Transgenic Medicinal Plants: Agrobacterium-Mediated Foreign Gene Transfer and Production of Secondary Metabolites

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TRANSGENIC MEDICINAL PLANTS: *AGROBACTERIUM*-MEDIATED FOREIGN GENE TRANSFER AND PRODUCTION OF SECONDARY METABOLITES

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ABSTRACT.—*Agrobacterium*-Ti/Ri plasmids are natural gene vectors, by which a number of attempts have been made in genetic engineering of secondary metabolism in pharmaceutically important plants in the last few years. Opines are biosynthesized by transformed crown galls and hairy roots integrated with T-DNAs of Ti/Ri plasmids. These opines are classified into five families according to their structures and biogenesis. The production of opines is a natural example of genetic engineering of the biosynthetic machinery of plant cells for the benefit of the bacterial pathogen.

One recent advance in transgenic technology of potential value to pharmacognosy is an application of transgenic organ cultures such as hairy roots and shooty teratomas to over-production and biotransformation of secondary metabolites. The hairy roots induced by Ri plasmid of *Agrobacterium rhizogenes* have been proved to be an efficient means of producing secondary metabolites that are normally biosynthesized in roots of differentiated plants. So far the specific metabolites produced by hairy root cultures and/or plants regenerated from hairy roots of 63 species have been analyzed and reported. As an alternative means of producing metabolites normally produced in leaves of plants, the shooty teratomas incited by the tumor-forming Ti plasmid or a shooty mutant of *Agrobacterium tumefaciens* have been used for the de novo biosynthesis and biotransformation of some specific secondary products.

A second and more direct way to manipulate secondary pathways is performed by transferring and expressing specifically modified genes into medicinal plant cells with *Agrobacterium* vector systems. The genes encoding neomycin phosphotransferase and β-glucuronidase have been used as model genes under the transcriptional control of appropriate promoters. Recently some specific genes that can eventually modify the fluxes of secondary metabolism have been integrated and expressed in medicinal plant cells. Future prospects are also discussed.

Transgenic technology provides enormous possibilities for genetic improvement of higher plants. In the last several years, remarkable progress has been made in the technology for stable integration and expression of engineered foreign genetic information in plant cells (1,2). In particular, an *Agrobacterium*-mediated gene delivery system has been developed and widely used. By using this technology, a number of studies have been carried out to clarify the molecular mechanism underlying the regulation of plant gene expression (3) and also to improve the quality of crop plants using a molecular approach (4). So far some agriculturally important traits, such as resistance to herbicide, viruses, and insects, have been successfully conferred to commercially important crop species (e.g., oilseed rape, potato, tomato, and cotton) by this transgenic technology.

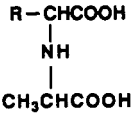
Several efforts have been also made to manipulate secondary metabolism in pharmaceutically important medicinal plants by transgenic techniques. Because secondary products, however, are biosynthesized in multi-step enzymatic reactions in specifically differentiated cells, it is not easy to control the production even by modern sophisticated molecular methods. One prerequisite for genetic manipulation is understanding the molecular mechanism of regulation of the secondary pathway and, in particular, gene expression of the rate-limiting step of the reaction. Although this was not always possible, some remarkable progress has been achieved in the last few years. This progress can be divided into two major categories: transgenic organ culture, such as hairy roots and shooty teratomas for high production of specific metabolites; and transfer and expression of artificially manipulated foreign genes, including model genes and specific genes capable of changing the metabolite pattern.

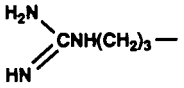
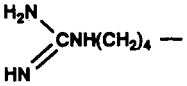
Plant cells transformed with *Agrobacterium* usually produce opines, specific secondary products in crown galls and hairy roots. The production of opines is actually a natural example of genetic manipulation of biosynthetic machinery by *Agrobacterium*. In this review, we first give a brief biochemical note on the production of opines, and second, an overview of recent advances of genetic manipulation of secondary metabolism by transgenic technology.

