MARKER GENE TRANSFER TO OPIUM POPPY
(*Papaver somniferum* L.)

A THESIS SUBMITTED TO
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UĞUR UZAY SEZEN

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Prof. Dr. Tayfur Öztürk
Director

I cerify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Gürdal Alaeddinoğlu
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Prof. Dr. Zeki Kaya
Co-supervisor

Assoc. Prof. Dr. Sertaç Önde
Supervisor

Examining Committee Members

Prof. Dr. Zeki Kaya

Prof. Dr. Murat Özgen

Assoc. Prof. Dr. Tülin Güray

Assoc. Prof. Dr. Sertaç Önde

Dr. Vehbi Eser

ABSTRACT

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SEZEN, Uğur Uzay
M.S. Department of Biology
Supervisor: Assoc. Prof. Sertaç Önde
Co-Supervisor: Prof. Dr. Zeki Kaya
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A gene transfer procedure to Opium poppy (*Papaver somniferum* L.) using *Agrobacterium tumefaciens* and a regeneration scheme for the transformed hypocotyl sections have been investigated in this study. Co-cultivation temperature experiments supported the proposed conjugational T-DNA transfer mechanism and best results were obtained at 19 ° C. Organogenesis temperature for both shoot and root formation was determined as 16 ° C. Previously unsuccessful attempts for root induction was achieved at 0.01mg.l^{-1} IBA concentration. Since the whole regeneration process were carried out under lethal Kanamycin selection, the Kanamycin resistance displayed by the recovered plantlets were accepted as the sole proof of the putative transgenicity. However, our attempts to confirm this putative transgenicity by PCR and Southern Blot analysis revealed negative results

due to the severe genomic DNA degradation that took place during longterm storage.

Results obtained from this dissertation will form the basis for conducting an unconventional breeding program involving genetic engineering of opium poppy. The procedure developed for marker gene transfer might be utilised for the transfer of genes conferring herbicide, pest and cold resistance as well as disease tolerance to the opium poppy plantlets in the future.

Keywords: *Papaver somniferum*, gene transfer, *Agrobacterium tumefaciens*, co-cultivation, organogenesis, regeneration.

ÖZ

HAŞHAŞA İŞARET GENİ TRANSFERİ
(*Papaver somniferum* L.)

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Kültür haşhaşı'na (*Papaver somniferum* L.) *Agrobacterium tumefaciens* yolu ile işaret geni olan neomisin fosfotransferaz'ın (NPTII) aktarılması ve transforme edilmiş dokuların tam bireyler haline organ oluşumu yoluyla dönüştürülmesi icin gerekli yöntemler araştırıldı. *Agrobacterium* ile ortak kültüre alınan haşhaş hipokotil parçalarına en verimli gen transferinin 19 °C sıcaklıkta gerçekleştiği saptandı. Bu bulgu *Agrobacterium* için T-DNA'nın aktarımının konjugasyon yolu ile gerçekleştiğine ilişkin öne sürülen hipotezi desteklemektedir. Transforme edilen bitki dokularının sürgün ve kök organ oluşum sıcaklığı ise 16 °C olarak belirlendi. Daha önce gerçekleştirilemeyen kök oluşumu için gereken uyarılma 0.01mg.l^{-1} derişimindeki IBA ortamında başarı ile gerçekleştirildi. Tüm rejenerasyon işlemleri öldürücü Kanamisin seleksiyonu altında gerçekleştirildiğinden elde edilen bitkiciklerin gösterdiği bu Kanamisin direnci transgenik adaylığı için tek kanıt olarak kullanılmıştır.

Buna karşın, transgenik adayı bitkiciklerin gerçekte transgenik olduklarının moleküler ispatı amacı ile gerçekleştirildiği PCR ve Southern Blot analizleri uzun süreli saklama işlemi sırasında genomik DNA örneklerinin ileri derecede parçalanması sonucu negatif sonucu vermiştir.

Bu çalışmanın sonuçları haşhaşın genetik mühendisliği için gerekli alt yapıyı oluşturmaktadır. İşaret genlerinin aktarılmasında kullanılan yöntem, ileride tarım ilaçlarına, soğuğa ve hastalıklara dayanıklılık genlerinin haşhaşa aktarılması yolunda da izlenebilecektir.

Anahtar Kelimeler: *Papaver somniferum*, gen aktarımı, *Agrobacterium tumefaciens*, ortak kültür, organ oluşumu, rejenerasyon.

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ABBREVIATONS

bp	base pairs
2,4-D	2,4-Dichlorophenoxyacetic acid
DIG	Digoxygenin-dUTP
DNA	Deoxyribonucleic acid
L-dopa	L-dihydroxyphenylalanine
DOPADC	Dihydroxyphenylalanine decarboxylase
EDTA	Ethylenediamine tetra acetic acid
GFP	Green Fluorescence Protein
IBA	Indole butyric acid
KIN	Kinetin
LB	Luria - Bertani
μg	micrograms
μl	microlitres
MS	Murashige-Skoog Basalt salt medium
NAA	α-Naphtylamide acetic acid
NPTII	Neomycine phosphotransferase II
OD	Optical density
PCR	Polymerase chain reaction
RNAse	Ribonuclease
(SAM-S)	S-adenosyl-L-methionine synthetase
SDS	Sodium dodecyl sulfate
TDC	Tyrosine decarboxylase
T-DNA	Transfer DNA
Ti- plasmid	Tumor inducing plasmid
TMO	Turkish Opiate Board
Tn5	Transposon 5
u	Enzyme unit

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I am deeply grateful to my supervisor Dr. Sertaç Önde for that he has been a fully patient person till the very end of this study tolerating the hyper mood of mine especially the "cross-border operations" that have never been outside his intelligence. Without his contribution in lab work as well as "proof reading" and scrutiny, this thesis would never be completed.

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To the enchanting forest of METU,
a place to hide, take shelter, make love, think, and imagine.
To the elements of my dream-time imagination: inhabitants of the forest itself

the pine, the woodpecker, the turtle, the bumble bee, the fox, the oak, the jay, the swallow tail, the snake, the rabbit, the buzzard, the mullein, the praying mantis, the badger, the kestrel, the poppy, the ant, the owl,the mice,the grasshoper, the hedgehog
and the countless
living body that have given me the inspiration and joy of life before and during this study and to the stalker spirit within me that will continue to do so.

CHAPTER 1

INTRODUCTION

1.1 Brief history of opium poppy

Opium poppy (*Papaver somniferum* L.) is a medicinally important plant that has been cultivated extensively as an oil seed crop throughout the history. The earliest record of the plant is found in the non-semitic language of the Summerians roughly between 6000-5000 BC (Neligan 1927). The medicinal fathers of the western world such as Hippocrates and Dioscoroides have described poppy as a painkiller in the 4th century BC. The plant is also mentioned in Homer`s epic poem "The Illiad" as "the cup of Helen" or "opium drought". There, it is stated that a person under the influence of opium would not be dismayed nor sorrowful even though his parents died and his brother or son were slain which explicitly reflects todays worldwide problem of drug addiction. The 18th century poets as Byron, Shelly, Keats, Wordsworth, Sir A.Conan Doyle and Edgar A. Poe were known by their addiction to opium. This property of poppy comes from its alkaloidal chemistry. In 1815 the major alkaloid from poppy was isolated by F. Serturner, a German drug clerk. He has named the chemical as *morphine* referring to the Greek goddess of dream, Morpheus. Later, narcotine and codeine were discovered by a French chemist Robiquet in

1817 and 1832 respectively. *P. somniferum* contains around 30 alkaloids (Fairbairn and Steele 1980) and among these morphinan alkaloids morphine, codeine, papaverine and thebaine manifest anticholinergic, antispasmodic and sedative activities and are therapeutically important.

1.2 Taxonomy and biology of opium poppy

The taxonomical grouping of the opium poppy is as follows:

Division..... .Spermatophyta

Class.....Dicotyledonae

Order.....Papaverales

Family.....Papaveraceae

Genus.....*Papaver*

Section.....*Papaver*

Species.....*somniferum*

1.2.1 Classification within genus level

The taxonomical grouping within the genus *Papaver* L. is not clearly defined. Based on karyotype analysis, the genus has seven sections (Srivastava and Lavania 1991) of nearly 120 species. On the other hand restriction site analysis of chloroplast DNA reveals eleven sections with about 80 species (Kadereit and Sytsma 1992). Members of the genus *Papaver* L. are annual, biannual or perennial plants bearing solitary, racemic or paniculate flowers. The structure of

the gynoecium is distinctive with a radially arranged sessile stigmata on a conical to flat stigmatic disc.

1.2.2 Classification within species level

P. somniferum is native to Asia minor and is grown largely in Pakistan, Afghanistan, Iran and Tasmania. It is a herbaceous annual plant. The flowers posses superior ovary with some luxurious corolla exhibiting variation in petal colour. Petal colour is monogenically controlled with four alleles, violet being dominant over red, red over pink and pink over white (Bhandari 1989). The capsule is a characteristic structure recognised upon maturation and has the alkaloid-bearing laticifer vessels. Laticifers are highly specialised internal secretory systems. These vessels are articulated and anastomosing, forming an extensive network through gradual dissappearence of common cell walls closely associated with phloem (Nessler and Mahlberg 1981). Leaf margins are incised and show a wide morphological variation between lacerate or pinnatisect to doubly serrated or weakly pinnately lobed with the lobes serrated. The former leaf incision was found to be dominant over the latter (Sharma *et. al.* 1991). Poppy seeds are rich in protein ranging from 23% (Srinivas and Rao 1986) to 37.8% (Statham 1984). The oil content of the seeds reaches to 50% (Srinivas and Rao 1986) and digestable energy content of 13.1MJ/kg dry matter (Statham 1984). For that reason it is an excellent food source for animals including humans.

1.3 Chronology of *in vitro* studies on opium poppy

Cultures of *P.somniferum* L. *in vitro* have been reported by various researchers. First report of poppy tissue grown in culture is by Pontowich (1959) for studying the growth of sterile placentae cultures. Formation of callus (Ranganathan *et al.* 1963, Rangachari *et al.* 1966, Furuya *et al.* 1972, Ikuta *et al.* 1974, Yoshikawa and Furuya 1983, Ilahi 1983, Bajwa and Wakhlu 1985, Seyrantepe 1992), suspension cultures (Tam *et al.* 1980, Nessler 1982, Schuchmann and Wellmann 1983, Bajwa and Wakhlu 1985, Curtis 1991, Siah and Doran 1991), and re-differentiation of those tissues through organogenesis by root formation (Staba *et al.* 1965 ,Nessler and Mahlberg 1970), shoot formation (Nessler and Mahlberg 1970, Ikuta *et al.* 1974, Yoshikawa and Furuya 1982, Bajwa and Wakhlu 1985) and emryogenesis (Nessler 1982, Schuchmann and Wellmann 1983, Galewski and Nessler 1986) have been investigated.

1.4 Genetic engineering of opium poppy

1.4.1 Preliminary works for improvement of alkaloid metabolism

Biochemistry of the alkaloid biosynthesis metabolism in *P. somniferum* L. was investigated in some detail. Genes of the two initial rate-limiting enzymes L-tyrosine (EC 4.1.1.25, TDC) and L-dopa (EC 4.1.1.26, DOPADC) decarboxylase have been screened, isolated and characterised (Facchini and De Luca 1994).

