# Genetic transformation and metabolic engineering of poppy

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## Introduction

Alkaloids are pharmacologically active, nitrogen-containing compounds produced predominantly, although not exclusively, in higher plants. The benzylisoquinoline alkaloids are the largest group in the field of plant alkaloids in terms of structural diversity and are found primarily in plants that belong to five families: the Berberidaceae, Fumariaceae, Menispermaceae, Papaveraceae, and Ranunculaceae. A variety of benzylisoquinoline alkaloids that remain important as pharmaceuticals are still isolated from plants, because of the complexity of their chemical structures.

The functions of benzylisoquinoline alkaloids in plants may include self-defense against herbivory and infection by pathogens. The pharmacological activity of benzylisoquinoline alkaloids that renders them useful as pharmaceuticals is often a clue to their biological role in the plant. For example, the effectiveness of morphine as an analgesic, colchicine as a microtubule disrupter, and (+)-tubocurarine as a neuromuscular blocker suggests that these alkaloids function as animal feeding deterrents. The antibiotic nature of sanguinarine suggests that it' s constitutive or inducible accumulation confers protection to the plant against challenges imposed by a pathogen. Many plants, such as opium poppy and California poppy, *Eschscholzia californica Cham* producing benzylisoquinoline alkaloids, invest considerable amounts of nitrogen and metabolic energy in the biosynthesis of numerous and structurally diverse alkaloids suggesting that these natural products play additional and essential ecochemical and/or physiological roles that remain to be discovered .

All benzylisoquinoline alkaloids share a common biosynthetic origin, beginning with the condensation of two aromatic units both derived from the amino acid L-tyrosine.

The enzymatic steps of particular interest in this proposal are: tyrosine decarboxylase (TYDC) which represents an entry point into the alkaloid pathway; *N*-methylcoclaurine hydroxylase (NMCH) which is the first of many P450-dependent enzymes in the early alkaloid pathway;

NADPH: cytochrome c reductase (CCR) which donates electrons to P450-dependent enzymes and is essential for their function; and berberine bridge enzyme (BBE) which operates at an important branch-point in sanguinarine biosynthesis. However, despite our extensive appreciation for the chemistry and enzymology of benzylisoquinoline alkaloid biosynthesis, the control architecture that regulates metabolic flux through the numerous biosynthetic pathways is only beginning to be understood. The application of molecular techniques to the study of benzylisoquinoline alkaloid biosynthesis will expand the frontiers of our ability to understand and manipulate these pathways in plants. The prospect to engineer the benzylisoquinoline alkaloid metabolism of plants for the 'custom' biosynthesis of pharmaceuticals will require both a thorough knowledge of the regulation of biosynthetic enzymes and genes, and the availability of cloned genes for the genetic transformation of plants and other organisms.

## Results

**Plant regeneration system in California poppy,** *Eschscholzia californica* Cham

The development of a rapid protocol for high efficiency somatic embryogenesis and plant regeneration from seed-derived embryogenic callus cultures of California poppy (*E. californica* Cham.) is established. The optimized procedure required less than 13 weeks from the initiation of seed cultures to the recovery of plantlets, and involved the sequential transfer of cultures onto solid Murashige and Skoog basal medium containing three different combinations of growth regulators. All steps were performed at 25 ° C. Friable primary callus was induced from seeds of *E. californica* cultured on medium supplemented with 1.0 mg $L^{-1}$ 2,4-D. The primary callus was transferred to medium containing 1.0 mg $L^{-1}$ NAA and 0.5 mg $L^{-1}$ BAP to establish embryogenic callus and promote somatic embryogenesis. Regenerated plantlets were recovered after germination of somatic embryos on medium containing 0.05 mg $L^{-1}$ BAP and showed normal development. Embryogenic callus was induced at a frequency of 85%, an average of 45 somatic embryos were produced per callus, 90% of the somatic embryos germinated, and virtually all showed normal development. The growth rate of somatic embryos could be further increased using gibberellic acid, but the resulting shoots were vitrified.