PRODUCTION OF OPINES.—*Agrobacterium* harboring tumor-inducing (Ti) or root-inducing (Ri) plasmids can transfer a specific DNA fragment called transferred DNA (T-DNA) from Ti/Ri plasmids to the plant nuclear genomes. The plant cells transformed by Ti and Ri plasmids proliferate to form, respectively, unorganized tumors called crown galls and adventitious organs called hairy roots. The neoplastic cells also produce opines, which are specific to tumor cells transformed with the specified types of *Agrobacterium*. From structural and biogenetic characters, five families of opines are identified (Table 1). All opines are built up from quite common compounds in plant cells such as α-keto acids, amino acids, and simple sugars. The opines in the Octopine (Scheme 1) and Nopaline (Scheme 2) families are imino acids formed by reduction of Schiff bases of α-keto acid and amino acids. The opines in the Agropine family (Scheme

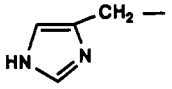
TABLE 1. Production of Opines by *Agrobacterium*-Transformed Plant Tissues.

Family of opines (Representative *Agrobacterium*) Opine	Possible precursors	Reference
Octopine Family		
(*Agrobacterium tumefaciens* B6, Ach5, 15955)		
Octopine	pyruvate, arginine	5
Lysopine [1]	pyruvate, lysine	5
Octopinic acid	pyruvate, ornithine	5
Homooctopinic acid	pyruvate, homoarginine	5
Canavanooctopine	pyruvate, canavanine	5
Histopine	pyruvate, histidine	5
Nopaline Family		
(*Ag. tumefaciens* C58, T37)		
Nopaline	α-ketoglutarate, arginine	5
Nopalinic acid (Ornaline)	α-ketoglutarate, ornithine	5
Succinamopine	α-ketoglutarate, asparagine	6
Leucinopine	α-ketoglutarate, leucine	7
Pyronopaline	α-ketoglutarate, arginine	8
Agropine Family		
(*Ag. tumefaciens* BO542, A281, *Agrobacterium rhizogenes* 8196, 15834, A4)		
Mannopine	mannose, glutamine	9
Agropine [2]	mannose, glutamine	10
Mannopinic acid	mannose, glutamic acid	9
Agropinic acid	mannose, glutamic acid	9
Agrocinopine Family		
(*Ag. tumefaciens* C58, T37)		
Agrocinopine A [3]	sucrose, arabinose, phosphate	11
Agrocinopine B [4]	sucrose (fructose), arabinose, phosphate	
Cucumopine Family		
(*Ag. rhizogenes* NCPPB2659, NIAES1724)		
Cucumopine [5]	α-ketoglutarate, histidine	12
Mikimopine [6]	α-ketoglutarate, histidine	13



Octopine R=  — Homooctopinic acid R=  —

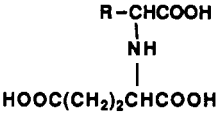
Lysopine [1] R= $H_2N(CH_2)_4$ — Canavanooctopine R=  —

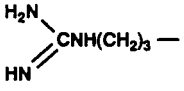
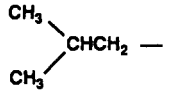
Octopinic acid R= $H_2N(CH_2)_3$ — Histopine R=  —

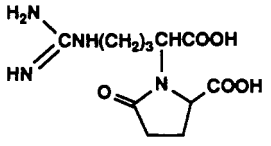
SCHEME 1

3) are formed in a similar manner from the amino acids and mannose instead of α-keto acids. Cucumopine [**5**] and mikimopine [**6**] (Scheme 4) are formed by condensation-cyclization of α-ketoglutaric acid and histidine. Agrocinopines A [**3**] and B [**4**] (Scheme 4) are phosphodiesters of the common sugars in plant cells.