Major steps of enzymatic pathway involved in biosynthesis of morphine from (S)-Reticuline have been hypothetically demonstrated (De-Eknamkul and Zenk 1992). Two enzymic reactions in this proposed pathway have also been demonstrated in *P. somniferum* cell-free system (figure 1.1) (Gerardy and Zenk 1993, Lenz and Zenk 1995). The first enzyme is salutaridine synthase, a membrane associated cytochrome P-450 enzyme specific to *P. somniferum* and the other is codeine:NADP oxidoreductase which plays an important role in two metabolic pathways and converts codeinone into codeine as well as morphinone into morphine.

1.4.2 Factors affecting field performance of opium poppy

Improvement of field performance is an important issue in poppy plantations. Weed problem is the initial question. The practice of herbicides to overcome weed problem is not possible because of extreme sensitivity of opium poppy to such chemicals. Therefore, traditionally poppy plantations need frequent weed picking and this requires labor. Major loss of crop comes from herbivory by certain insects and can be considered as the secondary problem.

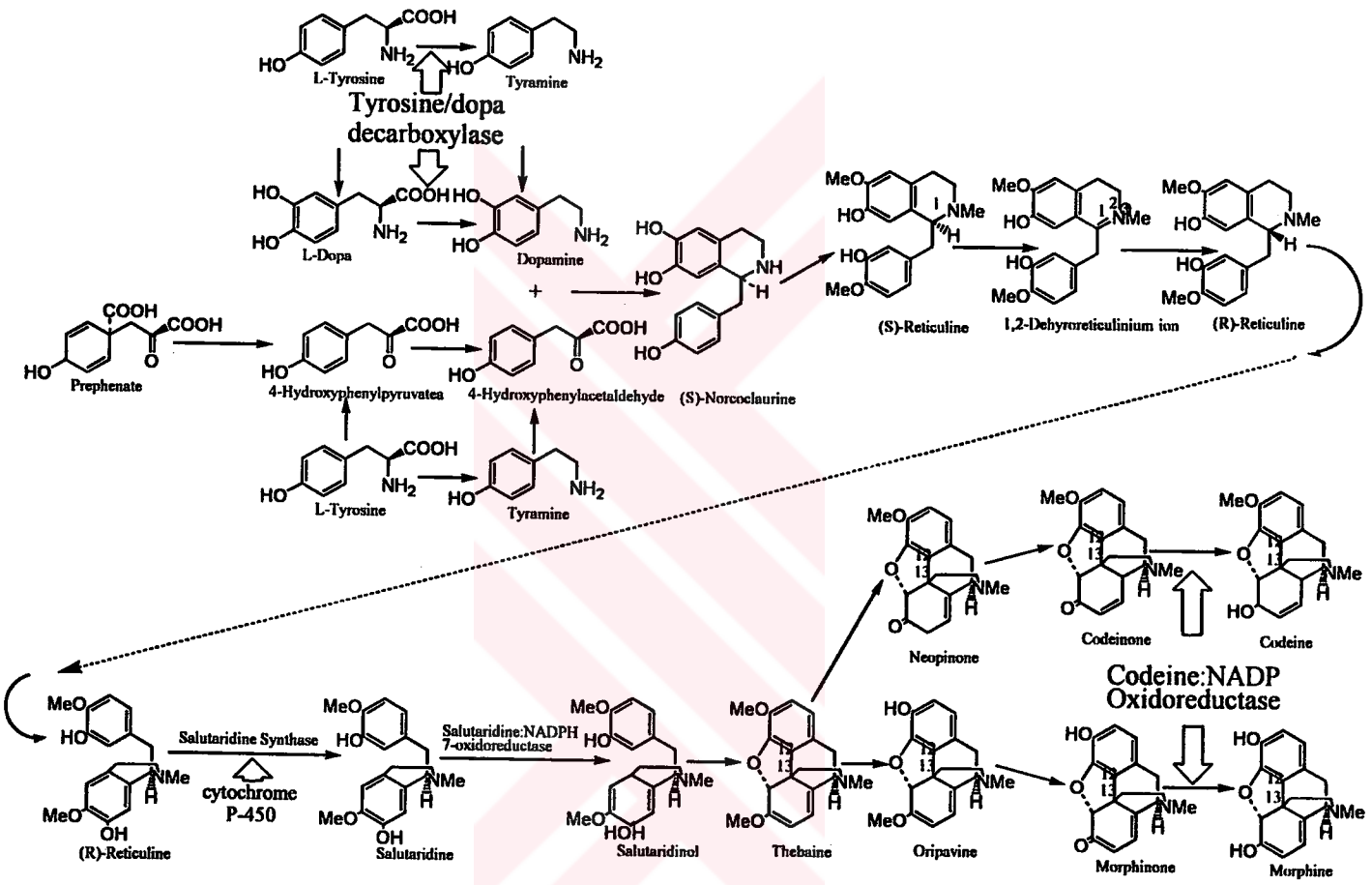


Figure 1.1 Proposed biosynthetic pathway for alkaloid biosynthesis. Rate limiting enzymes may be key elements of high morphinan alkaloid synthesis.

Poppy root worm (*Ceuthorrynchus denticulatus* Schrank), aphid (*Aphis fabae* Scopoli), thrips (*Thrips tabaci* Lind.), tettigometra (*Tettigometra hexaspina* Kolenati), mole cricket (*Gryllotalpa gryllotalpa* L.), cut worm (*Agrotis ipsilon* Hufn.) are common pests of *P. somniferum.* Among these, poppy root worm (*Ceuthorrynchus denticulatus*) causes the most extensive damage. It is reported that, in Central Anatolia, this pest leads to 45% loss in capsule weight and 55 % loss in seed weight in fields where the pest density comprises 77-79% (Erdurmuş and Öneş 1990). Fungal diseases like powdery mildew (*Peronospora arborescens* Berk.), and root junction rotting disease (*Dendryphion papaveris* Saw) gives extensive leaf and capsule damage. Plant is also vulnerable to low temperatures in the early stages of development. If young plants are exposed to -5^0 C prior to the rosette stage (6-7 leaf stage) farmers can experience absolute mortality rates. Proper design of a genetic transformation scheme can be an intelligent solution to the problems outlined. Applying the procedures for marker gene transfer in this work will be super-beneficial for improving certain characteristics as herbicide, pest and cold resistance as well as disease tolerance.

1.4.3 Genetic transformation of *P.somniferum* L.

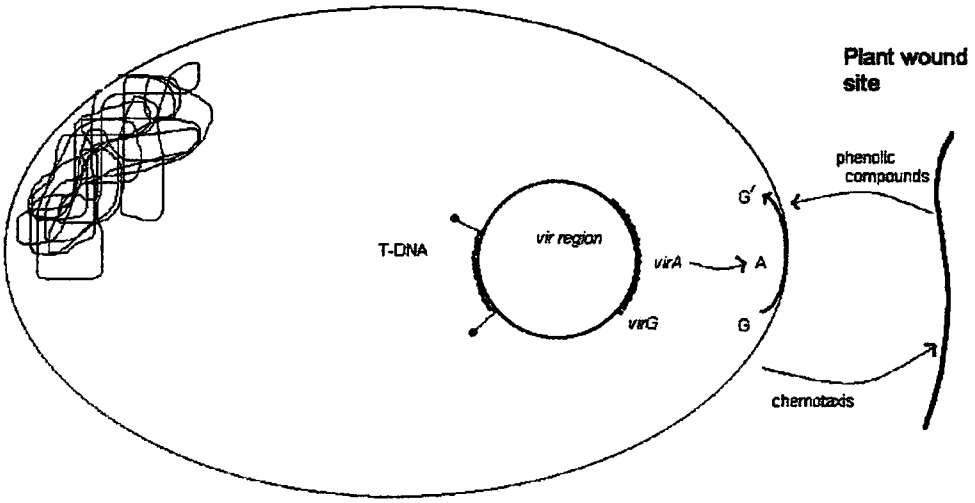
Genetic manipulation of *P. somniferum* cells for improvement of its pharmaceutical contents and field performance have not been studied sufficiently. Poppy tissues have been infected with *Agrobacterium rhizogenes*

and the resulting hairy roots were analysed (Yoshimatsu and Shimomura 1992, Williams and Ellis 1993). Only recently *P. somniferum* cell suspension cells have been transformed with *sam1* gene coding for the enzyme S-adenosyl-L-methionine synthetase (SAM-S) from *Arabidopsis thaliana.* The *sam* genes are vital in metylation reactions of nucleic acids in all living bodies. SAM-S enzymatically converts L-metionine to S-adenosyl methionine and the latter form is a principal methyl group donor. The enzyme also takes part in the synthesis of polyamines and ethylene in plant cells. Ethylene, as a phytohormone has profound physiological effects on the maturation and decaying of plant tissues. The use of *Agrobacterium tumefaciens* and the results obtained from such studies are promising for the genetic engineering of the opium poppy (Belny *et al* 1997).

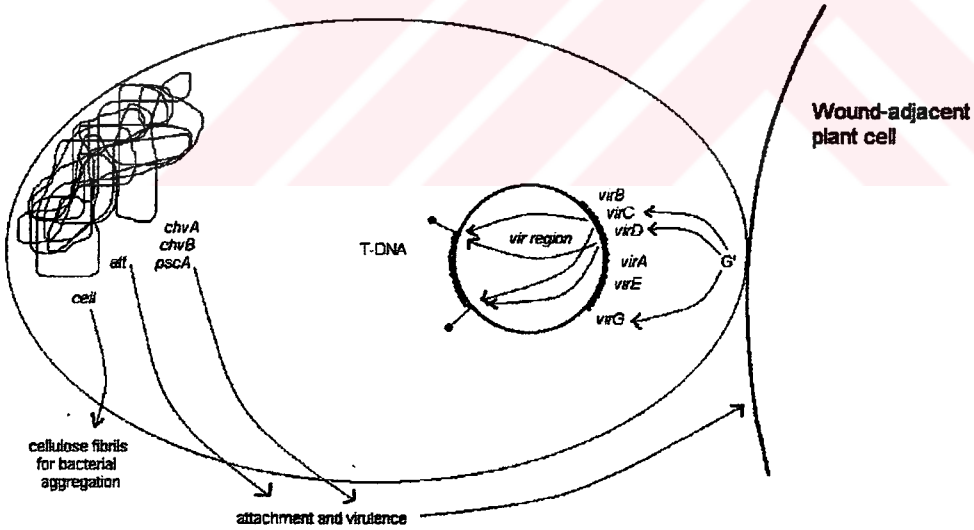
1.4.4 *Agrobacterium tumefaciens* mediated gene transfer

Agrobacterium tumefaciens is a gram negative soil bacteria which has the unique ability to transfer DNA to plant cells. A discrete DNA molecule designated as T-DNA (transfer DNA) is transferred from the tumor inducing (Ti) plasmid. T-DNA carries genes that encode protein products for the biosynthesis of auxin and cytokinin type phytohormones. These are indole acetic acid and isopentenyl-AMP respectively. Signals transduced from these two type of phytohormones cause a neoplastic growth on the wound sites of most dicotyledonous plant tissues known as crown gall disease

