The overexpression of the *Or*-gene in potato tubers

by

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B. Sc. (Agriculture Honours)

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In the
Faculty of Agriculture and Life Sciences

LINCOLN UNIVERSITY

2010

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Abstract

Vitamin A deficiency remains one of the major nutritional problems and affects an estimated 250 million people worldwide. Plant carotenoids are the primary dietary source of provitamin-A (β-carotene) and play a critical role in human nutrition and health. Potato is the third most important food crop in the world following wheat and rice. It represents a major proportion of daily calorie intake in many countries, with more than a billion people consuming potato tubers on a daily basis. Potato is therefore a key target crop where enhancing carotenoid concentration can have real impact on human health.

Introducing a worthwhile trait such as the cauliflower *Or*-gene into potato is hypothesised to have a positive impact on vitamin-A status. The *Or*-gene was isolated from an orange cauliflower mutant and encodes a plastid-targeted protein containing a cysteine-rich zinc finger domain. The *Or*-gene controls carotenoid accumulation by inducing the formation of chromoplasts, which provide a metabolic sink to sequester and deposit carotenoids. This research focused on enhancing carotenoid accumulation in potato tubers by expressing the cauliflower *Or*-Wild transcript.

Potato cultivars Iwa, Desiree, Summer Delight and Agria representing diverse genetic backgrounds and tuber colours ranging from white, cream and pale yellow through to yellow were used in this study. The *Or*-wild transcript under the control of the patatin promoter was constructed into the pMOA33 binary vector and transgenic potato plants have been produced by *Agrobacterium*-mediated transformation expressing the cauliflower *Or*-Wild transcript.

The efficient transformation procedure using disarmed *Agrobacterium* strain EHA105 was applied to produce around 40 transgenic lines from each cultivar through independent transformation events. Stable integration and expression of the *Or*-Wild transgene in independently derived potato transgenic plants were confirmed through molecular analyses. Transformation efficiency analysis showed that the potato transformation protocol used is genotype-dependent. Of the four genotypes tested, Iwa and Summer Delight were highly responsive genotypes, whereas Agria and Desiree were less responsive.

All the rooted transgenic plantlets were successfully acclimatized to a greenhouse. The majority were observed to have a phenotypically normal appearance. However, a range of off types was observed and attributed to somaclonal variation. These may have been due to the *in vitro*

establishment techniques (medium constituents) or may have pre-existed from the sources of explants.

The RT-PCR analysis of RNA from tubers of selected transgenic lines confirmed expression of *Or*-Wild gene in tubers. Total carotenoid concentrations were determined in tubers of the above selected transgenic lines and non-transgenic control. Among the controls, Agria, the yellow-fleshed cultivar, had the highest total carotenoid content (mean 0.134, SD 0.022 mg g^{-1} DW), substantially higher than all other cultivars tested. The lowest total carotenoid concentration was found in Iwa, a white-fleshed cultivar (mean 0.009, SD 0.002 mg g^{-1} DW). The total carotenoid content shows a positive relationship with the colour intensity of tuber flesh.

The transgenic tubers carrying the *Or*-Wild transgene show markedly higher carotenoid levels compared with respective non-transgenic controls. The transgenic lines from cultivars Agria and Summer Delight show 2-3-fold higher total carotenoid content compared with controls. Whereas, the transgenic lines from cultivars Desiree and Iwa show 2-fold higher total carotenoid content compared with controls. Among the transgenic lines, Agria line -7 (mean 0.326, SD 0.055 mg g^{-1} DW); Desiree line - 3 (mean 0.078, SD 0.018 mg g^{-1} DW); Iwa line - 35 (mean 0.026, SD 0.002 mg g^{-1} DW); Summer Delight line - 2 (mean 0.156, SD 0.017 mg g^{-1} DW) produced substantially higher total carotenoid content than all other transgenic lines tested in each cultivar.

The differences in total carotenoid content between lines may be due to different levels of expression of the same transgene in tubers. It is recognized that total carotenoid composition varies as a function of such as stage of maturity, cultivar, sample handling, and analytical method. Since the tubers were harvested well before its full maturity, the total carotenoid content reported in this study may be an approximation of the potential to be derived from the expression of the *Or*-Wild transgene function in those transgenic tubers.

The *Or*-gene is hypothesised to control carotenoid accumulation by inducing the formation of chromoplasts which provide a metabolic sink to sequester and deposit carotenoids. The successful demonstration of increased carotenoid accumulation represents a promising strategy for maximally improving the nutritional quality of food crops. This research developed potato plant material, genetic and biochemical data that increased our understanding of carotenoid accumulation in potato tubers of selected cultivars and set essential infrastructure for future detailed studies in this field.

Keywords: Carotenoids, β-carotene, *Or*-gene, phenotype, potato tuber, somaclonal variation, transgenic, molecular cloning, constructs, vectors, promoters

Dedication

I dedicate this thesis in memory of my loving mother Sivanammal and father Vellasamy who taught me the richness of learning and sharing in this world.

Acknowledgements

My sincere appreciation and gratitude to my supervisor, Professor Tony Conner for his guidance, encouragement, advice and most of all his constructive criticisms during this research. It was a great privilege working with him. Without his help and interest, I would not have completed this thesis. I am indebted to Professor Conner for his enormous help and understanding during the difficult phase of my indisposition. Thank you does not seem sufficient but it is said with great respect.

Special thanks to my other supervisors Professor John Hampton and Dr Philippa Barrell for their kindness, academic advice and continuous support throughout my research.

I would like to acknowledge with gratitude the Plant & Food Research Ltd., for the full logistic support in this research.

It is a pleasure to thank Julie Pringle for her invaluable assistance in the laboratory, molecular technical guidance in developing constructs and her warm friendship.

I am also thankful to Dr Meiyalagan for his friendship, support and valuable practical information rendered during the establishment of tissue culture plants and RT-PCR analysis.

It is also a pleasure to thank Martin Shaw and Michelle Gatehouse for their enthusiastic assistance and support in carotenoid analysis.

Many thanks and appreciation is also extended to my friends Azmi, Sara Mohan, Seelan and Rashidi for their sincere friendship, help and encouragement.

My deepest appreciation and love go to my wife Dharshini for her unrelenting support, help and understanding throughout the involvement of this research. Without her encouragement, this research would not have come to reality.

A very special recognition and love is due to my children Shankari, Shankar and Shivannee for being with me as a comfort and joy throughout this research.

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Glossary

°C	degrees centigrade
µg	microgram
µl	microlitre
BAP	benzylaminopurine
bp	base pair
CTAB	cetyl trimethyl ammonium bromide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
g	force of gravity
g	gram
h	hour
kb	kilobase
kDa	kilodaltons
L	litre
LB	Luria Bertani medium
mg	milligram
min	minute
mL	millilitre
mM	millimolar
NAA	napthaleneactic acid
ng	nanogram
nm	nanometer
nM	nanomolar
PCR	polymerase chain reaction
Pmol	picomole
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
T-DNA	transfer DNA
Tris	tris (hydroxylmethyl)-methylamine

1: Chapter: Literature Review

1.1 Introduction

Potato (*Solanum tuberosum* L.) is grown worldwide in about 125 countries and more than a billion people consume potato tubers on a daily basis. Potato is the third most important food crop in the world following wheat and rice (Camire *et al.*, 2009; Visser *et al.*, 2009). A wide range of potato cultivars have been bred and developed to meet this demand and new cultivars have improved traits such as pre or post-harvest disease resistance, superior processing characteristics, extended storage quality or the ability to meet certain nutritional requirements. In addition to providing starch, potato is rich in protein, potassium, ascorbic acid and is an excellent source of fibre. Although it represents a major proportion of daily calorie intake in a number of countries, potato contains very low levels of vitamins, minerals, fatty acids and antioxidants. Of the many important antioxidants identified, carotenoids play an essential role in cell communication, drought tolerance and are implicated in the prevention of degenerative diseases such as cancer, cardiovascular diseases and inflammatory disorders (Mayne, 1996).

The adaptability of the potato enables it to be grown in most parts of New Zealand in a wide variety of soils and climates (Logan, 1983) and 10,050 hectares were harvested in 2008, yielding 477,000 tons (Aitken & Hewett, 2008). Most of the potatoes produced in New Zealand are consumed locally and the New Zealand market for fresh potatoes is relatively stable. New Zealand's fresh potato exports go mostly to South Pacific Islands, Pacific Rim markets and Middle East (Petrie & Bezar, 1998).

1.2 Potato genome and genetic engineering

Potato (*Solanum tuberosum* L.) originated in the highlands of South America, where it has been consumed for more than 8000 years. Potato belongs to the *Solanaceae* family and the *Solanum* genus contains about 1000 species. The commonly cultivated potato belongs to the series *Tuberosa*, one of the 19 series of the subsection *Potatoe*, which comprises 225 wild tuber-bearing species (Hawkes, 1992). One complement of the potato genome consists of twelve chromosomes with an estimated DNA content in the order of 1 x 109 base pairs (D'Arcy, 1976; Ganal *et al.*, 1990). Cultivated potato is an extremely heterozygous autotetraploid (2n=4x=48) plant, having four genome complements and 48 chromosomes (Hawkes, 1994). The four homologous chromosomes pair during meiosis and segregate independently (tetrasomic inheritance). The alleles at any given genetic locus can occur in up to five allelic states: nulliplex (homozygous absent), simplex (present one time at the locus), duplex (present two times), triplex (present three times) and quadruplex (present four times; homozygous state).

Sterility is a major constraint in potato breeding. The autotetraploid nature gives the possibility of breeding tetraallelic (heteroallelic) genotypes over a long breeding period. The importance of tetraallelism for yield and other polygenic characters has long been evident (Ross, 1986). Because of its tetraploid nature and the vegetative mode of propagation, breeding is a cumbersome and time consuming process. As a result potato breeding has been less successful when compared to other crops. All the above aspects make potato an excellent crop plant to improve by genetic transformation. By using a gene modification approach the quality of the potato crop can be improved considerably in a relatively short time, especially by using the potato plant as a factory for the production of high value compounds such as vitamins and proteins (Camire *et al.*, 2009;

Visser, 2000; Visser *et al.*, 2009). Horticultural practices coupled with *Agrobacterium*-mediated transformation methodology make the potato an ideal candidate for genetic improvement via molecular biology techniques.

1.2.1 Plant genetic engineering

Plant genetic engineering can be defined as a process where the DNA is altered in a way that does not occur naturally by mating or natural recombination. It involves with the transformation of foreign genes isolated from plants, viruses, bacteria or animals into a new genetic environment (Hartl & Jones, 2009; Heldt & Heldt, 2005). The ultimate aim of GE is to regenerate plants identical to the parental material, except for the newly inserted genes (Draper & Scott, 1991). The inserted DNA is transcribed and the resulting mRNA translated for a new protein to be expressed, thereby giving an organism a new characteristic/trait. However, during this process, the cell culture phase of plants usually generates somaclonal variation that leads to individuals with different phenotypes resulting in changes in morphology, behaviour, or macro- or microconstituents. These differences are thought to be the result of genome instability in the *in-vitro* stages which results in plants (clones) with different phenotypes from one and the same parent plant (Custers, 2001).

1.2.2 T-DNA Binary Vector System

Researchers have designed DNA vectors based on the tumour-inducing Ti plasmid of *Agrobacterium tumefaciens* to transfer foreign genes into the plant. The development of DNA vectors using *A. tumefaciens* only requires the 25 bp border repeats, with none of the other T-DNA sequences being required for transfer and integration. That means the T-DNA genes can be replaced by any other DNA of interest, which will be transferred into

the plant genome (Hamilton *et al.*, 1996). Also, it has been found that T-DNA and *vir* genes do not have to be in the same plasmid for transfer of T-DNA (Hoekema *et al.*, 1983; Hoekema *et al.*, 1984). This has allowed development of a binary vector system to transfer foreign DNA into plants via *Agrobacterium*-mediated transformation.

1.2.3 *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation has been used internationally to introduce a wide variety of transgenes into potato. A variety of protocols are used in a *Agrobacterium*-mediated transformation to regenerate transgenic plants following inoculation of potato tissue and various explant sources (Rockhold *et al.*, 2001). Potatoes are one of the first crops in which transgenic plants were successfully regenerated (An *et al.*, 1986; Shahin & Simpson, 1986). Potato plants are easily regenerated in tissue culture and highly susceptible to *Agrobacterium* infection, which allows easy genetic modification (Mitten *et al.*, 1990). Although potato transformation can be accomplished by direct uptake of DNA into protoplasts (Feher *et al.*, 1991), *Agrobacterium* mediated transformation using binary vectors has been reported as an effective means of transferring foreign genes into important New Zealand potato cultivars (Conner *et al.*, 1997; Conner *et al.*, 1991). Manipulation of transgenes is simpler and more efficient in binary vector plasmids, which are capable of replication in both *Escherichia coli* and *Agrobacterium*. Most of the binary vectors also have convenient cloning sites that simplify the introduction of engineered transgenes.

In order to identify the rare transformed plant cells, genes conferring resistance to specific phytotoxic chemicals such as antibiotics are incorporated into the vectors as selectable markers in the transformation of a specific gene. The selectable marker gene is important

to enable transformed cells to survive in the media while non-transformed cells will not (Draper & Scott, 1991). Transformed cells are then selected in cell culture by their resistance to normally phytotoxic concentrations of the appropriate chemical (Grant *et al.*, 1991). Transformation systems for genetic engineering in potato are well established throughout the world and routinely rely on kanamycin resistance as a selectable marker (Conner *et al.*, 1997; Rockhold *et al.*, 2001; Vayda & Belknap, 1992). Selectable marker genes other than kanamycin resistance such as hygromycin, methotrexate, phosphinothricin and phleomycin resistance, have only rarely been used for potato transformation. Overall, kanamycin resistance is the preferred selection system resulting in the rapid recovery of large numbers of independently derived transgenic potato lines (Barrell *et al.*, 2002). The most commonly used selectable marker gene for plant transformation is *NPT* II (neomycin phosphotransferase II), which originates from the bacterial transposon *Tn5* (Conner & Meredith, 1989), and confers resistance to kanamycin and related antibiotics when transferred to and expressed in plant cells (Flavell *et al.*, 1992; Nap *et al.*, 1992). Plant cells expressing these genes are capable of phosphorylating kanamycin and related antibiotics, and thereby become resistant to the toxic effect of these chemicals (Conner *et al.*, 1991), serving as a convenient system to select the transformants effectively. Furthermore, the availability of promoters that differ in their ability to regulate the temporal and spatial expression patterns of the transgene increased the successful application of transgenic technology. A promoter is a region of DNA that facilitates the transcription of a particular gene. Promoters is necessary at all levels of genetic engineering in plants and, safety and containment of transgenic plants in the environment (Potenza *et al.*, 2004).

1.3 Carotenoids

Carotenoids are chemicals with nutritive properties that occur as pigments that colour plant and animal cells. They are also components of the photosynthetic machinery, and intermediates in the biosynthesis of abscisic acid and other apocarotenoids. As a fat-soluble material, carotenoids are ingested by humans mainly from vegetable green leaf tissue, fruits, seeds, roots and tubers. Plant carotenoids are the primary dietary source of pro-vitamin A for humans due to the fact that humans cannot synthesize carotenoids *de novo* and rely upon diet as the source (Krinsky *et al.*, 1994). Carotenoids with a β-ring end group are required for the synthesis of vitamin A. More than 600 different carotenoids have been isolated and characterized from natural sources, with about 50 being precursors of vitamin A based on their structural considerations (Gross, 1987). In plants, carotenoids are synthesized and sequestered in plastids, but accumulate in high levels in chromoplasts and in chloroplasts (Howitt & Pogson, 2006).

1.3.1 Health benefits of carotenoids

The health benefits of dietary carotenoids, especially β-carotene, are important in human and animal nutrition (Bramley, 2002; Fraser & Bramley, 2004; Van den Berg *et al.*, 2000). About 400 million people in developing countries currently suffer from dietary vitamin A deficiency because of the lack of carotenoids in their diets. Yearly, half a million people, mainly children, become blind as a consequence of vitamin A deficiency, fifty percent of whom may die within a year of becoming blind (ILSI-International-Food-Biotechnology-Committee, 2008; Ye *et al.*, 2000). Vitamin A deficiency severely affects the immune system; hence it is involved in many of these children's deaths in the guise of multiple diseases, like measles and malaria (Caulfield *et al.*, 2004). It has been calculated that simple measures, like breastfeeding, combined with vitamin A and zinc supplementation,

could save 25 % of the affected children (UNICEF, 2003). Some carotenoids have been linked to an array of health benefits in humans and animals, with β-carotene at the top of the list.

Van den Berg *et al.*, (2000) reported that positive effects of β-carotene were found in many ecological and observational cardiovascular disease studies, and Machlin (1995), Van Poppel & Goldbohm (1995) and Giovanucci (1999) found that lycopene and β-carotene could reduce cancer risk, especially prostate cancer. Cataract risk was reported to be increased in patients with low plasma β-carotene levels (Knekt *et al.*, 1992). Low levels of carotenoids or lycopene were reported to be associated with age related macular degeneration (Mares-Perlman *et al.*, 1995; Seddon *et al.*, 1994; Van den Berg *et al.*, 2000).

Evidence shows that different carotenoids have different beneficial effects. For example, lycopene appears to have a protective effect against prostate cancer (Gann & Khachik, 2003; Hadley *et al.*, 2002) and is effective in reducing the amount of DNA damage in white blood cells and prostate tissues of prostate cancer victims (Chen *et al.*, 2001). High dietary intake of zeaxanthin and lutein can protect against age-related macular degeneration disease (Krinsky *et al.*, 2003; Seddon *et al.*, 1994). The major dietary sources of lutein are dark green leafy vegetables but zeaxanthin is found in significant levels in relatively fewer dietary sources such as maize (Quackenbush *et al.*, 1963) and yellow orange pepper (Minguez-Mosquera & Hornero-Mendez, 1994).

Consuming natural sources of vitamin A rarely results in toxicity. There is no report of high β-carotene intake ever causing vitamin A toxicity (Allen, 2002). Retinol and other retinoids can cause acute toxicity. β-carotene and other pro-vitamin A carotenoids from foods are not toxic, because the absorption slows down as carotenoid intake increases

(Brubacher & Weiser, 1985). The upper safe intake level for β-carotene is 20 times that of retinol or 100 times the RDA (recommended daily allowance) for vitamin A. On this basis, it is better to manipulate provitamin A carotenoid synthesis in plants which has become the target for human health, rather than use systemic retinol synthesis (Khachatourians *et al.*, 2002).

1.3.2 Carotenoid biosynthesis

Carotenoid biosynthesis is a multifaceted and highly regulated process in plants. Carotenoids consist of eight isoprene units and can be divided from two major groups, carotene and xanthophylls. Carotenes are linear or cyclized hydrocarbons such as α-carotene and β-carotene. Xanthophylls are oxygenated derivatives of carotenes, lutein and zeaxanthin. Chloroplasts of higher plants typically accumulate lutein, β-carotene, violaxanthin and neoxanthin in the thylakoid membrane-bound photosystems (Peter & Thornber, 1991). β-carotene is generally found in the reaction center which quenches triplet chlorophyll and singlet oxygen (Young, 1993). Lutein can be found adjacent to the reaction centers. Finally, this reaction forms a complex that contains xanthophylls (lutein, violaxanthin and neoxanthin) (Peter & Thornber, 1991).

Carotenoids, the C_{40} tetraterpenoid compounds are biosynthesised by the isoprenoid pathway from mevalonic acid (MVA). Isoprenoids are formed from the basic C_5 isoprenoid isomers, isopentyl diphosphate (IDP) and dimethylallyl disphosphate (DMADP), that are condensed by the action of prenyl transferase enzymes to form the monoterpenes precursor, C_{10}-geranyl disphosphate (GDP). The addition of a second IDP unit produces C_{15}-farnesyl diphosphate (FDP), the sesquiterpene precusors. The reaction of FDP with a third molecule of IDP forms the C_{20}-geranylgeranyl phrophosphate (GGPP), the precursor

to carotenoids formed by the condensation of two molecules of GGPP to produce phytoene, the C_{40}-skeleton of carotenoids (Britton, 1998).

The detailed biosynthetic pathway of carotenoids from the GGPP precursor involves a series of stages (Britton, 1998; Matthews & Wurtzel, 2007). The first stage is the formation of phytoene, via the C_{40} intermediate prephytoene diphosphate (PPDP) involving a final loss of hydrogen to form a central double bond. Phytoene has a short (triene) chromophore and is therefore colourless. The extension of the conjugated double bond system by a series of desaturation reactions results in the formation of the coloured carotenoids due to the loss of two hydrogen atoms and the extension of the chromophore by two conjugated double bonds in each sequential reaction. This involves four sequential desaturations, alternating in the two halves of the molecule to give phytofluene, ξ-carotene, neurosporene and lycopene. Following desaturation the cyclization involves the rearrangement of an acyclic end group to form either β, ε or γ end groups. If cyclisation occurs after desaturation is completed, then lycopene undergoes two cyclizations to form γ-carotene and then β-carotene. The cyclic xanthophylls are then formed by hydroxylation of the cyclic end groups such as, the hydroxylation of β-carotene and β, γ-carotene gives zeaxanthin and lutein respectively.

In plants, the xanthophyll cycle involves the forward reaction which is the depoxidation of violaxanthin into antheraxanthin and antheraxanthin into zeaxanthin in the presence of light, and the reverse epoxidation of zeaxanthin into antheraxanthin into violaxanthin in the dark. The forward and reverse reactions are catalysed by two different enzymes, violaxanthin deepoxidase and zeaxanthin epoxidase. The synthesis of neoxanthin from violaxanthin is catalysed by neoxanthin synthase.

1.3.3 Regulation of carotenoid biosynthesis

Regulation within the chloroplasts and the chromoplasts is distinctly different. In chromoplasts, developmental regulation is strongly controlled at a transcription level with minimal flexibility and the presence of sequestering storage structures accounting for the carotenoid accumulation which occurs (Howitt & Pogson, 2006). In contrast, chloroplastic regulation of carotenogenesis is primarily light –dependent with regulation proceeding largely on both a transcriptional and translational level and to a lesser extent via end product feedback (DellaPenna & Pogson, 2006; Howitt & Pogson, 2006).

1.3.4 Genes encoding enzymes in the carotenoid biosynthetic pathway

The biosynthesis of plant carotenoids (Figure 1.1) can be divided into parts involving enzymes and their activities as listed in Table 1.1. Most genes encoding enzymes in the carotenoid pathway have been cloned in various plant species, such as tomato, *Arabidopsis*, and pepper (Cunningham & Gantt, 1998; Matthews & Wurtzel, 2007; Sauret-Güeto *et al.*, 2003; Vallabhaneni & Wurtzel, 2009; Ye *et al.*, 2000). Cloning of carotenoid biosynthesis genes provides better understanding of the characterization of the pathway and the identification of the respective gene functions and their relationship to the inheritance of phenotypes. Furthermore, cloned genes can be used as transgenes in a variety of crops to modify the carotenoid biosynthetic pathway for the production of high levels of natural or novel carotenoids with maximum health promoting activity (Giuliano *et al.*, 2000; Sandmann, 2001).

Marrs (1981) and Armstrong *et al.* (1990) genetically characterized the carotenoid pathway in *Rhodobacter capsulatus* and established the sequences of genes encoding the enzymes of the carotenoid biosynthesis. This was followed by Misawa *et al.* (1990) reporting on the

sequences and functions of the products of carotenogenic genes in *Erwinia uredovora*. Several carotenoid accumulations are known to be regulated by transcriptional or post-transcriptional levels of the carotenoid biosynthetic genes (Van den Berg *et al.*, 2000). In tomato, phytoene synthase (*PSY*) converts two molecules of GGPP into phytoene in the first committed step of the carotenoid biosynthetic pathway. Two isoforms of *PSY* were identified in tomato (*PSY1* and *PSY2*); *PSY1* expression increased markedly during fruit ripening encoding an enzyme in the tissues containing chromoplast, whereas *PSY2* was considered a vegetative gene which is abundantly expressed in mature leaves and it was not induced by fruit ripening.