Opines have no function in plant cells. They are produced at the expense of the precursor amino acids, α-keto acids, and carbohydrates. Agropine [**2**] may constitute as much as 7% of the tissue dry wt (14), and lysopine [**1**] may contain as much as 80% of the nitrogen of the free amino acid pool (15). The genes responsible for opine biosynthesis and secretion are located in T-DNA regions of Ti/Ri plasmids close to the genes for autonomous cell growth. The genes controlling opine catabolism in *Agrobacterium* also lie on the plasmids but outside of the T-DNAs (16). The transformed plant cells

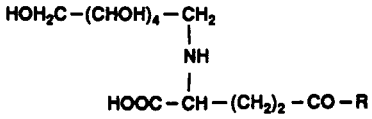


Nopaline R=  — Leucinopine R=  —

Nopalinic acid R= $H_2N(CH_2)_3$ — Pyronopaline 

Succinamopine R= H_2NOCCH_2 —

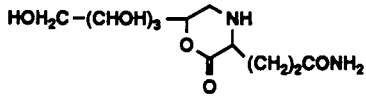
SCHEME 2



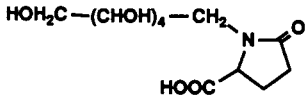
Mannopine R=NH_2

Mannopinic acid R=OH

Agropine [2]



Agropinic acid

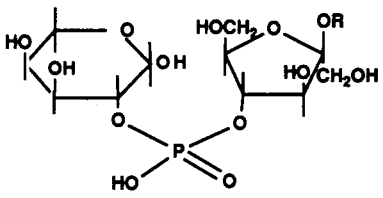


SCHEME 3

proliferate to form tumors and adventitious roots in which specific opines are produced and released. The *Agrobacterium*, but not host plant cells, can selectively utilize the opines as energy, carbon, and/or nitrogen sources. These give an evolutionary advantage to *Agrobacterium* and Ti/Ri plasmids over other soil bacteria. This idea was formulated as a general concept called "genetic colonization" (17) or "opine concept" (9). According to this concept, the Ti/Ri plasmids are natural gene vectors for plant cells and offer opportunities for genetic manipulation to change the fluxes of secondary metabolism.

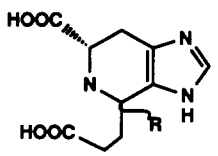
No studies have been reported on the pharmacological activities of opines and on effects of their production on endogenous secondary metabolism in plant cells. These issues will be more important as practical applications are sought for the use of transformed crown galls and hairy roots for the commercial production of pharmaceutical compounds.

PRODUCTION BY TRANSGENIC ORGAN CULTURES.—*Hairy roots.*—Ri plasmids present in *Agrobacterium rhizogenes* induce so-called hairy roots in most dicot plants. The phenomenon is due to the transfer and expression of T-DNA from the Ri plasmid in the plant nucleus (18). Hairy roots transformed with Ri plasmid grow rapidly without addition of any exogenous phytohormones. A number of reports have appeared since 1985 for the successful production of specific secondary metabolites. Table 2 summarizes the



Agrocinopine A [3] R=α-D-glucopyranoside

Agrocinopine B [4] R=H



Cucumopine [5] R= 

Mikimopine [6] R= 

SCHEME 4

TABLE 2. Production of Secondary Metabolites by Hairy Root Cultures and Regenerated Plants.[a]

Plant species	Plasmid strain	Investigated product	Plant regeneration	Reference
		Tropane alkaloids		
Atropa belladonna	15834		yes	19
	A4, 8196		yes	20
	A4, 8196		no	21
	15834		yes	22
Atropa caucasia	A4, 8196		no	21
Catystegia sepium	A4, 8196		no	20
Datura candida	15834		no	23, 24
Datura candida x *urea*	LMA9402		no	25
Datura chlorantha	A4, 8196		no	21
Datura ferox	A4, 8196		no	21
	NBA9402		no	25
Datura fastuosa	LBA9402		no	25
Datura fastuosa var. *violacea*	A4, 8196		no	21
Datura innoxia	A4, 8196		no	21
	LBA9402		no	25
Datura metel	A4, 8196		no	21
Datura meteloides	A4, 8196		no	21
Datura quercifolia	A4, 8196		no	21
	LBA9402		no	25
Datura rosei	A4, 8196		no	21
Datura sanguinea	A4, 8196		no	21
	LBA9402		no	25
Datura stramonium	A4, 8196		no	21
	LBA9402		no	25, 26
	15834		no	27
Datura stramonium var. *inermis*	A4, 8196		no	21
Datura stramonium var. *stramonium*	A4, 8196		no	21
Datura stramonium var. *tatula*	A4, 8196		no	21
Datura wrightii	LBA9402		no	25
Duboisia leichhardtii	A4, 15834		no	28
Duboisia myoporoides	HR1		no	29
Duboisia hybrid	A4, 8196		no	21
Hyoscyamus albus	A4, 8196		no	21
	15834	7β-hydroxyhyoscyamine	no	30
	LBA9402		no	25
Hyoscyamus aureus	A4, 8196		no	21
Hyoscyamus bohemicus	A4, 8196		no	21
Hyoscyamus desertorum	LBA9402		no	25
Hyoscyamus muticus	A4		no	31
	A4, 8196		no	21
	LBA9402		no	25
Hyoscyamus niger	A4		no	31
	A4, 8196		no	21
	15834		no	26
Hyoscyamus niger var. *pallidus*	A4, 8196		no	21
Hyoscyamus pusillus	LBA9402		no	25
Scopolia carniolica	A4, 8196		no	21
Scopolia japonica	15834		no	32
Scopolia lurida	LBA9402		no	25
Scopolia stramonifolia	A4, 8196		no	21
	LBA9402		no	25