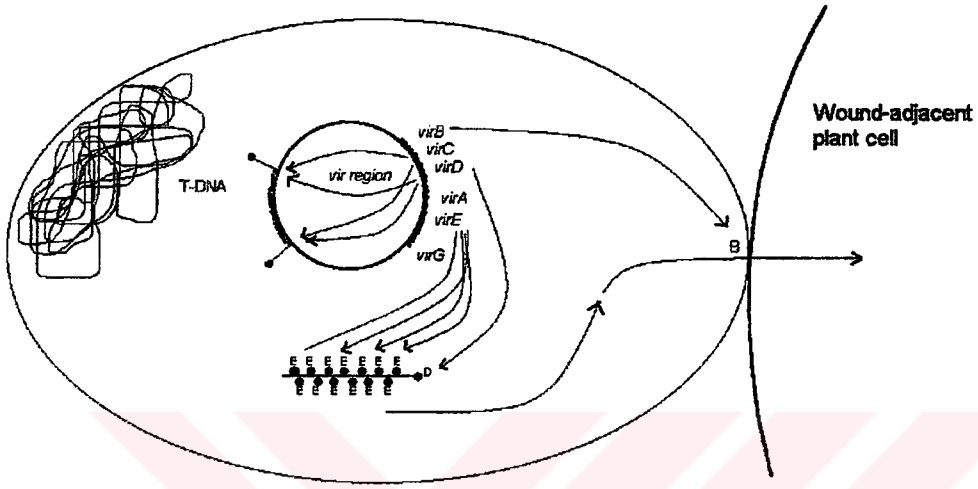
(Nester *et al.* 1984). Chemotactically *Agrobacterium* traces the wound response chemicals secreted from wound sites (figure 1.2a). The transformation of host plant starts by the attachment of *Agrobacterium* to wound-adjacent plant cells mediated by bacterial chromosomal gene region *att* (Douglas *et al.* 1982, Cangelosi *et al.* 1987). There are two distinct genetic regions on the Ti-plasmid. First is the T-DNA border sequences, consisting of 25 base-pair imperfect direct repeats that defines the ends of the T-DNA. Second is the virulence (*vir*) genes encoding proteins function to recognise that an appropriate host site is available. Virulence genes generate the T-DNA intermediate and transport this intermediate across the bacterial envelope (Hoykaas and Beijersbergen 1994). *Vir* gene region contains seven operons (*virA, virB, virC, virD, virE, virG,* and *virH*) that encode proteins required for virulence (figure 1.2b). The transformation initiates soon after recognition of plant wound signals by the membrane protein (VirA) and this signal is transduced to VirG which then expresses other *vir* genes. VirD1 and virD2 gene products act on the generation


Plant wound site
phenolic compounds
G'
T-DNA
vir region
virA
A
virG
G
chemotaxis

(a)


Wound-adjacent plant cell
virB
virC
virD
chvA
chvB
pscA
T-DNA
vir region
G'
att
virA
virE
cell
virG
cellulose fibrils for bacterial aggregation
attachment and virulence

(b)



(c)

Figure 1.2 (a) *Agrobacterium* senses the phenolic compounds secreted from the wound-site of the plant. *Vir* A gene product of the Ti-plasmid *virulence* region is a trans membrane protein. *Vir* A gene product phosphorylates *Vir* G product and the bacteria chemotactically directs itself into the wound-site. **(b)** Chromosomal genes *cell, att, chvA, chvB, pscA* start virulence and attachment of bacteria on the plant cell with their protein products. Phosphorylated *virG* product activates the transcription of operons *vir C* and *virD.* Their products introduces single stranded nicks at right and left border sequences that define the T-DNA ends. **(c)** A single stranded T-DNA complex is generated coated by virE2 proteins. VirD2 endonuclease nicks the right hand border and attaches to the 5' end as a pilot protein. *Vir B* operon expresses proteins and these form a T-DNA export channel.

of the single stranded T-DNA segment (figure 1.2c). A T-DNA complex is formed with VirD and VirE proteins and this complex is exported out of the bacteria into the plant cell.

The trans-kingdom DNA transfer ability of *A. tumefaciens* have been widely exploited in laboratories (de Framond *et al.* 1983). Disarmed Ti-plasmids have been constructed by deleting tumor inducing genes from the T-DNA and replacing with selectable marker genes or with any desired isolated genes engineered to be expressed in plant cells. *Agrobacterium* has been improved to obtain functional avirulent strains. The efficiency of gene delivery into plant genome is enhanced by using binary Ti-vectors in which the disarmed T-DNA carrying the foreign DNA and the vir functions are provided, *in trans*, on separate plasmids.

1.4.5 *Agrobacterium*-mediated gene transfer strategy for *P. somniferum*

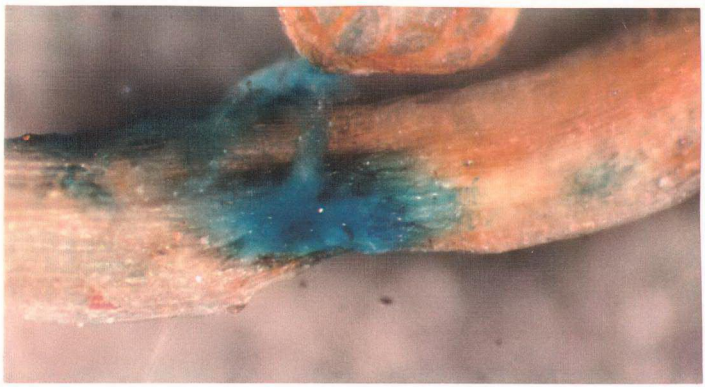
In 1983, the first tranformation of plant cells by using genetically modified *A. tumefaciens* was reported (Zambryski *et al.*1983). Since then many crop species of commercial value have been transformed with a variety of genes to improve agronomical characteristics such as pest resistance, herbicide resistance, disease tolerance, and better fruit storage capacity. *Agrobacterium*-mediated transformation is preferred by many researchers because it is a natural system of trans-kingdom gene delivery that has been conserved through evolution.

Once the wound signals of the plant is recognised by the bacterial chemotactic receptors the gene delivery is successfully accomplished. Even in the absence of this phenomenon, the wound response can be artificially created by exogenously supplying acetocyringone, a well known activator of virulence genes. Majority of the dicotyledonous species have the ability to respond to wounding by releasing such chemicals.

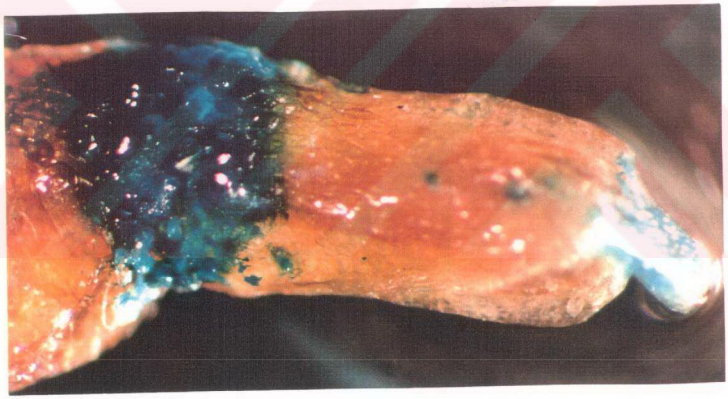
Based on the preliminary infection trials it has been demonstrated that, at transient gene expression level *P. somniferum* can be transformed by *Agrobacterium tumefaciens* (Önde 1995 unpublished data) (figure 1.3). Thus, in this study *Agrobacterium*-mediated transformation strategy has been chosen.

1.5 Aim of the study and proposed research strategy.

Aim of this study is to establish both a transformation and regeneration procedure by using marker genes and, by further manipulating the previously established tissue culture media as well as the physical settings, to develop an efficient organogenesis for transformed opium poppy tissues. Transformation research of opium poppy also includes testing of *Agrobacterium* T-DNA transfer efficiency in lower co-cultivation temperatures.



(a)



(b)

Figure 1.3 a, b GUS assays on leaf (a) and hypocotyl (b) sections of *P. somniferum* after co-cultivation with *Agrobacterium*. (Önde 1995, unpublished data)

The background of this study will form the basis for future research and an unconventional genetic breeding program can be conveyed to improve the agronomical as well as pharmaceutical qualities of opium poppy. Agronomical qualities involve herbicide, pest and cold resistance as well as disease tolerance and such qualities can be introduced once the gene in interest are isolated and cloned. Pharmaceutical qualities are high alkaloid content and directed biosynthetic metabolism into medicinally desired products such as codeine and thebaine. Directed biosynthesis of morphinan alkaloids can be achieved through either increasing the copy number of major rate limiting enzymes or blocking the pathways of undesired alkaloids by *Agrobacterium*-mediated gene delivery system developed in this dissertation.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used in this study are molecular biology grade and tissue culture tested. Main international/local commercial sources are:

- Aldrich, Sigma-Aldrich Chemical Co. Gillingham, Dorset, England.
- Merck, 64271 Darmstadt, Germany.
- Oxoid, Unipath LTD, Basingstoke, Hampshire, England.
- Sigma-Aldrich GmbH, Deisenhofen, Germany.

Local suppliers of the companies above are:

- Prizma Ltd. Yenişehir 06420 Ankara, Turkey.
- Tokra Medikal Ltd. Çevre sok. 22/8, 06680 Ankara, Turkey.

Medium contents

Sucrose (Sigma cat. no. S-5390), MS (Sigma cat. no. 5519), Growth regulators; NAA (Sigma cat. no. N-0640), IBA (Sigma cat. no. I-5386), 2,4-D (Sigma cat. no. D-4517), Kinetin (Sigma cat. no. K-3253), Agargel (Sigma cat.no. A-3301) ,

Trypton (Oxoid cat. no. L42), Yeast Extract (Oxoid cat. no. L21), Sodium chloride (Aldrich cat. no. 22,351-4),

Antibiotics

Kanamycin (Sigma cat. no. K-0129), Augmentin (amoksisilin sodium for intravenous use) Beecham Research Laboratories, Brentford, U.K. Sulperazon (sulbactam sodium and sephaperazon sodium for intravenous use) Pfizer Inc. Ortaköy, İstanbul, Ampicillin (ampicillin sodium for intramuscular and intravenous use) Mustafa Nevzat İlaç Sanayii A.Ş., Yenibosna, İstanbul.

Vitamin supplements

Pyridoxine (Sigma cat. no. P-8666), Thiamine (Sigma cat. no. T-3902), Nicotinic acid (Sigma cat. no. N-0765), myo-inositol (Sigma cat. no. I-3011), Agarose, Trizma Base (Sigma cat. no. T-1503), Trizma.HCl (Sigma cat. no. T-3253), EDTA (Sigma cat. no. E-5134), Sodiumhydroxide (Merck Art. 6462), Hydrochloric Acid (Merck Art. 314), Boric Acid (Sigma cat. no. B-0252/B-7901), Sodium Acetate (Sigma cat. no. S-8625), Bromophenol blue (Sigma cat.no. B-8026), Glucose (Sigma cat.no. G-7528), Acetic Acid (Merck Art.56), Ethidium Bromide (Sigma cat. no. E-8751), SDS (Lauryl Sulfate Sigma cat. no. L-4390), Phenol (Sigma cat. no. P-4557), Equilibration buffer associated with phenol (Sigma cat. no.B-5658) Chloroform (Merck UN1888), Isopropanol (Sigma cat. no. I-9516).

2.1.2 Stock solutions and buffers

•T_{10} E_1 (Tris-EDTA) buffer

per liter

Trizma Base 1.211g (10 mM)

EDTA 0.372g (1 mM)

pH 7.6

•Genomic DNA extraction buffer

Tris.HCl 50mM (pH 8.5)

SDS 0.5 % w/v

• Plasmid DNA extraction buffer

Sodium hydroxide 0.2M

SDS 0.5 % w/v

• GTE (Glucose-Tris EDTA) buffer

Glucose 50mM

Tris.HCl 25mM

EDTA 10mM

• TBE (Tris-Borate-EDTA) buffer

per liter 5x stock

Trizma base 54.0g

Boric acid 27.5g

EDTA 20 ml (0.5M pH 8.0)

pH 8.3

- Gel loading buffer

125 mM EDTA (pH 8.0)

50% v/v Glycerol

0.1% w/v SDS

Bromophenol Blue (few crystal particles per 10ml solution)

- Sodium Acetate buffer (3M)

per 100 ml

Sodium acetate 24.6g

After dissolving in 50ml dH_20 pH was adjusted to 4.8 with glacial acetic acid and volume completed to 100ml prior to steam sterilisation.

