenoids in the fruit should be done.

Since the ultimate goal is to enhance cooking bananas which are utilised before ripening, mature green fruit was used to analyse the promoter activity. However, enhancement of micronutrients may be expanded to include other economically important dessert bananas such as the popular introduced dessert banana cultivar, Sukali Ndizi. To do this, further research is needed to investigate the promoter activity at pre- and post-ripening to compare activity at the two stages of fruit maturation. In addition, carotenoid content at the pre- and post-ripening stage of fruit development could be quantified in order to establish correlations between peak promoter activity and the timing of *Psy* expression in banana fruit and therefore carotenoid accumulation. In a related study, (Mlalazi 2010) recommended future quantitative analysis of *Psy* transcription during fruit ripening. Such studies have been done during tomato fruit development (Fraser *et al.,* 1994) and during the development of the maize endosperm (Vallabhaneni and Wurtzel 2009). In maize, specific studies to establish the timing of gene expression in relation to carotenoid content demonstrated a positive correlation between *Psy* transcript levels and endosperm carotenoid content at specific stages of development (Vallabhaneni and Wurtzel 2009). These reports show that the timing of gene expression is vital when investigating the pVA biosynthesis. In banana fruit, therefore, such studies would provide correlations upon which predictive future strategies for improvement of pVA carotenoid content in banana could be made.

This research also showed that a banana-derived ferritin gene was functional in rice callus resulting in the accumulation of high levels of iron (Chapter 5). However, the results must be treated with caution, since the function of banana ferritin was determined *in-vitro* where the dynamics of iron uptake in rice callus, with readily available iron in media, may be different compared to iron uptake interactions in whole plants in the soil environment. Ultimately, future work needs to be carried out to assess the accumulation of iron, using fruit-specific promoters, in the fruit of stably transformed banana plants under field conditions. In addition, although research at QUT has indicated that banana utilises a strategy I mechanism for iron uptake (Rosier – PhD thesis in preparation), the mechanisms of iron transportation and storage in a banana plant are not well understood. Therefore, a functional native ferritin gene could be exploited for future research into understanding iron homeostasis in banana and ultimately improving the storage capacity of the fruit. One of the concerns regarding iron biofortification in food crops is the bioavailability of ferritin-bound iron. Reported bioavailability studies that were conducted in non-anaemic and anaemic women, showed that animal-derived ferritin (Davila-Hicks *et al.,* 2004) and plant (soybean)-derived ferritin (Lonnerdal *et al.,* 2006) were equally absorbed as ferrous sulphate – a highly bioavailable source of iron. Therefore, ferritin may serve as an efficient delivery medium for iron from biofortified staple foods. To complement iron biofortification strategies, a universal ELISA-based method for measuring ferritin concentrations in food crops has been developed (Lukac *et al.,* 2009). These methods will facilitate future rapid screening of ferritin-rich plants.

The outputs from this research make a significant contribution towards efforts to enhance micronutrient intake in communities that depend on bananas, such as the East African highland bananas in Uganda, as staple food. The development of biofortified food crops, however, goes beyond biotechnology research in the laboratory, and extends into investigating areas such as postharvest processing, nutrient bioavailability and retention, consumer acceptability, dietary intake, efficacy and large scale dissemination of the biofortified crop and the effectiveness of the biofortification strategy in a specific target country (Boy and Miloff 2009). The Ugandan Government

has put in place institutions such as the Uganda National Council for Science and Technology (UNCST) with a *mandate* to develop, coordinate and implement policies and strategies for integrating science and technology into the national development policies (www.uncst.go.ug). Through the UNCST, guidelines that support the use of biotechnology to facilitate in-country generation of genetically modified crops for Uganda have been developed. Additionally, a National Biotechnology and Biosafety policy was approved by the Uganda Government in 2008 (GOU 2008). A draft bill to put in place a law to regulate application of biotechnology and associated biosafety is before the Parliament of the government of Uganda for approval.

The EAHB remains Uganda's main staple food crop followed by sweet potatoes, cassava and maize. It is consumed both in rural and urban communities (UBOS 2000) and, as such, represents an appropriate target for biofortification. However, the successful adoption and use of any micronutrient-enhanced GM bananas in Uganda is likely to be dependent on several factors. Firstly, consumers have preferences for taste, appearance and textural quality of cooked bananas and it is not known if enhanced iron levels will alter these characteristics. This will obviously need to be examined once biofortified fruit has been generated. Increased levels of pVA carotenoids, however, will likely turn the colour of the fruit pulp from the pale yellow to darker yellow. It may be prudent, therefore, to establish nutrition education programs to encourage farmers and consumers to adopt the banana cultivars with nutrient enhanced traits. In Uganda, orange-fleshed sweet potatoes (OFSP) cultivars with enhanced pVA carotenoids have been successfully bred by conventional means, introduced and adopted by farmers in the existing agricultural system (Potts and Nagujja 2007). Some of the reasons put forward for accepting the new varieties were their good yielding capacity and the appealing flesh colour which adult farmers and children associated with improved nutrient content (Tumwegamire *et al.,* 2007). The successful education campaign adopted for the OFSP cultivars in Uganda may also be useful as an incentive for the acceptance of "orange-fleshed" pVA enriched banana varieties in the future.