TABLE 2. (Continued).

Plant species	Plasmid strain	Investigated product	Plant regeneration	Reference
		Nicotine alkaloids		
Nicotiana africana	LBA9402		no	33
Nicotiana cavicola	LBA9402		no	33
Nicotiana hesperis	LBA9402		no	33, 34
Nicotiana rustica	LBA9402		no	33–35
Nicotiana tabacum	A4		no	31
	LBA9402		no	33
			no	36
	A4, 15834		yes	37
	8196		no	38
	15834		yes	39, 40
Nicotiana umbratica	LBA9402		no	33
Nicotiana velutina	LBA9402		no	33
		Indole alkaloids		
Amsonia elliptica	A4		no	41
Catharanthus roseus	LBA9402		no	42
Catharanthus trichophyllus	15834		no	43, 44
		Other alkaloids		
Cinchona ledgeriana	LBA9402	quinoline alkaloids	no	45
Lobelia inflata	15834	lobeline	no	46
Spartium junceum		lupine alkaloids	no	36
Solanum tuberosum	15834	steroidal alkaloids	no	40
		Terpenoids		
Digitalis lanata	A4	cardenolides	no	47
Digitalis purpurea	15834	cardenolides	no	48
Glycyrrhiza uralensis	15834	glycyrrhizin	no	49
Lippia dulcis	A4	hernandulcin and other mono- and sesquiterpenes	no	50
Panax ginseng	A4	saponins	no	51
		biotransformation of digitoxigenin		52
	15834	saponins	no	49
		Anthraquinones		
Cassia torosa	A4, 15834		no	37
Cassia obtusifolia	A4, 15834		no	37
Cassia occidentalis	A4, 15834		no	37
Rubia tinctorum	15834		no	53
		Miscellaneous		
Beta vulgaris	LBA9402	betalain pigments	no	35
Echinacea purpurea	15834, R1601	alkamides	shoot formation	54
Geranium thunbergii	A4	tannins	no	55
Lithospermum erythrorhizon	A4	shikonin	no	56
Swertia japonica	15834	phenyl glucosides xanthones and amarogentin	no	57, 58
Tagetes patula	43057	thiophenes	no	59
	—	thiophenes	no	60

[a]This table lists the hairy roots in which the production of specific secondary metabolites was confirmed. For the whole plant species infected with *Agrobacterium rhizogenes* examined so far, readers should consult references 61–66.

induction of hairy root cultures and the specific secondary metabolites produced in the resulting hairy root cultures and regenerated plants. The solanaceous alkaloids have been most successfully produced with hairy root cultures. In particular, for nicotine alkaloids **9** and **10** and tropane alkaloids **12–14**, some biochemical and technological factors affecting biosynthesis and release of the alkaloids have been investigated in the established hairy root cultures (67–72). Some other pharmaceutically useful metabolites such as indole alkaloids (41–44), cardenolides (47,48), ginseng saponins (51), and shikonin (56) are also successfully produced with hairy roots.