- Ethidium Bromide

Prepared as a stock solution by dissolving 10mg Ethidium bromide in 10 ml dH_20 and stored at room temperature in darkness.

- 2M NaOH Solution

- 2M NaCl Solution

- Phenol/Chloroform

Phenol was equilibrated by the full volume of associated equilibration buffer and allowed to rest for at least four hours for the phases to separate. Phenol is then mixed with equal volume of chloroform. Some of the overlayering tris buffer is also added on the surface to minimise oxidation. Solution is kept in darkness at 4^0 C.

2.1.3 Glassware

Standard 10cm glass petri petri dishes (Labor İldam), baby jars (Sigma cat. no. V-8630) with autoclavable caps (Sigma cat. no. B-8648)

2.2 Instrumentation

2.2.1 Tissue culture setting

Laminar flowhoods, Holten Laminair TL, 2472 Allerot, Denmark, equipped with UV sterilisation lamp Philips TUV 30W G30 T8 U.V.Long life, Holland. BassAire 04HB Southampton, UK.

Illumination, growth room equipped with timer programmed light regime providing 2000 lux, KRL-710 control panel, KRL Sistem Tasarım Ankara, Turkey.

Rotational incubators, Gallenkamp Orbital incubator with refrigeration.

G24 Environmental incubator shaker, New Brunswick Scientific Co. Inc., Edison, N.J., USA.

2.2.2 Instruments used in molecular analysis

Electrophoresis unit, Sigma-Aldrich Horizontal Sub-marine Mini-gel 7.5x10cm tray size (Sigma cat. no. E-0638). UV transilluminator TFX-20.M Vilber Lourmat, Cedex, France. Power supply SAA-310 KRL Sistem Tasarım Ankara, Turkey. Gel photography unit, Polaroid MP-4 Camera System, Polaroid (UK) Ltd., St.

Albans, Hertfordshire, England. PCR, Techne Genius with a heated lid, Techne Limited, Duxford, Cambridge, CB2 4PZ, United Kingdom.

2.2.3 Visual macro analysis and photography

Nikon dissection microscope type 102 with camera attachement. Nikon Corporation, Fuji Bldg., 2-3, Marunouchi 3-Chome, Chiyoda-Ku, Tokyo 100, Japan. Macro photography Minolta Auto Bellows Macro 100mm, 55mm (diameter) skylight Light source Intralux 5000-1 Volpi Ag, Switzerland.

Films Fuji 100ASA 36 color print films,. Fuji Photo Film Co., LTD., Tokyo 106.

Kodak 100ASA 24x36mm color print films, Kodak Limited, England.

Ilford FP4 plus black/white print films

Polaroid 667 (print only) Coaterless black/white (8.5x10.8 cm) instant pack film. Polaroid (UK) Ltd., St. Albans, Hertfordshire, England.

2.3 Methods

2.3.1 Tissue culture

2.3.1.1 Plant material

Seeds from a registred cultivar of *Papaver somniferum* L. SARI 118 have been used and were obtained from Turkish Grain Board (TMO).

2.3.1.2 Surface sterilization

Seeds of opium poppy were sterilised first by 70% (v/v) ethanol for three minutes and then by 20% (v/v) commercial bleach for five minutes. After each step seeds were rinsed five times with sterile distilled water.

2.3.1.3 Germination of seeds

Surface sterilised seeds are spreaded on hormone-free half strength MS (Murashige and Skoog 1962) medium supplemented with 30% (w/v) Sucrose and 4% (w/v) high gel strength agar. The pH of the medium was adjusted to 5.8 prior to autoclaving at 120 ^{0}C for 20 minutes. Seeds were allowed to germinate at room tempereture under regular room illumination for seven to ten days.

2.3.1.4 Induction of callus

Hypocotyl sections of 10 days old poppy seedlings were used as explant material for callus induction. Hypocotyl sections were excised from root-shoot junction and below the cotyledons discarding the root and epicotyl parts (Figure 2.1). Explants were incubated at 23 ^{0}C in dark on callusing medium (Appendix A-2). Callus induction was achieved within 10 days.

2.3.2 Regeneration of whole plants

2.3.2.1 Organogenesis: shooting

Shooting was induced on shoot induction medium containing the same ingredients as the callus induction medium but supplemented with 1mg.l^{-1} Kinetin as the sole growth regulator (Appendix A-4). Augmentin and kanamycin were used as selective agents in 500 and 150 mg.l^{-1} doses. Incubation was done under continuous light at 6 temperature settings programmed to provide a 24h thermonastic cycle (table 3.1). Subsequently, shoot induction was carried out at 16°C under continuous light. Composition of the vitamin solution remained unchanged. Organogenesis of the explants were allowed to proceed on shoot proliferation medium (Appendix A-5) upon the emergence of the green shoot meristemoids. Proliferation medium contained 0.15 mg.l^{-1} NAA having the same composition with that of shooting medium.

2.3.2.2 Organogenesis: root formation

Root induction medium was a modified form of callusing medium (Appendix A-6). Sucrose concentration was decreased to 15 g.l^{-1} and the strength of the MS basal medium was reduced to one fourth. Composition of the vitamin solution remained unchanged. Plantlets were treated in 0.01 mg.l^{-1} IBA overnight and then transferred to hormone free root induction medium.

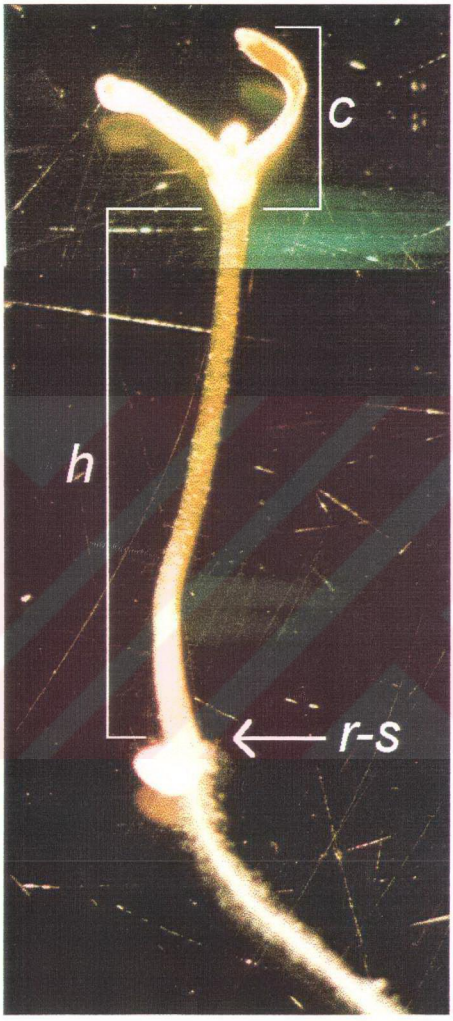


Figure 2.1 10 days old hypocotyl stage *P. somniferum* seedling used as the explant source. Hypocotyl sections above the root-shoot junction were taken as explants. Cotyledons (*c*), hypocotyl (*h*), root-shoot junction (*r-s*).

2.3.3 Bacterial strains

The bacterial strains used in this study and the corresponding marker genes cloned in these strains are given in Table 2.1.

Table 2.1 List of bacterial strains involved in this study.

Bacterium	Strain	Plasmid	gene
A. tumefaciens	LBA4404	pBIN 35S-mGFP4	GFP4 + NPTII
E. coli	xL-1 Blue	$pBSK^+$	mGFP4
E. coli	xL-1 Blue	pUC18	NPTII
E. coli	HB 101	pBIN 35S-mGFP4	GFP4+ NPTII

2.3.3.1 Storage of bacterial strains

Bacteria were maintained on LB agar plates with necessary antibiotics at 4 ^{0} C. Cryogenic storage for longer periods were done by freezing the overnight grown liquid LB cultures (Maniatis *et al.* 1982) at -70 ^{0} C in 15-20 (v/v) glycerol.

2.3.4 Transformation procedure

A desired strain of *Agrobacterium tumefaciens* were streaked on standard LB medium solidified by agar (1% w/v) supplemented with 50 mg.l^{-1} kanamycin and grown for 48 h at 30 ^{0}C. A single colony was then inoculated to 2 ml liquid LB with 50 mg.l^{-1} kanamycin and incubated overnight on an orbital incubator at 30 ^{0}C with 250 rpm speed. Poppy hypocotyls cultured in callus induction medium

for at least 10 days were immersed in liquid medium (Appendix A-2) containing *Agrobacterium* diluted to 1-5 x10^6 cells/ml for 10 minutes. Meanwhile wounds are introduced on induced swollen hypocotyl sections by a sharp razor (Figure 2.2). Hypocotyls infected with *Agrobacterium* were blotted dry on a sterile filter paper and incubated in on callus induction medium for 48 h at 19 ^{0}C in darkness. *Agrobacterium* was removed from the plant material by rinsing in liquid callus induction medium containing 500 mg/L augmentin.

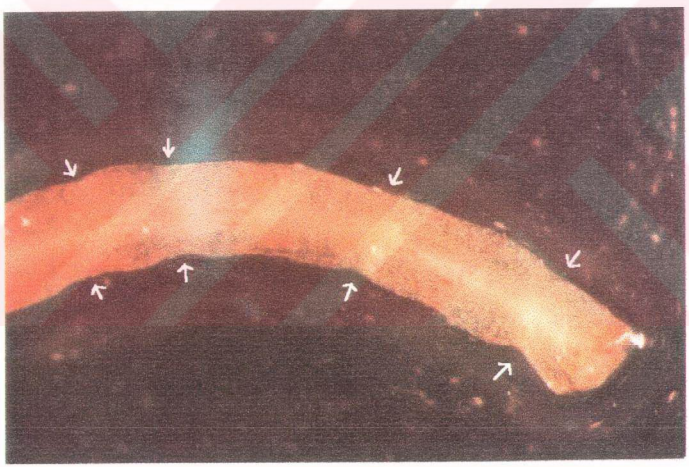


Figure 2.2 Wound-sites on hypocotyl sections of *P. somniferum*. Arrows show the cuts introduced by a razor blade.

2.3.5 Selection of putative transformants

Selection of transformed cells were carried out on solid callus induction medium supplemented with 150 mg/L kanamycin and 500 mg/L augmentin (Figure 2.3). Culture conditions were remained as described in section 2.3.1.4.

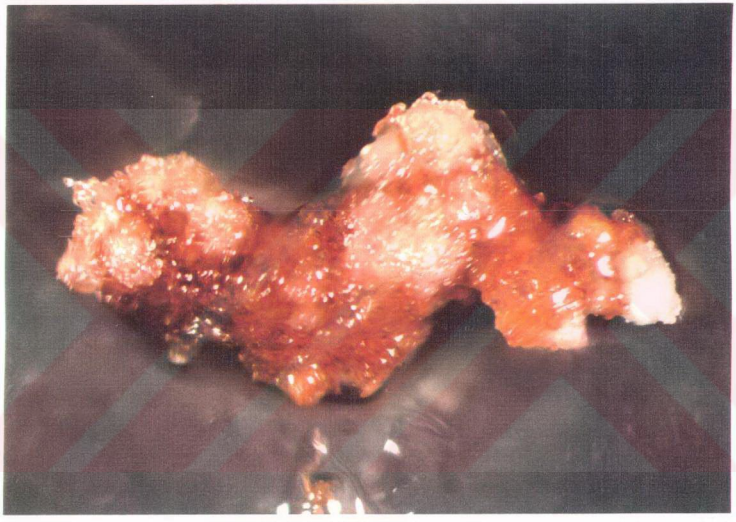


Figure 2.3 Proliferation of putatively transformed wound-adjacent cells on selective medium. Non-transformed tissues undergo necrosis and are eventually died.