Phytoene desaturase (*PDS*) catalyzes the desaturation steps, sequentially producing phytofluene and ζ-carotene (Van den Berg *et al.*, 2000) from phytoene. It was reported that expression of *PSY* and *PDS in* tomato started to increase at the breaker stage and slightly declined during fruit ripening (Giuliano *et al.*, 1993; Pecker *et al.*, 1996; Ronen *et al.*, 1999). ζ-carotene desaturase (*ZDS*) with carotenoid isomerase (*CRTISO*) are both involved in the steps which sequentially convert ζ-carotene to prolycopene and to lycopene. Isaacson *et al.* (2002) investigated *CRTISO* to elucidate two types of mutants by map-based cloning in tomato; *tangerine3183* and *tangerinemic*. Both mutants showed orange fruit colour, but *tangerine3183* accumulates prolycopene whereas *tangerinemic* accumulates prolycopene and ζ-carotene. The deletion of 24 bp in the exon and 258 bp in the intron region including on RNA splicing site of *tangerinemic* were detected and an early stop codon resulted in abolishing the function of the *CRTISO* gene. The mutated function of *tangerine3183* was due to the 348-bp deletion in the promoter region of the *CRTISO* gene.

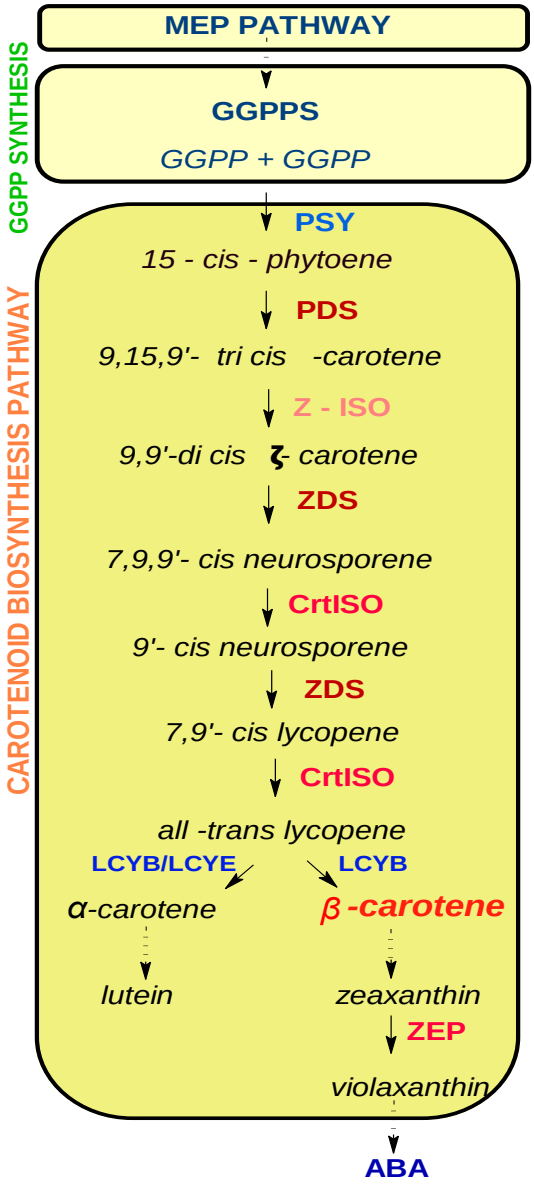


Figure 1.1 Simplified carotenoid biosynthetic pathway in plants

Developed based on Mathews & Wurtzel (2007; 2009). Enzyme abbreviations and enzyme activities are defined in Table 1.1

Table 1.1 Enzymes and substrates involved in carotenoid biosynthetic pathway in plants (Matthews & Wurtzel, 2007).

	Enzyme	Name	Substrate
GGPS	GGPPS	Geranylgeranyl pyrophosphate synthase	
Carotenoid Biosynthesis	PSY	Phytoene synthase	Geranylgeranyl pyrophosphate (GGPP)
	PDS	Phytoene desaturase	15-*cis*-phtoene; 9,15-*cis*-phytofluene
	Z-ISO	15-*cis*-ζ-carotene isomerase	15-*cis*-ζ-carotene
	ZDS	ζ-carotene desaturase	9,9′-di- ζ-carotene; 9′-*cis*-neurosporene
	CRTISO	Carotene isomerase	7,9,9′-*cis*-neurosporene; 7,9′-*cis*-lycopene
	LCYB	Lycopene β-cyclase	All-*trans* Lycopene; λ-carotene; γ-carotene
	LCYE	Lycopene ξ-cyclase	All-*trans* lycopene
	ZEP	Zeaxanthin epoxidase	Zeaxanthin; antheraxanthin

Lycopene cyclases convert lycopene to α or β-carotene with a ring structure. There are two lycopene β-cyclases; *LCYB* and chromoplast-specific lycopene β-cyclase (*CYCB*) in tomato (Hirschberg, 2001). The *Beta* (*B*) mutant of tomato has β-carotene as the principal carotenoid and six additional sequence elements in the promoter region of the *B* allele. Amino acid sequence analysis of the *B* gene showed 98% identity to the *b* allele in the coding region of the wild type. In *old-gold* (*og*) mutant of tomatoes, a frame shift was detected in the coding region resulting in null mutation (Ronen *et al.*, 2000). Lycopene cyclase (*CrtL-b*) was downregulated during fruit ripening in both wild type and *Beta* (B)

mutant fruit, whereas expression of *B* increased with maturity. The expression of *B* was detected only in chromoplast-containing tissues, such as flowers and fruits as compared to *CrtL-b*, which was expressed in both leaf and fruit (Pecker *et al.*, 1996). The level of expression in wild type fruit was not significant. The deduced amino acid sequence comparison showed 53 % identity between *CrtL-b* and *B* and 86 % between *B* and capsanthin-capsorbin synthase (*CCS*).

Currently, considerable interest has been demonstrated in the manipulation of carotenoid content and composition in plants to improve the nutritional value for human and animal consumption; a plethora of studies have been reported in the past decade (Matthews & Wurtzel, 2007).

1.3.5 Carotenoid metabolic engineering

The market for carotenoids has increased with the new discoveries on their anticarcinogenic and antioxidant properties (Demmig-Adams & Adams, 2002; Fraser & Bramley, 2004; Handelman, 2001; Stahl & Sies, 2005). The elucidation of the carotenoid biosynthetic genes in plants has paved the way to the biotechnological overproduction of carotenoids of nutritional crops (Table 1.2). The advantage of enhancing carotenoid levels in plants by genetic engineering over supplementing the diet with single components either chemically synthesized or extracted from natural sources, is the health benefits provided by carotenoid mixtures and their synergy with other antioxidants and components. Furthermore, farming of engineered carotenoid rich staple foods is a simple solution to alleviate carotenoid deficiency in the diet of the countries struggling with malnourished populations.

To date, the metabolic engineering of carotenoids has been largely conducted in tomato, potato, canola and rice, are reviewed.

Table 1.2 Cloned genes for carotenoid biosynthesis enzymes in plants

Enzyme	Plant source	Gene	Clone type	Accession no.
IPP isomerase	*Arabidopsis thaliana*	*GGPS2*	cDNA	U47324
		GGPS3	cDNA	U49259
GGPP synthase	*A. thaliana*	*PSY*	cDNA	U44876
		PSY	cDNA	U44877
Phytoene synthase	*A. thaliana*	*PDS*	cDNA	AY056287
	Capsicum annum	*PDS*	cDNA	X68017
Phytoene desaturase	*C. annuum*	*ZDS*	cDNA	X68058
	Glycine max	*ZDS*	cDNA	M64704
	Hordeum vulgare		cDNA	AY062039
Zeta-carotene desaturase	*Solanum lycopersicum*	*LCY*	cDNA	AF195507
	Zea mays	*LCY*	cDNA	AF047490
Lycopene β-cyclase	*A. thaliana*	*LCY*	Genomic	AF117256
	Solanum lycopersicum	*LCY*	cDNA	AF254793

IPP- IPP isomerae;GPPS- GGPS synthase; PSY- Phytoene synthase; PDS- Phytoene desaturase and z-carotene desaturase;- LCY -Lycopene b-cyclase (Naik *et al.*, 2003).

1.3.5.1 Tomato

Phytoene synthase (PSY) which catalyzes the first committed step in the carotenoid biosynthesis pathway was the first target for the biotechnological manipulation of the carotenoid composition in tomato (Fray *et al.*, 1995). The expression of the PSY1 gene encoding the chromoplasts specific isoform of tomato PSY resulted in carotenoid rich vegetative tissues and in the premature accumulation of lycopene in young fruit. The ripe

fruit of transgenic plants produced less lycopene than untransformed plants likely due to co-suppression (Fray *et al.*, 1995). To circumvent the undesired effects of both co-suppression and depletion of GGPP precursors for the production of essential isoprenoids, a bacterial *crtB* gene encoding PSY was fused to a chromoplast targeting sequence and was overexpressed in a fruit-specific fashion (Fraser *et al.*, 2002). Total fruit carotenoid levels were doubled compared with the controls, while biosynthetically related isoprenoids remained unchanged. The phytoene content of ripe *crtB* fruit showed the greatest increase (up to 3-fold), whereas increased levels of lycopene and β-carotene were 2.1 and 2.7-fold respectively as compared to the wild type. Although a significant increase in PSY activity was measured (up to 10-fold), it was proposed that the metabolic flux co-efficient was low in transgenic plants, shifting the regulatory step from PSY to another step in the pathway (Fraser *et al.*, 2002).

Gene expression studies showed that the endogenous genes encoding β-cyclases were upregulated in *crtI* transgenic fruit, likely causing the formation of β-carotene at the expense of lycopene (Romer *et al.*, 2000). The *PDS* and *ZDS* genes were also upregulated in the transgenic fruit, whereas PSY gene expression and activity were reduced, suggesting the existence of a feedback regulation from a carotenoid metabolite. Increases in β-carotene levels in tomato fruit were also achieved by expressing the *Arabidopsis* gene encoding LCYB under the control of a ripening-induced promoter (Rosati *et al.*, 2000).

The antioxidant properties of xanthophylls and the difficulty of their chemical synthesis have prompted recent metabolic engineering approaches to convert the large pool of lycopene found in wild-type tomato ripe fruit into high value-added oxygenated carotenoids. Transformation of tomato plants with constructs to overexpress the genes encoding *Arabidopsis* LCYB and pepper CHYB under the control of a ripening-induced

promoter resulted in no changes in the carotenoid profile of transgenic leaves, whereas in ripe fruit, lycopene was converted to β-carotene. Although β-carotene accumulated at levels up to 63 μg g^{-1} fresh weight in transgenic fruit (an impressive 13-fold increase relative to control fruit), total xanthophylls were also increased 10-fold, suggesting that the *CHYB* transgene could actively convert part of the newly synthesized β-carotene into downstream xanthophylls such as zeaxanthin, which was undetected in the wild type fruit but reached high levels (up to 13 μg g^{-1} fresh weight) in transgenic fruit (Dharmapuri *et al.*, 2002).

1.3.5.2 Potato

The carotenoid content of potatoes has been significantly increased by targeted overexpression of the bacterial *crtB* gene in tubers of *S. tuberosum* (a species with low carotenoid levels) and *S. phureja* (a carotenoid accumulating species). Tubers from both species contain violaxanthin as the most abundant carotenoid, with lower amounts of other xanthophylls (lutein, antheraxanthin, neoxanthin, and zeaxanthin) and virtually no carotenes. When the patatin promoter and a plastid targeting sequence were used to specifically increase PSY activity in the plastids of developing tubers, *S. tuberosum* and *S. phureja* lines accumulated around 7-fold and 3-fold higher carotenoid levels, respectively, than untransformed lines (Ducreux *et al.*, 2005). The carotenoid profile changed dramatically in transgenic *S. tuberosum* tubers, which were highly enriched in lutein (19-fold increase) and β-carotene (more than 10 μg g^{-1} dry weight). Higher α-tocopherol levels were also observed in some of these lines. Carotenoid accumulation in transgenic *S. phureja* tubers was higher than in transgenic *S. tuberosum* tubers (78 vs. 35 μg g^{-1} dry weight, respectively), but β-carotene levels were lower (6.5 μg g^{-1} dry weight).

Xanthophylls (including zeaxanthin) also accumulated at higher levels in transgenic *S. phureja* tubers compared with untransformed lines. Despite the observed changes in carotenoid composition, no increases in the expression of carotenoid biosynthetic genes were detected in the transgenic tubers (Ducreux *et al.*, 2005).

Xanthophyll composition of potato tubers has also been successfully changed by using antisense and co-suppression technologies (Romer *et al.*, 2002). To produce zeaxanthin-rich potatoes, a cDNA encoding potato ZEP was cloned in antisense and sense orientation and expressed in transgenic potato (*S. tuberosum*) plants under the control of a tuber-specific promoter (Romer *et al.*, 2002). As expected, potato tubers from antisense and co-suppressed lines showed a dark yellow to orange coloration due to higher levels of zeaxanthin (up to 40 µg g^{-1} dry weight, a 130-fold increase compared with untransformed lines), which became the most abundant carotenoid at the expense of violaxanthin. However, deregulation of the endogenous carotenoid pathway upon the expression of a transgene was observed. Analysis of gene expression showed an upregulation of *PSY* and *CHYB* genes, which may explain the observed increase in total tuber carotenoids (up to 6-fold) and the reduced levels of lutein in some transformants.

Van Eck *et al.* (2007) employed the RNA interference (RNAi) to increase the carotenoids in *Yema de Huevo* potato. Using this method, they targeted silencing of the β-carotene hydroxylase gene (*bch*) which converts β-carotene to zeaxanthin. They found that 86 % of the silenced lines had altered carotenoid profiles and the β-carotene content was increased from trace amounts in wild type tubers up to 331 µg 100 g^{-1} fresh weight and some transformants exhibited a significant decrease in zeaxanthin content and an increase in lutein.

Diretto *et al.* (2007) silenced the β-carotene hydroxylases *CHY1* and *CHY2* in potato tuber (cv Desiree). Real Time RT-PCR measurements confirmed the tuber-specific silencing of both genes. *CHY* silenced tubers showed significant changes in carotenoid content than *LCY-e* silenced tubers, with beta-carotene increasing up to 38-fold and total carotenoids up to 4.5-fold. Diretto *et al.* (2007) also transformed potato with three genes (driving the synthesis of β-carotene in bacteria) from *Erwinia* bacteria, encoding phytoene synthase (*CrtB*), phytoene desaturase (*CrtI*) and lycopene beta-cyclase (*CrtY*). Expression of all three genes resulted in tubers with a deep yellow "golden" phenotype without any adverse leaf phenotypes. They found that the tuber, carotenoids increased by 20-fold, to 114 mcg g^{-1} dry weight and beta-carotene 3600-fold, to 47 mcg g^{-1} dry weight.

1.3.5.3 Canola

Overexpression of a bacterial *crtB* gene extended with a plastid-targeting sequence under a seed-specific promoter increased up to 50-fold the total carotenoid content of mature seeds (Shewmaker *et al.*, 1999). The predominant carotenoids accumulation in the transgenic rape seeds were β-carotene (up to 950 μg g^{-1} fresh weight, a 316-fold increase compared with untransformed seeds), α-carotene (up to 450 μg g^{-1} fresh weight), and, a lower level of phytoene. Lutein (the major carotenoid in untransformed seeds) remained almost unchanged. A decrease in the levels of other plastid isoprenoids such as tocopherols and chlorophylls was also observed, consistent with a limiting pool of the common isoprenoid precursor GGPP. The structural alteration of plastids and the change in the fatty acid composition of transgenic seeds could be the consequence of alternative strategies developed to sequester carotenoids in lipoprotein complexes (Shewmaker *et al.*, 1999). The levels of carotenoids in the transgenic rape seeds were above 1.5 mg g^{-1} fresh

weight. Increasing carotenoid amounts in a tissue that does not accumulate them to levels that high confirms the flexibility of the pathway and the accumulation mechanisms in some species (Kumagai *et al.*, 1998; Shewmaker *et al.*, 1999).

1.3.5.4 Rice

The production of β-carotene in carotenoid free rice endosperm has represented a breakthrough in the biotechnology of carotenoids (Kroger, 2008; Paine *et al.*, 2005; Ye *et al.*, 2000). Five enzyme activities appeared to be required for β-carotene synthesis from GGPP (Figure 1.1): PSY, PDS, ZDS, CRTISO, and LCYB. To provide such activities, the daffodil (*Narcissus pseudonarcissus*) genes encoding PSY and LCYB (under the control of the endosperm-specific glutelin promoter) and the bacterial *crtI* gene fused to a plastid-targeting sequence (constitutively expressed) were transferred to Japonica rice plants. When a construct carrying only the *PSY* and *crtI* transgenes was first used for transformation, the transgenic rice grains showed a distinctive yellow colour (golden rice) and accumulated carotenoids. The synthesized carotenoids were mostly β-carotene and to some extent lutein and zeaxanthin, whereas none of the lines accumulated detectable amounts of lycopene (Ye *et al.*, 2000). Co-transformation of rice plants with an additional construct containing the *LCYB* gene increased the production of β-carotene. The total carotenoid amount of 1.6 μg g^{-1} dry weight was achieved in heterozygous plants (Ye *et al.*, 2000). The highest increase in carotenoid levels in rice was recently accomplished when the daffodil PSY of the original design was substituted by a maize enzyme with a higher activity in maize and rice tissues. Overexpression of the monocot PSY together with the bacterial desaturase encoded by *crtI* in the endosperm of the grain was shown to boost

the production of carotenoids, reaching up to 37 μg g^{-1} dry weight (Kroger, 2008; Paine *et al.*, 2005).

1.4 Orange cauliflower

Mutant analysis provides a wealth of information in plant biology. One of the interesting mutations is the orange cauliflower (*Brassica oleracea* L. var. botrytis) (Figure 1.2) mutant gene designated as the *Or*-gene, which causes large quantities of carotenoids to accumulate in the edible curd (Crisp *et al.*, 1975; Dickson *et al.*, 1988; Li *et al.*, 2001). The original mutant plant was found in the Bradford Marsh in Canada. The orange cauliflower arose from a spontaneous, semi-dominant mutation of a single gene. This gene represents the first identified regulatory gene serving as the controlling switch of the carotenoid accumulation in specific tissues of the plant through involvement in the differentiation of non-photosynthetic plastids into chromoplasts. The *Or*-gene may provide an ideal model to reveal the carotenoid regulatory control as it switches on carotenogenesis in tissues where the activity of this pathway is normally repressed.

1.4.1 The *Or*-gene

The *Or*-gene was isolated and studied by Cornell University plant geneticist Li and colleagues using a map-based cloning strategy (Li & Garvin, 2003; Li *et al.*, 2003). A 50 kb BAC clone harbouring the *Or*-gene was isolated. Sequencing of this BAC clone revealed 15 putative genes. Using fine genetic mapping, a single gene encoding for *Or* was identified and verified by phenotypic complementation in the wild type cauliflower and Arabidopsis ap1-1 cal-1 "cauliflower" mutant. The wild type *Or*-gene consists of 8 exons and encodes a protein of 33 kDa with a putative transit peptide and two transmembrane domains. Sequence analysis revealed that the mutation is due to a 4.7 kb insertion of a

copia-like LTR (Long Terminal Repeat) retrotransposon in the *Or* allele, which resulted in alternative RNA splicing (Li & Garvin, 2003; Li *et al.*, 2003). Three alternatively spliced *Or* mutant transcripts (*Or*-del, *Or*-LDel and *Or*-Ins) were produced following excision of the retrotransposon (Table 1.3).

All of the spliced transcripts can read through, and they share the same start and stop codons as the wild type gene. The *Or*-gene encodes a plastid-associated protein containing a DnaJ cysteine-rich zinc binding domain (Lu *et al.*, 2006). The *Or*-gene appears to be plant-specific and shares high sequence identity with orthologs from *Arabidopsis*, tomato, potato, maize, and rice. However, the *Or*-gene shares no sequence homology with the carotenoid biosynthetic genes and appears to exert no direct effect on the capacity of carotenoid biosynthesis (Lu *et al.*, 2006). By contrast analyses of the gene, the gene product and the cytological effects of *Or* indicate that *Or* induced carotenoid accumulation is associated with a metabolic process that triggers the differentiation of non-coloured plastids into chromoplasts (Li & Van Eck, 2007). Therefore the *Or*-gene appears to regulate the differentiation of proplastids or other non-colored plastids into chromoplasts, which provide a metabolic sink for carotenoid accumulation (Li & Van Eck, 2007).

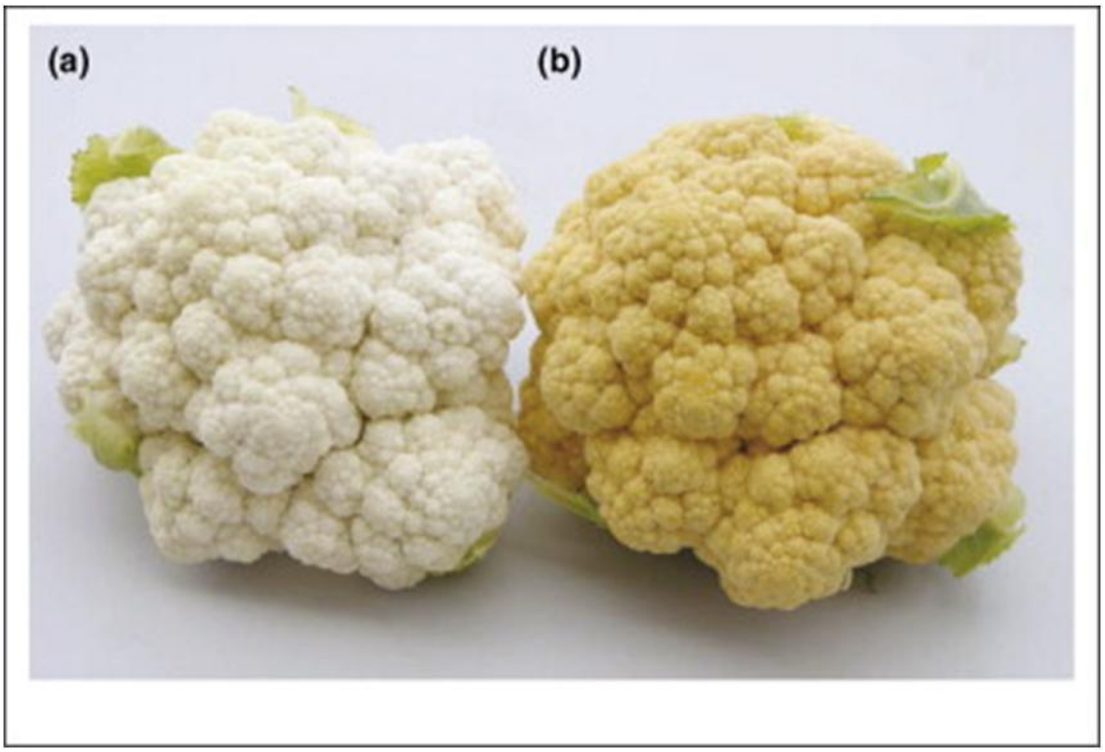


Figure 1.2 Normal (a) and *Or* mutant (b) cauliflowers (Giuliano & Diretto, 2007)

The cauliflower in (b) is yellow because the dominant *Or* mutation induces the formation of chromoplasts containing β-carotene.

Table 1.3 Cauliflower *Or*-genes reported in the NCBI 2008 (Lu *et al.*, 2006)

Accession No.	Name	Description	Size bp
DQ482460	*Or*-gene(native)	*Or*-mutant allele, complete sequence; and LTR retrotransposon	9240
DQ482459	*Or*-Del mRNA	*Or*-mutant allele, complete cds, alternatively spliced	897
DQ482458	*Or* -Ins mRNA	*Or* -mutant allele, complete cds, alternatively spliced	954
DQ482457	*Or* -LDel mRNA	*Or* -mutant allele, complete cds, alternatively spliced	810
DQ482456	*Or*-Wild mRNA	*Or* -wild type allele, complete cds	915

1.4.2 Use of *Or*-gene as a tool to enhance carotenoid accumulation

Recently, Lopez *et al.* (2008) transformed the *Or*-gene into potato plants under the control of a tuber-specific GBSS promoter. They found that the *Or* transgenic potato plants produced tubers with a deep orange-yellow flesh. HPLC analysis showed that these transgenic tubers exhibit more than a 6-fold increase in total carotenoid content. The transgenic tubers contained not only increased levels of the violaxanthin and lutein that are normally present in nontransformed controls, but also accumulated significant levels of β-carotene. In addition, three other metabolic intermediates, phytoene, phytofluene and ζ-carotene were accumulated which were not detected in the controls. The accumulation of these metabolic intermediates suggests a hindrance in desaturation in the carotenoid biosynthetic pathway. Such a hindrance may restrain the extent of *Or* induced carotenoid accumulation in the transgenic potato tubers.