The advantages of hairy root culture for high production are as follows: (a) these are rapidly-proliferating root cultures with no need of the addition of phytohormones, and (b) there is a concomitant high yield of specific secondary metabolites. Nevertheless, the successful products are strictly limited to those which are normally produced in roots of differentiated plants. Thus, for the production of metabolites normally biosynthesized in green parts of plants, it is necessary to explore modified hairy roots, such as "green hairy roots," or alternatively transformed shooty teratomas. Green hairy roots are chloroplast-containing hairy roots with photosynthetic activity (62,66). These green hairy roots have been induced in certain plant species and produced some metabolites that are normally synthesized in green parts of plants (48, 50, 62, 66).

Crown galls and shooty tetratomas.—Ti plasmid in *Agrobacterium tumefaciens* causes crown gall disease, which forms unorganized proliferated plant tumors on the site of infection of the bacterium. This is also due to the integration and expression of a T-DNA fragment of ca. 20 kb length from the Ti plasmid into the chromosomal DNA of plant nuclei. At least four oncogenes are identified inside the T-DNA. These oncogenes encode the enzymes responsible for biosynthesis or mobilization of plant hormones, auxin and cytokinin, in transformed plant cells (73,74).

The crown gall and derived cell suspension cultures incited with wild type Ti plasmids have been used for production of some specific secondary metabolites (Table 3). Transformed shooty teratomas of some medicinal plants are formed with several strains of *Ag. tumefaciens* (Table 3). The induction of shooty teratomas was performed by a mutant of the Ti plasmid lacking the genes for auxin biosynthesis (38,40) and by the shoot-forming Ti plasmid pTiT37 (79,80). These transgenic organ cultures are also useful for de novo production and biotransformation of some specific metabolites which are usually produced or biotransformed in leaves and/or green stems of differentiated plants. The shooty teratomas of *Atropa* and *Nicotiana* failed to produce the alkaloids. However, the shooty teratomas had the abilities to store and metabolize the alkaloids, indicating the separation of source and sink organs of secondary metabolism. The number of reports of the successful production of these kinds of chemicals in shooty teratomas is still limited, compared with that of hairy roots. This might be due to the difficulty of establishing rapidly growing shooty teratomas in liquid cultures.

TRANSFER AND EXPRESSION OF ENGINEERED FOREIGN GENES.—*Integration of model genes.*—Several genes have been used as models to investigate the different elements governing gene expression in transgenic plants of interest. The genes encoding chloramphenicol acetyltransferase (*cat*), neomycin phosphotransferase II (*kan*), and β-glucuronidase (*gus*) are widely used as the model reporter genes for plant transformation because the translational products of these prokaryotic genes are stable in most plant cells and their enzymatic activities can be easily detected in vitro and in vivo in transformed plant tissues.

These model genes were also transferred into several medicinal plants and their mode of expression was investigated (Table 4). Up to now, the binary vector system based on an Ri plasmid has been widely used for integration of foreign genes into

TABLE 3. Production and Biotransformation by Crown Galls and Shooty Teratomas Induced with Tumor-inducing (Ti) Plasmids.

Plant species	*Agrobacterium*/Ti plasmid	Type of culture	Specific metabolites	Reference
Cinchona ledgeriana . .	*Agrobacterium tumefaciens* A6	Gall cell suspension	Quinoline alkaloids	75
Bidens sp.	Ti plasmid	Gall	1-Phenylhepta-1,3,5-triyne and other polyines	76
Lupinus polyphyllus, *Lupinus hartwegii* .	*Ag. tumefaciens* DSM 30150	Cell suspension derived from gall	Isoflavonoid glucosides orobol-3′-7-*O*-diglc and genistein-4′-7-diglc	77
Digitalis lanata . . .	pTiC58, pTiB6S3 pTiA6NCtmr-338::Tn5	Gall	Cardenolides	78
Nicotiana tabacum . .	pGV3845 (*aux*$^-$)	Shooty teratoma	Biotransformation of nicotine to nornicotine	38
	pGV3304 (*cyt*$^-$)	Rooty teratoma	Nicotine, nornicotine	
Mentha citrata	pTiT37	Shooty teratoma	Mint oil terpenes	79
Mentha piperita . . .	pTiT37	Shooty teratoma	Mint oil terpenes	80
Atropa belladonna . .	pGV2215 (*aux*$^-$)	Shooty teratoma	Biotransformation of hyoscyamine to scopolamine	40

TABLE 4. Transfer and Expression of Engineered Model Genes into Medicinal Plants.