2.3.6 Molecular confirmation of putative transformants

2.3.6.1 Genomic and plasmid DNA isolation

The genomic DNA were isolated using the procedure by Spiers & Brady (1981).The plasmids were isolated by employing the protocol by Birnboim & Doly (1979).

2.3.6.2 Determination of size and concentration of nucleic acids

Determination of DNA concentration were carried out by spectrophotometric readings at 260 nm as follows: OD value of 1.0 at 260 nm is equivalent to 50$\mu g.ml^{-1}$ of DNA. Size determination of DNA were done by using two standard size markers having the fragment sizes of:

Hind III-cut λ DNA (23.1, 9.4, 6.7, 4.4, 2.3, 2.0, 0.56, 0.125 kbp)

Hae III-cut ϕX174 DNA (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72 bp)

DNA were run on ethidium bromide supplemented agarose gel (Maniatis *et al.* 1982) and size determination were done by comparing the banding pattern of the size markers.

2.3.6.3 PCR Amplification

The forward and reverse primers used for PCR amplification were specific to the coding region of NPTII. The sequences of the 21-mer primers are

F: 5'GAGGCTATTCGGCTATGACTG-3'

R: 3'-ATCGGGAGCGGCGATACCGTA-5' respectively.

PCR reactions were performed in a reaction mixture consisting of 25ng of each primer, 200μM dNTP mixture 2mM $MgCl_2$, 50ng DNA template, 1u Taq DNA polymerase, in 25μl total volume. PCR amplification protocol included 4 cycles of 1-min 30s at 95 °C, 1-min 30s at 55 C, 2-min at 72 °C followed by 35 cycles of 1-min at 95°C, 1-min at 55 °C, 1-min at 72 °C and a final extention at 72 °C for 10-min.

CHAPTER 3

RESULTS

3.1 Effect of co-cultivation temperature

The mechanism underlying the T-DNA transfer from *Agrobacterium* to the plant cells has been a hot topic. A conjugational transfer mechanism has been proposed based on the amino acid sequence similarities between plasmid genes such as Tra genes of F pilus of *Escherichia coli* and VirB genes of *Agrobacterium tumefaciens* and Ptl operons of *Bacillus pertussis* (Lessl and Lanka 1994). Quite recently, pilus assembly by *Agrobacterium* cells grown at 19 ° C has been demonstrated by electron microscopy (Fullner *et al.* 1996). This finding is quite important to emphasise the need of lower co-cultivation temperatures for efficient plant transformation through appropriate pilus assembly.

In this study three temperature settings (19, 23, 25°C) were tested for best transformation efficiency. Co-cultivation at 19°C was found to be the best temperature relative to the others. Putative transformants selected after 19° C co-cultivation showed a significantly higher number of tissue proliferation. This result is also supporting the optimum temperature in T-DNA transfer

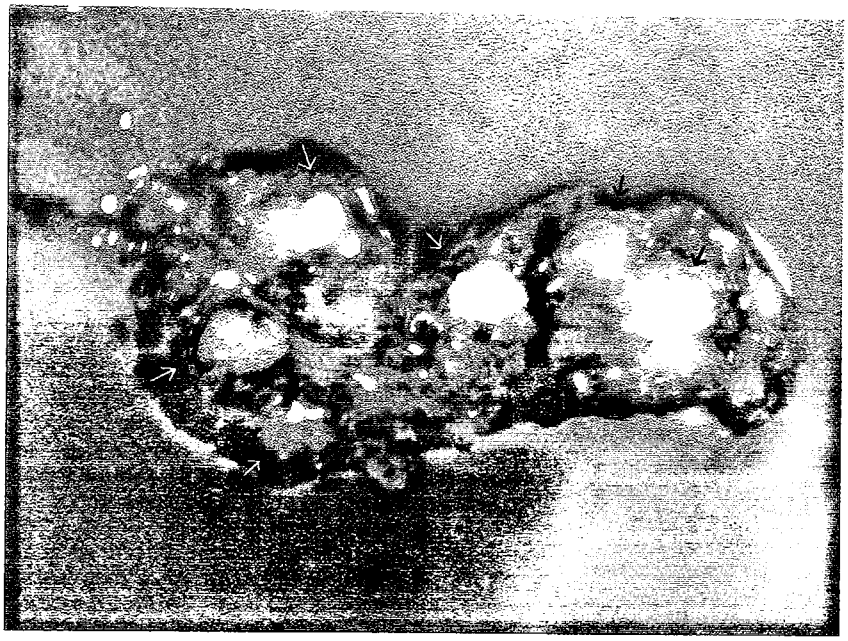


Figure 3.1 (a)

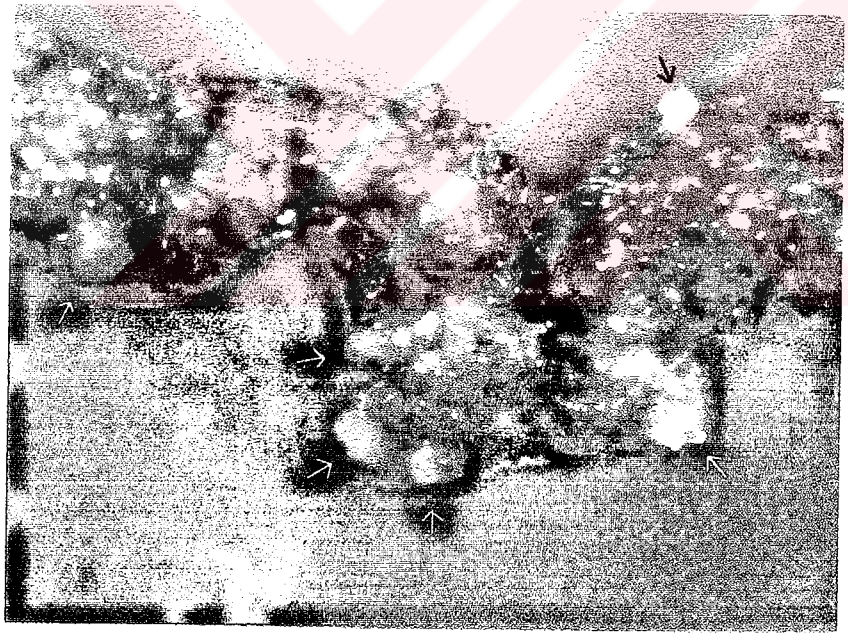


Figure 3.1 (b) (continued)

Figure 3.1 (c)

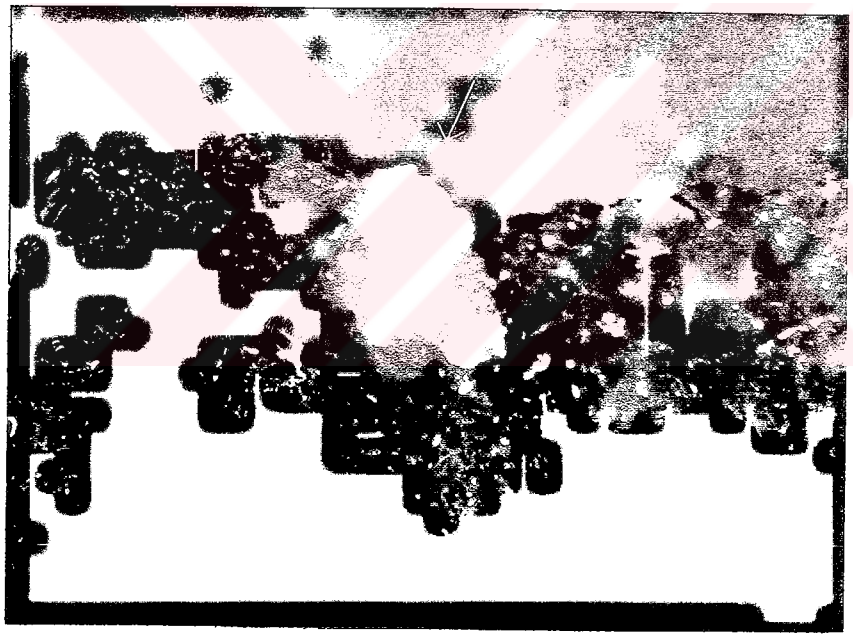


Figure 3.1 (d)

Figure 3.1 Effect of co-cultivation temperatures on the proliferation performance of wound-sites. Arrows indicate proliferating wound-sites. Co-cultivation at 19 ° C resulted in highest transformation rates with 6-7 proliferation site per hypocotyl section (**a,b**). Co-cultivation at 23 ° C gave intermediate results with 3-4 proliferation site per explant (**c**). Poorest results were obtained at 25 ° C with only 1 or no proliferation per hypocotyl (**d**).

machinery of *Agrobacterium tumefaciens* being 19 °C (Fullner and Nester 1996). Typically, the total number of wound sites introduced per explant including the cut sites on both ends of hypocotyl sections were 6. On average when cultured at 25 °C there were one or no tissue proliferations per hypocotyl explant (Figure 3.1 d) while at 19 °C this number could reach as high as 7 (Figure 3.1 a,b). Co-cultivation at 23 °C has given consistent transitional values around 3 tissue proliferations per explant (Figure 3.1 c).

3.2 Effects of antibiotics used for eliminating *Agrobacterium*

Removal of *Agrobacterium* from the culture medium is an absolute necessity. Due to this fact an antibiogram was performed to decide which antibiotics to use. Among the 20 antibiotics tested Sulbactam yielded the largest inhibition zone (Data not shown). As a consequence, sulbactam was used to kill the bacteria. However, it was found to be cytotoxic on poppy plant material and caused severe necrosis starting at 350 $mg.l^{-1}$. For that reason, it's use was discontinued. Augmentin was used later on successfully at 500 $mg.l^{-1}$ concentration and no bacterial relapse was observed.

3.3 Organogenesis

Transformed tissues should be regenerated into complete individuals to obtain stable bodies to further test whether the transmission of transgene is in Mendelian fashion. The developmental program of most plants starts with the emergence of the radicle and subsequent root formation and proceeds with the

unfolding of photosynthetic structures to become primary leaves. Contrary to the developmental program of most plants, organogenesis approach almost always favours the shoot formation for the generation of photosynthetically self-sufficient plantlets. Root formation follows after photosynthetically active organised shoots are obtained.

Induction of organised shoots from meristems on calli under selection was the first obstacle faced in this study. Meristemoids never responded to increased kinetin concentrations before the calli have reached a proper size. Therefore, after co-cultivation the tissues were allowed to proliferate at least for two sub-culture periods until they have a diameter of 6 to 8mm. During this period supply of vitamins was found to be a critical necessity (Appendix A-8). Calli that are ready for shoot formation can be visually identified by the distinct meristemoids on callus surface (Figure 3.2)

3.3.1 Effect of temperature in organised shoot formation

Transformed calli were selected by Kanamycin and cultured at 23°C in darkness. Shoot induction was the first obstacle in regeneration attempts. Despite of the changing growth regulator composition the response of calli towards shoot formation was not strong and a synchronous development was lacking. This observation marked the necessity of some physical requirement to initiate the parallel development of dormant shoot meristems on growing calli.