Another consideration for the successful development of pVA-enriched EAHB is the fact that carotenoids are liable to degradation after harvesting, during storage or during

thermal processing (Rodriguez-Amaya 2004). Similar to cooking bananas, sweet potatoes are commonly steamed or boiled prior to eating. Studies investigating the retention and bioavailability of pVA carotenoids, in particular β-carotene, in OFSP showed that between 33-75% is retained and bioavailable after boiling, steaming or frying (Boy and Miloff 2009; Tumuhimbise *et al.,* 2009). There will need to be similar studies with genetically modified bananas.

Finally, improved banana cultivars such as those modified with traits for enhancing micronutrient content will need to be multiplied and distributed to the farmers. Tissue culture is the internationally accepted method for the movement of *Musa* germplasm (Diekmann and Putter 1996). In East Africa, national and commercial tissue culture laboratories produce and distribute tissue cultured banana planting materials to farmers nationally and interstate thus reducing the possible spread of diseased planting material. In Uganda, the NARO's banana research program has established effective means for dissemination of tissue culture-derived banana plants, both introduced hybrids and local cultivars, through farmer groups, government extension services and NGOs (Kikule *et al.,* 2007). Although tissue-culture derived banana plants are clean from fungal diseases and pests, they need to be screened for viruses such as *Banana streak virus* which is endemic in the region (Kubiriba *et al.,* 2001). For this reason, one of the major complementary activities of the Bill and Melinda Gates Foundation-funded banana biofortification project in East Africa is to develop reliable and sensitive diagnostic tests for banana viruses and to develop the capacity to undertake virus indexing so that banana plants generated and multiplied through tissue culture can be screened for viruses before they are distributed to farmers.

In summary, the outputs from this research project demonstrated that the banana-derived genes, *APsy*2a and *BanFer*1, are functional and therefore can be exploited to generate enhanced levels of pVA carotenoids and iron content in banana. Furthermore, out of a range of banana-derived fruit-related promoters transiently examined for transgene expression in banana fruit and in stably transformed plants, candidate fruit-active promoters were characterised. Ultimately, however, analysis of the fruit from field-grown transgenic banana plants will be required to fully evaluate the suitability of

these genes and promoters for banana fruit biofortification. As part of the banana biofortification project activities at QUT, approximately 1000 Cavendish bananas transformed with various pVA-biosynthesis genes and promoter combinations have been regenerated and planted in the field in North Queensland to assess the levels of micronutrients in fruit. The first fruit from these plants has recently been harvested and should provide an indication of the most appropriate promoter-gene combinations for future use. Uganda has a functional agricultural research infrastructure that would facilitate generation, analysis, release and distribution of biofortified bananas. Therefore, an interdisciplinary exploitation of available research tools is expected to have impact, not only for Uganda, but also the communities in neighbouring countries that depend of cooking bananas as a staple food crop.

Appendix 1

Stock solutions for plant tissue culture media

MS salts stock solutions (Murashige et al., 1962)	
MS Macronutrients stock	**Quantity**
NH_4NO_3	1.65 gL^{-1}
KNO_3	1.90 gL^{-1}
$CaCl_2 .2H_2O$	0.44 gL^{-1}
$MgSO_4 .7H_2O$	0.37 gL^{-1}
KH_2PO_4	0.17 gL^{-1}
MS Micronutrients stock	
$MnSO_4 .4H_2O$	2.230 mgL^{-1}
$ZnSO_4 .7H_2O$	0.860 mgL^{-1}
H_3BO_4	0.620 mgL^{-1}
KI	0.083 mgL^{-1}
$NaMoO_4 .2H_2O$	0.025 mgL^{-1}
$CuSO_4.5H_2O$	0.0025 mgL^{-1}
$CoCl_2 .6H_2O$	0.0025 mgL^{-1}
MS Fe-EDTA stock	
$Na_2EDTA .2H_2O$	37.24 mgL^{-1}
$FeSO_4 .7H_20$	27.80 mgL^{-1}
MS vitamin stock	
Myo-inositol	100.0 mgL^{-1}
Nicotinic acid	0.50 mgL^{-1}
Pyridoxine HCl	0.50 mgL^{-1}
Thiamine HCl	0.10 mgL^{-1}
Glycine	2.0 mgL^{-1}