Plant species	Model gene construct	Delivery system	Production of metabolite	Reference
Nicotiana rustica	Pnos-*kan* (pBin19) Pnos-*hyg* (pAGS125)	Ri binary (pRi1855)	Nicotine alkaloids (+)	81
Beta vulgaris	Pnos-*kan* (pBin19) Pnos-*hyg* (pAGS125)	Ri binary (pRi1855)	Betalain pigments (+)	81
Glycyrrhiza uralensis . . .	TR1′-*kan* TR2′-*gus* (pGSGluc1)	Ri binary (pRi15834)	Glycyrrhizin (−)	82
Glycyrrhiza glabra	Pnos-*kan* 35S-*gus* (pBI121)	Ri binary (pRi15834)	Glycyrrhizin (−)	83
Digitalis purpurea	TR1′-*kan* TR2′-*gus* (pGSGluc1) Pnos-*kan*, 35S-*gus* (pBI121)	Ri binary (pRi15834)	Cardenolides (+)	48
Digitalis lanata	Pnos-*kan* 35S-*gus* (pBI121)	Ti binary (pGV2260)	Not determined	84
Atropa belladonna	TR1′-*kan* TR2′-*gus* (pGSGluc1)	Ri binary (pRi15834)	Tropane alkaloids (+)	85

medicinal plants, because this technique has the following advantages. First, one can easily obtain transgenic tissues integrated with any desired foreign gene on a binary vector without selection of transformed cells using growth inhibitors such as antibiotics and herbicides. This is due to the high probability of double transformation with both an Ri plasmid and a binary vector. Second, this technique induces hairy roots which grow quite rapidly and produce secondary metabolites in high yield. Third, some plant species can regenerate shoots from transgenic hairy roots to give mature plants and set seeds.

The production of specific metabolites in different plant species has been analyzed in some transgenic tissues integrated with the chimeric model genes as shown in Table 4. These tissues should be appropriate targets for further work on transfer and expression of specific genes to modify the secondary pathway.

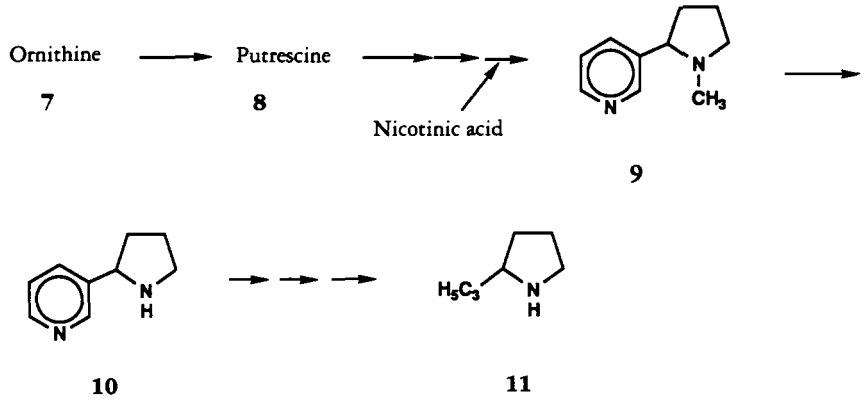
Integration of specific genes.—The specific genes encoding enzymes possibly involved in the key steps of biosynthetic pathway must be transferred and expressed in medicinal plants. Several attempts along this line have been already made in the last couple of years (Table 5). The over-expression of a yeast ornithine decarboxylase gene committed to the first step, **7**↦**8**, for nicotine biosynthesis has been able to enhance the nicotine accumulation two-fold in transgenic roots of *Nicotiana rustica* (90) (Scheme 5). The mammalian drug-metabolizing cytochrome P-450 gene has been expressed in *Nicotiana tabacum* and has led to changes in the biosynthetic pattern of alkaloid production (91), i.e., production of **11**, although this may be due to an indirect effect of early senescence specific in these transgenic plants. The genes for bacterial lysine decarboxylase (92) and hyoscyamine 6β-hydroxylase, **12**↦**13** (Scheme 6), from *Hyoscyamus niger* (94) have been transferred and expressed in transgenic *N. tabacum* and in *Atropa belladonna*, re-