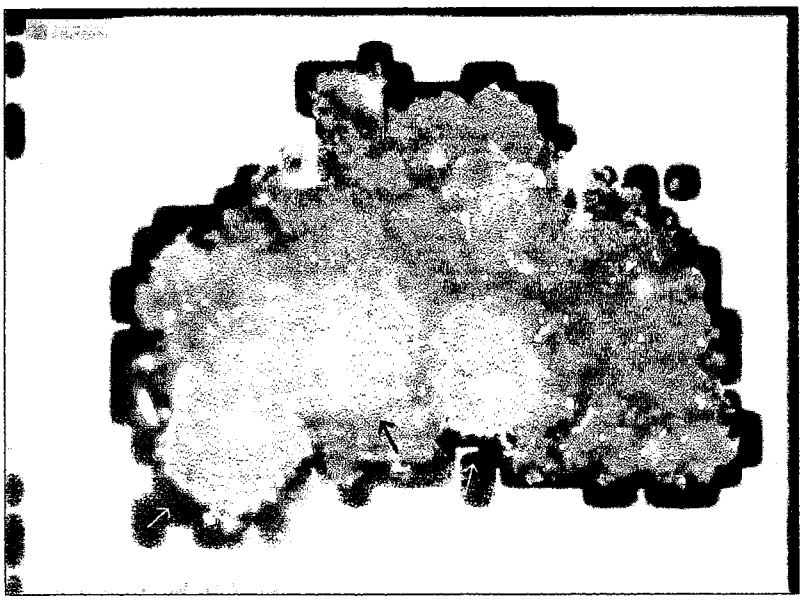


Figure 3.2 Callus with three distinct meristemoids ready for shoot induction.

Temperature appeared as the most imminent physical parameter for achieving a strong synchronous shoot induction. For this a 24-h thermonastic cycle consisting of 6 temperature settings was started in a programmed incubator. The calli were cultured under continuous light. The expectation from such a temperature profile was to simulate diurnal temperature variation to trigger shooting and mimic a kind of circadian rhythm experienced in nature (Table 3.1).

Table 3.1 Thermonastic program cycles.

Program number	Temperature (° C)	Duration (h, min)	Time of the day
1	20	4.00	05:00 - 09:00
2	25	4.00	09:00 - 13:00
3	27	2.30	13:00 - 16:30
4	24	3.30	16:30 - 19:00
5	16	9.00	19:00 - 04:00
6	14	1.00	04:00 - 05:00

Although thermonastic cycle was seemed to be working at the beginning, some anomalities in shoot organisation especially the morphology of developing leaves were noted to appear later. Shoot induction was achieved but, some unknown fact was interfering with the leaf development. When the program were reviewed, program number 5 and 6 were elucidated to be the inducing parts of the whole program cycle, which was responsible from the shoot induction. This result is similar to that observed in a previous study in which the shoot induction were achieved at lower temperature settings at 16° C (Yoshikawa and Furuya 1982).

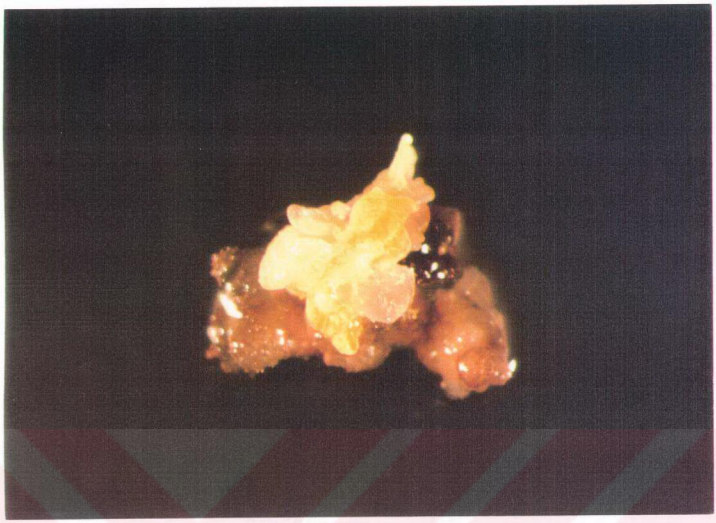


Figure 3.3 Induction of shoot meristem on poppy callus grown at 16 °C.

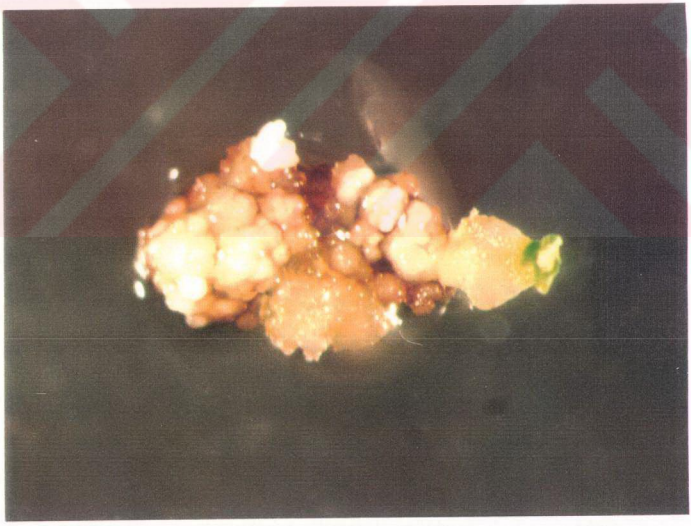


Figure 3.4 Development of chlorophyll on shoot meristem and formation of first leaves at 16 °C.

Induction of shoot meristems were achieved at 16 °C, a lower temperature setting than employed in callusing (Figure 3.3). Regeneration of healthy shoots were achieved in three weeks (Figure 3.4, 3.5, 3.6). Culture temperatures higher than 25 °C resulted in deformed shoot development where the growing leaves were cylindirical and cup shaped (Figure 3.7 a,b).

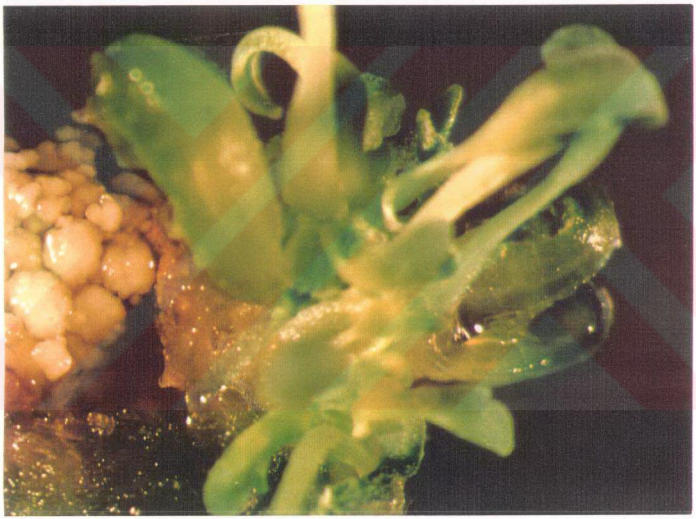


Figure 3.5 Healthy shoot structure with multiple leaves at 16 °C.

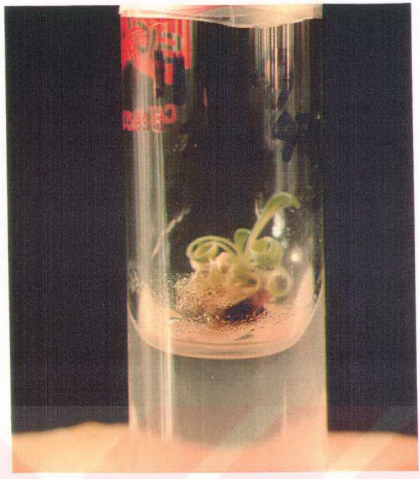
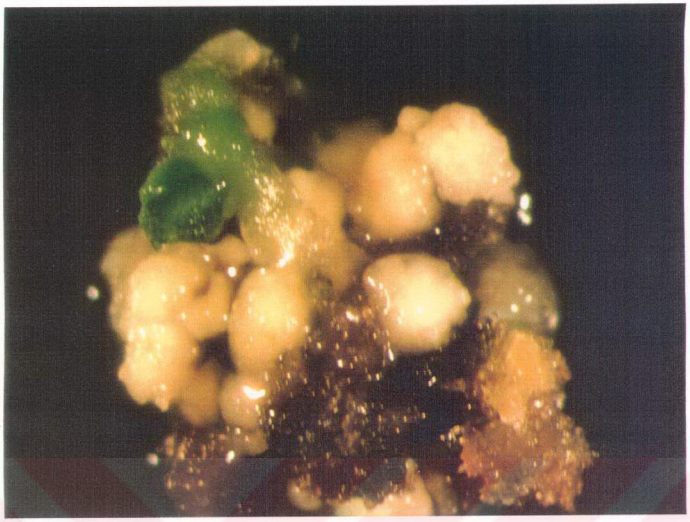
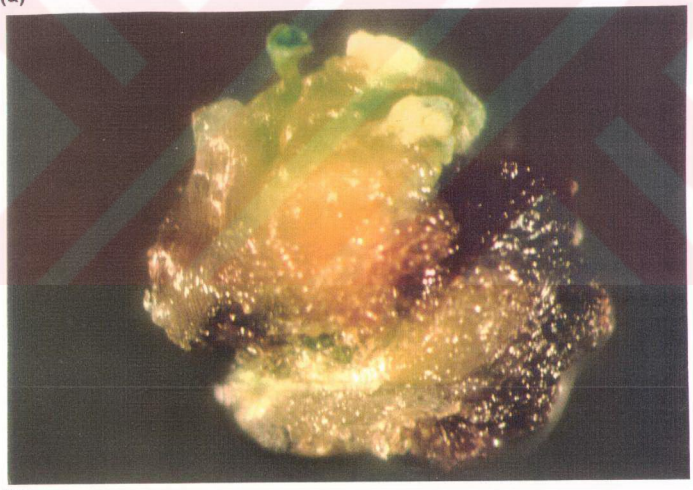


Figure 3.6 General view of shoot structure in poppy plantlet.

Root induction was a problematic issue in previous tissue culture studies in our laboratory. Root formation was encountered as merely a spontanous event and directed synchronous organogenesis could not be achieved. In this study inducers for organised root formation was sought and two auxin type growth regulators were tested. The course of action taken as in root induction emanates from a pre-determination period during shoot proliferation. The success of root formation is based on the gradual tissue determination provided by the NAA present in shoot proliferation medium (Appendix A-5).



(a)



(b)

Figure 3.7 Deformed leaf structure at culturing temperatures higher than 25 °C. Cup shaped (a) and/or cylindirical (b) leaf structures are characteristic morphological anomalities at this temperature setting.

3.3.2 Organised root formation

3.3.3 Effect of auxin-type growth regulators in organogenesis

Two auxin type growth regulators, NAA and IBA were tested as inducers of root formation. NAA and IBA were used in 0.1, 0.05 and 0.01mg.l^{-1} concentrations (Appendix A-6). NAA produced a strong rooting signal and repressed shoot development. In 0.1mg.l^{-1} concentration aberrations in root formation were observed. Signal transduced by NAA was so strong that "punky" root hair formations and de-differentiation into white meristemoids appeared as an immediate response (Figure 3.8). IBA produced better results than that obtained from NAA. In high concentration (0.1 mg.l^{-1}) immediate root hair formation on white meristemoids were observed in plantlets treated overnight with slight interference to the shoot development (Figure 3.9). IBA used at 0.05 mg.l^{-1} concentration enhanced meristemoids near the solid medium surface (Figure 3.10) and roots were initiated on these meristemoids (Figure 3.11)

Excellent root initiation occurred in 0.01mg.l^{-1} IBA concentration, with minimum interference on shoot development and leaf formation (Figure 3.12). A simultaneous growth in both root and shoot tissues have been observed (Figure 3.13).