CHU (N6) salts stock solutions (Modified from (Chu *et al.,* 1975)	
2N6 Macronutrients stock	**Quantity**
KNO_3	2.83 gL^{-1}
$(NH4)_2SO_4$	0.463 gL^{-1}
KH_2PO_4	0.40 gL^{-1}
$MgSO_4.7H_2O$	0.185 gL^{-1}
$CaCl_2.2H_2O$	0.166 gL^{-1}
Substituting Chemicals	
$CaCl_2$	0.125 gL^{-1}
$MgSO_4$	0.09 gL^{-1}
2N6 Micronutrients stock	
$MnSO_4.4H_2O$	0.0044 gL^{-1}
$H3BO_3$	0.00160 gL^{-1}
$ZnSO_4.7H_2O$	0.0015 gL^{-1}
KI	0.0008 gL^{-1}
2N6 Vitamin stock	
Myo-inositol	0.10 gL^{-1}
Glycine	2.0 mgL^{-1}
Thiamine-HCl	1.0 mgL^{-1}
Nicotinic acid	0.50 mgL^{-1}
Pyridoxine-HCl	0.50 mgL^{-1}
or Pyridoxine	0.41 mgL^{-1}

Stock solutions for antibiotics

Reagent	Concentration	Solvent
Ampicillin	100 $mgmL^{-1}$	de-ionised water
Carbenicillin	100 $mgmL^{-1}$	de-ionised water
Geneticin	50 $mgmL^{-1}$	de-ionised water
Hygromycin	50 $mgmL^{-1}$	pre-dissolved in PBS
Kanamycin	100 $mgmL^{-1}$	de-ionised water
Rifampicin	25 $mgmL^{-1}$	DMSO
Tetracycline	9.402 $mgmL^{-1}$	50% ethanol (v/v)
Timentin	200 $mgmL^{-1}$	de-ionised water

Stock solutions for plant growth regulators

Reagent	Concentration	Solvent
2, 4-D	1 $mgmL^{-1}$	Ethanol
2iP	0.1 $mgmL^{-1}$	1M NaOH
BAP	1 $mgmL^{-1}$	1M NaOH
Biotin	1 $mgmL^{-1}$	Ethanol
IAA	1 $mgmL^{-1}$	1M NaOH
Kinetin	1 $mgmL^{-1}$	1M NaOH
NAA	1 $mgmL^{-1}$	1M NaOH
Zeatin	0.5 $mgmL^{-1}$	1M NaOH

Other stock solutions

Reagent	Concentration	Solvent
Acetosyringone	19.62 $mgmL^{-1}$	in DMSO
Ascorbic acid	10 $mgmL^{-1}$	De-ionised water
K_2HPO_4	10% w/v	De-ionised water
KH_2PO_4	10% w/v	De-ionised water
$MgSO_4.7H_2O$	10% w/v	De-ionised water
NaCl	10% w/v	De-ionised water
Thiamine	10 $mgmL^{-1}$	De-ionised water

Composition of the media

Tissue culture media for Rice transformation

CHU N6 media for callus induction from rice mature seed was adopted from the rice transformation protocol according to Cambia as modified from (Chu *et al.,* 1975). Bacterial re-suspension and culture co-cultivation media was according to Khanna *et al.,* 2004.

2N6 Rice callus induction and multiplication media

1X 2N6 Macronutrients; 1X 2N6 Micronutrients; 1X Fe-EDTA; 1X 2N6 Vitamins; 30 gl^{-1} sucrose; 1.0 gl^{-1} casein enzymatic hydrolysate; 0.5 gL^{-1} proline; 0.5 gL^{-1} glutamine; 2 mgL^{-1} 2,4-D. pH was adjusted to 5.8, solidified with 2.5 gL^{-1} phytagel before autoclaving.

Bacterial re-suspension media (BRM)

1/10X MS Macronutrients; 1/10X MS Micronutrients; 1/10X Fe-EDTA; 1X MS vitamins; 0.4 gL^{-1} L-cysteine; 10 mgL^{-1} thiamine; 36 gL^{-1} glucose; 68.4 gL^{-1} sucrose; 100 µM acetosyringone; pH 5.2

Co-cultivation media (CCM)

1/10X MS Macronutrients; 1X MS Micronutrients; 1/10X Fe-EDTA; 1X MS vitamins; 0.4 gL^{-1} L-cysteine; 0.1 gL^{-1} glutamine; 0.1 gL^{-1} malt extract; 1.0 mgL^{-1} biotin; 10 gL^{-1} PVP; 10 mgL^{-1} ascorbic acid; 300 mgL^{-1} proline; 0.5 mgL^{-1} 2,4-D; 0.5 mgL^{-1} kinetin; 2.5 mgL^{-1} NAA; 30 gL^{-1} sucrose; 30 gL^{-1} maltose; 10 gL^{-1} glucose; 100 µM acetosyringone; 7.0 gL^{-1} agar; pH 5.3.