Lopez *et al.* (2008) examined the cytological effects of the *Or*-transgene by light microscopy and found that expression of the *Or*-transgene in the heterologous system leads to the formation of chromoplasts with orange structures in transgenic potato tubers. The *Or*-transgene caused no significant changes in the transcript levels of endogenous carotenoid biosynthetic genes (Lopez-Juez, 2007). Collectively, these results demonstrate that the induction of chromoplast formation is the cause for the *Or*-associated carotenoid accumulation.

1.5 Impact of altered amyloplasts on chromoplasts

Plastids are dynamic and versatile double-membrane-bounded organelles that are classified into at least six distinct categories - chloroplasts, leucoplasts, elaioplasts, amyloplasts, chromoplasts and undifferentiated plastids or proplastids (López-Juez & Pyke, 2005; Waters & Pyke, 2004). They have a range of size, shape, and number per type of cell. They are related to the age of a cell and its developmental state (Osteryoung & McAndrew, 2001). Plastids are the sites of many agricultural important processes. For example, chloroplasts are important for photosynthesis, amyloplasts for starch synthesis, while chromoplasts contain vitamin A precursors of many fruits and vegetables. Other important biological pathways including lipid and amino acid synthesis are partially compartmentalized in the plastids. Understanding plastid physiology at molecular level is therefore pivotal to future improvement in agricultural productivity.

Earlier research has focused on chloroplast or differentiation of proplastids into chloroplasts, due to the pivotal role played by leaf chloroplasts in photosynthesis. Relatively few studies have focused on developmentally controlled differentiation of proplastids and chloroplasts into chromoplasts, as the later studies have mainly been

limited to systems such as plastids of tomato fruits, pepper, capsicum and Arabidopsis. Therefore the *Or*-gene systems in potato may have very unique aspects that warrant further study on amylo-to-chromoplast transformation and the impact on the balance in potato starch quality.

1.5.1 Amyloplasts

Since starch constitutes the major source of calories in the human diet, the starch storage function of amyloplasts has a direct impact on human nutrition. Amyloplasts are mature plastids and most of their internal volume is filled with starch. They are a specialized type of leucoplasts found in roots and storage tissues such as cotyledons, endosperm and tubers (Tetlow *et al.*, 2004). Amyloplasts function as storage plastids and they presumably synthesize starch as a reserve when carbohydrates are available in excess and break down their starch into free sugars or sugar derivatives when the plant is in need of carbohydrates (Neuhaus & Emes, 2000; Pyke & Waters, 2004).

More than 80 % of potato tuber dry matter is carbohydrate which occur mainly as starch stored in amyloplasts. Potato amyloplasts take up about 15 % of the relative compartment volume of tuber cells as compared to vacuoles 67 %, plastids 14.8 %, cytosols 12 %, cell walls 5 %, mitochondria and nucleus 1.2 % (Farre *et al.*, 2001). For potato tuber cells, there is a spatial separation of sucrose metabolism and starch metabolism; the former takes place outside the amyloplast, and the latter is confined to the amyloplast. The separation of synthesis of starch and of sucrose into two distinct compartments allows independent regulation of these two important storage and transport metabolites in the cell (Dennis & Greyson, 1987). Since it is such a distinct

feature of plant sink cells, it is vital to study the impact of amyloplasts on chromoplast differentiation when potato is engineered for enhanced carotenoid content.

1.5.2 Chromoplasts

Carotenoids are synthesized in all types of plastids in plants, but accumulate in high levels in chromoplasts and in chloroplasts (Howitt & Pogson, 2006). Chromoplasts are special among heterotrophic plastids because they develop from photosynthetic (autotrophic) chloroplasts during fruit ripening or petal development (Camara *et al.*, 1995). These changes also occur in certain roots and underground stems without passing through the chloroplast stage (amyloplast to chromoplast). The primary function of the chromoplast is carotenoid synthesis and storage that contribute to the characteristic colour of fruits and flowers (Kirk & Tilney-Bassett, 1978). Chromoplasts develop a unique mechanism to accumulate massive amounts of carotenoids by generating novel carotenoid sequestering structures (Bartley & Scolnik, 1995; Vishnevetsky *et al.*, 1999). These structures serve as a metabolic sink to sequester carotenoids and may also prevent the end-products of the carotenoid biosynthetic pathway from overloading chromoplast membranes, the site of carotenoid biosynthesis (Deruere *et al.*, 1994; Rabbani *et al.*, 1998). Carotenoid accumulation is not dependent solely upon the catalytic activities of carotenogenic enzymes, but also involves a network of other processes such as metabolite turnover and the stable storage of end products. The study of the *Or* mutant gene provides strong evidence showing that an increase in sink strength exerts an influence on carotenoid accumulation, and thus sink strength offers a novel approach for genetically engineering the carotenoid content in food crops.

Frey-Wyssling and Schwegler (1965) showed that chromoplasts can possibly be differentiated from non-photosynthetic plastids such as amyloplasts. Caiola and Canini (2004) studied the structure, origin and pigment localization of the chromoplast and other plastids of saffron (*Crocus sativus* L. Iridaceae). They found that the development of the stigma occurs concomitantly with the amyloplast-to-chromoplast transition and the stigma never turns green during this process. They also showed that stigma development parallels carotenoid accumulation.

Paolillo *et al.* (2004) studied the *Or* mutation in cauliflower (*Brassica oleracea* L. var. *botrytis*) that leads to abnormal accumulations of β-carotene in orange chromoplasts. The electron microscopy study revealed that carotenoid inclusions in *Or* chromoplasts resembled those found in carrot root chromoplasts in their optical activity and angular shape. This study also revealed that the inclusions were made up of parallel, membrane bound compartments. These stacks of membranes are variously rolled and folded into three-dimensional objects and they classified the *Or* chromoplasts as "membranous" chromoplasts.

Horner *et al.* (2007) studied tobacco floral nectaries developmental changes occurring in the plastids up to maturity and found that the large, engorged amyloplasts are initially converted into intermediate plastids amylochromoplasts (plastids containing both starch and β-carotene crystals)- and finally to chromoplasts. The nectaries shifted from quiescent anabolism to active catabolism resulting in starch breakdown and production of nectar sugars. Starch was replaced by osmiophilic bodies, which contain needle-like carotenoid crystals. All the above information serves as the basis for studying the impact of the amyloplasts on chromoplast differentiation in the potato *Or*-gene system.

1.6 Aim of this work

The overall goal of this thesis is to make use of the cauliflower *Or*-gene as a tool to study plastid differentiation for carotenoid accumulation in potato. To achieve this goal the following objectives were set:

- To construct *Or* coding sequences (four transcripts) and assemble the resulting constructs into plant expression cassettes and binary vectors for plant transformation.
- To produce transgenic lines with *Or*-Wild transcript by transforming four potato cultivars using *Agrobacterium* - mediated gene transfer.
- To study the phenotypic characterisation of these transformed plants in the greenhouse, including transgene expression and total carotenoid levels in tubers.

2: Chapter: The design and construction of vectors for potato transformation

2.1 Introduction

Plant genetic engineering has contributed considerably to the understanding of gene regulation and their effects in the generation of transgenic plants for widespread usage in agriculture. It has been one of the successful methods to incorporate gene(s) from one species to another, based on the fact that the exact genetic blueprint is transcribed and translated during the cell division. The process of introducing a gene into an organism via recombinant DNA technology is known as gene transformation and these genetically engineered plants are called transgenic and categorised as genetically modified organisms (GMOs).

Producing a GMO with a desirable gene starts with the selection of gene with specific characters and tailoring the gene into a vector before plant transformation. This process mainly involves the identification of a useful gene, choosing appropriate promoters to express the gene, construction of the gene and promoter into a vector and the final validation of the construct. Once these important steps are completed, the construct can be used for plant transformation and gene expression studies. The constructs containing various components useful for expressing the particular genes in plants are commonly known as expression cassettes. Therefore the genes of interest can easily be inserted into expression cassettes previously validated using assayable for reporter genes allowing the accurate quantification of gene expression for pre-determined specific patterns of gene expression.

2.1.1 Identification of gene of interest

The first step in genetic engineering is the identification of the source of the useful gene. A gene can be isolated from the genome or can be easily synthesised. Alternatively, the relevant mRNA can be isolated and the corresponding gene can be synthesised by using the reverse transciptase enzyme (Strachan & Read, 1999). Purified genes can be transferred to target cells with the help of the vectors to have their expression manipulated and controlled.

2.1.1.1 Isolation of gene of interest from genomic DNA

Isolation of the gene of interest from the genomic DNA is one of the most common and direct methods. Genomic DNA is extremely long and is obtained from tissues, cells or *in vitro* grown cultivated cells. Generally, the information on the gene of interest remains as an element of a 'genomic library'. Entire genomes are being sequenced including *Arabidopsis*, rice, maize, poplar tomato, cucumber cassava and potato (Delmer, 2005) [e.g. *Arabidopsis* (www.arabidopsis.org); potato (www.potatogenome.net)] and the manufacture of DNA banks are playing major role to facilitate the isolation of the gene of interest. Genomic information can be accessed via databases such as National Center for Biotechnology Information [NCBI (www.ncbi.nlm.nih.gov). Once the sequence is known, appropriate primers can be designed and gene can be isolated as a PCR product. The gene of interest can also be isolated using the known protein sequence of other genes using inverse genetics approaches. RNA-DNA hybridization and gene walking methods also can be used to isolate the gene of interest (Buchanan *et al.*, 2000; Strachan & Read, 1999).

2.1.1.2 Organochemical synthesis of gene of interest

Organochemical synthesis of double stranded DNA of known base sequence is rapidly becoming the most efficient way to make functional genetic constructs and enables applications such as codon optimization, RNAi resistant genes and in protein engineering (Villalobos *et al.*, 2006). This technique was developed by Hargovind Khorana in 1956 (Nishimura *et al.*, 1964; Sinha & Sinha, 1982; Söll & RajBhandary, 2006) that provided an easy way of manipulating and modifying the nucleotide sequence. Khorana also introduced many of the techniques that allowed scientists to decipher the genetic code and show how RNA can specify the structure of proteins. Currently, commercial gene synthesis services are available from companies worldwide, comprising the completed *de novo* production of structural genes. It has become an important tool in many fields of recombinant DNA technology including vaccine development, gene expression analysis, gene therapy and molecular engineering. Some major companies that provide such services are GeneScript (www.genscript.com), Geneart (www.geneart.com), DNA2.0. (www.dna20.com) and Epoch Biolabs (www.epochbiolabs.com).

2.1.1.3 Enzymatic synthesis of gene of interest

Given that mRNA can be isolated in a pure form, its corresponding gene can be easily synthesised with the help of suitable enzymes. Reverse transcriptase can be used to form single-stranded complementary DNA (cDNA) on the mRNA template (Buchanan *et al.*, 2000; Strachan & Read, 1999). The cDNA can be used to synthesise a double-stranded DNA with the help of suitable replicating enzymes. This technique has been widely used now as what requires only a small amount of purified mRNA as a starting material. However, it has a serious limitation that the initiation and termination signals at either end

of a structural gene are not recovered, although the cDNA can be multiplied to be used as a probe to isolated fragments of cellular DNA containing the nearby sequences.

2.1.2 Selection of a vector

Selection, isolation and purification of a suitable vector (gene carrier) is essential for a successful genetic transfer (Choi *et al.*, 2002; Preston, 2003). Though the vector, it is possible to give a gene fragment additional characteristics that may expand it use considerably. The vector is capable of replication within the host along with the inserted gene. Since the vector is responsible for the multiplication or cloning of the inserted gene in its host, it is known as a cloning vector. A vector should have specific restriction sites so that the circular genome is cut open to facilitate cloning of the DNA insert; a start of replication (*origin of replication* - ori) to promote autonomous replication; contain a genetic marker (usually dominant) for selection; be able to pull out the vectors with inserted DNA segments from a mixture of pure insert DNA and pure vector DNA and a combination of both; the size of the vector should be such that after combining with the heterologous DNA it should be able to get transferred to the host; the cells carrying the modified cloning vector should be identifiable with certainty and ease; be amenable to stringent biological containment so that they may not escape with any undesirable DNA fragment.

Pure preparation of DNA fragments is important as substances acquired during the preparation of inserts and vector that are often inhibitory to ligation and transformation (Bothwell *et al.*, 1990; Lodish *et al.*, 2000). Usually small amount of vector (25-50 ng) is required for individual ligation. During vector preparation for ligation, if it is cut with single restriction enzyme, two compatible ends emerge that can re-ligate with one

another. This self-ligation of the vector can be reduced by removing the 5-phosphate groups at the ends of the insertion site by shrimp alkaline phosphatase or calf intestine alkaline phosphatase (CIAP) (Altenbuchner *et al.*, 1992; Ausubel *et al.*, 1999). On the other hand, if the vector is digested with two restriction enzymes the intervening region, usually a small piece of polylinker, will be released. These polylinkers disturb the following ligation because such small DNA fragments are far better ligated into the vector than the DNA insert fragment (Lodish *et al.*, 2000; Strachan & Read, 1999). This can be eliminated by purifying the vector by means of gel electrophoresis.

Plasmid vectors are 2.5 to 5 kb long, depending on which characteristics they exhibit. In principle it is possible to clone around 15 kb DNA into them, but the difficulty of cloning usually increases with longer fragments (Altenbuchner *et al.*, 1992; Preston, 2003; Strachan & Read, 1999). Generally, the cloning sites involve Dibasic restriction sites that occur only once in the vector. This allows cutting the vector for cloning without decomposing it into smaller pieces. These sites vary between vectors. Some plasmid vectors contain 10 to 20 of these restriction sites over a short region, known as multiple cloning site (MCS) (Lodish *et al.*, 2000).

The number of plasmid copies within a bacterium depends on the origin of replication. The *ori* is a specific DNA sequence of 55-100 bp must be present in the plasmid to replicate. The host-cell cell enzymes bind to *ori* and initiate the replication. Once the replication is initiated at *ori*, it continues around the circular plasmid regardless of its DNA sequence (Lodish *et al.*, 2000). If the copy number is less than 20, it is known as a low-copy plasmid. High-copy plasmids can produce in several hundred copies per bacterium. Usually high-copy plasmids are used, because the yield is higher in plasmid preparation (Buchanan *et al.*, 2000; Strachan & Read, 1999). However, high copy number vector also

can cause problems when the cloned DNA encodes proteins that are toxic to cell when present at high levels (Preston, 2003).

This main aim of using expression vectors is to produce controlled expression of the gene inside a convenient host organism such as *E. coli*. The controlled expression in plant vectors is achieved by inserting the gene of interest into a site that is under control of a particular promoter such as the 35S promoter from cauliflower mosaic virus etc. A selectable marker such as an antibiotic resistance (eg *NPT* II gene resistant to kanamycin) is often carried by the vector to allow the selection of positively transformed cells (Glick & Pasternak, 2005). Several vectors are available for cloning depending on the aim of the research. Vectors used in this study include pUC57, pART7 and Dibasic pMOA33.

2.1.2.1 pUC57 Vector

The pUC57 plasmid (2,710 bp) is a commonly used cloning vector in *E. coli* (See Appendix B for vector map). The vector is a derivative of pUC19 vector, resistant to ampicillin and has high copy number in *E. coli*. The multiple cloning site of pUC57 contains 6 restriction sites with protruding 3'-ends that are resistant to *E. coli* exonuclease III (Genscript Corporation, USA). All four *Or*-transcripts used in this study were synthesized by Genescript and supplied inserted into pUC57 vector via blunt-end ligation. The GBSS promoter/terminator expression cartridge was also synthesized from Genescript inserted into pUC57 vector.

2.1.2.2 pART7 Vector

The expression cassette of the pART7 vector (4900 bp) comprises a multiple cloning site between the cauliflower mosaic virus 35S promoter and the transcriptional termination

region of the octopine synthase gene (OCS3'). The entire cassette can be removed from pART7 as a *Not* I fragment and introduced directly into the binary vector (Gleave, 1992). The patatin promoter/terminator expression cassette used in this study was supplied in the pART7 vector instead of the 35S-OCS3' expression cassette (4438 bp).

2.1.2.3 pMOA33 Vector

The pMOA33 (10014 bp) is a minimal T-DNA binary vector (Barrell & Conner 2006) that has features to overcomes the limitations found in other types of minimal T-DNA vectors (See Appendix B for vector map). The pMOA33 has a MCS with 12 unique restriction sites (85 bp) for insertion of the gene of interest located between the right T-DNA border and a selectable marker gene conferring kanamycin resistance to plants cells (Barrell & Conner, 2006). Due to the right-to-left orientation of T-DNA transfer (Zambryski, 1992), it is preferable to clone the gene of interest next to the right border. Also, virtually identical pMOA 34-37 vectors with similar small T-DNAs (1660-2140 bp) provide alternative selectable marker genes for plant transformation, including resistance to hygromycin, methotrexate, phosphinothicin, and bleomycin. A further key advantage of these binary vectors is that sufficient plasmid can be extracted from 1.5 mL overnight cultures of *E.coli* due to high copy number *colE1* replicon on the backbone (Barrell & Conner, 2006).

2.1.3 Promoters

Regulation can occur at many different stages of gene expression, and it is particularly important during transcription. The promoter that drives transgene expression ensures the control of transcription. Promoters are regions of the DNA located upstream of a gene's coding region that contains specific sequences recognized by proteins involved in the initiation of transcription (Buchanan *et al.*, 2000). As the promoters affect transcription

both quantitatively and qualitatively, the success of gene transfer technologies in crop improvement depends on their efficacious selection and use. New promoters of known expression patterns are invaluable tools in plant biology research.

Promoters are a set of transcription control modules clustered around the initiation site of RNA polymerase II (Russell, 1996). They are important in the control of the overall expression profile of a gene, either driving or preventing transcription at appropriate times and places. Generally, the basal or core promoter is located about 40 bp upstream of the start of transcription and the upstream promoter region may extend as many as 200 bp farther upstream. Although initiation of transcription is dependent on sequences found in the core and upstream promoter region, many other DNA sequence motifs, which occur within the surrounding DNA, are also involved in the regulation of gene expression (Potenza *et al.*, 2004).

Plant promoters that drive high, constitutive expression have become a valuable tool in plant genetic engineering. The choice of the promoter used within a transgene construct is essentially dependent on the objectives of the research. Potato is a tuber crop and the best characterized tuber storage organ specific are from genes involved in the starch deposition and the storage of highly abundant glycoproteins (Potenza *et al.*, 2004).

2.1.3.1 Patatin promoter

Patatin promoter is widely used in potato tuber research. The patatin genes in potato encode proteins that compose up to 40 % of the soluble proteins (40-43 kDa) in the tubers (Barta & Bartova, 2008; Prat *et al.,* 1990). The potato class I patatin family (B33 and PAT21) are highly expressed at early stages of tuber development in the vascular tissues and in later stages of development, in both parenchyma and vascular tissues (Jefferson *et*

al., 1990; Liu *et al.*, 1991). They are tuber-specific, and the presence of *cis*-acting sucrose-responsive elements within the class I patatin promoters allows for continued gene expression because of sucrose uploading into the tuber during growth and development (Liu *et al.*, 1990). In reporter gene studies, 1.5 kb of 5' flanking DNA of the B33 promoter and 2164 bp of the PAT 21 promoter appeared to contain all the elements necessary for tuber-specific expression when transformed back into potato (Potenza *et al.*, 2004).

2.1.3.2 Granule-bound starch synthase promoter

The granule-bound starch synthase promoter (GBSS) is also used in potato tuber research. Potato tubers contain vast quantities of starch as main polysaccharide reserve. Starch in potato tubers consists of up to 25% amylose, and granule-bound starch synthase (GBSS) is the key enzyme in amylose biosynthesis. Visser *et al.* (1991) found that the 5′ promoter region of 800 bp flanking the potato GBSS gene was sufficient to cause high levels of reporter gene expression in both stolons and tubers with little to no activity in leaves. Like patatin promoters, sugars can induce the GBSS promoter's expression in leaves, but not to levels as high as that of the patatin promoters (Potenza *et al.*, 2004).

2.1.4 Restriction enzymes

Restriction enzymes are restriction endonucleases (purified bacterial enzymes) that recognize specific double stranded DNA sequences and cleave the DNA in both strands (Lodish *et al.*, 2000; Pingoud *et al.*, 1993). Depending on their specificity, they generally recognize four to eight base pairs in a DNA strand in which only a small pile of phosphates and residual glucose can be seen. There are three types of restriction endonucleases. Type I, recognize a specific DNA sequence but cut by chance therefore the application is limited. Type II, sever DNA at defined sites and are used extensively in

DNA cloning. Type III, recognize specific sequences and sever 20-25 nucleotides and have limited use in molecular biology (Strachan & Read, 1999). The restriction enzyme digestions are utilized for the preparation of vectors and DNA inserts and for confirmation of inserts during sub-cloning (Sambrook & Russell, 2001).

2.1.5 Ligation of DNA

Ligation of the desired DNA segment to the vector is the crucial step in vector construction for transformation. This involves cutting and joining of DNA duplexes with the help of ligase enzyme (Lodish *et al.*, 2000). A DNA preparation is an assemblage of randomly sheared fragments (Strachan & Read, 1999). For optimal and controlled use of DNA, it is often desirable to cleave DNA at specific and defined points, thereby producing a unique set of discrete fragments. Many site-specific endonucleases (restriction enzymes) are available for this purpose and they recognises a particular sequence of nucleotides on a DNA duplex in the form of an inverted palindrome (Lodish *et al.*, 2000; Strachan & Read, 1999). Some restriction enzymes break the phosphodiester bonds between base pairs that are directly opposite and generate blunt-ended breaks. Others cause breaks a few base pairs apart within the target sequence generating sticky-ends. The sticky ends of the same or different DNA segments join together due to simple base complementarities. The gaps can be sealed with a help of polynucleotide ligase. The vector and the DNA fragment can thus be joined together to give rise to a recombinant heterologous DNA. When this foreign DNA containing information for a desired character is attached to the vector DNA with help of enzymes, a recombinant DNA is formed which is ready to be transferred to a suitable host environment for its replication and cloning. DNA fragments with blunt-ends also can be joined with the help of suitable enzymes facilitating them to stick to one another (Bothwell *et al.*, 1990; Lodish *et al.*, 2000).

The usual enzyme used for ligations is the T4 DNA ligase and the ligation function starts as soon as the enzyme is added (Bothwell *et al.*, 1990). Typically, the ligation is carried out at 14 ^{0}C to 16 ^{0}C for 1 to several hours. Fragments with overlapping ends normally have to be incubated for 1 h at room temperature. The ligations for blunt end are more difficult because the reaction is less effective. Therefore the incubation for blunt end ligation is generally carried out at 16 ^{0}C for 4 to 18 h.

2.1.6 Ascertaining the orientation of a DNA fragment insert

The construct validation process confirms whether the construct has the components necessary for gene expression in a useful order and ascertains the orientation of the DNA fragment inserted. This is accomplished though restriction digestion and sequencing. The final validation of binary vector can be transformed into *Agrobacterium* for plant transformation when the presence of all components in the construct is confirmed in their desired positions.

2.1.7 Objectives of this Chapter

There are two objectives:

- To construct cauliflower *Or*-Wild type coding sequence (*Or*-Wild) under the transcriptional control of the regulatory regions of the potato patatin gene and transfer the resulting chimeric gene into the binary vector pMOA33 for plant transformation.
- To construct the coding sequences of four cauliflower *Or*-transcripts (*Or*-Wild, *Or*-Del, *Or*-LDel and *Or*-Ins) under the transcriptional control of the regulatory regions of the potato GBSS gene and independently transfer the chimeric genes into pMOA33 binary vector for plant transformation.

2.2 Materials and methods

2.2.1 General methods in nucleic acid manipulation, amplification and cloning

2.2.1.1 Synthesis of *Or*-transcripts inserts

The cauliflower *Or* transcripts (GenBank Accession no: DQ482456 -*Or* -Wild; DQ482457 -*Or*-LDel; DQ482458 - *Or*-Ins; DQ4824590 -*Or*-Del) were synthesized by Genescript Corporation, USA, based on GenBank (NCBI) published sequences. *Hin*DIII restriction sites were added in the sequence for cloning purpose (See Figure 2.1 for sequence details).

2.2.1.2 Primer synthesis

Primers were designed based on GenBank (NCBI) published sequences using the web-based program Primer-3 (biotools.umassmed.edu/bioapps/primer3www.cgi). Unique restriction sites were included when required.

Primers were synthesised by Invitrogen, New Zealand (Table 2.1) and diluted to 100 mM with sterile H_2O and stored at -20 ^{0}C. A 10 mM working solution was prepared from this stock solution.