Figure 3.8 "Punky" root hair formations as an effect of strong root signal induced by 0.1 $mg.l^{-1}$ NAA.

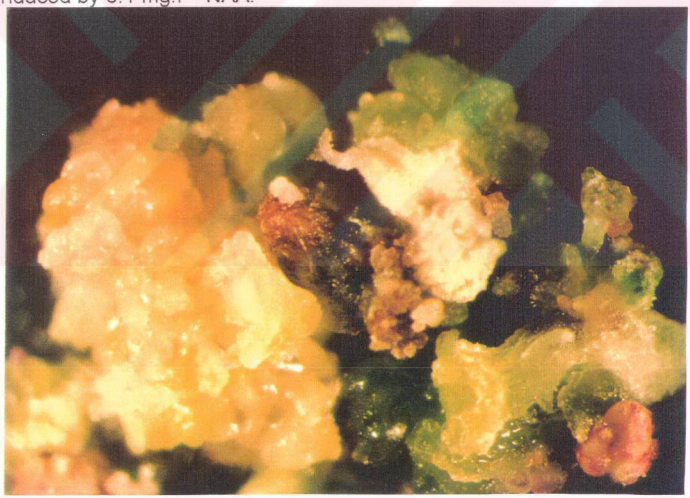


Figure 3.9 Repressed shoot development by NAA in 0.05$mg.l^{-1}$ concentration. Root hair are visible on meristemoids and chlorophyllic regions lack expected shoot morphology tending to be more callus-like structures.

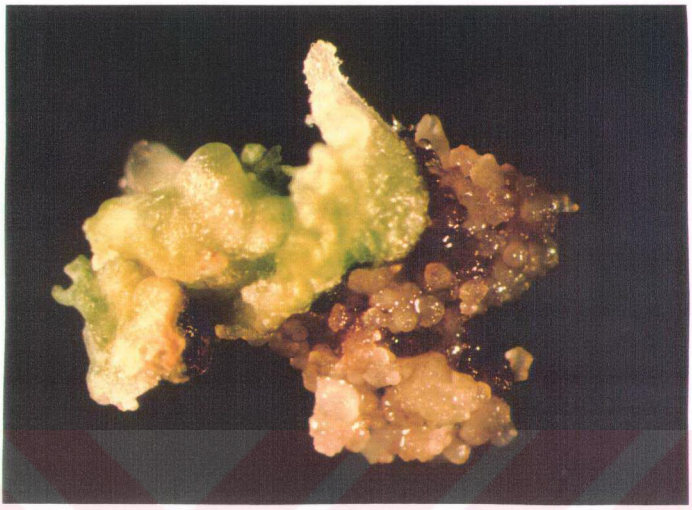


Figure 3.10 Enhanced meristemoids near the solid medium surface by 0.05 $mg.l^{-1}$ IBA. Green tissues less were less influenced by the callusing effect of IBA.

Figure 3.11 Proper root formation on developing plantlets by 0.05 $mg.l^{-1}$ IBA.

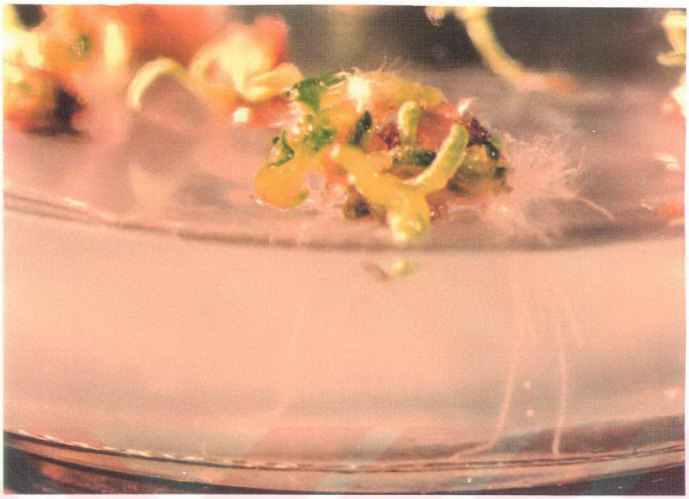


Figure 3.12 Best root development resulted in 0.01 $mg.l^{-1}$ IBA with no appearent interference on shoot development and leaf morphology.

Figure 3.13 Parallel development of root and shoot on hormone free medium treated overnight with 0.01$mg.l^{-1}$ IBA.

3.4 Molecular analysis of putative transformants

3.4.1 PCR analysis of bacterial plasmid DNA

PCR analysis were carried out on original plasmid pBIN 35S-mGFP4 isolated from *E. coli* strain HB101 harbouring GFP4 and NPTII in order to optimise reaction conditions. NPTII coding reagion could be amplified in plasmid preparations (Figure 3.14).

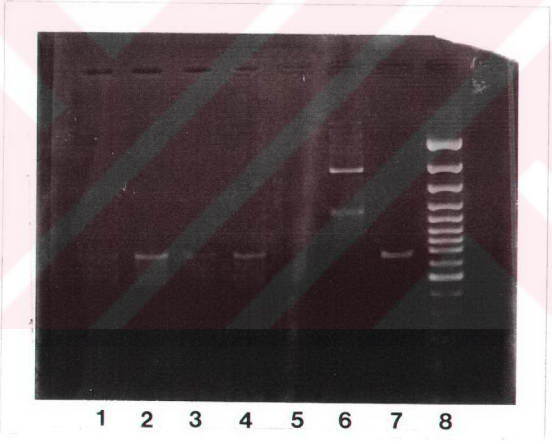


Figure 3.14 PCR amplification results of plasmid DNA preparations. Lane 8 100bp DNA ladder, lane 7 previously amplified NPTII fragment, lane 6 control pBSK^{+} DNA, Lanes 1 to 4 amplified NPTII fragments from pBIN 35S-mGFP4.

3.4.2 PCR analysis of putatively transformed plant genomic DNA

Genomic DNA from 8 cell lines isolated from callus subjected to kanamycin selection (Figure 3.15) were analysed for the presence of NPTII. Surprisingly no amplification could be detected in all of the DNA preparations. The concentrations of putatively transformed poppy genomic DNA were drastically reduced after RNAse treatment prior to PCR amplifications and some samples were found to be almost undetectable or in trace amounts (Figure 3.16). Losses in sample numbers 1,2,3 and 8 were significantly high especially 3.

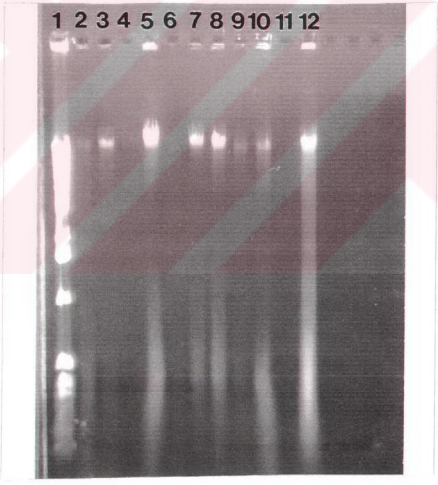


Figure 3.15 Appearence of DNA samples from potatively transformed poppy calli. Lane 12 C: Control untransformed poppy genomic DNA. Lanes 2-10 putatively transformed poppy DNA. Lane 1 1kb size marker. Lanes 4 and 11 were left blank intentionally.

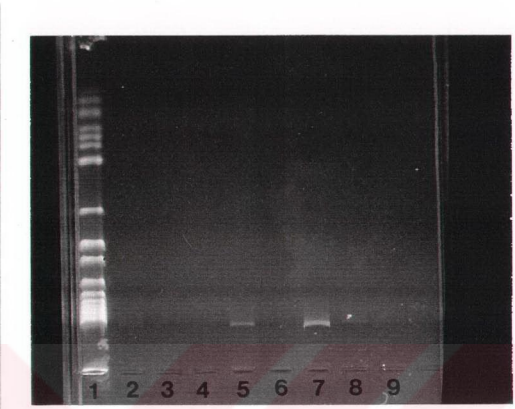


Figure 3.16 Putatively transformed poppy genomic DNA as they appear on the gel following RNAse treatment with subsequent phenol/chloroform extraction and ethanol precipitation. Lane 1 100bp DNA ladder. Lanes 2-9 putatively transformed poppy genomic DNA.

Table 3.2 DNA concentrations (μg/ml) before and after RNAse treatment. PGU: Poppy genomic untransformed, PT: Putatively transformed poppy genomic.

DNA Labels	Before RNAse treatment	After RNAse treatment
PGU	0.39	0.25
PT-1	0.38	-0.04
PT-2	0.32	-0.06
PT-3	0.52	-0.06
PT-4	0.68	-0.04
PT-5	0.05	-0.08
PT-6	1.24	0.03
PT-7	0.44	-0.05
PT-8	0.07	-0.03

CHAPTER 4

DISCUSSION

Transfer of foreign genes have been well established in many plants so far. *Arabidopsis thaliana,* members of the *Solanecea* family *e.g.* tobacco, tomato, potato, and major crop species such as rice, wheat, and maize have been sucessfully transformed. *A. thaliana* and *N. tabacum* are currently accepted as model plants by the scientific community. These models have built an invaluable body of experience leading to the manipulation of other important agricultural species. Based on the existing tissue culture background, a gene transfer procedure for opium poppy using *Agrobacterium tumefaciens* have been investigated and established throughout this study. The transferred marker gene was consisted of NPTII from Tn5 which confers resistance against kanamycin. For transferring this marker gene to opium poppy, the *Agrobacterium tumefaciens*-mediated transformation procedure has been employed. Since the procedure differs from direct gene transfer strategies by its biological nature, understanding the relevant parameters concerning the *Agrobacterium* biology became essential. One of the key factors effecting the success of gene transfer from *A. tumefaciens* to plant cells is the co-cultivation temperature. As a consequence, co-cultivation temperatures ranging from 22 to 28° C have been used for a variety of plant species (Table 4.1).

Table 4.1 Co-cultivation temperatures employed in various species.

Species	Co-cultivation temperature (°C)	Reference
Papaver somniferum L.	22	Belny *et al.* (1997)
Brassica carinata	22	Babic *et al.*(1998)
Eucalyptus camaldulensis	25	Chen (1998)
Persea americana Mill.	25	Cruz-Hernandez *et al.*(1998)
Paspalum vaginatum Schwartz	26	Cardona and Duncan (1998)
Mentha X piperita L.	26	Niu *et al.*(1998)
Diospyros kaki Thunb	28	Nakamura *et al.*(1998)
Tabernaemontana pandacaqui	28	Cardoso *et al.* (1997)
Catharanthus roseus	28	Meijer (1998)
Cicer arietinum L.	28	Kar *et.al.*(1996)

Although the *vir* genes are optimally induced at 25 ^{0}C, there exist considerable amount of evidence that later stages in *Agrobacterium* virulence system require lower temperatures for optimal transfer of T-DNA across the trans-kingdom membrane boundaries (Fullner and Nester 1996). A conjugation model has been proposed for *Agrobacterium* T-DNA delivery to plant cells. The framework of this model is based on the sequences deduced from Vir B and Vir D4 proteins which are homologous to membrane associated proteins for conjugative transfer of broad host range plasmids and F plasmid of *E. coli* as well as export of toxins by the human pathogen *Bordetella pertussis* (Lessl and Lanka 1994). There is a great deal of homology between amino acid sequences of Ti Vir B and Tra proteins of broad host range plasmids of IncP, IncN, and IncW groups. Additionally some of the Tra genes function in assembly of F pilus in *Escherichia coli* and therefore Vir B proteins were suspected elements of a proposed pilus in *Agrobacterium.* Assembly of pilus in *Agrobacterium* has been visually

demonstrated on cells grown at 19 °C by electron microscopy (Fullner *et al.* 1996). In this study best co-cultivation temperature was also noted to be 19 °C and, this is supportive of conjugative T-DNA transport mechanism to plant cells. As far as the literature has been cited this is the first report expressing the importance of temperature effect on *Agrobacterium* T-DNA transfer efficiency to plant cells since A. J. Riker (1926). On the optimistic side, the need to incubate both *Agrobacterium* and plant cells at lower temperatures will become more appearent by the accumulating data.