A somatic embryogenesis protocol was developed for *E. californica* using embryogenic cell suspensions and optimized media conditions. Rapidly-growing, finely-dispersed embryogenic cell suspension cultures were established from embryogenic callus and maintained in B5

liquid media supplemented with 0.5 mg $L^{-1}$ 2,4-dichlorophenoxyacetic acid. Culture conditions were optimized by investigating the effect of basal media composition, gyratory shaker speed, various carbon sources, different cytokinins, and $AgNO_3$ on the efficiency of somatic embryogenesis. After 40 d in culture, the somatic embryos that formed were counted and their overall growth expressed as packed cell volume. The selected media consisted of either Gamborg (B5) or Murashige and Skoog (MS) salts and vitamins supplemented with 40 g L-1 sucrose, 0.05 mg L-1 6-benzylaminopurine, and 10 mg $L^{-1}$ $AgNO_3$. Somatic embryo production was substantially reduced at shaker speeds above 40 rpm. Glucose and sucrose were the most effective carbon sources, whereas fructose, galactose, and maltose resulted in a reduced yield and growth of somatic embryos. The development of somatic embryos was promoted by $AgNO_3$ at concentrations below 10 mg $L^{-1}$. A semi-solid medium containing 1.5 g $L^{-1}$ Gel-rite produced the highest frequency of somatic embryo conversion, and promoted the efficient growth of plantlets. Using the reported protocol, over 500 viable somatic embryos were produced per 25 mL of embryogenic cell suspension culture.

## Plant genetic transformation in California poppy, E. californica

An efficient Agrobacterium-mediated protocol for the stable genetic transformation of E. californica via somatic embryogenesis is developed. Excised cotyledons were co-cultivated with A. tumefaciens strain GV3101 carrying the pBI121 binary vector. Except for the co-cultivation medium, all formulations included 50 mg $L^{-1}$ paromomycin as the selective agent and 200 mg $L^{-1}$ timentin to eliminate the *Agrobacterium*. Four to five weeks after infection, paromomycin-resistant calli grew on 80% of explants in the presence of 2.0 mg $L^{-1}$ 1-naphthaleneacetic acid (NAA) and 0.1 mg $L^{-1}$ 6-benzylaminopurine (BAP). Calli were cultured on somatic embryogenesis induction medium containing 1.0 mg $L^{-1}$ NAA and 0.5 mg $L^{-1}$ BAP, and somatic embryos were visible on 30% of the paromomycin-resistant calli within 3 to 4 weeks. Three to four weeks after the somatic embryos were transferred to phytohormone-free plant regeneration medium, 32% converted to paromomycin-resistant plants. Detection of the neomycin phosphotransferase gene and high levels of $\beta$-glucuronidase (GUS) mRNA and enzyme activity, and the cytohistochemical localization of GUS activity in all plant tissues confirmed the integrative transformation of the regenerated plants. The normal alkaloid profile of California poppy was unaffected by the transformation process; thus, the

established protocol could serve as a valuable tool to investigate the molecular and metabolic regulation of the benzophenanthridine alkaloid pathway.

## Plant regeneration and transformation in opium poppy, Papaver somniferum L

An efficient Agrobacterium-mediated protocol has been developed for the stable genetic transformation of intact opium poppy, *Papaver somniferum* L., plants via shoot organogenesis. Excised cotyledons were cocultivated with the disarmed *A. tumefaciens* strain GV3101 carrying the pBI121 binary vector, and incubated on an optimized shoot induction medium consisting of B5 salts and vitamins, 30 g $L^{-1}$ sucrose, 2 mg $L^{-1}$ 6-benzylaminopurine, 5 mg $L^{-1}$ $AgNO_3$, and 3 g $L^{-1}$ Gelrite. Except for the cocultivation medium, all formulations included 30 mg L-1 paromomycin as the selective agent, and 200 mg $L^{-1}$ timentin to eliminate the Agrobacterium. Eight-week-old, paromomycin-resistant shoots were transferred to an optimized root induction medium consisting of B5 salts and vitamins, 0.5 mg $L^{-1}$ indole-3-acetic acid, 0.5 mg $L^{-1}$ indole-3-butyric acid, and either 5 mg $L^{-1}$ $AgNO_3$ or 40 mg $L^{-1}$ putrescine. About 15% of the regenerated shoots developed roots within eight weeks. Regenerated plants were transferred to soil, where they grew normally and set seed. Detection of the neomycin phosphotransferase gene, the high levels of $\beta$-glucuronidase (GUS) mRNA and enzyme activity, and the cytohistochemical localization of GUS activity in all organs, confirmed the genetic transformation of the regenerated plants. The transformation process did not alter the normal alkaloid content of opium poppy; thus, the developed protocol could serve as a valuable tool to investigate the molecular and metabolic regulation of benzylisoquinoline alkaloid biosynthesis.