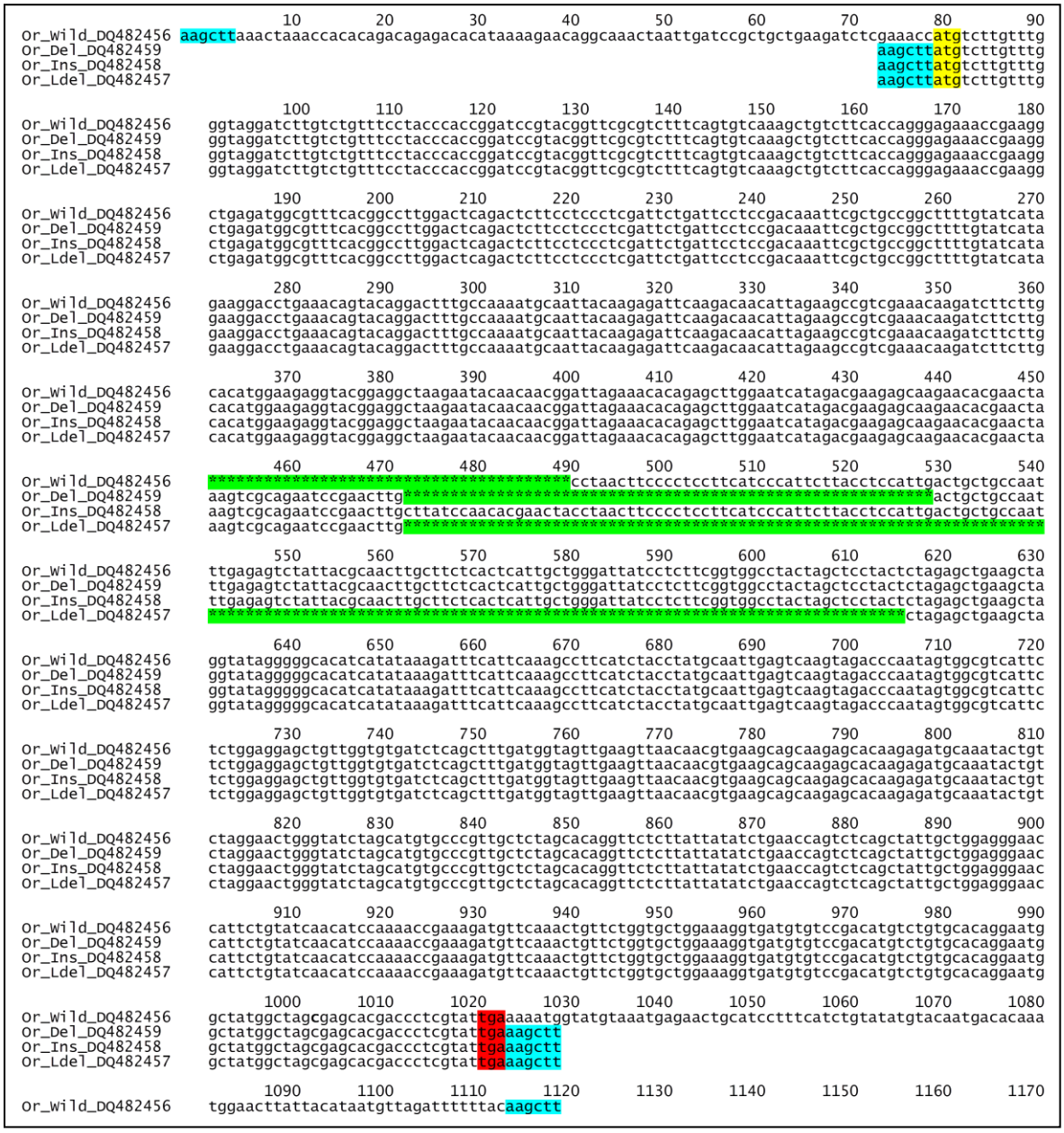


Figure 2.1 Multiple sequence alignments of *Or*-Wild and the three alternatively spliced transcripts.

Alignments indicate the presence of identical nucleotides at given positions. *Or*-Wild 1148 bp; *Or*-Del 899 bp; *Or*-Ins 956 bp; *Or*-L Del 812 bp; ATG - start codon; TGA - stop codon; *Hin*DIII - restriction site (added in sequence for cloning purposes when ordered with Genescript). The figure was generated using Gap4 Genome Assembly Program.

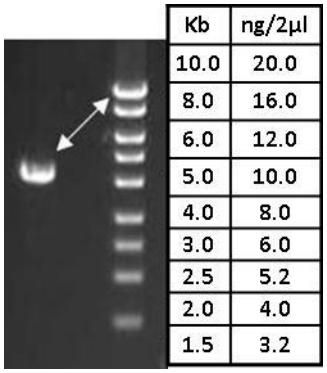
Table 2.1 Primers for PCR and expected product size

Target Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Product Size (bp)	Optimal Annealing Temp (^{0}C)
Actin	GATGGCAGAAGGCGAAGATA	GAGCTGGTCTTTGAAGTCTCG	1069	60
NPT II	ATGACTGGGCACAACAGACAATCGGCTGCT	CGGGTAGCCAACGCTATGTCCTGATAGCGG	612	60
Or Tran-scripts	ACTGCTGCCAATTTGAGAGTCTATTA	CTTTCGGTTTTGGATGTTGATACAG	442	61.9
PatCor	AACTCGAGGAATTCGGTACCC	TGCATTTTGGCAAAGTCCTG	351	61

2.2.1.3 Determining the concentration of nucleic acid (DNA and RNA)

DNA and RNA concentrations were determined using GE NanoVue spectrophotometer (GE Healthcare, New Zealand). GE NanoVue also measures the purity of nucleic acids by wavelength scanning to detect the impurities. Samples of 1 µl were pipetted directly onto a novel sample plate for measurement and then the sample plate was wiped clean after the spectroscopic reading.

DNA concentrations were also measured by running 5 µl aliquots of DNA samples in agarose gel using a dilution series of a DNA molecular-weight marker (MassRuler, Fermentas, USA). DNA was estimated based on a comparison of the band intensities (Figure 2.2).



Kb	ng/2µl
10.0	20.0
8.0	16.0
6.0	12.0
5.0	10.0
4.0	8.0
3.0	6.0
2.5	5.2
2.0	4.0
1.5	3.2

Figure 2.2 DNA estimation based on a comparison of the band intensities

The example shows approximate quantification of GBSS vector (≤ 20 ng) according to Fermantas MassRuler (Fermantas, USA) protocol. Images of 1 % agarose gel. Lane 1- Sample DNA (2 µl DNA+1 µl 6X Mass Ruler Loading Dye + 3 µl H_2O); Lane 2 – empty; Lane 3 - 5µl MassRuler DNA Ladder High Range (Fermantas, USA)

2.2.1.4 PCR amplification

To amplify a specific region of DNA, gene specific primers (Appendix 2.2) were used in conjunction with the Taq DNA Polymerase with ThermoPol Buffer (New England Biolabs, USA). In general, for *Or*-transcripts, an optimized amplification program on the thermocycler (Eppendorf Mastercycler) consisting of an initial denaturation step at 94 ^{0}C for 4 min, followed by 35 cycles of 94 ^{0}C 30 s; 61.9 ^{0}C for 30 s; 72 ^{0}C for 1 min per kb of expected amplicon, followed by a final extension step at 72 ^{0}C for 5 min. The PCR products were then analyzed by agarose gel electrophoresis.

2.2.1.5 Agarose gel electrophoresis

Nucleic acids were separated and analyzed using a 1 % (w/v) agarose gel made with 1xTAE buffer. After electrophoresis at 100V for approx. 1 h, the gel was then soaked in ethidium bromide (5 mg mL^{-1}) for 15-30 min. The nucleic acids were the visualized under UV light and digitally photographed (Quantity One, BioRad).

2.2.1.6 Purification of PCR products and or restriction enzyme digested DNA fragments

2.2.1.6.1.Gel purification

Following separation by agarose gel electrophoresis (0.8 % agarose gel) and staining with ethidium bromide, the DNA fragments were briefly visualized using low UV light. The DNA fragment of interest was then excised using a sterile razor blade, weighed in a 1.5 mL microfuge tube and purified using Qiagen Gel Extraction Kit (Qiagen, USA) according to manufacturer's instructions.

2.2.1.6.2.PCR column purification

Each 50 µl DNA mixture was purified using High-Pure PCR Purification Kit (Roche, USA) according manufacturer's instructions to remove salts, buffers, and restriction enzymes. The DNA was eluted in 30 µL Rnase-free sterile water.

2.2.1.7 DH5-α competent cells

The DH5-α competent cells were prepared according to the protocol by Nishimura *et al.* (1990). Briefly, 6 fresh colonies of DH5-α were inoculated in 100 mL LB media (containing 10 mM $MgSO_4$ + 0.2 % w/v glucose) and incubated at 37 ^{0}C in a temperature controlled shaker. When the OD_{600} was around 0.6, cells were chilled on ice for 20 min. Then the cells were spun at 4000 *g* at 4 ^{0}C for 5 min. The cell pellet were resuspended in 20 mL ice cold SEM buffer and left on ice for 10 min. The cells were then spun at 4000 *g* for 5 min at 4 ^{0}C and the pelleted cells were resuspended in 6 mL SEM and 460 µl DMSO. Aliquots of x ml were then snap frozen in liquid nitrogen and stored in -80 ^{0}C. The DH5-α Max Efficiency competent cells (Invitrogen, Carlsbad, CA, USA) were also used whenever required.

2.2.1.8 Transformation of competent cells

As recommended in Sambrook & Russell (2001) and the Max Efficiency (Invitrogen, Carlsbad, CA, USA) protocol, 5ng of purified DNA was incubated with 50 µL of competent cells on ice for 30 min. Following a 42 ^{0}C heat shock for 45 s, the mix was placed back on ice for 2min and 900 µL SOC medium without antibiotics was added. The cells were incubated at 37 ^{0}C at 250 rpm for 1 h and then plated onto LB agar medium containing

100 μg mL^{-1} ampicillin or 100 μg mL^{-1} spectinomycin depending on the selection requirement. The plates were incubated at 37 ^{0}C for about 16 h.

2.2.1.9 Mini preparation of plasmid DNA

A single bacterial colony containing a plasmid was inoculated in 1.5 mL of LB media containing the appropriate antibiotic. Cultures were grown for 16 h at 37 ^{0}C with shaking at 250 rpm, following which the plasmid DNA was extracted using alkaline lysis (Good & Nazar, 1997; Sambrook & Russell, 2001) (see Appendix A for details). When required, Roche Minipreps DNA Purification kit (Roche, USA) was used to prepare high quality DNA as per manufacture's protocol.

2.2.1.10 DNA -alkaline lysis

The pellet derived from a 1.5 mL bacterial culture was resuspended in 150 µl of solution 1 and incubated on ice for 5 min. A 1.5 mL aliquot of solution II was added to this preparation, thoroughly mixed and incubated for 30 sec until the bacteria are fully lysed. Then 150 µl solution III was added and the suspension mixed well again. Two drops of chloroform was added to this solution to precipitate the potassium-SDS flakes. The suspension was centrifuged for 5 min and the clear supernatant was poured into a new 1.5 mL microfuge tube. An equal volume of isopropanol was added to the solution (approx. 400 µl), mixed well and centrifuged at 15, 000 *g* at 4 ^{0}C for 15 min. The pellet was centrifuged with 70 % ethanol, dried and dissolved in 50 µl TE buffer which contained DNAse-free RNase (10mg mL^{-1}).

2.2.1.11 DNA miniprep - 96 Well Block Protocol

When required, a 96-well block protocol (modified alkaline lysis method-see Appendix A for details) was used to screen large number of colonies to identify the positive clones. Briefly, the blocks were inoculated with 1 mL LB medium containing appropriate antibiotics. Sterile plastic lids were used to cover the blocks. The culture was grown overnight at 37 ^{0}C. The following day blocks were spun at 4,000 rpm for 15 min. The block was quickly inverted to tip off the supernatant and drained well on paper towels. Then 150 µl P1 buffer was added and the pellet resuspended. After adding 150 µl P2 buffer, the sealed plate was quickly inverted to mix, then kept at room temp for 5 min before 150 µl P3 buffer was added. The resealed plate was inverted to mix, incubated on ice for 20 min and then spun at 4,000 rpm for 30 min. The supernatants were transferred to a new sterile block and 0.5 mL 100 % ethanol added, followed by thorough mixing and incubation on ice for 1 h. After spinning at 4,000 rpm for 30 min the supernatant was removed by quickly inverting the plate. Pellets were washed with 150 µl 70 % ethanol, air dried and resuspended in 50 µl dH_2O.

2.2.1.12 Restriction enzyme digestion

Restriction digestion was performed using NEB restriction enzymes and the recommended buffers (New England Biolabs, USA) (Table 2.2). In general, reactions were performed in 25 µL volumes (400 ng of DNA) and 5 units of restriction enzyme, and reaction incubation times and temperatures followed enzyme supplier's guidelines. The digestions were analyzed by agarose gel electrophoresis.

Table 2.2 Restriction enzymes used

Enzyme	Buffer	Heat Inactivation (^{0}C)	Incubation Temp (^{0}C)
*Bam*HI	NEB Buffer 3 + BSA	No	37
*Cla*I	NEB Buffer 4 + BSA	65	37
*Dra*I	NEB Buffer 4	65	37
*Eco*RV	NEB Buffer 3 + BSA	80	37
*Hin*DIII	NEB Buffer 2	65	37
*Nhe*I	NEB Buffer 2 + BSA	65	37
*Not*I	NEB Buffer 3 + BSA	65	37
*Sma*I	NEB Buffer 4	65	25
*Spe*I	NEB Buffer 4 + BSA	80	37

Source: www.neb.com/nebecomm/tech_reference/restriction_enzymes

2.2.1.13 DNA blunting reaction

The Quick Blunting Kit (New England Biolabs, USA) was used to convert DNA with incompatible 5´or 3´overhangs to 5´phosphorylated, blunt-ended DNA for efficient blunt-end ligation into DNA cloning vectors. Briefly, a 50 µl blunt reaction consisted of 38 µl digested DNA, 5 µl NEB Blunting buffer, 5 µl NEB dNTP's and 2 µl NEB Quick blunting enzyme mix. The mixture was incubated at room temperature for 15 min, then treated at 70 ^{0}C for 10 min to inactivate the enzyme as per manufacturer's protocol. This DNA was used directly in ligations.

2.2.1.14 Dephosphorylation of blunted DNA

When required, the blunted DNA was dephosphorylated using the RAP (rAPID Alkaline phosphatise, Roche, USA) according to manufacturer's protocol. A 40 µl total volume consisted of 28 µl digested/blunted/purified DNA, 4 µl 10 X RAP buffer, 2 µl RAP enzyme and 6 µl H_2O. The mix was mixed thoroughly and incubated at 37 ^{0}C for 10 min. The

phosphatase was then heat inactivated at 75 ^{0}C for 2 min. The DNA was used directly in ligations.

2.2.1.15 Ligation of digestion products for vector construction

DNA fragments and the cloning vector were be prepared by appropriate restriction digestion followed by DNA purification. For blunt-end and sub-cloning of DNA, fragments were ligated directly or after gel purification with appropriated vector DNA. Since blunt-end cloning works well with ligations of 1:5 molar ratio, the insert DNA amounts were calculated as

$$\frac{\text{ng of vector x Kb size of insert}}{\text{Kb size of vector}} \times \text{Insert: Vector molar ratio} = \text{ng of insert}$$

Usually, a total volume of 10 µl ligation mixture was prepared by adding 2 µl of T4-DNA ligase and 1 µl of 10 X Ligase buffer (New England Biolabs, USA). This was incubated at 16 ^{0}C for 16 h as recommended by the manufacturer. After ligation, 5 ng DNA was used to transform into DH5-α competent cells. A positive vector only control was included in every ligation.

2.2.1.16 DNA Sequencing

When required, DNA was sequenced using Big-Dye. For a 10 µL sequencing reaction, 200 ng of pure DNA, 0.5 µL Big Dye, 0.5 µL (4 pmol) of appropriate sequencing primer, 2 µL 5 x Buffer and water was added as required. The sequencing amplification program on the thermocycler (Eppendorf Mastercycler) consisted 96 ^{0}C for 1 min, followed by 25 cycles of 96 ^{0}C 10 s; 50 ^{0}C for 5 s; 60 ^{0}C. The sequencing output was visualized using Vector NTI Advance 11 software (Invitrogen, Carlsbad, CA, USA).

2.2.1.17 Transformation of constructs in *Agrobacterium*

The pMOA33-derived constructs were transferred to the disarmed *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.*, 1993) by the freeze-thaw method (Hofgen & Willmitzer, 1988) for potato transformation. Briefly, 0.5 mL *Agrobacterium* competent cells were thawed on ice for 20 min, then gently mixed with 0.5-1 µg plasmid DNA and incubated on ice for 5 min. The mix was immersed in liquid nitrogen for 5 min, followed by thawing at 37 ^{0}C for 5 min, then 1 mL LB medium was added to the mixture. Following incubation for 3 h at 28 ^{0}C, 0.5 mL of the mixture was plated onto LB plates (3 plates) containing 100 mgL^{-1} spectinomycin. The plates were incubated at 28 ^{0}C for 36-48 h.

2.3 Results

All four *Or*-transcript inserts were received from Genscript cloned into pUC57 constructs. DNA was bulked up by a miniprep of 4 X 2 mL cultures. The DNA from each pUC57 construct was digested with *Hin*DIII to release the *Or*-transcripts from pUC57 construct. Each of the released *Or*-transcripts were separated by gel purification and blunted them individually using NEB Quick blunting enzyme mix (Figure 2.3). The DNA was quantified and used directly for ligations after inactivating the blunting enzyme.

The pUC57-GBSS vector was digested with *Eco*RV, pART7-patatin vector was digested with *Hin*DIII and the pMOA33 vector was digested with *Not*I (Figure 2.3). All digested DNAs were purified and, were blunted using the NEB Quick blunting enzyme mix. The blunted DNAs were dephosphorylated using the Roche rAPID Alkaline phosphatase. The vector DNAs were then quantified and used for ligation after inactivating the phosphatase.

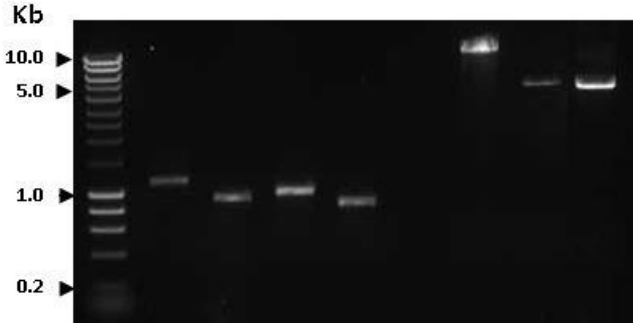


Figure 2.3 Gel purified *Or* -transcripts and vectors after appropriate digestions (showing single bands)

The example shows *Or*-transcripts and vectors prepared for ligation. Images of 1 % agarose gel - Lane 1-HyperLadder I (Bioline, USA), Lane 2- *Or*-Wild transcript (1148 bp), Lane 3- *Or* Del (899 bp), Lane 4- *Or* Ins (956 bp), Lane-5 *Or* L Del (812 bp), Lane 6- empty, Lane 6- pMOA33 vector (10014), Lane 8- pART7-Patatin vector (4438 bp), Lane 9- pUC57-GBSS vector (4275 bp). *Or* transcripts were digested with *Hin*DIII, pMOA33 digested with *Not*I, pART7-Patatin vector digested with *Hin*DIII and pUC57-GBSS vector digested with *Eco* RV.

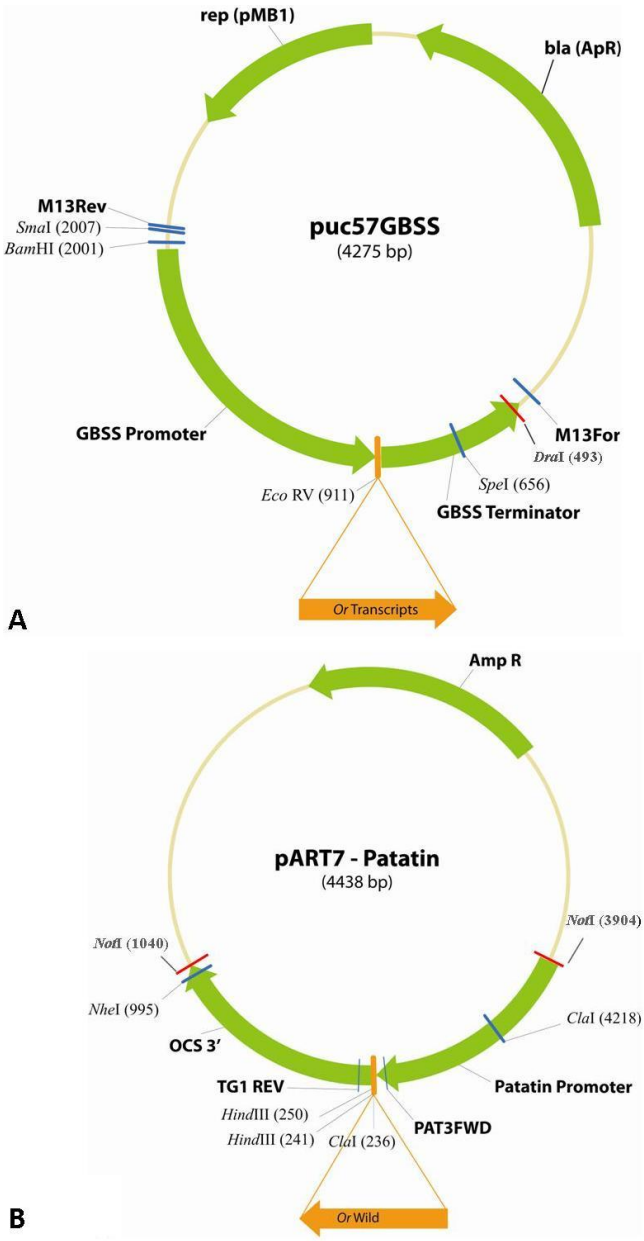


Figure 2.4 Map of the pART7-patatin vector and pUC57-GBSS vector containing *Or*-transcripts.

A. *Or*-Wild, *Or* -Del, *Or* -LDel and *Or*-Ins were individually ligated into pUC57-GBSS vector that was digested with *Eco*RV. B. *Or*-Wild transcript was ligated into pART7-patatin vector that was digested with *Hin*DIII. The Figures was generated using Vector NTI Advance ® 11 program.

2.3.1.1 Constructing the cassettes and transferring into pMOA33 vector

The blunted *Or* inserts were ligated with the blunted and dephosphorylated pUC57-GBSS vector DNA (Figure 2.4 A) at a molar ratio of 5:1 using 50 ng vector DNA. A 5 ng ligation aliquot was transformed into DH5-α competent cells and plated onto an LB plate containing 100 mgL^{-1} ampicillin and incubated at 37 ^{0}C overnight. PCR-screening of selected colonies was used to confirm successful ligations. A restriction digest with *Bam*HI digestion was set up for positive clones to screen for insert orientation (the correct orientation produces a 1141 bp fragment, whereas an incorrect orientation produces a 3232 bp fragment).

The GBSS Promoter/ terminator cassette with *Or*-transcripts (2660 bp) were cleaved from the pUC57 vector by restriction digestion with *Dra*I and *Sma*I. The resulting DNA fragments were then cloned into the *Not*I digested/dephosphorylated pMOA33 vector by standard cloning protocol as described earlier. The transformed DH5 α competent cells were plated onto a LB plate containing 100 mgL^{-1} spectinomycin and incubated at 37 ^{0}C overnight. Set up miniprep and a colony PCR for selected colonies to screen for positive clones. A restriction digest with *Spe*I digestion was set up for to screen for right orientation (2489 bp) (Figure 2.6 B).

Similarly, the blunted *Or*-Wild insert was also ligated into the blunted and dephosphorylated pART7-patatin vector DNA (Figure 2.4 B). A 5 ng ligation aliquot was transformed into DH5-α competent cells and plated onto an LB plate containing 100 mgL^{-1} ampicillin and incubated at 37 ^{0}C. A restriction digestion with *Nhe*I restriction enzyme was set up to screen for the insert orientation (the correct orientation produces a 924 bp fragment, whereas an incorrect orientation produces a 1711 bp fragment). The *Or*-patatin promoter/ terminator cassette (2707 bp) was separated from the pART7 vector by

digesting the DNA with *Not*I. The resulted cassette was then cloned into the *Not*I digested/dephosphorylated pMOA33 binary vector (Figure 2.5 A). Set up miniprep and a colony PCR for selected colonies to screen for positive clones. A restriction digest with *Cla*I digestion was set up for to screen for the correct orientation (3723+456+8550 bp).