2N6-S Rice selection media

This was the same as callus multiplication media above but containing 200 mgL^{-1} Timentin and appropriate antibiotic selection agent (geneticin for *nptII* or hygromycin for *hptII*) at pH 5.2 and solidified with 8 gL^{-1} agar.

2N6-R Rice regeneration media

1X 2N6 Macronutrients; 1X 2N6 Micronutrients; 1X Fe-EDTA; 1x 2N6 Vitamins; 30 gL^{-1} sucrose; 0.5 gL^{-1} glutamine; 0.5 gL^{-1} proline; 0.3 gL^{-1} casein enzymatic hydrolysate; 3

mgL^{-1} BAP; 0.5 mgL^{-1} NAA; pH 5.8; 8 gL^{-1} agar; 200 mgL^{-1} Timentin; appropriate antibiotic selection agent.

1/2MS Rice rooting media

1/2X MS Macronutrients; 1/2X MS Micronutrients; 1X Fe-EDTA; 1X 2N6 vitamins; 10 gL^{-1} sucrose; 0.2 mgL^{-1} IAA; pH 5.8; 7 gL^{-1} agar (or 2.5 gL^{-1} phytagel); appropriate antibiotic selection agent.

MS media. 1X MS macronutrients; 1X MS micronutrients; 1X MS Fe-EDTA; 1X MS vitamins; 30 gL^{-1} sucrose; pH 5.8.

Bacterial media . (Sambrook and Russel 2001)

YM (Yeast Mannitol) media for growth of *Agrobacterium*

10 gL^{-1} mannitol; 0.4 gL^{-1} yeast extract; 0.1 gL^{-1} K_2HPO_4; 0.4 gL^{-1} KH_2PO_4; 0.1 gL^{-1} NaCl; 0.2 gL^{-1} $MgSO_4.7H_2O$; pH 6.8

LB [Luria Bertani] media for growth of *E.coli*

10 gL^{-1} tryptone; 5 gL^{-1} yeast extract; 5 gL^{-1} NaCl; pH 7.5.

LB(Agar) was solidified with 15 gL^{-1} agar

SOB

2% tryptone; 0.5% yeast extract; 5 gL^{-1} NaCl. These compounds were first dissolved in 990 mL of MilliQ water. Then 10 mL of 250 mM solution were added to media. pH was adjusted to 7.0 with 5 M NaOH before autoclaving at 121°C, 1.5 bars for 15 min. After autoclaving, 5 mL of 2 M $MgCl_2$ was added to the media. Note: To make 250 mM KCl solution, 1.86 g of KCl was dissolved in 100 mL MilliQ water. Similarly, 2 M $MgCl_2$ was prepared by dissolving 19 g in 100 mL of MilliQ water and sterilised by autoclaving.

SOC media

2% tryptone; 0.5% yeast extract; 5 gL^{-1} NaCl ; 10 mL of 250 mM KCl (was mixed together in 980 mL MilliQ water and pH adjusted to 7.0 then autoclaved for 15 min. Before use, 20 mM glucose was added (i.e. 20 mL of filter sterilised solution of 1 M glucose).

2x YT media. 16 gL^{-1} tryptone; 10 gL^{-1} yeast extract; 5 gL^{-1} NaCl. PH adjusted to 7.0 before autoclaving**.**

Source and storage of tissue culture chemicals

Tissue culture chemicals were obtained as cell culture tested grade from Sigma Aldrich and or PhytoTechnology Laboratories™. All macronutrient and micronutrient stock solutions were stored at 4°C while growth regulators, vitamins and antibiotics as well as Timentin were stored at -20°C. The PH for all tissue culture media was adjusted to 5.8 unless otherwise stated.

All media was autoclaved at 121°C, 1.5 bars for 15 min. Antibiotics and heat labile compounds were filter sterilised through 0.2 µM syringe filter (Acrodisc® syringe filter fitted with a Supor® membrane). All antibiotics and heat labile plant growth regulators were added to media after autoclaving at 55°C before dispensing media into culture vessels. Geneticin and Hygromycin were used at a concentration of 50 mg/l for selection against *npt*II and *hpt*II selection marker genes respectively.

Buffers

(Sambrook and Russel 2001)

Alkaline Lysis solutions for pDNA isolation

Solution 1: 100 mL. 0.9% Glucose; 0.025 M Tris-HCl (2.5 mL of 1.0 M stock, PH 8.0), 0.01 M EDTA (2.0 mL of 0.5 M stock, PH 8.0), top up with dH_20 to 100 mL

Solution 2: 10 mL. 0.2 M NaOH (2 mL of 1 M NaOH stock); 1% Sodium dodecyl sulphate (1 mL of 10% SDS solution); 7 mL dH_20. This solution was always prepared fresh before use.