## Transgenic hairy root cultures in E. californica and P. somniferum

An efficient protocol for the establishment of transgenic *P. somniferum* L. and *E. californica* root cultures using *A. rhizogenes* is reported. Five strains of *A. rhizogenes* were tested for their ability to produce hairy roots on wounded opium poppy seedlings and California poppy embryogenic calli. Three of the strains induced hairy root formation on both species, whereas two others either caused the growth of tumorigenic calli or produced no response. To further characterise the putative transgenic roots, explant tissues were co-cultivated with

the most effective *A. rhizogenes* strain (R1000) carrying the pBI121 binary vector. Except for the co-cultivation medium, all formulations included 50 mg $L^{-1}$ paromomycin to select for transformants and 200 mg $L^{-1}$ timentin to eliminate the *Agrobacterium*. Four weeks after infection, paromomycin-resistant roots appeared on 92 to 98% of explants maintained on hormone-free medium. Isolated hairy roots were propagated in liquid medium containing 1.0 mg $L^{-1}$ indole-3-acetic acid to promote rapid growth. Detection of the neomycin phosphotransferase gene, high levels of $\beta$-glucuronidase (GUS) transcripts and enzyme activity, and GUS histochemical localisation confirmed the integrative transformation of root cultures. Transgenic roots grew faster than wild type roots, and California poppy roots grew more rapidly than those of opium poppy. With the exception of a less compact arrangement of epidermal cells and more root hairs, transformed roots of both species displayed anatomical features and benzylisoquinoline alkaloid profiles that were virtually identical to those of wild type roots. Transgenic root cultures of opium poppy and California poppy are a simple, reliable and well-defined model system to investigate the molecular and metabolic regulation of benzylisoquinoline alkaloid biosynthesis, and to evaluate the genetic engineering potential of these important medicinal plants.

## Metabolic engineering in transgenic cell and root cultures of E. californica

*E. californica* cell cultures produce several benzophenanthridine alkaloids, such as sanguinarine, chelirubine, and macarpine, with potent pharmacological activity. Antisense constructs of genes encoding two enzymes involved in benzophenanthridine alkaloid biosynthesis, the berberine bridge enzyme (BBE) and N-methylcoclaurine 3' -hydroxylase (CYP80B1), were introduced separately into California poppy cell cultures. Transformed cell lines expressing antisense-BBE or antisense-CYP80B1 constructs and displaying low levels of BBE or CYP80B1 mRNAs, respectively, showed reduced accumulation of benzophenanthridine alkaloids compared to control cultures transformed with a -glucuronidase gene. Pathway intermediates were not detected in any of the transformed cell lines. The suppression of benzophenanthridine alkaloid biosynthesis using BBE or CYP80B1 antisense RNA constructs also reduced the growth rate of the cultures. Two-dimensional $^{1}$H-NMR and *in vivo* $^{15}$N-NMR spectroscopy showed no difference in the abundance of carbohydrate metabolites in the various transgenic cell lines. However, transformed cells with reduced benzophenanthridine alkaloid levels contained larger cellular pools of several amino acids including alanine, leucine, phenylalanine,

threonine, and valine compared to controls. The relative abundance of tyrosine, from which benzophenanthridine alkaloids are derived, was less than twofold higher in antisense-suppressed cells relative to controls. These results show that alterations in the metabolic flux through benzophenanthridine alkaloid biosynthesis can affect the regulation of amino acid pools. These data provide new insight into the metabolic engineering of benzophenanthridine alkaloid pathways.

*E. californica* root cultures produce a variety of benzophenanthridine alkaloids, such as sanguinarine, chelirubine and macarpine, with potent biological activity. Sense and antisense constructs of genes encoding the berberine bridge enzyme (BBE) were introduced into California poppy root cultures. Transgenic roots expressing BBE from opium poppy displayed higher levels of BBE mRNA, protein and enzyme activity, and increased accumulation of benzophenanthridine alkaloids compared to control roots transformed with a $\beta$-glucuronidase gene. In contrast, roots transformed with an antisense-BBE construct from California poppy had lower levels of BBE mRNA and enzyme activity, and reduced benzophenanthridine alkaloid accumulation, relative to controls. Pathway intermediates were not detected in any transgenic root lines. Suppression of benzophenanthridine alkaloid biosynthesis using antisense-BBE also reduced the growth rate of the root cultures. Two-dimensional $^1$H-NMR spectroscopy showed no difference in the abundance of carbohydrate metabolites in the various transgenic roots lines. However, transformed roots with low levels of benzophenanthridine alkaloids contained larger cellular pools of certain amino acids compared to controls. In contrast, cellular pools of several amino acids were reduced in transgenic roots with elevated benzophenanthridine alkaloid levels relative to controls. The relative abundance of tyrosine, from which benzophenanthridine alkaloids are derived, was only marginally altered in all transgenic root lines; thus, altering metabolic flux through benzophenanthridine alkaloid pathways can affect cellular pools of specific amino acids. Consideration of such interactions is important for the design of metabolic engineering strategies that target benzophenanthridine alkaloid biosynthesis.