TABLE 5. Transfer and Expression of Specific Genes to Modify Secondary Metabolism and to Confer Other Useful Traits in Higher Plants.[a]

Plant species	Engineered gene	Delivery system	Change of metabolism	Reference
Petunia hybrida . . .	35S-A1 gene (maize)	Ti cointegration	Pelargonidin-3-glucoside (brick red flower color)	86
Petunia hybrida . . .	35S-antisense CHS	Ti binary	Partial inhibition of flower pigmentation	87
	35S-CHS 35S-DFR	Ti binary	Change of flower pigmentation	88, 89
Nicotiana rustica . . .	Enhanced 35S-yeast ODC	Ri binary (pRi1855)	Nicotine production 2-fold enhanced	90
Nicotiana tabacum . .	TR2′-rabbit liver cytochrome P-450	Ti cointegration (pGV2260)	Degradative metabolite of nicotine	91
	TR-LDC rbcS-LDC	Ti binary		92
	A resveratol synthase gene	Ti cointegration	Resveratol biosynthesis	93
Atropa belladonna . .	35S-*bar*	Ri binary	Herbicide resistance	22
	35S-hyoscyamine 6β-hydroxylase	Ti binary	Scopolamine production	94[b]
Zea mays	Enhanced 35S-*Lc* cDNA (*R* gene family)	MB	Anthocyanin pigmentation	95

[a]Abbreviations: CHS, chalcone synthase; DFR, dihydroflavonol reductase; ODC, ornithine decarboxylase; LDC, lysine decarboxylase; rbcS, promoter of small subunit of ribulose-1,5-biphosphate carboxylase and transit peptide; MB, microprojectile bombardment.

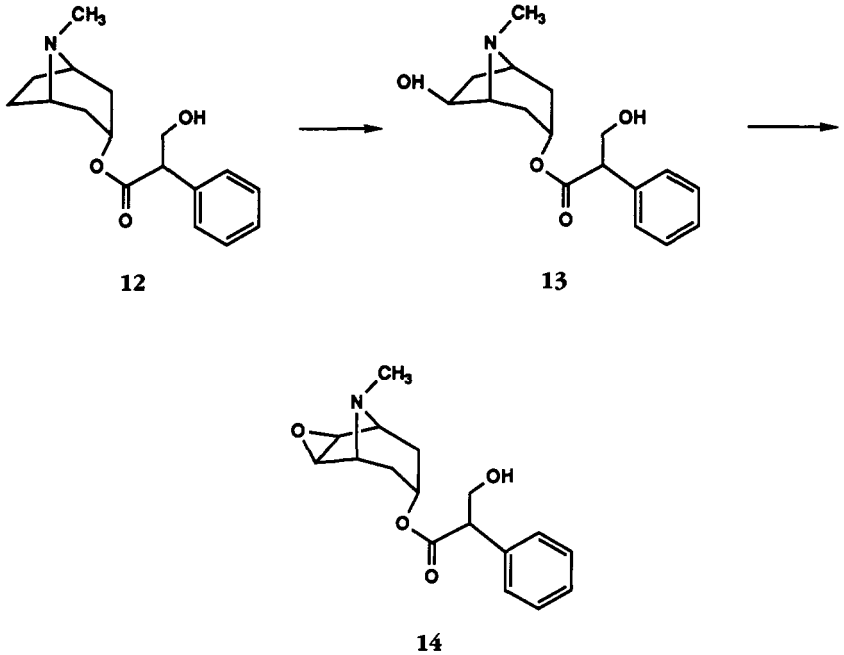
[b]Hashimoto *et al.*, personal communication.