Solution 3: 100 mL. 3 M Potassium acetate (29.44 g dissolved in 60 mL MilliQ water), 11.5% (11.5 mL) Glacial acetic acid, top up to 100 mL with MilliQ water.

Loading buffer (Bromophenol blue (BPB) dye – 10 mL (6X)

0.25% BPB (*25 mg*); 0.15 M EDTA pH 8.0 (*3 mL 0.5 M EDTA*); 30% glycerol(*3 mL glycerol*); top up to 10 mL with MilliQ water

0.8% CTAB buffer for DNA extraction – 100 mL

1.0 % sarcosine (N-lauryl sarcosyl); 0.8 M NaCl (*4.67 g*); 0.0022 M EDTA pH8.0 (*4.4 mL of 0.5 M stock*); 0.22 M Tris-HCl pH 7.8 (*22 mL of 1M stock*); 0.8% CTAB; 0.14 M Mannitol *(2.55 g*); 1.4% mercaptoethanol (14 μlmL^{-1}). NaCl was added after mixing all the other ingredients in solution [except 2-mercaptoethanol].

2% CTAB buffer for Rapid DNA extraction

2% CTAB (20 gL^{-1}); 0.1 M Tris-HCl (100 mL 1 M Tris-HCl pH 8.0); 0.05 M EDTA (100 mL 0.5 M EDTA pH 8.0); 1.4 M sodium chloride (81.7 $6gL^{-1}$); 0.08 M sodium sulphite (10 gL^{-1}); 20% PVP-40. (No β-mercaptoethanol is used with this buffer).

2% CTAB for RNA extraction buffer

2% CTAB (20 gL^{-1}); 2% PVP40 (20 gL^{-1}) ; 100 mM Tris HCl (pH 8); 25 mM EDTA; 2 M NaCl; 0.5 gL^{-1} spermidine (added after autoclaving); 2% β-mercaptoethanol (20 μlmL^{-1} added just before use). All components were mixed (except spermidine and mercaptoethanol), then 1 mL of DEPC (per litre of buffer mixture) was added to the buffer before autoclaving at 1.5 bars, 121°C, for 15 min (Chang *et al.,* 1993)

GUS extraction buffer (with Dowex1X2-400 Resin) - 500 mL

50 mM Na_2HPO_4 pH 7.0, 10 mM Na_2EDTA, 0.1% (v/v) SDS, 0.1% (v/v) Triton X-100, 500 nM DTT, 27.2 $mgmL^{-1}$ (dry weight) Dowex 1X2-400 resin.

[Note: Na_2HPO_4 (3.549 g) and Na_2EDTA (10 mL of 0.5 M stock at pH8.0) were mixed and topped up to 480 mL then pH was adjusted to 7.0 before adding 5 mL of 10% SDS and 5 mL of 10% Triton]. Buffer was then topped up to 500 mL. Prior to use, the required amount of GUS extraction buffer was decanted, then added DTT to a final concentration of 500 nM (7.72 mg DTT/100 mL buffer). In addition, to a required volume of GUS extraction buffer containing DTT, Dowex 1X2-400 resin was added to a

final concentration of 27.2 mg equivalent dry weight/mL. Dowex resin was added into buffer for sample extraction step only.

GUS stain (Histochemical stain) – 100 mL

0.1 M phosphate buffer (10 mL of 1M phosphate buffer stock); 880.5 mg Ascorbic acid—dissolve and adjust pH back to 7.1]; then 0.15% triton X-100 (1.5 mL), 50 mg of X-gluc (dissolved in dimethyl formamide).

4-Methylumbelliferone (4-MU) stock solution – 10 mL

4 mM 4-Methylumbelliferone Sodium Salt, 100 mM Sodium Acetate buffer pH 5.0, and 0.02% Sodium Azide.

4-Methylumbelliferone (4-MU) working solution – 100 mL

10 µM 4-Methylumbelliferone Sodium Salt, 100 mM Sodium Acetate buffer pH 5.0, and 0.02% Sodium Azide

1 M Phosphate buffer-100 mL

8.191 g Na_2HPO_4; 6.60 g NaH_2PO_4 (or 7.59 g $NaH_2PO4.H_2O$); pH7.0 with 5 M KOH. Autoclaved at 121°C, 15 min and stored at room temperature.

Stop buffer – 500 mL

1 M NaOH (20 g), 1 M Glycine (35.53 g). [This is a high pH buffer. MUG doesn't fluoresce at high pH hence the essence of using this to stop the reaction].