2.3.1.2 Validation of patatin-pMOA33-*Or* -Wild construct for potato transformation

The patatin-pMOA33-*Or* -Wild construct was further sequenced and confirmed orientation of the ligated components. Only this construct was transformed with the disarmed *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method (Hofgen & Willmitzer, 1988) for potato transformation.

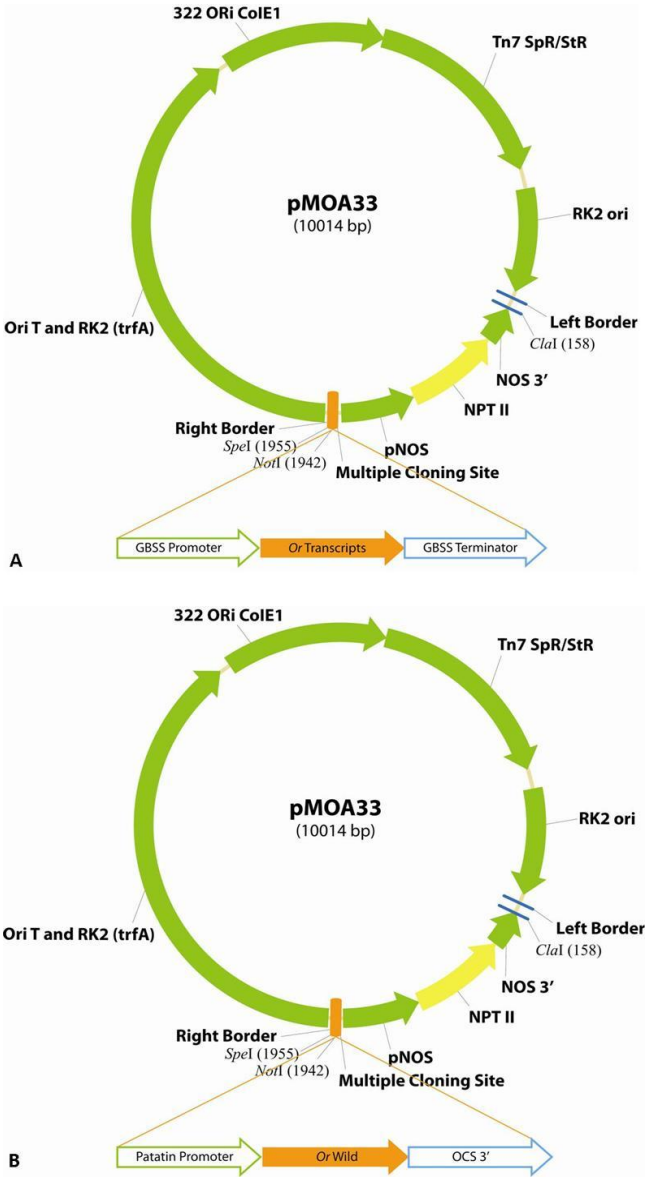


Figure 2.5 Map of the pMOA33 vector containing cassettes of the *Or*-transcripts.

A. pUC57-GBSS- transcript constructs were (*Or*-wild, *Or*-Del, *Or*-LDel and *Or*- Ins) individually digested with *Dra*I and *Sma*I to release the cassette. Resulting cassettes were ligated individually into pMOA33 binary vector. B. pART7-patatin- *Or*-Wild construct was digested with *Not*I to release the cassette. Resulting cassettes was ligated into pMOA33 binary vector. The figures was generated using Vector NTI Advance ® 11 program.

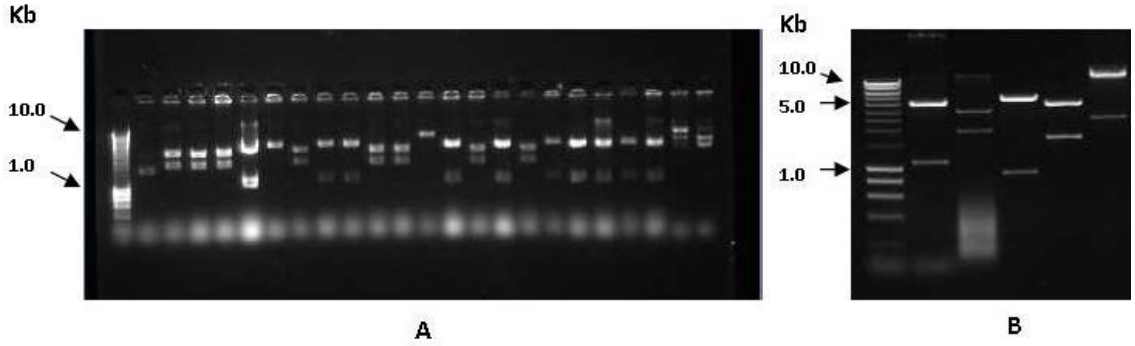


Figure 2.6 Selected examples of digestions to confirm the orientations.

A. The example shows a *Bam*HI digest to check the orientation of pUC57-GBSS-*Or*-LDel construct, using 96-Well Block Miniprep protocol. Images of 1 % agarose gel- Lane 1- Gene Ruler Ladder Mix (Fermentas, USA), Lane 3- Incorrect orientation and Lane 6- Correct orientation

B. The example shows some selected digestions for orientation: Images of 1 % agarose gel - Lane 1- Hyper Ladder I (Bioline, USA), Lane 2- GBSS-*Or* -LDel construct digested with *Bam*HI (correct orientation - 1141+3944 bp), Lane 3 *Or*-LDel construct digested with *Bam*HI (incorrect orientation - 1853 +3232 bp), Lane 4- pART7-patatin-*Or*-Wild digested with *Nhe*I (correct orientation - 940 + 4647 bp), Lane 5- pART7-patatin-*Or*-Wild digested with *Nhe*I (incorrect orientation - 1711+3860 bp), Lane 6-pMOA33-GBSS *Or*-LDel construct- digested with *Spe*I (correct orientation -2489 + 10206 bp)

2.4 Discussion and conclusion

The use of plasmids as cloning and expression vectors in prokaryotes has been instrumental in the development of new biotechnological applications for crop improvement. The construction of different binary vectors for potato transformation using the standard protocols and cloning are discussed.

The tuber-specific GBSS and patatin promoters were used in this study for the expression of the *Or*-gene in the potato tubers. Previous studies have shown that these two promoters were successfully used in tuber specific carotenoids studies in potato (Diretto, Al-Babili *et al.*, 2007; Diretto *et al.*, 2006; Diretto, Welsch *et al.*, 2007; Ducreux *et al.*, 2005; Lopez *et al.*, 2008; Potenza *et al.*, 2004; Van Eck *et al.*, 2007).

Gene synthesis is an efficient and easy way to make functional genetic constructs and enables simple sequence manipulations such as the inclusion of restriction enzymes or desired sequence elements (Villalobos *et al.*, 2006). The cost for synthesis is approximately 0.49 US cents per bp (www.genscript.com/gene_synthesis.html). In this study, all four *Or* transcripts were synthesised from Genescript Corporation with specified sequence including the restriction enzyme *Hin*DIII site to facilitate cloning. The company provided quality control documents such as sequence verification, synthesis process and ISO certificates. This establishes the synthetic gene is highly reliable and a convenient technology to use in genetic engineering.

Selection of a cloning vector depends on insert size, copy number, incompatibility, selectable marker, cloning sites and specialized vector functions (Preston, 2003). The pMOA33 (10 kb) binary vector was selected considering the above criteria and the extensive use of the vector in potato transformation. It has unique restriction sites in the

multiple cloning region with a kanamycin-resistant selectable marker gene for plant transformation and other features such as small T-DNAs, high copy number colE1 replicon that are all important for efficient plant transformation and binary vector manipulations (Barrell & Conner, 2006). Importantly, vector pMOA33 has high transformation efficiency and does not need special strains for *Agrobacterium* mediated transformation.

In this study, many colonies isolated after transformation did not contain the desired insert. This may be due to the direct insertion of random DNA fragments into a bacterial cloning vector which might have played a major role in not achieving the insertion of the gene of interest (Altenbuchner *et al.*, 1992; Preston, 2003; Strachan & Read, 1999). Large fragments of insert DNA (2-5 kb) ligation have low probability of successful vector construction in comparison to smaller DNA fragments. In this study the size of the vectors used were ranged from 4-10 kb (patatin-pART7- 4 kb and GBSS-pUC57- 4kb, pMOA33 10 kb) and the cassettes with promoter/terminator sequences ranged up to 2.7 kb.

In terms of replication, larger fragments can pose additional problems, especially when the copy number is high which may cause insert instability (Preston, 2003). This may be another reason for a low frequency of cloning success, as the vectors used in this study all had high copy number. Under such circumstances screening method became very industrious and requires performing a great number of mini-preparations. In this study, a 96 well block digestion protocol was employed to screen large number of the clones to eliminate those which did not contain the desired insert.

The quality and quantity of the DNA used in cloning is important for restriction digestion and successful ligation (Ausubel *et al.*, 1999; Strachan & Read, 1999). The use of five times more insert DNA material than the quantity of vector essentially improved the blunt

ended ligation process. The quantity refers to number of molecules and not the amount in nano grams. Therefore the quantity required for every ligation was always calculated using the appropriate equation (see 2.2.1.15). The quantity was measured using the gel quantification method and the NanoVue photospectrometer to ensure the relative quantification. After every restriction digestion, only gel purified DNA or the DNA purified with PCR purification kit (or restriction enzyme was heat inactivated at recommended temperature) was used directly for successful ligations. Gel purification after digestion of vectors can improve the ligation process by eliminating the polylinkers. Polylinkers are small pieces of DNA (10-15 bp) often released during concurrent digestion with restriction enzymes. The polylinkers disturb the ligation process as small DNA fragments and can easily become ligated into the vector relative to the DNA fragment of interest (Strachan & Read, 1999).

During gel purification, care was taken to minimize the damage to DNA by the UV light while visualizing DNA bands separated on agarose electrophoresis gels. Any DNA damage may significantly affect ligation and transformation efficiency.

The self-ligation of the vector can be reduced by removing the 5'-phosphate groups at the ends of the insertion site by alkaline phosphatase. Additional processing steps such as this would reduce the ligation efficiency (Altenbuchner *et al.*, 1992; Ausubel *et al.*, 1999). When the vector was cut with single restriction enzyme, two compatible ends emerge and the phosphate remnants remain at the 5' ends of the DNA fragment. These can ligate with one another resulting in self-ligation events, thereby reducing the number of desired clones with the intended insert. This problem was reduced by dephosphorylating the vector DNA using shrimp alkaline phosphatase (SAP). Therefore,

the ligation was improved as the insert DNA fragment still had phosphate remnants at both ends to ligate with the vector DNA

The inefficient ligation may also result from the lack of ATP in the 10 X ligation buffer. The ATP in the buffer degrades if the buffer is old or undergo more than a few freeze-thaw cycles. Using a fresh buffer or an addition of some fresh ATP can improve the ligation.

In conclusion, four cauliflower *Or*-transcripts under the transcriptional control of the GBBS promoter and the *Or*-Wild transcript under the control of the patatin promoter were successfully constructed into the pMOA33 binary vector. This design of the vectors provides an approach to specifically target the expression of the *Or*-transcripts to tubers upon *Agrobacterium*-mediated transformation of potatoes.

The pMOA33 construct, *Or*-Wild coding region with patatin promoter/terminator was transformed into *Agrobacterium*, for potato transformation (Chapter 3).

3: Chapter: The transformation of potato with patatin-*Or*-Wild construct

3.1 Introduction

Genetic engineering of potato offers a directed method of plant breeding that selectively targets one or many traits for introduction into the crop plant. It has many advantages over conventional breeding methods, and potato (*Solanum tuberosum* L.) was one of the first crops to be transformed (An *et al.*, 1986; Chakravarty *et al.*, 2007; Shahin & Simpson, 1986). The development of transgenic potato plants relies on two basic requirements. The first is a method that can transfer a gene or genes into the potato genome and regulate the expression of these genes in progeny. The main gene delivery system for achieving this is via *Agrobacterium*-mediated transformation (Grant *et al.*, 1991). The second requirement is the ability to regenerate fertile plants from transformed cells. This is achieved by regenerating plants through tissue culture (S. S. Bhojwani & M.K. Razdan, 1996; Conner *et al.*, 1994). The following presents reviews of these topics in detail.

3.1.1 Tissue Culture

Plant tissue culture is the most widely used application to create copies of plants from a single plant through micro-propagation to produce cloned plants that are genetically identical to the parent plant. This technique has excellent potential for use in the genetic engineering of plants, as transformation of plants involves the stable incorporation of DNA of interest into the nuclear genome. In 1902, Haberlandt introduced the concept of totipotency: that all living cells containing a normal complement of chromosomes should be capable of regenerating an entire plant (S. S. Bhojwani & M. K. Razdan, 1996; Taji *et al.*,

2002). Plant tissue culture can be used to produce large quantities of plant lines 'true-to-type' from desirable plant lines.

An efficient tissue culture system for regenerating shoots is vital in the development of transgenic potato plants. This involves developing successful tissue culture protocols for developing plants from transformed cells or tissues. Much work has been carried out on tissue culture in potatoes, and a range of protocols and procedures have been established by researchers since tissue culture gained an importance in plant propagation, conservation and breeding (Ahloowalia, 1982; Wareh *et al.*, 1989).

3.1.2 Somaclonal variation

A number of external factors, including the tissue culture process itself, can induce heritable genetic changes. The variations can originate from the genetic heterogeneity of somatic cells of the source plant or due to the variations of structural alterations of chromosome induced during tissue culture. Larkin and Scowcroft (1981) adopted the term 'somaclonal variation' to describe the genetic variation occurring in *in vitro* cultured plants (Taji *et al.*, 2002). Somaclonal variation has been observed in essentially all plant species that have been regenerated from tissue culture. Scowcroft and Larkin (1988) found that the somaclonal variants derived from tissue culture are the mutants that result from changes in pre-existing loci. Some genetic events which occur at a very low frequency (spontaneously); these events normally occur far more frequently during tissue culture and give the impression that somaclonal variation creates new genes (Scowcroft & Larkin, 1988).

The main causes of somaclonal variations are: reduced regulatory control of mitotic events in culture; use of growth regulators; other medium components; culture conditions;

inherent genetic instability; changes in the structure of number of chromosomes; noticeable point mutations; activation of transposable elements; chromatin loss; DNA amplification; somatic crossing over (Kaeppler *et al.*, 2000; Taji *et al.*, 2002). Tissue cultures can be maintained indefinitely, but they are genetically and biochemically unstable due to the high degree of somaclonal variation exhibited between cultures (Phillips *et al.*, 1994; Scowcroft & Larkin, 1988).

For obtaining true-to-type propagules from a selected genotype, somaclonal variation is undesirable, as the variation obtained is not always stable and heritable (Sahijram *et al.*, 2003). Some negative consequences of somaclonal variations are: introduction of deleterious genes (dominant alleles such as albinos, pollen sterility or recessive alleles such as off-types that show up in progeny of seed-propagated cultivars) and carry-over effects from tissue culture (such as excessive and multiple branching of regenerated plants due to lack of establishment of apical dominance) (Kaeppler *et al.*, 2000; Karp, 1995).

On the other hand, somaclonal variation offers prospects for the recovery of useful mutants (novel sources of variability) for crop improvement. One benefit is the creation of additional genetic variability in co-adapted, agronomically useful cultures, without the need to resort to hybridisation or the production of transgenic plants (Cocking, 1990). It can also be used to introgress alien genes from wild relatives into crop species via chromosome rearrangements that occur in plants regenerated from tissue culture (Scowcroft & Larkin, 1988).

There are two general types of somaclonal variants, namely genetic changes and epigenetic changes (Cassells & Curry, 2004). Genetic changes can occur due to point mutations, cytoplasmic inheritance, gene amplification and activation of transposable

element leading to cytogenetic changes. The cytogenetic changes leads to changes in the genome structure such as aneuploidy (gain or loss of one or more chromosomes), polyploidy (gain or loss of an entire genome) and translocation (arms of chromosomes switched or a piece of chromosome inverted).

Epigenetic changes are changes in phenotype that are not stable during sexual propagation and may or may not be stable during asexual propagation (Cassells & Curry, 2004). The factors that can cause epigenetic changes are habituation (loss of exogenous requirement for a growth factor e.g., auxin, cytokinin) and detection (callus may lose requirement for a plant growth regulator in the process of several transfers to fresh medium). Epigenetic changes can occur gradually and are reversible in nature.

Variations can be characterized on the basis of morphological, biochemical (isozymes) and DNA markers such as Randomly Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and Inter-Simple Sequence Repeat (ISSR) (Jain *et al.*, 1997).

3.1.3 Somaclonal variations in potato

Solanaceous plants are easily amenable to tissue culture, and have held a unique place in the study of regeneration phenomenon and variation among regenerated plants. The causes that underline somaclonal variation have also been more intensively studied in Solanaceous plants than any other (Jain *et al.*, 1997).

Plant genotype has important effects on somaclone regeneration and frequency. This is evident in potatoes, as significant differences were observed in the number of regenerated plants of distinct cultivars grown under identical conditions (Evans *et al.*,

1986; Gunn & Shepard, 1981). This suggests the involvement of a genetic component on the susceptibility to somaclonal variation (Karp & Bright, 1985).

Explant source is considered one of the critical factors for somaclonal variation. Plants regenerated from chrysanthemum petal epidermis-induced calli showed greater somaclonal variation than those from apex induced calli (De Jong & Custers., 1986). Addition of growth regulators to culture such as 2,4-D in potato culture medium increases the frequency of abnormal plants in potato (Shepard, 1981).

In potato, somaclonal variation occurred frequently in dedifferentiated cultures (callus and suspension cultures) than in cultures where organized plant structures, such as pre-existing meristems (Anthony, 1999). Berljak (1991) reported a wide range of somaclonal variation among potato plants (cv Bintje) regenerated from protoplasts, stem and tuber tissue callus cultures. Differences in general appearance, height, leaf size and shape, and also in number, size, shape and skin colour of tubers were reported.

Shepard *et al*. (1980) studied somaclonal variation in 10,000 protoplasts-derived potato clones from cv Russet Burbank. They found that many types of morphological variants and somaclones exhibited more disease resistance than the parent to *Phytopthora infestans*. In another study of somaclones pre-selected for normal appearance, Secor & Shepard (1981) found statistically significant differences for 22-35 characters examined; each of 65 somaclones differed from the parent cultivar for at least one trait.

Pijnacker & Sree Ramulu (1990) reported frequent variation for chromosome number in potato somaclones. In another study, Sree Ramulu *et al*. (1983) found that most of the abnormal protoclones of cv Bintje were octoploid or mixoploid. The duration of culture phase and type of culture can affect the frequency of abnormal re-generants. Sree

Ramulu *et al*. (1986) found that protoclones from cell suspension cultures of a diploid potato clone exhibited more somaclonal variation than those from shoot cultures.

Bordallo *et al*. (2004) studied somaclonal variation on *in vitro* callus cultures of potato cultivars, Achat, Baraka, Baronesa, Bintje and Contenda. Seventy and 90 days after callus induction, DNA samples of 40 calli were compared concerning the effects of the two explants (leaf and stem) and two growth regulators sources on five cultivars. The RAPD pattern suggested a high percentage of polymorphic fragments among the give genotypes, indicating high level of genetic variation among cultivars.

3.1.4 *Agrobacterium* - mediated transformation

Agrobacterium-mediated transformation has revolutionized plant molecular biology by enabling the genetic modification of a wide variety of plant species (Banta & Montenegro, 2008). Recently, *Agrobacterium* was reported to also transfer DNA to human cells (Gelvin, 2003). The *Agrobacterium* tumefaciens-mediated transformation has advantages such as stable gene expression as the insertion of the foreign gene directly into the host plant chromosome; simple segregation by low copy number of the transgene; ability to transfer large size DNA segments and preferred by breeders over direct transformation (Gelvin, 2003).

Agrobacterium tumefaciens is a gram negative soil phytopathogen that causes crown gall in a wide range of broad-leaf plants (Nester *et al*., 1984; Otten *et al*., 2008). *Agrobacterium* infects plants through wounds. It has the unique ability to genetically transfer a defined fragment of its Ti (Tumour-inducing) plasmid, the T-DNA (transfer-DNA), into the host genome at an essentially random location (Slater *et al*., 2008). The genes for opine synthesis and tumour inducing factors in T-DNA are transcribed in the

infected cells (Lin *et al.*, 2008; Nester *et al.*, 1984). Tumour induction is initiated by bacterial recognition of monosaccharides and phenolic compounds secreted by the plant wound site. Chemical signals released from the wounded tissue activate a series of *vir* genes and, in turn, initiate the infection process. Expression of *vir* genes is triggered by a phenolic compound, which is secreted from the wound site of the host plant. The main functions of *vir* proteins are to mediate the T-DNA excision from the Ti plasmid, export the T-DNA piece from the bacteria, and insert it into the host plant chromosome (Gelvin, 2003). At least 24 *vir* genes in nine operons (*vir*A, *vir*B, *vir*C, *vir*D, *vir*E, *vir*F, *vir*G , *vir*H and *vir*J) have been identified (Atmakuri & Christie, 2008; Hellens *et al.*, 2000; Lin *et al.*, 2008; Winans, 1991).

Several steps must be optimized in both bacteria and plant material before *Agrobacterium* transformation. These include: the identification of an *Agrobacterium* strain that infects the appropriate plant genotype; the design and construction of modified T-DNA to allow gene expression in plant cells; the transfer to and maintenance of the modified T-DNA in a specified *Agrobacterium* strain; the frequency of T-DNA transfer events to plant cells being high enough to be detected; and the selection and regeneration of transformed plant cells (Grant *et al.*, 1991).

In order to identify the rare transformed plant cells, genes conferring resistance to specific phytotoxic chemicals such as antibiotics are incorporated into the vectors used for the transfer of the targeted gene. Transformed cells are then selected in cell culture by their resistance to normally phytotoxic concentrations of the appropriate chemical (Grant *et al.*, 1991).

3.1.5 *Agrobacterium* strains and transformation efficiency

The efficiency of *Agrobacterium*-mediated transformation is affected by various factors, including plant species, explants, medium, antibiotics, *Agrobacterium* strains, co-cultivation, and selection methods. The bacterial strain plays an important role in transformation. The efficiency and infection capacity of different *A. tumefaciens* strains could be related to their different genetic background. The widely used *Agrobacterium* strains for plant transformation are LBA4404, EHA101, EHA105, AGL0, and AGL1; they contain non-oncogenic *vir* helper plasmids (Gelvin, 2003; Otten *et al.*, 2008). LBA4404 harbours the disarmed Ti plasmid pAL4404 with chromosomal background TiAch5 (Cheng *et al.*, 2004; Hellens *et al.*, 2000; Otten *et al.*, 2008). Super-virulent strain EHA101 is an A281 derivative harbouring pEHA101. EHA105 is a derivative of EHA101, containing plasmid pEHA105, a derivative of the plasmid pTiBo542. The strain AGL1 with C58 background also carries plasmid pTiBo542 (Lazo *et al.*, 1991; Otten *et al.*, 2008).

3.1.6 Studies on gene transfer in potato

Although potato transformation can be accomplished by direct uptake of DNA into protoplasts (Feher *et al.*, 1991), *Agrobacterium*-mediated transformation using binary vectors is the preferred method and has been reported as an effective means of transferring foreign genes into important New Zealand potato cultivars (Conner *et al.*, 1991). The techniques of plant cell and tissue culture are then used to regenerate complete plants from the individually transformed cells (Conner *et al.*, 1994).

A variety of explant sources - such as leaf disc, tuber disc, and stem - have been used for genetic transformation of potato (Rockhold *et al.*, 2001). Regeneration of plants from tuber discs was reported to lead to a lower level of somaclonal variation than that

observed in plants derived from other somatic tissue sources (Scowcroft & Larkin, 1988; Shepard *et al.*, 1980). Conner *et al.* (1991) however, reported that the co-cultivation of *Agrobacterium* with leaf segments from axenic potato plants is a convenient approach and has been successfully used for potato transformation. The use of axenic plants as the source of explants eliminates the need for surface sterilisation, saves time, reduces contamination and eliminates the potential stress on the plant tissue induced by the chemical treatment.