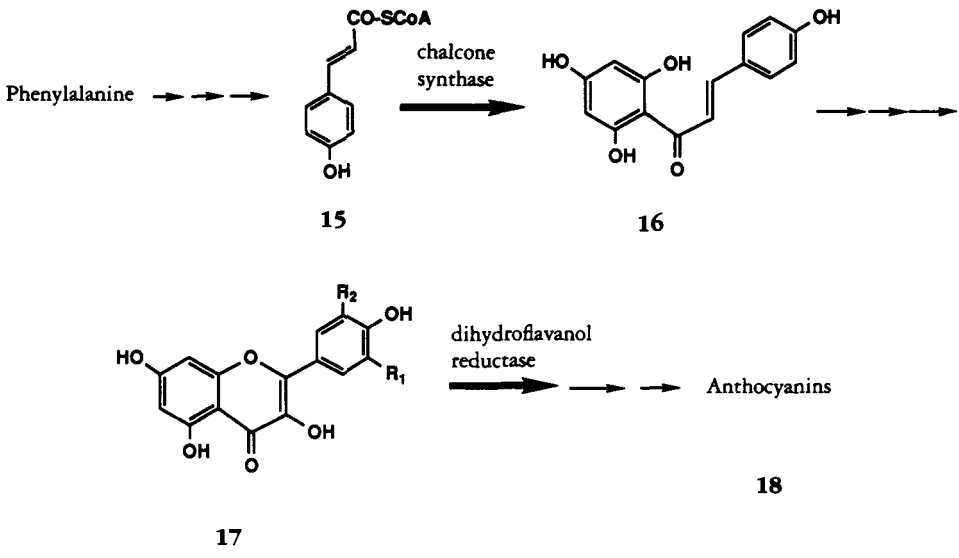
SCHEME 5

spectively. Transgenic *At. belladonna* expressing the exogenous 6β-hydroxylase gene accumulated high amounts of **14** (T. Hashimoto, private communication). The engineering of agronomically useful trait herbicide resistance was also achieved in *At. belladonna* using an Ri vector/plant regeneration system (22).

Equally successful results (86–89) have been obtained from the manipulation of floral color by expression of engineered genes for anthocyanin biosynthesis [chalcone synthase (CHS), **15**↦**16**, and dihydroflavanol reductase (DFR), **17**↦**18**] (Scheme 7), although for the moment no special medicinal application has been considered for this change in floral color.



SCHEME 6



SCHEME 7

FUTURE PROSPECTS AND CONCLUSIONS

In the last several years, some effort has been devoted to genetic manipulation of secondary metabolism in medicinal plants. Transgenic techniques definitely offer promising possibilities for future research. However, we need more detailed knowledge, in particular, of basic plant molecular and cellular biology. The reason for successful genetic manipulation of flavonoid pathway in floral color is the long-term accumulation of a basic understanding of the chemistry, biochemistry, and molecular biology of flavonoid biosynthesis.

We can point out the following prerequisites for future success:

(a) Isolation and characterization of enzymes and genes for regulatory steps of each secondary pathway.

(b) Understanding of the tissue- and developmental-regulation of the expression of secondary pathway genes.

(c) Identification of cis and trans acting factors that regulate the temporal and spatial gene expression of each secondary pathway. The study of anthocyanin pigmentation by transfer of regulatory *R* gene is a good illustration of the power of this line of research (95).

(d) Development of suitable tissue specific promoters (96), since some gene products under non-specific promoters show adverse effects on the normal physiological homeostasis in plant cells (97,98).

(e) Development of reproducible methods for regeneration of whole plants of each medicinal plant. This problem might be overcome by application of newly developed physical gene delivery techniques, i.e., microprojectile bombardment and other innovative methods (99–101). In some cases, transgenic mature plants can be obtained by DNA delivery with high-velocity microprojectiles into meristems of immature seeds (100) or into embryogenic callus (101). This physical method is much simpler than the *Agrobacterium*-mediated technique, although the transformation frequency is still not high.

In conclusion, although we need more detailed information of the biochemistry, cell biology, and molecular biology of plant secondary pathways, the first steps to the genetic manipulation of secondary metabolism in medicinal plants have been already made in the last few years. These included transgenic organ cultures induced with *Agrobacterium* and transfer of specific genes, which can eventually change the fluxes of secondary metabolism. In the near future, increased numbers of specific gene transfers will be done with the aim of manipulation of plant secondary metabolism.

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