TAE buffer

4.84 gL^{-1} Tris; 1.14 mL glacial acetic acid; 0.372 gL^{-1} EDTA. PH 7.8 with glacial acetic acid.

TE buffer. 10 mM Tris-HCl (1211 mgL^{-1}, pH8.0); 1 mM EDTA (372.24 mg, pH 8.0)

Transformation buffer (TB) - 500 mL

10 mM Pipes (1.512 g) [or 10 mM Hepes (1.19 g)]; 15 mM $CaCl_2$ (0.832 g); 250 mM KCl (9.32 g); pH 6.7 with KOH. Then add 55 mM $MnCl_2$ (5.44 g). The mixture was sterilized by filtration through a 0.2 µm filter.

Appendix 2 Operating the flame atomic absorption spectrometry instrument for analysis of iron in plant tissue

Instrument specifications: SpectrAA220-FS, SPS-5

The FAAS instrumentation comprises four primary parts; the light source (hollow cathode lamp), the flame apparatus, the detector, and the data analysis system.

Operating Procedure

1. Switch on power to the Instrument. Switch on the auto-sampler SPS-5
2. Turn-on the inlet taps for air and acetylene on the mains.
3. Firmly fit the specific hollow cathode lamp in position [Varian, SpectrAA hollow cathode lamp for AAS producing a wavelength of 248.3 nm specific for iron]
4. Refresh the 'rinse container' with distilled water. Place standard solutions in the "standards rack position". Always include a blank i.e. 10% nitric acid in position 1 of the standards' rack.
5. Set up samples in the sample tubes on the sample rack, place test samples in the "samples rack position".
6. Connect the tubing that draws sample from the auto sampler to the instrument. This is connected to the nebuliser.
7. Open SpectrAA-**220 software** on computer connected to the spectrometer. Open Worksheet, choose **New worksheet** from the desk top, give the worksheet a name and associated details.
8. **Select Add methods** (this allows for selection of the metal of interest – select Iron. More elements can be added if desired).
9. **Go to "Develop", Choose "edit Methods"** form the menu. Set up parameters as follows:
    a. Under "standards' -- Input units – i.e. mgL^{-1} (default)
    b. Flame type; choose air acetylene (Note: Air flow is set by default for element selected in 8 above).
    c. Instrument mode absorbance – select absorbance
    d. Measurement – select 1% precision every 10 s [Note: Percentage precision is an indication of signal: noise ratio in respect to relative standard deviation (RSD). Percentage precision should be less than or equal to 1% except for the blank

sample whose precision is normally greater because its RSD is too high. However, as metal concentration increases, the RSD stabilises.

e. Measurement mode – choose prompt

f. Optical – UltrAA lamp – tick box once lamp is in position. The wavelength for iron is auto-set at **248.3 nm** .

g. Background correction – Select 'ON' [this ensures limited interference with readings in case the sample has organic material in it). Background correction is provided by a deuterium lamp.

h. Under 'sampler"; go to "sampling set up" section, input the following: **probe height** (set at 0mm); **Rinse rate** (enter 1 to rinse between each sample); Rinse time (3 s); Tick "smart rinse".. to allow for finish of rinse at minimum absorbance of 0.010 Absorbance Units. This ensures that a clean probe is used in the next sample.

10. **Select standards** from the menu. Insert the concentrations of standards in the table including a blank (10% nitric acid) in first position. At least 3 standards are sufficient for measurement of one element].
11. **Select labels** from the menu – This provides columns where to type the identification of test samples. Enter the identity of all samples in rows.
12. **Clean flame slit** using paper card provided. This paper has circular print on it against which the light beam can be aligned to pass through the middle of the flame path.
13. **Under Instrument, set up the following parameters**
    a. Select **flame optimisation**
    b. **Adjusting the flame alignment**: Before turning on the flame, check flame alignment with the card provided. Place and Hold the card upright against the flame path. The light from the cathode lamp should align to the centre of the circle on the card. This can be adjusted back and forth or up-down by turning the flame adjustment button (i.e. Black-grey Knob (in front of Instrument below the flame window) accordingly
    c. **Adjusting the hollow cathode lamp (HC) gain** to a maximum level. To adjust the gain, turn the black buttons at the bottom of the cathode lamp. Click on "rescale" to restore the adjusted gain.
    d. **Switching on the flame**: Press and hold down the black button on left hand front side of instrument until the flame comes on. The flame is the right

type if the bottom is light blue, with a middle thin layer of pale yellow 'flicker' very close to the blue, and the top part should be purplish/orange.