Cultivars vary in their physiological and agronomic characteristics, and these differences appear to affect the efficiency of *Agrobacterium*-mediated transformation. Round cultivars with white tubers such as Atlantic and Lenape (Akeley *et al.*, 1968) have been easier to transform with *Agrobacterium* than russeted varieties such as Russet Burbank, Lemhi Russet and Ranger Russet. Wenzler *et al.* (1989) experienced a similar difference between Russet Burbank and a proprietary chipping variety. Ishida *et al.* (1989), in early experiments into hormone ratios, produced no dramatic difference in the effect of auxin-to-cytokinin ratio on transformation frequencies. Instead, sensitivity to the kanamycin used for selection was the key factor. While tuber disks from cultivars with round, white tubers withstand a minimal 100 µg ml^{-1} of kanamycin during regeneration, the russeted cultivars often fail to thrive (Ishida *et al.*, 1989).

Similarly, Ishige *et al.* (1991) compared transformation efficiency of 21 Japanese cultivars and found that 11 could be transformed using binary vectors and *Agrobacterium*-mediated transformation, but four lines failed to regenerate whole plants after transformation. Filho *et al.* (1994) showed that only one of three Brazilian cultivars could be successfully transformed when using a binary vector and *Agrobacterium*-mediated transformation.

3.1.7 Vectors and *Agrobacterium* strains used in potato transformation

Davidson *et al.* (2004) suggested that, for *Agrobacterium*-mediated transformation of potato, LBA4404 strain is more suitable than AGL1 strain as a higher proportion of plants transformed using the more virulent AGL1 strain have an abnormal appearance. Therefore, the suitability of an *Agrobacterium* strain must be determined not only by transformation efficiency, but also through phenotypic evaluation of greenhouse-grown plants.

Potato transformation systems routinely rely on kanamycin resistance as a selectable marker (Conner *et al.*, 1997; Rockhold *et al.*, 2001). Selectable marker genes other than kanamycin resistance - such as hygromycin, methotrexate, phosphinothricin and phleomycin resistance - have only rarely been used for potato transformation (Barrell & Conner, 2006; Barrell *et al.*, 2002). Overall, kanamycin resistance is the preferred selectable marker, resulting in the rapid recovery of larger numbers of independently derived transgenic potato lines (Barrell *et al.*, 2002). The most commonly used selectable marker gene for plant transformation is *NPT* II (neomycin phosphotransferase II) (Conner & Meredith, 1989); it confers resistance to kanamycin and related antibiotics when transferred to and expressed in plant cells (Flavell *et al.*, 1992; Nap *et al.*, 1992). Plant cells expressing this gene are capable of phosphorylating kanamycin and related antibiotics, and thereby become resistant to the toxic effect of these chemicals (Conner *et al.*, 1991).

It is important to define the lowest concentration of the selective agent that repeatedly inhibits the development of non-transformed cells in order to minimise the recovery of false positives (Conner, 1986). It is also important that the selection intensity is not too high, as this may lead to false negatives resulting from the failure to recover transformed

plants, especially lines with low expression of the selectable marker gene. Barrell *et al.* (2002) reported that, for efficient *Agrobacterium*-mediated transformation of leaf segments from *in vitro* cultured potato plants, it was vital to delay the addition of the selection agent to the culture medium until 5-days after co-cultivation, to allow initiation of cell divisions prior to the application of selection for transformed cells. It was also important to remove the cell colonies from the leaf explant before transferred to the regeneration medium to allow. This is important to allow immediate shoot regeneration from transformation events to minimise the opportunity for somaclonal variation (Mitten *et al.*, 1990), 1990), even in the absence of transformation (Belknap *et al.*, 1994).

3.1.8 Objectives of this Chapter

The objectives of this Chapter were:

- To transfer the *Or*-Wild gene into four potato cultivars via *Agrobacterium*-mediated transformation.
- To establish their transformation efficiency; to confirm the transgenic status of putatively transformed lines.

3.2 Materials and methods

3.2.1 Plant material for transformation

Plant material for transformation of potato cultivars Agria, Desireé, Iwa and Summer Delight were grown axenically *in vitro* on a multiplication medium consisting of MS salts and vitamins (Murashige & Skoog, 1962), plus 30 g L^{-1} sucrose, 40 mg L^{-1} ascorbic acid, 500 mg L^{-1} casein hydrolysate, and 10 g L^{-1} agar. The agar was added after pH was adjusted to 5.8 with 0.1 M KOH; then the medium was autoclaved. Aliquots of 50 mL were dispensed into (80 mm diameter x 50 mm high) pre-sterilized plastic containers (Vertex, New Zealand). Plants were routinely subcultured as two to three node segments every 3-4 weeks and incubated at 20 ^{0}C under cool white fluorescent lamps (80-100 µmol m^{-2} s^{-1}; 16 h photoperiod).

(All media used in the experiments were sterilized by autoclaving at 121 ^{0}C at 103 kPa for 15 min. Filter-sterilized antibiotics and plant growth regulators were added as required, just before the media were dispensed into culture vessels. See Appendix A for recipe).

3.2.2 *Agrobacterium* strains and plasmid vectors

For co-cultivation, the *Agrobacterium* cultures harbouring the pMO33-*Or*-Wild patatin binary vector (Chapter 2) was grown overnight at 28 ^{0}C in LB medium containing 300 mg L^{-1} of Timentin, on a temperature controlled shaker, to an OD_{600} 0.6 - 0.7. The grown culture was centrifuged at 5,000 rpm at 4 ^{0}C for 10 min. The pellet was re-suspended in an equal volume LB medium without any antibiotics.

3.2.3 *Agrobacterium*-mediated transformation of *Or*-constructs

Fully expanded leaves from the *in vitro* plants were cut in half across the midribs, dipped for about 30 sec in the liquid *Agrobacterium* culture, then blotted dry on sterile filter paper (Whatman ®No 1, 100 mm diameter). To initiate calli on these leaf segments they were cultured on callus induction medium [potato multiplication medium supplemented with 0.2 mg L^{-1} napthaleneactic acid (NAA) and 2 mg L^{-1} benzylaminopurine (BAP)] in standard plastic Petri dishes (9 cm diameter x 1 cm high). The Petri dishes were then incubated under reduced light intensity (5-10 µmol m^{-2} s^{-1}) by covering them with white paper. Two days later, the leaf segments were transferred to the medium supplemented with 200 mg L^{-1} Timentin to prevent *Agrobacterium* overgrowth. Five days later, they were transferred onto the same medium further supplemented with 100 mg L^{-1} kanamycin. The resulting kanamycin resistant calli (0.5-1 mm diameter) developed within 2-6 weeks were picked and maintained on regeneration medium (potato multiplication medium with reduced sucrose level (5 g L^{-1}) and induced shoot regeneration by replacing the NAA with 5 mg L^{-1} GA_3 plus 1.0 mg L^{-1} zeatin (Sigma), both were added after autoclaving). All cultures were incubated under 30-40 µmol m^{-2} s^{-1} until shoots had regenerated. These individual shoots were then excised and transferred to the potato multiplication medium supplemented with 100 mg L^{-1} Timentin.

3.2.4 Selection of transformants

Once complete potato plants had developed, they were subcultured and re-challenged with the selective agent (100 mg L^{-1} kanamycin) under full light (80-100 µmol m^{-2} s^{-1}). Non-transgenic controls were included for each cultivar. Individual shoots (one per original shoot clump) that rooted in kanamycin supplemented medium were labelled and

further subcultured on to potato multiplication medium with 100 mg L^{-1} Timentin for micro-propagation in plastic containers as described earlier. All the independently derived lines that initiated roots on kanamycin selection media were recorded as putative transgenic lines.

3.2.5 PCR screening of putative transformed lines

Genomic DNA was isolated from *in vitro* shoots of putative transgenic and control plants using the modified CTAB protocol based on the method described by Saghai-Maroof *et al.* (1984). CTAB buffer was prepared by adding of 20 mL 0.5M EDTA (pH 8.0), 10 mL 1M Tris (pH 8.0), and 40.9 g NaCl to sterile double-distilled water up to 500 mL. After autoclaving and cooling down, 10 g CTAB (Cetyl-Trimethyl-Ammonium Bromide) was added with incubation at 60 ^{0}C until it dissolved. Two-three leaves from *in vitro* plants, or an equivalent amount of callus, were crushed in a 1.5 mL tube under liquid nitrogen and 0.5 mL CTAB buffer (preheated to 60 ^{0}C) was added, followed by incubation at 60 ^{0}C for 1 h. After cooling down, 0.5 mL chloroform/sec-octanol (24:1) was added and microfuge tubes were inverted 20 times to mix well. Following centrifugation at 10,000 rpm at 4 ^{0}C for 5 min. The upper aqueous phase was transferred to a fresh tube and 50 µl 3 M sodium acetate (pH 5.2) and 0.5 mL ice-cold isopropanol were added to each sample. This was mixed slowly, then centrifuged at 14,000 rpm at 4 ^{0}C for 10 min. The DNA pellet was washed with 0.5 mL 70 % ethanol and air dried, then dissolved in 50 µl TE-4 and stored at -20 ^{0}C for further analysis.

DNA was amplified by PCR with primers specific for the *NPT* II (neomycin phosphotransferase gene providing the kanamycin resistance) and the *Or*-gene (primers were designed in the junction between the patatin promoter and *Or*-gene), each

multiplexed with primers for the endogenous potato actin gene as an internal control (Meiyalaghan *et al.*, 2006) (see Table 2.1. for primer sequence details). The expected PCR product for *NPT* II primers was 612 bp, for actin primers was 1069 bp, and for the *Or*-Wild-patatin was 351 bp.

PCRs were carried out in a Mastercycler (Eppendorf, Germany). The reaction included 2 µl 1xThermoPol Reaction Buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$ 10 mM KCl, 2 mM $MgSO_4$, 0.1 % Triton X-100, pH 8.8 @ 25 ^{0}C), 0.4 µl dNTPs (10mM), 0.2 µl Taq DNA Polymerase (5u μl^{-1}) (Biolabs, New England), 0.4 µl of each primer (10µM), 2.0 µl template DNA (10-50 ng) and water to a total volume of 20 µl. The conditions for PCR were: 1 min at 94 ^{0}C, followed by 40 cycles of 92 ^{0}C 30 s; 60 ^{0}C 30 s; 72 ^{0}C 90 s; followed by a 5 min extension at 72 ^{0}C. Amplified products were separated by electrophoresis in a 1 % agarose gel and visualized under UV light after staining with ethidium bromide.

Lines confirmed as transgenic were transferred to the containment glasshouse and grown to maturity. The phenotype of the transformed plants and their tuber production were evaluated. Transcriptional expression of *Or*-Wild gene and patatin promoter were characterized at the RNA level by RT-PCR analysis.

3.3 Results and discussion

3.3.1 Potato transformation

The cultivars Agria, Desireé, Iwa and Summer Delight were chosen as they posses different tuber colour and carotenoid profiles (Othman *et al.*, 2006). The *Or*-Wild-patatin-pMOA33 construct was constructed and transformed successfully into these selected potato cultivars. After 3-4 weeks, the transformed leaf discs produced small cell colonies (green

and/or pale coloured) along the cut edges, on callus induction medium. When these colonies were transferred onto potato regeneration medium, most of the cell colonies remained as callus for 3-4 weeks. Following a transfer to regeneration medium, some calli colonies slowly started to produce shoots, as reported by Meiyalaghan *et al.* (2006) and Chan (2006). Rapid and early shoot bud induction is a key step for success in transformation. The friable calli generally failed to regenerate shoots. However, a number of other callus colonies continued undifferentiated growth for nearly 10-14 weeks, and regeneration of plantlets from callus took place at 12-14 weeks (Figure 3.1.). This late plantlet regeneration potentially increases the frequency of somaclonal variation, as demonstrated by Tican *et al.* (2008). Austin and Cassells (1983) demonstrated that the callus stage of regeneration is the phase in which somaclonal variation arises in potato. Organogenesis is highly dependent on genotype, origin of the explant, interaction between endogenous growth hormones, and exogenous growth regulators added to the medium. Many studies have reported that, along with explant source, the medium composition - in particular different concentrations of auxin (NAA is a synthetic phytohormone) and zeatin - is the cause of abnormal prolonged callus growth and somaclones in potato (Beaujean *et al.*, 1998; Hussain *et al.*, 2005; Tican *et al.*, 2008; Turhan, 2004). Hussain *et al* (2005) found that a lower concentration of BAP reduced shoot formation (BAP has significant role in cell multiplication), and increased concentration of IAA reduced the shoot regeneration potential in potato. Razdon (1996) reported that such prolonged callogenesis may be due either to lack of the growth hormone essential for induction of organogenesis and shoot induction, or to selective pressure exerted against the explant source. The *in vitro* culture cycle of the callus phase may have increased the frequency of somaclone incidence that will become apparent in greenhouse-grown plants.

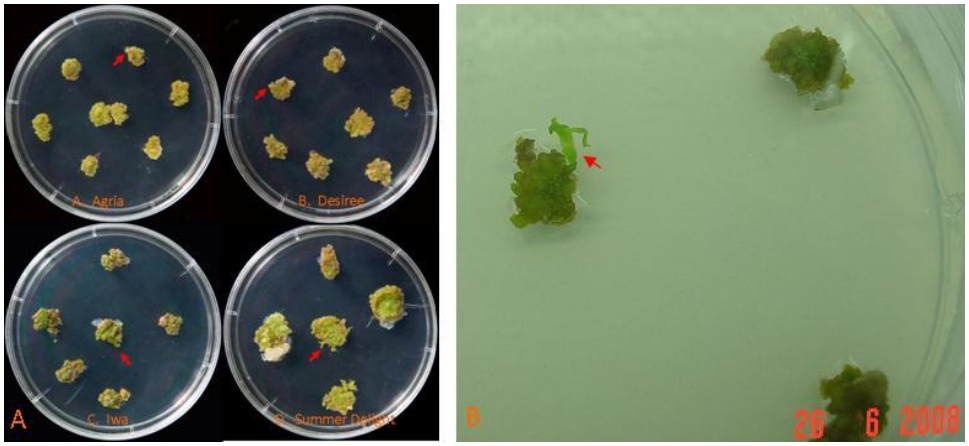


Figure 3.1 Transformed callus showing continued callogenesis and late plantlet regeneration.

A. Transgenic cell colonies of Agria, Desiree, Iwa and Summer Delight showing continuous friable callus growth in potato regeneration medium.

B. Friable Iwa callus in potato regeneration medium showing plantlet regenerated 12 weeks later.

In order to confirm the T-DNA integration in these variant transgenic calli that potentially failed to regenerate, a PCR analysis was performed using the genomic DNA (Figure 3.2). The presence of *Or*-Wild and *NPT* II genes in these lines was confirmed using multiplex PCR with actin gene as an internal control. The representative results are presented in Figure 3.2.

The cell colonies were transferred with regenerated shoots to potato multiplication medium. Single healthy shoots excised from each shoot clump were grown in kanamycin selection medium, and root induction was observed as early as 4–5 days after transfer to potato multiplication medium. All rooted plants exhibited a normal phenotype. Some of the shoots that did not form any roots on the rooting media supplemented with kanamycin 50 mg L^{-1} withered and died after 2–3 weeks. Rooting on kanamycin selective medium was found to be a good indicator of transformation, as nearly 99 % of rooted shoots were positive when tested by PCR for *NPT* II gene. The kanamycin concentration of up to 50 mg l^{-1} in the rooting medium was efficient for screening against non-transformed shoots. In a similar study, Banerjee *et al.* (2006) found that an increase of the kanamycin concentration to 75 mg L^{-1} in the rooting media delayed shoot regeneration by 12–14 days. The escapes recorded in this study may have been due to the low level of kanamycin available in the medium.

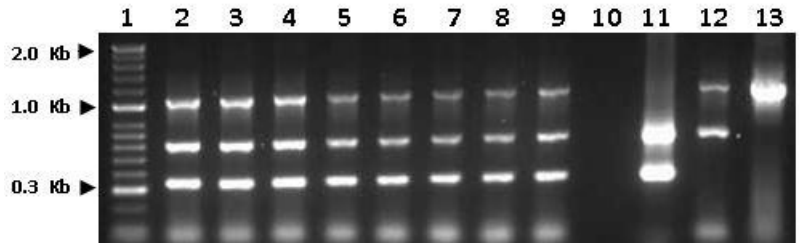


Figure 3.2 PCR analysis for the integration of the T-DNA into callus of putative transformation events.

Lanes 1-13 represent a multiplex PCR reaction producing the following expected size products: *Or*-Wild primers - 351 bp, *NPT* II primers - 612 bp, actin primers 1069 bp (as an internal control). Images of 1 % agarose gel - Lane 1-HyperLadder II (Bioline), lanes 2-3, putative transformed Agria calli; lanes 4-5, putative transformed Desireé calli; lanes 6-7, putative transformed Iwa calli; lanes 8-9, putative transformed Summer Delight calli; ;ane 10, empty; lane 11, control plasmid DNA from pMOA33 Or-Wild patatin binary vector; lane 12, positive control genomic DNA from potato plant containing *NPT* II (give name of line and source or citation); lane 13, non-transgenic Iwa DNA (negative control).

3.3.2 Transformation efficiency

Transformation efficiency was also studied for all four cultivars: Agria, Desireé, Iwa and Summer Delight. Three transformation experiments were carried out for each cultivar, with the aim of producing at least 35 lines to study the effect of *Or*-Wild gene on potato carotenoids. Controls of untransformed plant material were also prepared under the same conditions.

The most appropriate indicator of the effectiveness of the transformation event is the transformation efficiency derived from the number of transformed calli produced and the number of explants co-cultivated (Table 3.1). The transformation efficiency is affected by various factors, including potato cultivars, explants sources, medium, antibiotics, *Agrobacterium* strains, co-cultivation, and selection methods. However, many studies have reported that *the* frequency of calli colony formation is genotype dependent (Banerjee *et al.*, 2006; Beaujean *et al.*, 1998; Hussain *et al.*, 2005; Tican *et al.*, 2008; Turhan, 2004). The *Agrobacterium* strain EHA105 used for transformation also played a significant role in determining the efficiency of infection. In general, calli are produced 4 -5 weeks after *Agrobacterium* infection. Of the four genotypes tested, Iwa (15.2 %) and Summer Delight (11.8 %) were highly responsive genotypes (Table 3.1). Agria (5.8 %) and Desiree (5.0 %) were less responsive, with lower number of calli produced (Table 3.1).

3.3.3 PCR analysis of regenerated lines for T-DNA integration

PCR analysis was performed using the genomic DNA of the putatively transformed *in vitro* potato plants to confirm the T-DNA integration. The presence of *Or*-Wild and *NPT* II genes in these lines was confirmed using multiplex PCR with actin gene as an internal

control. Actin gene facilitates to determine failed PCR reaction and any presence of non-transgenic lines (escapes). The representative results are presented in Figure 3.3.

PCR results established that a total of 174 lines (Agria - 41, Desiree - 45, Iwa - 45, Summer Delight - 43) were positive for both *NPT* II and *Or*-Wild genes. Therefore, the independently derived, putative transgenic potato lines were confirmed as transgenic. Among the lines from all four cultivars, the PCR confirmed that 2 lines from Agria and 3 lines from Iwa were non transgenic (escapes), but they showed growth and rooting ability on kanamycin selection medium.

Table 3.1 Transformation efficiency of *Agrobacterium* EHA105 mediated transformation with *Or*-Wild gene in pMOA33 - patatin construct

Potato cultivar	Experiment number	Number of explants co-cultivated	Number of PCR positive transgenic plants	Transformation efficiency per experiment (%) *	Average transformation efficiency (%)**
Agria	1	232	12	5.2	5.8±2.9
	2	247	22	8.9	
	3	218	7	3.2	
Desiree	1	302	14	4.6	5.0±3.65
	2	285	24	8.4	
	3	324	7	2.2	
Iwa	1	150	22	14.7	15.2±2.4
	2	99	13	13.0	
	3	56	10	17.9	
Summer	1	177	15	8.5	11.8±3.0
Delight	2	126	18	14.3	
	3	80	10	12.5	

*****Transformation efficiency was calculated as the number of explants co-cultivated divided by the number of explants infected and produced callus. **Each value represents the mean of three independent experiment ± standard deviation (S.D)

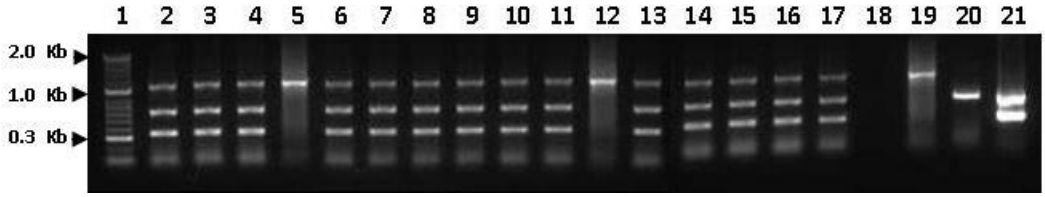


Figure 3.3 PCR analysis for the integration of the T-DNA in putative transgenic potato lines.

Lanes 1-21 represent a multiplex PCR reaction producing the following expected size products: *Or*-Wild primers - 351 bp, *NPT* II primers - 612 bp, actin primers 1069 bp (as an internal control). Images of 1 % agarose gel - Lane 1-HyperLadder II (Bioline, USA), Lane 2-5 Agria, Lane 6-9 Desiree, Lane 10-13 Iwa, Lane 14-17 Summer Delight, Lane 18- empty, Lane 19-Control Iwa genomic DNA, Lane 20-Control genomic DNA from plant containing *NPT* II, Lane 21-Control Plasmid DNA from pMOA33 *Or*-Wild patatin vector construct.

* Lanes 5 and 12 shows the escapes (false positive plants)

3.4 Conclusion

In conclusion, the efficient transformation procedure using disarmed *Agrobacterium* strain EHA105 containing pMOA33-*Or*-wild patatin construct was successfully applied to produce 178 transgenic potato plants (Agria - 41, Desiree - 45, Iwa - 45 and Summer Delight- 43) through independent transformation events, from the leaf fragments as explants. The transformation efficiency and reliability of the reported *Agrobacterium* transformation system make it possible to generate a large number of transgenic potato plants from different cultivars, as transformation is genotype dependent.

Stable integration and expression of the *Or*-Wild transgene in independently derived potato transgenic plants were confirmed through molecular analyses. The T-DNA integration shows that the pMOA33 vector construct with *Or*-Wild patatin promoter and the *Agrobacterium* strain EHA105 worked well together.

Tuber colour and phenotypic characters are discussed in Chapter 4.

4: Chapter: Phenotypic characterisation, transgene expression and tuber total carotenoid levels in transgenic greenhouse plants

4.1 Introduction

The final steps in developing transgenic potato for carotenoid fortification involve phenotypic and molecular assessment of plants in containment greenhouse followed by controlled field-testing for performance under natural conditions, open field-testing for agronomic performance, environmental and food/feed safety testing before the release and commercialization. This chapter reviews the containment greenhouse study for phenotypic assessment, molecular characterization and tuber total carotenoid levels in transgenic greenhouse plants.

4.1.1 Phenotypic characterization of transformed plants in the greenhouse

The greenhouse study of transgenic potato plants enables the measurement of changes in plant phenology from transgene expression and environmental inputs. The rooted transgenic plants from tissue culture are hardened prior to their transfer to the containment greenhouse for further growth and development. These plants allow the expression and inheritance of the introduced genes to be investigated (Navarro *et al.*, 2006). The molecular and genetic characterization will determine whether the expected traits are being expressed, the inheritance of the introduced gene is stable over generations and the expected phenotypic trait is being produced (Navarro *et al.*, 2006).

It is not possible to perform meaningful assessment of transgenic phenotypes under laboratory or greenhouse conditions (Conner, 2007), because under controlled environment of the greenhouse, the plants will be exposed to the constraints that they

have been designed to withstand and their performance is evaluated. Several years of field evaluation are required to thoroughly characterize individual transgenic clones constructed using genetic transformation (Conner, 2007). Transgenic potato lines suitable for the marketplace must have transgene-dependent improved characteristics without compromising key agronomic or quality properties.

Dale and McPartlan (1992) showed that a population of transgenic clones would be expected to contain a significant number of individuals with transformation induced variations severe enough to limit commercial viability. The stability of transgenic selections may also be dependent upon the propensity of the parental line to transformation-induced variation. For example, Jongedijk *et al.* (1992) field tested selections of cultivars Bintje and Escort transformed to express the coat protein of potato virus X (PVX). They showed that 18.2 % of the Escort and 82.1 % of the Bintje selections failed to maintain normal tuber yield and grading. The effect of such variations can be evaluated completely only when the transgenic plants are propagated under multiple plot field conditions.