## Discussion

Plant cell and tissue culture plays an important role in the manipulation of plants for improved crop varieties. Plant regeneration system is an essential part of molecular approaches leading to plant improvement in poppy family and acts as an intermediary whereby

advances made by the molecular biologists in gene isolation and modification are transferred to poppy plant cells. Ultimately, geneticists and plant breeders can then fully evaluate the transgenic plants that are regenerated in culture. Cells and tissues of poppy plant species are difficult to culture and establish optimal growing conditions in vitro. Therefore there continues to be an urgent need for extensive work in the field of basic tissue culture protocols for poppy plants before any practical utilization of molecular biology approaches can be achieved.

Plant transformation act as a core research tool in plant biology and a practical tool for plant improvement. Genetic engineering using plant gene transformation system has already been responsible for the production of plants with enhancement in a range of desirable traits, notably disease resistance, insect resistance, ripening properties and nutritional and commercial value. Progress is also being made towards longer term aims such as improvement of other polygenic mechanisms. For the establishment of genetic engineering of poppy family, plant transformation system is essential. We developed the transformation system of California poppy and opium poppy, but still that is difficult to produce transgenic plant easily. Therefore we need to make optimal protocol for poppy transformation. Based on poppy transformation protocol, metabolic engineering has allowed the production of poppy cell and root cultures with an altered content of alkaloids.

The expression of an antisense gene leads, regardless of the mechanism involved, to a specific interference with the taret RNA. Therefore, the antisense technology provides a simple but, nevertheless, specific tool for the suppression of a single gene. This process has been descrebed as "gene suppression", "genesilencing", "gene ablation", "knocking out" of a gene or "down-regulation" of gene expression. The presence of the antisense RNA creates a mutant phenotype in which the target gene is inactivated or greatly reduced in its activity. This is important especially since mutants in many cases provide a very valuable source of information for understanding biological processes. It may be able to use antisense methodology to poppy family. For example, we can reduce expression of deleterious genes and thereby enhance overall crop productivity of remove from plants chemicals that are hazardous to human health, such as morphine from opium poppy.

## Future prospects

This work is just the beginning of metabolic engineering in benzylisoquinoline alkaloid biosynthesis. There remains much to be done. Of all the numerous alkaloid biosynthesis genes in Papaveraceae, relatively few have been explored and for those that have the explores have only recently set out.

In the future we will see more success in modifying in benzylisoquinoline alkaloid biosynthesis. We just established this work using transgenic cell and hairy root cultures of California poppy. One of the challenges is developing the metabolic engineering of benzylisoquinoline alkaloid biosynthesis using transgenic plant. Therefore, it might be useful to also study the alkaloid biosynthesis gene function in Papaveraceae plants.

Particularly changing one single step, which can result in higher or lower levels of a desired compound. In the major pathway leading to morphine, thebaine is converted by enol-ether cleavage to codeinone, which is subsequently reduced to codeine. Ultimately, codeine is demethylated to yield morphine. The cytosolic enzyme codeinone reductase (COR), which catalyzes the NADPH-dependent reduction of (-)-codeinone to (-)-codeine, has recently been purified and the corresponding cDNA isolated from opium poppy. For example, increasing the levels of COR mRNA may be achievable by gene transfer with sense-COR construction. This can increase the production of morphine in opium poppy. On the contrary, reducing the levels of COR mRNA may be achievable by antisense technology. We can reduce expression of COR genes and thereby enhance overall crop productivity of remove from plants chemicals that are hazardous to human health, such as morphine from opium poppy.

The other future success of biotechnological metabolic engineering will depend on the molecular cloning of elusive regulatory genes that control the expression of a series of alkaloid biosynthesis genes. Enhanced and constitutive expression of such regulatory genes in transgenic medicinal plants is expected to increase significantly the total amount of useful alkaloids produced per plant.

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