e. **Adjusting the flame**: If the flame is too yellow, it can be adjusted by adjusting the gas flow and the oxidant. To adjust gas flow, still under Instrument (as above), use cursor to adjust the red arrow up and down between the black arrows on the "gas flow' dialogue box. Carefully watch the flame until it's appropriate as described in 13c above. If satisfied, click "OK" at the bottom of dialogue box. Adjusting the flame ensures that the signal is optimised [The right flame is obtained at about 1.70 to 2.0 litres/min acetylene gas with oxidant at 13.68 litres/min]**.**

f. **Auto sampler**: To align the auto sampler; Click on Instrument from menu, select flame facilities, then select **Align** on the dialogue box. [The auto sampler then starts to align itself to draw samples and standards from positions set under Standards and Labels in 10 and 11 above]. AAS automatically rinses between standards. Set it to rinse between samples as required e.g. insert 1 for AAS to rinse after each sample.

14. Starting sampling;

Click on "analysis" ---brings up analysis window

a. Click on highlighter (see vertical menu on the left hand side of the screen)
b. Hold cursor down starting at the first sample listed. Drag downwards to select sample list
c. Click start – the auto-sampler begins to take samples.

15. **Calibration curve**: Click on view, select calibration graph [left click on mouse, from curve fit, select Linear. [If during operation, the curve goes off the screen, double click screen to restore].

16. **Turning off the instrument**:

a. The flame turns off automatically once the sampling is complete
b. Switch off the instrument power points
c. Turn off the air and acetylene gas taps
d. Gently remove HC lamp from position taking care not to knock it!
e. Rinse sample tubes

17. **To retrieve Data;** Close operation and go back to start up. Choose reports

and find appropriate file as named in step 11 above. Save own results including absorbance for calculation of iron content in Excel.

Appendix 3 Operating the HPLC instrument for analysis of carotenoid in banana and rice

HPLC Equipment at QUT: Type: Agilent HP100 HPLC – Circa 2004

Location: QUT Chemistry analytical lab; **Software:** Atlas online

Operating Procedure: Turn the switches for the equipment on (normally they are all left on).

Start Atlas online from computer screen to open the "Method and Run Control" window

Cleaning up the solvent and tubing system before running samples

1. Set up solvents in the right position: Our method was set to use channels C and D
2. Set up and connect waste bottle for the correct solvents in use
3. Turn on Atlas online software. This starts in "LC OFF" mode for both Method and Sequence
4. Load method from file. Our method was set up as "PBCARO.M" [Go to File, Load Method and select PBCARO].
5. Open pump valve (middle black knob) before turning on the pumps. [This allows the solvents to bypass going into the pulse damper thereby cleaning out air from the system into the waste bottle].
6. Turn on pumps by Clicking 'on' from screen
7. Click on pump, choose "setup pump" to get the time table for solvents
8. Setting up timetable for the solvents [Use both channels for solvent C &D to clear the system C: Methanol: TMBE: 57%; D: Methanol: MBE: Water : 43%
9. Turn off pump and set flow rate at 5 $mlmin^{-1}$ (max). Turn on pump again to set system running. Once all the air bubbles are out of the system tubing, turn off pump [from screen]. Then close the pump valve (black middle knob).

10. Click into pump icon [while off], choose “setup pump” again, set flow rate at 1 $mlmin^{-1}$. Cross check set up of solvents in the solvents set up dialogue box to confirm that it is C is at 57% and D at 43%. Set stop time to 35 min [this should be equal to total run time as per method timetable]. Turn on pump. Let system run for approx. 2-5 min before connecting column. [This cleans all tubing through the flow cell of any other solvents that were used in the system in previous run]

Cleaning up and connecting the column:

1. With pumps off, connect column in position [to injector inlet and outlet to detector]. Ensure that the column is connected in the correct orientation of sample flow (there is an arrow on the column).
2. Turn on pumps [from the screen] the pressure changes automatically. Let the column sit for 15-30 min. This purges out anything that could be bound onto column from previous run. [Note: If samples to be run are the same as previous ones on the same column, clean column for 5-10 min]

Running samples/setting up sequence table

Method: PBCARO

1. In the “Method and Run Control” window,
2. Click on methods, Select Load existing method and select file name of saved method: PBCARO – (for carotenoids)--always cross check the method to confirm parameters. Place samples in position.
3. Always include a blank (*solvent used to dissolve samples*] in position 1. Place wash vial (water) in position (usually the last position on the sample rack) and indicate this sequence table and method. [Note: Position of wash vial can be changed. Go to method ~ Edit entire method~ change position; or can exclude wash step depending on samples being run]
4. From menu, select sequence parameters, and fill in details for Operator name, prefix (to identify type of samples; Subdirectory name (where data files will be saved). The data file counter and data path is automatically set up once these have been filled in.