4.1.2 Potato total carotenoid content and *Or*-Wild gene

The *Or*-gene offers a new molecular means to complement current approaches for carotenoid enhancement in potato tubers (Li & Garvin, 2003; Li *et al.*, 2006; Li & Van Eck, 2007; Lopez *et al.*, 2008; Zhou *et al.*, 2008). It has been demonstrated that the *Or*-gene is not a carotenoid biosynthetic gene, but represents a novel regulatory gene, perhaps controlling the creation of deposition sink (Li *et al.*, 2006; Li *et al.*, 2001). The carotenoid biosynthesis and the functions of the cauliflower *Or*-gene has been thoroughly reviewed in detail in Chapter 1.

Othman *et al.* (2006) have studied the genetic diversity in total carotenoid content of 32 potato cultivars grown in New Zealand. They found that the level of total carotenoid concentration was associated with a greater intensity of yellow pigmentation in potato tuber flesh, and the total carotenoid content ranged from 3.62 µg g^{-1} dry weight to 141.6 µg g^{-1} dry weight. Numerous other studies also reported that carotenoid content is highly correlated with the yellow-intensity of tuber flesh and is frequently used as a measure of the carotenoid levels in potato (Haynes, 2000; Lu *et al.*, 2001).

4.1.3 Broad screen for total carotenoid content by spectrophotometric method

The absorbance properties of pigments facilitate their qualitative and quantitative analysis. There are numerous different methods for quantifying antioxidant compounds in plants. For example, carotenoids are generally quantified by spectrophotometric methods although HPLC is an accurate method of measuring antioxidants. Spectrophotometric methods are being developed that results approximate to those obtained using HPLC.

The advantage of spectrophotometric method is that they are often simple, faster and cheaper to run as compared to the HPLC method. A spectrophotometric method essentially involves extraction of sample in a relevant solvent in order to release the dissolved carotenoid compounds. The absorbance of the extracted material is then read at a single wavelength specific to the particular compound or scanned between excitation and emission wavelengths for a group of compounds. The carotenoids in the broad screen are generally determined by absorbance of the hexane extracts at 446 nm (Goodwin, 1955; Scott, 2001)

Perhaps due to their complex nature in terms of structure and forms there is lack of a specified reference method available to analyze carotenoids. Different solvents are

reported useful to extract different carotenoid compounds and there are numerous published method for the extraction of the same compound. For example, the extraction of lycopene may be achieved by both a 2:1 mix of (tetrahydrofuran) THF: methanol (MeOH) or a 2:1:1 mix of hexane, acetone, ethanol as reviewed by Rodríguez-Bernaldo de Quirós & Costa (2006). The results from both methods have been published; however, data from different methods may not necessarily be compared. This appears to be a source of inconsistency between reported results. Total carotenoid levels are frequently reported with variation because of the inherent difficulties associated with the analysis of individual carotenoids (Chavez *et al.*, 2008).

4.1.4 Objectives of this Chapter

The objectives set for this Chapter were phenotypic characterization, transgene expression and total carotenoid analysis of tubers from transformed plants.

4.2 Materials and methods

Lines confirmed as transgenic were transferred to the containment glasshouse to be grown to maturity. The phenotypes of the transformed plants were evaluated. Transcriptional expression of *Or*-Wild gene under the transcriptional control of the patatin promoter were characterized at the RNA level by RT-PCR analysis. Tuber total (crude) carotenoids levels were studied in selected lines.

4.2.1 Plant transfer from *in vitro* culture to glasshouse and phenotypic evaluation

Lines confirmed as being transgenic were transferred to the containment glasshouse using the method described in Conner *et al.* (1994) and grown to maturity. Plants were transferred to soil 3-4 weeks after their last subculture. The gelled medium was washed from their roots in tepid water, and six plants of each line were transferred into plastic 6-plug cavity trays (Vertex, New Zealand). The soil mix consisted of 3 parts shredded bark (*Pinus radiata*); 2 parts sand (washed crusher dust), supplemented with 6.4 kg m^{-3} dolomite lime, 3.32 kg m^{-3} Osmocote 14-16.1-11.6 (Sierra), 1.4 kgm^{-3} superphosophate, 120 gm^{-3} calcium nitrate, 56 gm^{-3} Fetrilon Combil (BASF) and 1 kg m^{-3} Micromax (sierra). The plants were placed in shade under benches. After 1 week, plants were transferred to the top of the benches for a further 2-3 weeks. Two plants were then established in each of three PB5 bags (15 cm x 15 cm x 15 cm black polythene bags) per line, with each PB5 bag treated as replicate, and the bags placed in a randomized block design. The greenhouse conditions provided heating below 15 ^{0}C and ventilation above 22 ^{0}C. Day length was supplemented to 16 h when needed with 500 W metal halide vapour bulbs, and relative humidity was maintained above 60 %.

The phenotypes of the transformed plants were evaluated after 8-10 weeks in the greenhouse. The appearance of the foliage from each line was recorded using the categories: phenotypically normal, marginal leaf curl, leaf wrinkling, reduced vigour and/or stunted plants (Conner, 2007; Conner *et al.*, 1994). Tubers were harvested 18 weeks after before their maturity due to disease outbreak. They were immediately stored in cool dark storage (8 ^{0}C).

4.2.2 Reverse transcription (RT)-PCR analysis

Tubers from 5 phenotypically normal transgenic lines (with a control) grown in greenhouse were chosen for Agria, Desiree, Iwa and Summer Delight for RT-PCR analysis. Total RNA was isolated from 24 tuber samples using the Illustra RNAspin Mini Kit (GE Healthcare Life Sciences), according to the manufacturer's protocol. Tubers were sampled from dark storage and in the shortest possible time (<10 min) to minimize the light exposure. The membrane desalting step provided in-process DNase I treatment (supplied with kit) for the total RNA to be free of genomic DNA.

The RNA samples were assessed for genomic DNA contamination with actin gene specific primers previously used in PCR screening of putative transformed lines (Meiyalaghan *et al.*, 2006) (Table 2.1). A PCR product of 1069 bp rather than 709 bp is expected if DNA is present, since the primers flank two introns (Meiyalaghan *et al.*, 2006). The multiplex RT-PCR was performed on each DNA-free RNA sample using the SuperScriptR VILO™ cDNA Synthesis Kit (Invitrogen). For the *Or*-Wild gene, the nucleotide sequences of the primers (Table 2.1) (*Or*-Wild Forward ^{5}TAGAAGGACCTGAAACAGTACAGG^{3}, *Or*-Wild Reverse ^{5}GAGCTGTTGGTGTGATCTCAGC^{3}) that generate a RT-PCR product of 442 bp were used.

RT-PCRs were carried out in a Mastercycler (Eppendorf, Germany). The reactions included 4 µl 5X VILO™ Reaction Mix, 2 µl 10X SuperScriptR Enzyme Mix (Invitrogen), 1.0 µl RNA (2.5 µg) and DEPC-treated water to 20 µl. The conditions for RT-PCR were: 25 ^{0}C for 10 min, 42 ^{0}C for 60 min, terminate the reaction at 85 ^{0}C at 5 min (for cDNA synthesis). The conditions for PCR were: 2 min at 94 ^{0}C, followed by 40 cycles of 94 ^{0}C 15 s; 60 ^{0}C 30 s; 68 ^{0}C 90 s; followed by a 5 min extension at 68 ^{0}C. Amplified products were separated by electrophoresis in a 1 % agarose gel and visualized under UV light after staining with ethidium bromide.

4.2.3 Total carotenoid analysis

4.2.3.1 Potato tuber sample material

Tubers from 5 phenotypically normal transgenic lines (with a control), in triplicate were chosen for total carotenoid analysis. Selected tubers from the above lines (including control) were cut into half and photographed for tuber flesh colour comparisons, immediately after harvest. All tubers were harvested before maturity as early plant senesce occurred due to fungal disease outbreak. The tubers were sampled using a standard sampling core (10 mm diameter) and the skin at the both end of the tubers was removed with a surgical blade. The sample fresh weight was taken before the tuber samples were freeze-dried in sterile Whirl-Pak (3" x 7") sampling bags for 7 days. After which the samples were ground into fine powder and stored at 4 ^{0}C, in sealed Whirl-Pak bags for carotenoid extraction.

4.2.3.2 Spectrophotometric method

Samples for total crude carotenoid extraction were prepared as described by Knee (1972). 50-100 mg samples were placed into 1.7 ml Eppendorf tube and 1.5 mL 100% acetone (AnalaR grade) was added immediately. The tubes were inverted to mix and rotated on wheel in working cool room in dark for 1 hr to extract carotenoids. The samples were centrifuged at 3000 × *g* for 10 min at 4 °C. Aliquot of 1 mL acetone (100 %) placed into quartz curvette (capping curvette) and used as a blank and calibrated spectrophotometer (zero). Aliquot of 1 mL sample from carotenoid extract was placed into quartz curvettes and read the absorbance at 446 nm (β-carotene equivalents) and 550 nm (to correct for turbidity). Total carotenoid content was calculated by the following calculation:

$$\text{Total carotenoids (mg/g FW)} = \frac{D_{446} \times 10 \times V}{d \times E_{1cm}^{1\%} \times W}$$

D_{446} = the absorbance at 446 nm minus the absorbance at 550 nm; V = original extraction volume (mL of acetone) d = path length (1 cm); $E_{1cm}^{1\%}$ = extinction coefficient (2500; Goodwin 1955); W = weight of sample (g).

4.3 Results and discussion

4.3.1 Evaluation of the transgenic lines in glasshouse conditions

All transgenic potato lines of Agria, Desiree, Iwa and Summer Delight were grown with untransformed control plants in the glasshouse to study the phenotypic alteration, molecular characterization and tuber carotenoid analysis. All 174 lines were planted (Agria - 41, Desiree - 45, Iwa - 45 and Summer Delight - 43), with the expectation of a low

success in establishment as the majority of the transgenic callus and sub-cultured transgenic plants exhibited somaclonal variation in plant culture.

When plants were grown in 6 plug cavity trays in the greenhouse, all four cultivars showed very uniform growth. These plants were then planted into polybags, and morphological differences started to appear 6-10 weeks later. Majority of the plants were phenotypically normal (Figure 4.1). Plants that exhibited somaclonal variations such as aerial tubers, marginal leaf curl, leaf wrinkling, reduced vigour, dwarf/stunted plants or changes in leaf colour were categorized as abnormal phenotypes (Table 4.1). Figures 4.2 and 4.3 show the somaclonal variation exhibited by some transgenic potato lines.

Some of the plants established in the glasshouse were weak and died due to fungal wilt disease coupled with the winter climatic conditions (Figure 4.4). Fungal disease was mainly caused by *Fusarium* species. The initial symptoms observed were a yellowing and drooping or curling downward of the lower leaves. This was despite the routine spraying with appropriate chemicals was undertaken as a precautionary measure as well as in response to observing disease symptoms. Nonetheless, within a day or two, one side of the plant or the entire plant showed further symptom development. Leaves developed dark flecks or spots or a bronzed appearance, and the plant died within a week. Due to disease outbreak, plants were harvested before natural senescence and full maturity. Therefore majority of the tubers were smaller in size ranging from 2-3 cm in length.

The majority of the plants that died were altered phenotype variants (somaclonal variants), as they showed weak growth and high susceptibility to disease compared with phenotypically normal plants. Overall, 43 % Agria, 16 % Desiree, 22 % Iwa and 44 % Summer Delight plants (of the total plants established) had died by the time of harvest.

This shows that the phenotypically abnormal plants from Agria and Summer Delight were highly vulnerable to disease.

During tissue culture, various environmental factors may have caused not only genetic variation but also influenced gene expressions or caused variations in epigenetic interactions within the genome. The changes in gene expression, synthesis of certain proteins, cell differentiation, or protein distribution may have caused phenotypic variations in the transgenic plants. The frequency of variation and spectrum depended on the genotypes (Agria, Desiree, Iwa and Summer Delight).

Table 4.1 Phenotypic analysis of transgenic potato lines grown in glasshouse.

Type of variation	Number of transgenic lines			
	Agria	Desiree	Iwa	Summer Delight
Phenotypically normal	13	29	22	18
Phenotypically abnormal	10	9	13	6
Lines lost due to fungal disease	18	7	10	19
Total number of lines	41	45	45	43

*Lines with characters such as marginal leaf curl, variegated leaf, leaf wrinkling, aerial tubers, reduced vigour, stunted plants or with aerial tubers were categorized as abnormal

Figure 4.1 An example of phenotypically normal plants growing in the glasshouse.

A. Non transgenic control plants showing healthy growth with distinctive cultivar characters.

B. Putative transgenic potato lines showing normal growth with distinctive cultivar characters.

Figure 4.2 An example of aerial tubers produced due to somaclonal variation in putative transgenic potato lines growing in the glasshouse.

Pictures represent aerial tubers produced due to somaclonal variation.

A. Agria Line 16 showing splashed aerial tubers with growing buds (a sectoral or mericlinal variant).

B. Desiree Line 26 showing green aerial tuber from a plant with reduced vigour.

C. Iwa Line 44 showing stunted plant producing aerial tubers in between leaf nodes, growing against the gravity.

D. Summer Delight Line 18 showing green aerial tubers, stunted multi branched growth.

Figure 4.3 An example of abnormal leaves developed due to somaclonal variation in putative transgenic potato lines growing in the glasshouse.

Pictures represent variegated leaves produced due to somaclonal variation.

A. Agria Line 32 showing dwarf variant showing variegated marginal leave curl.

B. Desiree Line 38 showing lustreless cupping leaves with reduced vigour.

C. Iwa Line 18 showing leaf wrinkled dwarf plant.

D. Summer Delight Line 18 showing leaves changed from green to bronze colour and stunted growth.

Figure 4.4 An example of abnormal phenotype potato plants dying due to fungal disease.

A. Agria Line 32 Replicate 2 was dead due to fungal disease.

B. Summer Delight Line 13 R2 showing the commencement of fungal disease on leaves.

4.3.2 Analysis of *Or*-Wild gene expression in transgenic plants using RT-PCR

To confirm the expression of the *Or*-Wild transgene in these transgenic lines, RT-PCR was performed using tuber total RNA extracted from 5 phenotypically normal transgenic potato lines for each cultivar and one control for each cultivar. The cDNA was amplified using gene specific primer pairs of *Or*-Wild gene and potato actin, as described earlier. The *Or*-Wild gene was present on the pMOA33 construct used for transformation of these transgenic plants. The actin, an endogenous gene used as an internal control allowed failed reactions to be distinguished from non-expressing gene lines and provided a baseline for standardising gene expression between transgenic lines. Actin also provided a convenient check for genomic DNA contamination of the RNA, because the primers flank two introns in the actin gene (Chan, 2006; Meiyalaghan *et al.*, 2006).

RT-PCR analysis using *Or*-Wild gene primers produced the expected 442 bp product in the transgenic lines (Figure 4.5). Also the RT-PCR actin primers produced the expected size of 709 bp, as reported by Chan (2005) and Meiyalagan (2005). The RT-PCR results established that all 20 putatively transformed lines were positive for transcriptional expression of the *Or*-Wild gene (Table 4.2), as reported by Lopez *et al.* (2008). Thus, these data indicate that the transgene is well transcribed in the tubers of transgenic plants, as expected for the tuber-specific patatin promoter (cite reference for patatin promoter).

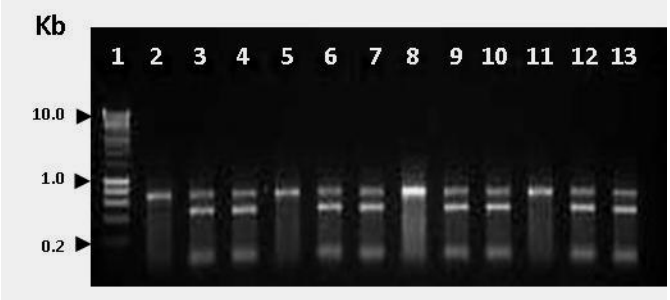


Figure 4.5 Tuber specific expression of *Or*-Wild gene in putative transgenic potato tubers.

Lanes 2-13 represent a multiplex RT-PCR reaction producing the following expected size products: *Or*-Wild primers - 442 bp, actin primers as an internal control producing 709 bp .

Images of 1 % agarose gel - Lane 1-HyperLadder II (Bioline, USA), Lane 2 Agria Control, Lane3-4 Agria lines 4 & 7, Lane 5- Desiree Control, Lane 6-7- Desiree Lines 1 & 3, Lane 8- Iwa Control, Lane 9-10 Iwa, Lines 23 &24. Lane 11-Summer Delight Control, Lane 12-13 Summer Delight Lines 4 & 10.

Table 4.2 Summary of tuber specific expression of *Or*-Wild gene in putative transgenic potato tubers.

Cultivar	Transgenic line number	RT-PCR +ve for *Or*-Wild gene
Agria	Line - 4	+
	Line - 7	+
	Line - 20	+
	Line - 30	+
	Line - 32	+
Desiree	Line - 1	+
	Line - 3	+
	Line - 4	+
	Line - 7	+
	Line - 10	+
Iwa	Line - 5	+
	Line - 17	+
	Line - 23	+
	Line - 24	+
	Line - 35	+
Summer Delight	Line - 1	+
	Line - 2	+
	Line - 4	+
	Line - 7	+
	Line - 10	+
	Total	*20*

4.3.3 Visual tuber colour characterization

All four potato cultivars selected in this study; Iwa, Desiree, Summer Delight and Agria represent diverse tuber flesh colours ranging from white, cream, pale yellow and yellow respectively (Othman *et al.*, 2006). Transgenic and non-transgenic tubers from each cultivar were cut and photographed immediately after harvest in order to avoid change in the flesh colour (Figure 4.6). The transgenic lines from all cultivars tested carrying the *Or*-Wild gene under the control of patatin promoter; produced colour changes anticipated for the expression of the *Or*-Wild gene compared with non-transgenic control (Figure 4.4). As reported in the previous studies (Li *et al.*, 2006; Li & Van Eck, 2007; Lopez *et al.*, 2008; Lu *et al.*, 2006; Zhou *et al.*, 2008), visual examination of the flesh colour of tubers revealed that the *Or* transgenic lines exhibited a dark yellow/orange flesh hue with secondary colour as flecks or rings. The colour change was markedly different between transgenic lines of Agria, Summer Delight, Desiree and Iwa respectively.

The visual assessment showed that the tuber colour intensity varied with the size of the tubers. Larger tubers generally showed high colour intensity than the smaller tubers from the same transgenic origin. Most tubers were smaller in size ranging from 2-3 cm long, with the shape ranged from oval-oblong or round. The colour intensity may be even higher if tubers attain maturity with natural plant senescence.

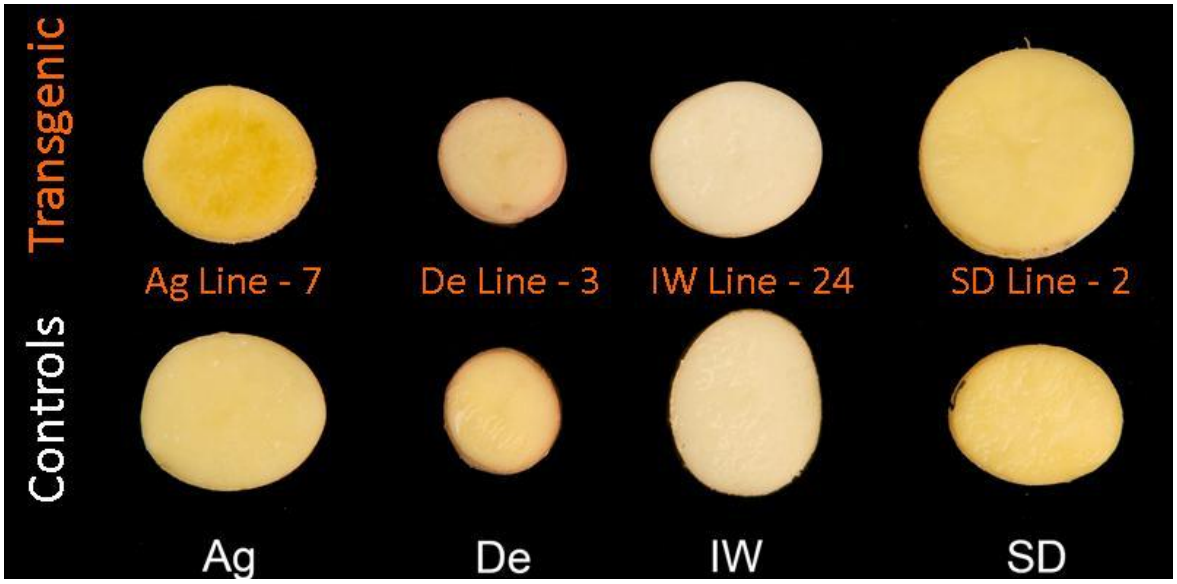


Figure 4.5 Increased carotenoid accumulation *Or*-gene in transgenic potato tubers expressing the *Or*-gene compared with respective controls.

Picture represents the cross section of the potato tubers transformed with the *Or*-gene and the non-transgenic controls of each cultivar.

Increased colour pigmentation was observed in the *Or* transformants compared with respective non-transgenic controls.

Photograph was taken immediately after harvesting. Ag - Agria; De - Desiree; IW – Iwa; SD – Summer Delight.

Table 4.4 Spectrophotometric analysis of total tuber carotenoid content and colour profiles of *Or*-transgenic lines

Cultivar	Sample name	Visual Tuber colour	Mean total carotenoid content (mg g^{-1} DW)		SD
Agria	Control	Yellow	0.134	±	0.022
	Line - 4	Dark Yellow	0.289	±	0.082
	Line - 7	Dark Yellow	0.326	±	0.055
	Line - 20	Dark Yellow	0.197	±	0.086
	Line - 30	Dark Yellow	0.245	±	0.078
	Line - 32	Dark Yellow	0.296	±	0.079
Desiree	Control	Cream	0.039	±	0.007
	Line - 1	Pale Yellow	0.073	±	0.019
	Line - 3	Pale Yellow	0.078	±	0.018
	Line - 4	Pale Yellow	0.064	±	0.012
	Line - 7	Pale Yellow	0.057	±	0.007
	Line - 10	Pale Yellow	0.057	±	0.031
Iwa	Control	White	0.009	±	0.002
	Line - 5	Cream	0.020	±	0.003
	Line - 17	White	0.018	±	0.003
	Line - 23	Cream	0.022	±	0.006
	Line - 24	Cream	0.025	±	0.001
	Line - 35	Cream	0.026	±	0.002
Summer Delight	Control	Pale Yellow/Cream	0.052	±	0.015
	Line - 1	Dark Yellow	0.143	±	0.034
	Line - 2	Dark Yellow	0.156	±	0.017
	Line - 4	Dark Yellow	0.136	±	0.044
	Line - 7	Dark Yellow	0.125	±	0.016
	Line - 10	Dark Yellow	0.143	±	0.039

Visual tuber colour observation was made immediately after cutting the tubers into halves. The colour intensity varied with tuber size as larger tubers showed more colour intensity. Values are the means of three replicates. DW - Dry Weight; SD – Standard Deviation.

4.3.4 Total carotenoid concentration determination

Total carotenoid concentrations determined in tubers on selected transgenic lines and non-transgenic control are shown in Table 4.2. All cultivars exhibited clear differences in total carotenoid content as previously reported by Othman *et al* (2006). Among the controls, Agria, the yellow-fleshed cultivar, had the highest total carotenoid content (mean 0.134, SD 0.022 mg g^{-1} DW), substantially higher than all other cultivars tested. The lowest total carotenoid concentration was found in Iwa, a white-fleshed cultivar (mean 0.009, SD 0.002 mg g^{-1} DW). The total carotenoid content shows a positive relationship with the colour intensity of tuber flesh as reported by previous studies (Burgos *et al.*, 2009; Lu *et al.*, 2001; Morris *et al.*, 2004; Othman *et al.*, 2006). Lu *et al.* (2001) surveyed 11 diploid potato clones and found the yellow-fleshed diploid potatoes contained four to 22 times more carotenoids than the white-fleshed cultivars.