As a domesticated plant, opium poppy is rather sensitive to many selective agents and necrosis is commonplace in cultured cells. In this study previous attempts for removing *Agrobacterium* from transformed calli by sulbactam failed because of its cytotoxic effect. Augmentin was found to be an effective antibiotic as well as a promoting agent in tissue proliferation. Augmentin was used as an essential antibiotic after co-cultivation.

Regeneration of the whole plants from transformed callus tissue is the penultimate goal of plant breeding. In order to start field applications, the plant material in question must have the functional organs to survive outside the *in vitro* conditions. Shoot induction with successive root formation has been the best strategy for plant regeneration. The need for fine adjustment in organogenesis temperature was evident from the germination performances of two *P. somniferum* varieties. Observations based on routine laboratory trials

have shown that, germination temperature preferred by SARI-118 were lower than the Danish variety DAN (Sezen and Önde, unpublished data). Germination success of SARI-118 were higher at low room temperatures below 25 ^{0}C. In this study, the temperature profile within the programmed incubation cycle having both high and low temperatures has provided an inventive means to reveal the importance of temperature as a physical parameter for both shoot induction and proliferation. Following this, synchronous shoot induction was achieved at 16° C, a comparably lower culture temperature than that of callusing. Effect of lower culture temperatures for the organogenesis of *P. somniferum* was also emphasised in a previous study (Yoshikawa and Furuya 1982). Indeed, application of lower temperatures is a physiologically proficient way for culturing a naturally overwintering plant such as opium poppy and may also be suitable for other recalcitrant species.

Root formation has been a rather problemmatic issue in organogenesis of *P. somniferum* and was unraveled in this study. Besides the constancy of 16 °C as the rooting temperature, two auxins were applied seperately. A relatively easy to metabolise auxin type growth regulator IBA, was found to be superior over NAA. For root induction, the metabolic half-life of the growth regulator employed appears to be an important feature. Since a brief auxin treatment was required, IAA might perform much better than IBA because of its quicker metabolic rate in plant tissues. Effect of IAA as root inducer in *P. somniferum* should be tested also to gain a better tuned biochemistry of root induction.

The success of rooting in this study was to a large extend depended on the auxin content of shoot proliferation medium (Appendix A-5). Presence of NAA in lower proportions with kinetin must have prevented poor effects of sudden changes in medium composition such as shifting to auxin based root induction medium by reducing cytokinin concentration to zero. It is getting more clear that, there should be a gradual transition in tissue culture media to provide the means for physiological determination during organogenesis as has been experienced in root induction phase of this study. Auxin shock, as commonly encountered in root induction procedures, had adverse effects as repressed shoot development (Figure 3.7), reversions into callusinogenic meristemoid formations (Figure 3.9) and even "punky" bursts of localised root hair formations incompatible with the shoot structure (Figure 3.8). Exogenous supply of the growth regulators must be gradually eliminated as the organogenesis of the plantlets go toward completion. This enables the establishment of the inherent balance of endogenous hormones leaving the plant independent in its habitual development. In his classical book, *On the Origin of Species by Means of Natural Selection* published in 1959, Charles H. Darwin made a remarkable statement which should be appreciated at this point: "Nature does not make leaps."-*Natura non facito saltum..*-

Although the final stage of transgenic plant technology, in laboratory environment, is to transfer regenerated plantlets from their *in* vitro environment to soil, due to the delicate nature of opium plantlets, all attempts for transfering

into soil have failed. Also time constraints prevented us to optimise this critical transition from *in vitro* to soil environment.

From the very beginning of this study the tissues in selection and isolated DNA samples were assumed as "putative transfomants" because of the fact that the selected plant material can not be named as "transgenic" by solely based on the evidence from the physiological survival capacity in lethal dose of the selective agent. Molecular analysis for the demonstration of stable integration of transgene to the poppy genome gave negative results in this study. Due to the scarcity of DNA samples PCR amplification followed by southern blot analysis was intended for molecular confirmation since the amount of DNA required for PCR amplification is in nanogram levels. Southern blot technique is essential in all transformation work because PCR amplification alone is not a reliable proof since the positive signal may originate from bacterial DNA that has remained viable throughout the selection period. This scientific paranoia can be eliminated by carrying out PCR using primers specific to the Ti-plasmid of *Agrobacterium.* The negative results from the PCR analysis should not be regarded strange because almost more than 40% (3 out of 7) of the DNA samples were lost upon phenol/chloroform extraction following RNAse treatment. The probability of leaving a transgene undetected becomes rather high in these low DNA concentrations (figure 3.16). Furthermore, the smeary appearence of genomic DNA on agarose gel is a clear sign of severe degredation (Figure 3.15).

Rate limiting enzymes on morphinan alkaloid biosynthesis pathway are key elements regulating the alkaloid titre in poppy capsules. Increased concentrations of alkaloids can be achieved by increasing the reaction rates of such enzymes via random mutagenesis with subsequent screening. Alternatively, multiple copy introduction of genes coding for enzymes in the biosynthetic pathway of desired morphinan alkaloids such as codeine: NADP oxidoreductase can be applied for increasing the yields in preferred way. Characterisation of the enzymes like L-tyrosine and L-dopa (Facchini and De Luca 1994) together with the others waiting to be characterised (Figure 1.1) on this biosynthetic pathway will provide a valuable background for the engineering of alkaloid synthesis. Blocking of the synthesis pathway via antisense RNA technology can also be a powerfull means to obtain therapeutically important chemicals such as codeine or thebaine.

Field performance of *P. somniferum* could also be increased by conducting an unconventional breeding program using the marker gene transfer and regeneration procedure established in this study. This approach could be successfully employed for improving the characteristics such as herbicide, pest and cold resistance as well as disease tolerance described in section 1.4.2. However, more work is required to accomplish the growth of regenerated plantlets in soil environment.

CHAPTER 5

CONCLUSION

The primary goal of this study was to establish a transformation procedure for *Papaver* somniferum L. using *Agrobacterium tumefaciens* mediated gene transfer. A commonly used marker gene NPTII was used in transformation.

Efficiency of transformation to *P. somniferum* was found to be higher at 19 ° C which was concluded to be the best co-cultivation temperature for the T-DNA delivery by allowing exact pilus assembly (Fullner *et al.* 1996). This study is the first report expressing the need of lower co-cultivation temperatures for *Agrobacterium* in order to maximise gene delivery to plant tissues since A.J. Riker 1926 with the molecular confirmation that the transgene is stably incorporated in plant genome (in preperation).

Augmentin was used as an efficient antibiotic for the removal of *Agrobacterium* after co-cultivation in 500mg.l^{-1} concentration. Sulbactam was found to be cytotoxic and was discontinued. Endogenous resistance of plant material to kanamycin selection was determined to be 150 mg.l^{-1}.

Callusing temperature was 23 °C and proliferation of transformed tissues under kanamycin selection was also maintained at the same temperature. Organogenesis temperature of 16 °C was found to be essential for both shoot and root formation. Temperatures higher than 25°C resulted in abnormalities in shoot organisation and leaf morphology and therefore, was avoided throughout organogenesis.

Vitamin solution described in this study and supplemented to the media was one of the indispensable components throughout the whole tissue culture work (Appendix-8).

Roles of the two auxins, IBA and NAA during and prior to root formation was quite significant. NAA content of shoot proliferation medium have provided the gradual tissue determination for root induction (Appendix A-5). Tissues responded to IBA, a relatively more rapidly metabolised auxin in plant tissues, by displaying excellent root initiation and development when briefly exposed to 0.01mg.l^{-1} concentration. In this study, the tissue culture strategy followed in later stages has allowed the tissues to implement their self-determination of inherent physiological machinery by gradually reducing exogenous growth regulators as much as possible. Hence the plantlets were avoided from sudden "leaps" such as "auxin shocks".

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APPENDIX

MEDIUM COMPOSITIONS

A-1 Germination medium

(per liter)
hMS (Murashige &Skoog mineral salts, half strength).....2.2g
Sucrose.....30g
Agar (high gel strength)..... ..3.1g
The pH was adjusted to 5.8 before autoclaving.
Vitamin supplements are listed in A-8.

A-2 Callus induction medium

(per liter)
hMS (Murashige &Skoog mineral salts, half strength).....2.2g
Sucrose.....30g
Agar (high gel strength).....3.1g
2,4-D/NAA..... ....0.5mg
Kinetin.....0.1mg
The pH was adjusted to 6.2 before autoclaving.
Vitamin supplements are listed in A-8.

A-3 Selective medium

(per liter)
hMS (Murashige &Skoog mineral salts, half strength).....2.2g
Sucrose.....30g
Agar (high gel strength).....3.1g
2,4-D/NAA..... ...0.5mg
Kinetin.....0.1mg
Kanamycin.....150mg
Augmentin.....500mg
The pH was adjusted to 6.2 before autoclaving.
Vitamin supplements are listed in A-8.

A-4 Shoot induction medium

(per liter)
hMS (Murashige &Skoog mineral salts, half strength)..... .2.2g
Sucrose..... .....30g
Agar (high gel strength).....3.1g
Kinetin.....1mg
Kanamycin.....150mg
Augmentin.....500mg
The pH was adjusted to 6.2 before autoclaving.
Vitamin supplements are listed in A-8.

A-5 Shoot proliferation medium

(per liter)
hMS (Murashige &Skoog mineral salts, half strength).....2.2g
Sucrose.....30g
Agar (high gel strength)..... 3.1g
Kinetin..... .....1mg
NAA 0.15mg
The pH was adjusted to 6.2 before autoclaving.
Vitamin supplements are listed in A-8.

A-6 Root induction (RI) medium

(per liter)
qMS (Murashige &Skoog mineral salts, quarter strength).....1.1g
Sucrose.....15g
Agar (high gel strength).....3.1g
Auxins NAA or IBA 0.1mg
..... 0.05mg
....0.01mg
Kanamycin.....150mg
Augmentin.....500mg
The pH was adjusted to 6.2 before autoclaving.
Vitamin supplements are listed in A-8.

A-7 Root proliferation medium

(per liter)
qMS (Murashige & Skoog mineral salts, quarter strength).....1.1g
Sucrose..... ....15g
Agar (high gel strength)..... .....3.1g
The pH was adjusted to 6.2 before autoclaving.
Vitamin supplements are listed in A-8. Cultured at 16° C.

A-8 Vitamin supplements

The following filter sterilised vitamin solution were added to the medium before the medium was dispensed:

(per liter)

Thiamine	0.4mg
Nicotinic acid	0.5mg
Pyridoxine	0.5mg
Myo-inositol	100mg

A-9 LB (Luria-Bertani) medium for *A. tumefaciens* and *E.coli* strains

(per liter)

Tryptone	10g
Yeast Extract	5g
NaCl	5g

pH was adjusted to 7.5 before autoclaving.
For solid LB standard agar was used as 14 g.