5. From Menu again, under sequence, select sequence table and fill in the details as required. Once sequence is finished, click Ok to close. Then Under Menu, Choose sequence, select "save sequence as" then name sequence appropriately in chosen folder.

   Starting the run: Click on auto-sampler sequence (*i.e. the multiple tubes at left hand corner*] to set up sequence]. Note: The auto-sampler should show up with the indication of where the samples are located. **Click on start to run**. Note: If you change sequence details, and save, the instrument will ask to replace already existing sequence - Accept.

Developing a method:

1. From Menu, Select Method
2. Edit entire method, ¬ok. First set up method to clean up the system
3. Set flow rate (depending on column size). 1 mL or 1.5 $mlmin^{-1}$. [Note: Default pressure limit-400 bar – a flow rate that creates high column pressure may damage the column
4. Set Stop time for the auto sampler: 35 min (this is how long the machine will take to collect the data from individual sample runs]
5. Set up time table for solvent percentages i.e. C: Methanol: TMBE: 57%; D: Methanol: TMBE: Water: 43%
6. Set up time table for solvents C and D according to specific protocol for analysing carotenoids (refer to time table below)

   Summary HPLC method used

   Method file name : **PBCARO**

   Separation module:

   Photodiode array detector DAD: Agilent 1100 set at **465 nm wavelength** [for visible lamp] and 287 nm **[**for - UV lamp] for detection wavelength for alpha tocopherol acetate.

   Column: C30 250x4.6 mm, 3 µm,

   Column Type: Waters

   Solvent C: Methanol: TMBE: [1: 1; v/v] ----------57%

Solvent D: Methanol: MBE: Water [5: 1: 1 v/v/v] -------43%

Timetable for carotenoid analysis according to PBCARO method

Time(min)	% Solvent C	% Solvent D	Flow pressure ($mlmin^{-1}$)
0	57	43	1
10	100	0	1
20	100	0	1.5
23.10	100	0	1.5
23.20	57	43	1.5
29.0	57	43	1.5
35	57	43	1

Note: When changing flow rate from 1.5 to 1 $mlmin^{-1}$, give the column sufficient time to adjust to this vol & pressure change – at least 5 min (this preserves the column).

7. Set up auto-sampler injection volume at 20 µL **.**
8. Set position for the wash vial
9. Auxiliary draw speed and ejection speed: 100 $\mu lmin^{-1}$
10. Set up diode array detector (DAD) at a wavelength of 465nm [uses visible lamp]. This is the wavelength at which β-carotene has maximum absorbance in Chloroform. Set the wavelength for alpha tocopherol acetate at 278 nm [uses UV lamp]
11. Once method is done; Go to file, --save as,-- method,-- insert method name
12. Checking solvent bottles: Click on the sign for solvent bottles - check the volumes, Ensure to tick the "Turn OFF " for volume below 0.1 L [ensures that when solvent volume goes below 100 mL, the system shuts down].

Closing the instrument

1. Set up pump to solvent B – this is usually Methanol at 100%, 1 $mlmin^{-1}$
2. Let Methanol run thought the column for 5 min [this cleans the system of current solvents. This is important for maintenance of the column. Note: Do not open the damper valve in this case.
3. Set method and sequence menu back to LC OFF and turn off all components from screen.

Data analysis and drawing calibration curves

1. Open Atlas software offline

2. Load method: PBCARO
3. From menu, choose view, Select data analysis, Go to file, Load signal
4. Find data – from known folder where it was saved for that particular run, then select files as stored/saved
5. From menu, Choose “Calibration”; Select “new calibration table”
6. Select appropriate peak (e.g. peak at 13 min retention time, at 465 nm is the peak corresponding to β-carotene)
7. Delete the other lanes in the table manually [To do this, Bring cursor directly to the side of row to highlight …then hit delete. Then feed in the right concentration (if standard sample) in appropriate rows
8. Go back to file, select load signal, click on the file name of next sample
9. Go to Calibration, Select …”Add level” to add the next level of calibration
10. Then add next STD files as they follow each other and fill in the concentrations values
11. <u>How to create two STD curves in one run</u>
12. After the first curve (as per 1-10 above)
13. Go to load signal again, Select next first file for the new calibration compound (e.g. Alpha tocopherol acetate)
14. Go to Calibration; Select “Add peak”. Note: all peaks of new compound will appear into the existing table. Then select peak of interest in appropriate row, (e.g. peak at RT= 7 min); feed in name of compound [e.g. α-tocopherol acetate]
15. Delete unwanted rows (as in 7 above); feed in concentration of the new STD compound.
16. Load all subsequent calibration data points for the second compound as before by selecting “add level”. Note: Leave the value of levels as they came up in the dialogue box and change later manually in the Calibration table to avoid overlapping the already existing levels of the first compound. The calibration curve comes up automatically.

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