The transgenic tubers carrying the cauliflower *Or*-Wild transgene show markedly higher carotenoid levels compared with respective non-transgenic controls. The transgenic lines from cultivars Agria and Summer Delight show 2-3-fold higher total carotenoid content compared with controls. Whereas, the transgenic lines from cultivars Desiree and Iwa show 2-fold higher total carotenoid content compared with controls. Among the transgenic lines, Agria Line -7 (mean 0.326, SD 0.055 mg g^{-1} DW); Desiree Line - 3 (mean 0.078, SD 0.018 mg g^{-1} DW); Iwa Line - 35 (mean 0.026, SD 0.002 mg g^{-1} DW); Summer Delight Line - 2 (mean 0.156, SD 0.017 mg g^{-1} DW) produced substantially higher total carotenoid content than all other transgenic lines tested in each cultivar. This increase in transgenic tuber total carotenoid content is associated with levels of *Or*-Wild gene expression. This increased is similar to the levels reported by other groups attempting to enhance carotenoid levels by transgenic approaches for different potato cultivars (Diretto,

Al-Babili *et al.*, 2007; Diretto *et al.*, 2006; Diretto, Welsch *et al.*, 2007; Ducreux *et al.*, 2005; Li *et al.*, 2006; Lopez *et al.*, 2008; Lu *et al.*, 2006; Zhou *et al.*, 2008). The differences in total carotenoid content between lines may be due to different levels of expression of the same transgene in tubers as reported by other groups (Diretto, Al-Babili *et al.*, 2007; Diretto *et al.*, 2006; Diretto, Welsch *et al.*, 2007; Ducreux *et al.*, 2005; Li *et al.*, 2006; Lopez *et al.*, 2008; Lu *et al.*, 2006).

It is recognized that total carotenoid composition varies as a function of such as stage of maturity, cultivar, sample handling, and analytical method (Chavez *et al.*, 2008). Since the tubers were harvested well before its full maturity, the total carotenoid content reported in this study may be an approximation of the potential to be derived from the expression of the *Or*-Wild transgene function in those transgenic tubers. The *Or*-gene is hypothesised to controls carotenoid accumulation by inducing the formation of chromoplasts which provide a metabolic sink to sequester and deposit carotenoids (Zhou *et al.*, 2008).

These changes in carotenoid content can be attributed to the specific effect of the introduced *Or*-Wild transgene, and not to non-specific events occurring during plant regeneration, such as somaclonal variation. However, somaclonal variation also can be accounted for variation in carotenoid levels such as the best-analyzed tangerine-virescent variant in tomato, as reported by Miller (1985). The somaclone variant observed in Summer Delight Line 18 with bronze/ yellow colour might be related with carotenoid accumulation.

4.4 Conclusions

The RT-PCR analysis of RNA from tubers revealed that the *Or*-Wild gene was detectable in tubers. This expression in tubers can be attributed to the tuber specific patatin promoter used in transformation of these transgenic potato lines.

Somaclonal variation is a common problem in potato transformation using leaf explants. All the rooted transgenic plantlets, including the somaclones, successfully acclimatized in the greenhouse. The majority were observed to have a phenotypically normal appearance. However, a range of off-types was observed and attributed to somaclonal variations. These may have been due to the *in vitro* establishment techniques (medium constituents) or may have pre-existed from the sources of explants. As somaclones can provide a novel source of useful variation, more detailed morphometric and chromosomal analyses are required, in particular the somaclone variant observed with bronze/ yellow colour (eg. Summer Delight - Line 18) which might be a random change associated with carotenoid accumulation.

Many of the transgenic lines were susceptible to fungal disease; 42 % of the Agria population and 44 % of the Summer Delight died due to fungal infection. However, enough tubers from genotypically normal plants were harvested for further analysis, from all four cultivars.

Or-Wild gene enhances the accumulation of high levels of carotenoids, which is a phenotypic trait of considerable agronomic importance in potatoes. The expression of *Or*-Wild transgene in this study influenced the total carotenoid content of transgenic tubers and the extent of this effect is highly variable in different cultivars. The total carotenoid content in transgenic lines reported was increased by 2-3 fold. The carotenoid

content in these lines may represent a minimum estimation, as these transgenic potatoes did not reach optimum maturity. The *Or*-Wild gene-associated carotenoid accumulation is hypothesised to result from forming carotenoid sequestering structures in chromoplasts (Lopez *et al.*, 2008; Lu & Li, 2008; Lu *et al.*, 2006; Zhou *et al.*, 2008). This may depend on the duration of plant growth to reach the equivalence of full canopy in order to provide the metabolic sink to facilitate further accumulation of carotenoids in storage tissues of potato.

5: Chapter: General discussion and conclusion

5.1 Background

The research described in this thesis was carried out to find means to enhance the carotenoid levels in potato cultivars.

The literature review in Chapter 1 documented that the development of healthy functional foods rich in natural vitamins and minerals is a major task for breeders of fruits and vegetables. The standard approach to this problem is the use of modern methods of plant breeding and genetic engineering to increase the content of such useful substances in food crops. Transgenic plants facilitate our understanding of the functional roles of genes and the metabolic processes in plants.

Plant carotenoids are the primary dietary source of provitamin-A (β-carotene) and play a critical role in human nutrition and health. Vitamin A deficiency remains one of the major nutritional problems and affects an estimated 250 million people worldwide. Therefore, developing carotenoid-enriched crops will be the most effective approach to maximize their nutritional and health potential. Several attempts have been made to genetically manipulate carotenoid biosynthesis in crop plants to increase the nutritional value, thus it has become a most interesting research areas in plant biotechnology.

Currently, significant progress has been made in understanding of carotenoid metabolism in plants. Nearly all the genes encoding the biosynthetic enzymes have been isolated and characterized from various organisms. So far the popular strategy is to overexpress the plant or bacterial gene that controls the committed steps of carotenoid biosynthesis in food crops. Biofortified golden rice is one of the best examples in using this strategy to

improve nutritional value and associated undesirable effects with this strategy. In golden rice 2, overexpression of genes [phytoene synthetase gene (*psy*) from maize and the carotene desaturase gene (*crtI*) from *Erwinia uredovora*] in carotenoid biosynthetic pathway accumulated 37 μg g^{-1} total carotenoids in rice endosperm. This is enough to provide the recommended dietary allowance of vitamin A for children in an average daily consumption of rice (Kroger, 2008; Mayer *et al.*, 2008).

The undesirable effect of the modification in golden rice 2 is the redistribution of metabolites that arise from GGPP, such as within the carotenoid pool or among carotenoids, tocopherols, and terpenoids. Perturbations in isoprenoid metabolite concentrations subsequent to pathway engineering have also been reported (Kroger, 2008; Mayer *et al.*, 2008). Such changes could, in turn, indirectly affect the overall composition and nutritional value. In many other cases, alteration of expression of carotenoid biosynthetic genes is insufficient to enhance higher carotenoid levels in transgenic plants or even leads to unexpected phenotypic changes (Kroger, 2008). This makes it essential to search for novel genes controlling plant carotenogenesis and to gain understanding of the mechanism underlying carotenoid biosynthesis and accumulation in potato crop.

Based on the above, the cauliflower *Or*-gene approach (Li *et al.*, 2006; Lopez *et al.*, 2008; Lu *et al.*, 2006; Zhou *et al.*, 2008) was selected for this study and the results are presented in Chapters 2, 3 and 4. This is a complementary to more classical approaches, which rely on the alteration of expression of structural genes in the carotenoid pathway. The cauliflower *Or*-gene encodes a DnaJ-related protein and is able to dramatically increase β-carotene levels in many low pigmented tissue of the plant, in particular the edible curd and shoot meristem (Lu *et al.*, 2006). This gene functions in increasing the sink capacity

rather than altering the expression of genes involved in carotenoid biosynthesis. This is thoroughly reviewed in Chapter 1.

The potato crop was selected in this study as the world's 3rd most important source of calories in the human diet (Camire *et al.*, 2009). Furthermore, the nutritional quality of potato tubers is an area of key interest to many plant scientists. Potato contains a number of important nutrients. It is a source of high quality protein, vitamin C, and niacin (Brown *et al.*, 2003). However, the potato tubers contain low levels of carotenoids, composed mainly of the xanthophylls lutein, antheraxanthin, violaxanthin, and of xanthophyll esters (Diretto, Al-Babili *et al.*, 2007; Diretto *et al.*, 2006; Diretto, Welsch *et al.*, 2007). The health benefits of different carotenoids become increasingly evident and so the types and amounts of carotenoids in potato tubers is of importance. Therefore, the different molecular approach using cauliflower *Or*-gene, holds good promise for the further increase of the provitamin A levels of potato cultivars.

Briefly, to achieve the above following key objectives were established for the research in this thesis:

- Construct chimeric genes to target the expression of *Or*-transcripts in potato tubers and insert these genes into a binary vector for plant transformation.
- Develop a large population of independently derived putatively transgenic lines in four potato cultivars with differing carotenoid levels (Agria, Desiree, Summer Delight and Iwa) and confirm their transgenic status for the *Or*-Wild gene.
- Study the phenotypic characterisation of these transformed plants in the greenhouse, including transgene expression and total carotenoid levels in tubers.

5.2 Summary of findings

- In this study, four cauliflower *Or*-transcripts under the transcriptional control of the GBBS promoter, plus the *Or*-Wild transcript under the control of the patatin promoter, were successfully constructed in the pMOA33 binary vector using a blunt-end cloning strategy (Chapter 2).
- This project has demonstrated that the transformation efficiency and reliability of the reported *Agrobacterium*-mediated transformation system for potato made it possible to generate a large number of transgenic potato plants through independent transformation events (Chapter 3).
- It has also demonstrated that the efficiency of the potato transformation protocol used is genotype-dependent with transformation frequencies and the ease of recovering transgenic lines varying markedly between the four potato cultivars (Chapter 3).
- The cultivar Iwa was highly responsive to *Agrobacterium*-mediated transformation, confirming earlier studies (Conner *et al.*, 1991). This project also established that Summer Delight cultivar was also highly responsive to *Agrobacterium*-mediated transformation (Chapter 3).
- The data from molecular studies confirmed the transgenic status of the recover plants and the transcriptional expression of the *Or*-Wild transgene in twenty independently derived potato transgenic plants. The RT-PCR analysis of RNA from tubers revealed that the *Or*-Wild gene was detectable in tubers which can be attributed to the use of tuber specific patatin promoter in this study. (Chapters 3 & 4).

- The study identified and established phenotypically normal transgenic plants for further studies. Other lines showed a range of off types attributed to somaclonal variation. This somaclonal variation may have been due to the *in vitro* establishment techniques (medium constituents) or may have pre-existed from the sources of explants (Chapter 4).

- The study also found that majority of the phenotypically normal transgenic lines maintained the expected resistance to fungal diseases, but that some phenotypic off types were more vulnerable to diseases (Chapter 4).

- The present study also showed that the four cultivars exhibited clear differences in total carotenoid content, confirming earlier studies (Othman *et al.*, 2006). The cultivar Agria, a yellow-fleshed cultivar, had the highest total carotenoid content (mean 0.134, SD 0.022 mg g^{-1} DW), substantially higher than all other cultivars tested. The lowest total carotenoid concentration was found in Iwa, a white-fleshed cultivar (mean 0.009, SD 0.002 mg g^{-1} DW). The total carotenoid content shows a positive relationship with the colour intensity of tuber flesh (Chapter 4).

- The transgenic tubers expressing the cauliflower *Or*-Wild transgene show markedly higher carotenoid levels compared with respective non-transgenic controls. The transgenic lines from cultivars Agria and Summer Delight show 2-3-fold higher total carotenoid content compared with the non-transgenic controls. Whereas, the transgenic lines from cultivars Desiree and Iwa show 2-fold higher total carotenoid content compared with the controls (Chapter 4).

- Among the transgenic lines, Agria line - 7 (mean 0.326, SD 0.055 mg g^{-1} DW); Desiree line - 3 (mean 0.078, SD 0.018 mg g^{-1} DW); Iwa line - 35 (mean 0.026, SD 0.002 mg g^{-1} DW); Summer Delight line - 2 (mean 0.156, SD 0.017 mg g^{-1} DW)

produced substantially higher total carotenoid content than all other transgenic lines tested in each cultivar (Chapter 4).

- This study also indicates that total carotenoid composition varies as a function of such as stage of maturity, cultivar, sample handling, and analytical method. The total carotenoid content reported in this study may be an approximation of the potential to be derived from the expression of the *Or*-Wild transgene function in those transgenic tubers. The *Or*-gene is hypothesised to controls carotenoid accumulation by inducing the formation of chromoplasts which provide a metabolic sink to sequester and deposit carotenoids (Chapter 4).

5.3 Contribution of this thesis

- In this thesis, the experimental work is described in three chapters. One chapter focuses on the construction of vectors for plant transformation which includes useful protocols for students. The other two chapters focus on *Agrobacterium*-mediated transformation, transgenic plant establishment and total carotenoid analysis.

- The experiments in this thesis have established the cauliflower *Or*-Wild transgene markedly increases the carotenoid levels in transgenic potato tubers. This is hypothesised to results from inducing the formation of chromoplasts which provide a metabolic sink to sequester and deposit carotenoids. The twenty lines established by molecular analysis to express the *Or*-Wild gene in tubers will be a useful reference for future studies on carotenoid in these potato cultivars.

- The transformation efficiencies reported in this thesis reiterates the need to modify the transformation system and the concentrations of growth nutrients to suit

particular cultivars in order to produce a high and reliable transformation frequency.

- The total carotenoid analysis reported in this thesis shows the importance of stage of maturity, cultivar, sample handling, and analytical method. The spectrophometric method used to analyse the total potato carotenoids may be improved by adding saponification step to reduce the chlorophyll and starch contamination.

5.4 The studies for future research

- Re-establishing the glasshouse study in order to confirm the total carotenoid content from tubers once they attain full maturity.
- Quantitative analysis of *Or*-Wild gene expression by Real Time RT-PCR to examine the differential gene expression levels with tuber age and investigate an association between the transcriptional expression of the *Or*-Wild gene and carotenoid accumulation.
- Use HPLC method to analyse the concentrations of individual carotenoid compounds at different growth stages of potato tuber growth.
- Analyze transgenic tubers for changes in carotenoid and starch content in order to investigate if the is any influence on starch accumulation from a possible conversion of amyloplasts to chromoplasts.
- Microscopic analysis of structural changes in the development of amyloplasts, amylo-chromoplasts and chromoplasts.

- Examine the expression of candidate potato endogenous carotenoid and starch biosynthetic genes.

5.5 Conclusion

In conclusion, this investigation has demonstrated that the cauliflower *Or*-Wild gene can be used as a powerful tool to enhance the carotenoids levels in potato cultivars.

Appendix A – Reagents and Media Recipes

Three solution miniprep reagents

Solution I	**For 50 mL**
0.5 M Glucose	5.00 mL
1M Tris HCl	1.25 mL
0.5M EDTA	1.00 mL
dH_2O	42.75 mL

Solution II (Prepare Fresh)	For 15 mL
10N NaOH	0.30 mL
10% SDS	1.50 mL
dH_2O	13.20 mL

Solution III	For 50 mL
5M Potassium acetate (14.721 g)	30.00 mL
Glacial acetic acid	5.75 mL
dH_2O	14.25 mL

1 x TE Buffer

10mM Tris HCl + 1mM EDTA

Add 5 µl RNAseA (10mg mL^{-1}) per every 1 mL of 1 x TE Buffer and , store at 4 ^0C

Alkanine lysis miniprep reagents

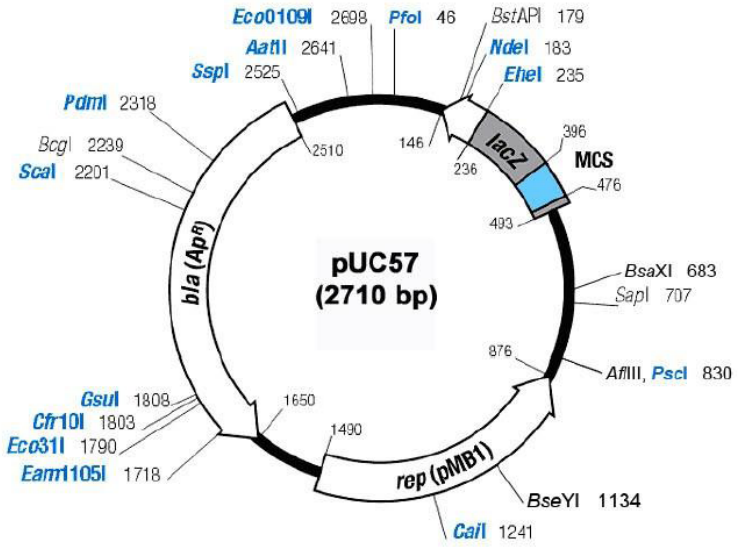
P1 Buffer			
Stock solution	**Final conc. Req.**	**Dilution factor**	**For 100 mL**
1M Tris	50 mm	1:20	5.0 mL
0.5M EDTA	10 mm	1:50	2.0 mL
RNAseA 10mg mL^{-1}	100 µg mL^{-1}	1:100	1.0 mL
dH_20			92 mL
P1 Buffer without RNase A can be stored long term at room temperature. Once RNaseA is added, store at 4 ^{0}C.			

P2 Buffer			
Stock solution	**Final conc. Req.**	**Dilution factor**	**For 100 mL**
5M NaOH	0.2M	1:25	4.0 mL
10% SDS	1%	1:10	10.0 mL
dH_20			86.0 mL
Store at room temperature, if the SDS precipitates (in cold weather) warm in a water bath at 40 ^{0}C until the precipitate dissolves back into solution.			

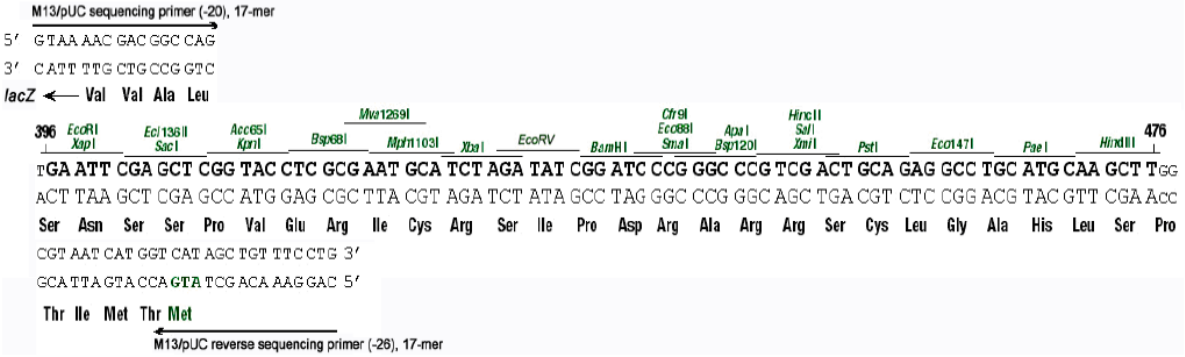
P3 Buffer			
Stock solution	**Final conc. Req.**	**Dilution factor**	**For 100 mL**
5M CH_3COOK*	3M		60.0 mL
Glacial Acetic Acid	5M		11.5 mL
dH_20			28.5 mL
Store at 4 ^{0}C			
* 5M CH_3COOK (Potassium Acetate) = 49.075 g in 100ml dH_20, autoclave			

		Potato Multiplication Media (PM)	Callus Induction Media I	Callus Induction Media II	Callus Induction Media III	Potato Regeneration Media (PRM)
		For maintaining non transgenic plants	*For co-cultivation explants*	*For co-cultivation explants*	*For co-cultivation explants s*	*For regenerating transgenic plants*
Stock Solution:	**Stock Conc.**	**For 1 Litre Add:**	**For 1 Litre Add:**	**For 1 Litre Add:**	**For 1 Litre Add:**	**For 1 Litre Add:**
MS Macro	20x	50ml	50ml	50ml	50ml	50ml
MS Micro	200x	5ml	5ml	5ml	5ml	5ml
MS Iron	200x	5ml	5ml	5ml	5ml	5ml
MS Vits	200x	5ml	5ml	5ml	5ml	5ml
Ascorbic Acid	40mg/ml	1ml	1ml	1ml	1ml	1ml
Casein Hydrolysate	0.5g/l	0.5g	0.5g	0.5g	0.5g	0.5g
Sucrose (3%)	30g/l	30g	30g	30g	30g	5g
Final volume		make up to 1 L	make up to 1 L	make up to 1 L	make up to 1 L	make up to 1 L
Final pH required		5.8	5.8	5.8	5.8	5.8
Additives:						
Napthaleneactic Acid (NAA)	0.2mg/ml		1ml	1ml	1ml	
Benzylaminopurine (BAP)	2mg/ml		1ml	1ml	1ml	
Timentin	200mg/ml			1ml	1ml	1ml
Kanamycin	50mg/ml				2ml	1ml
Zeatin	1mg/ml					1ml
Gibberelic Acid	5mg/ml					1ml
Agar	10g/l	10g	10g	10g	10g	10g
Containers		large pottles 50ml	Petri dishes 20ml	Petri dishes 20ml	Petri dishes 20ml	large pottles 50ml
Time on plates			2 Days	5 days	3-6 weeks	Until Shoots Develop

Appendix B – Vector Maps



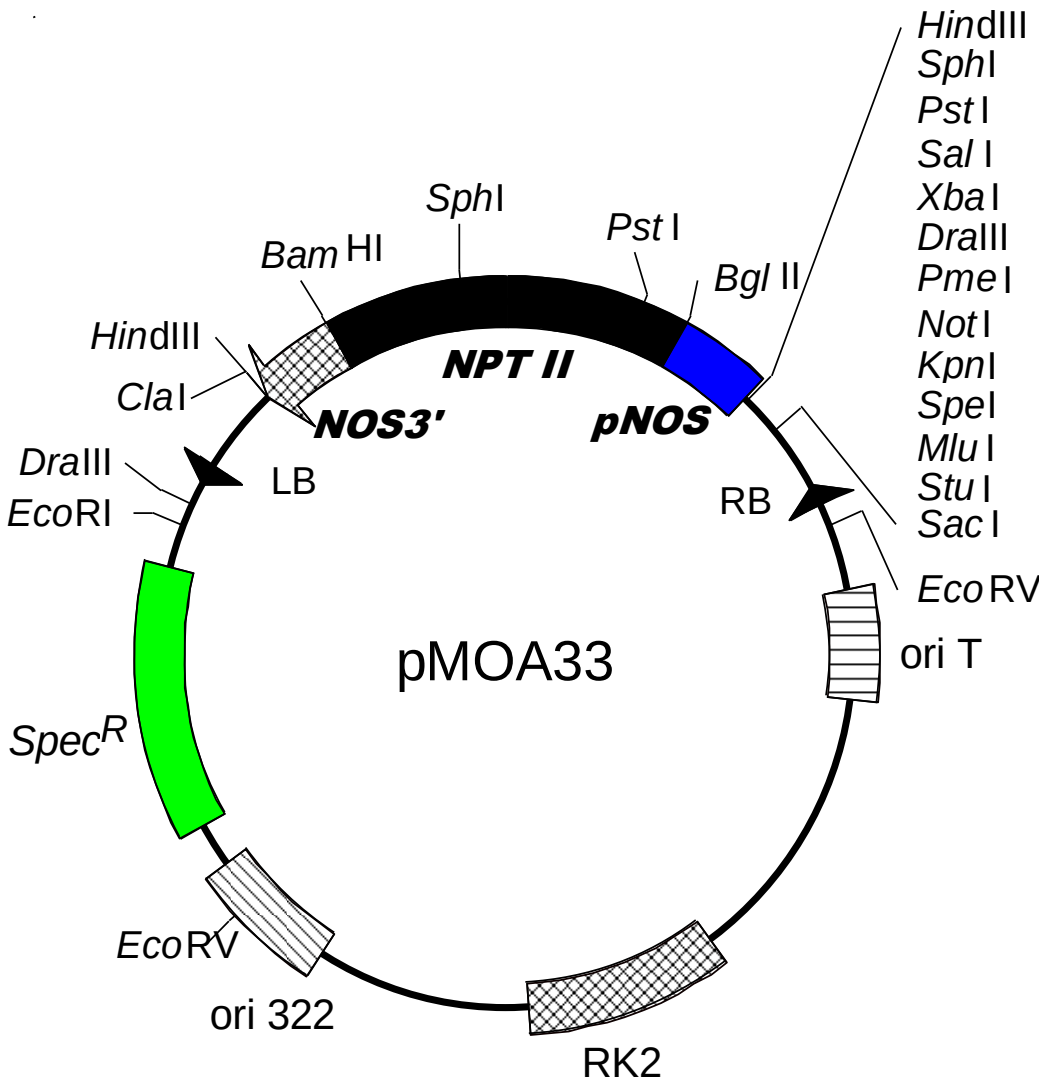
Cloning Region:



Sequencing Primers:

Forward primer DA0001: pUC57 Forward (GTAAAACGACGGCCAGTG)
Reverse primer DA0002: pUC57 Reverse (GGAAACAGCTATGACCATG)

pMOA33 Vector



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