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Genetic Engineering in Tissue Culture of Medicinal Plants

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Introduction

The stable introduction of foreign genetic information into plants represents one of the most significant developments in recent advances of plant biotechnology[1,2]. By using this transgenic technology, a number of studies have begun to clarify the molecular mechanisms underlying the regulation of plant gene expression[3] and to improve agriculturally important crops[4]. Traits such as resistance to herbicides, virus and insects have been successfully conferred to commercially important crop species. Attempts have also been made to manipulate pharmaceutically important medicinal plants and their secondary metabolic pathways by using transgenic techniques[5]. Secondary products are often biosynthesized in multi-step enzymatic reactions in specifically differentiated cells, and it is not a straightforward matter to alter metabolite production. It is becoming clear that a greater understanding of the molecular mechanisms of regulation of secondary pathways is required, in particular, the gene expression of key step reactions. Despite these difficulties, significant progress has been achieved in recent years. These advances can be divided into two major categories: (1) Transgenic organ culture such as hairy roots and shooty teratomas for stable production and biotransformation of specific metabolites by tissues grown *in vitro*; (2) Transfer and expression of manipulated foreign genes which may alter the metabolite pattern in medicinal plants.

In this review, I present an overview for recent advances in genetic manipulation of secondary metabolism in medicinal plants by transgenic technology.

Transgenic organ cultures

The production of secondary metabolites is often associated with cell differentiation. For example, the biosynthetic abilities of quinolizidine alkaloids in tissue culture of lupin plants are only detected in green callus and in multiple shoots of *Thermopsis*[6] and *Sophora*[7]. It is, therefore, a possible strategy to incite transgenic differentiated organ cultures and produce specific metabolites which are normally biosynthesized in these differentiated cells.

Derivatives of the plant pathogenes *Agrobacterium tumefaciens* and *A. rhizogenes* have proved to

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be efficient and highly versatile vehicles for the introduction of genes into plant genome. As a result of the transfer and integration of genes from the plasmids in the bacteria into plant DNA, the transformed neoplastic tissues, crown galls and hairy roots, are actually formed. These transformed tissues are potential sources for stable production of plant metabolites.

1. *Hairy roots*

The Ri (root inducing) plasmid present in *A. rhizogenes* induces so-called hairy roots in most dicotyledonous plants. This phenomenon is due to the transfer and expression of T-DNA (transferred DNA) from Ri plasmid to plant nuclear genome[8]. Hairy roots transformed with Ri plasmid grow rapidy *in vitro* without the addition of any exogenous phytohormones. A number of reports have appeared on the successful production of secondary metabolites. Recent review papers[5,9] summarized the molecular biology of hairy root induction from a range of species and reported on the production of specific secondary metabolites.

The advantages of hairy root culture for high production are: (1) They often are rapidly-proliferating differentiated root cultures which grow in defined media without the need to add phytohormones; (2) They show concomitant high production of specific secondary metabolites with fast growth rate. 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 alternatives such as chlorophyll-containing hairy roots or transformed differentiated shooty teratomas.

2. *Crown galls and shooty teratomas*

Ti (tumor inducing) plasmid in *A. tumefaciens* causes crown gall disease which usually forms an unorganized proliferating plant tumor at the site of infection with the bacterium. This is also due to the integration and expression of T-DNA fragment of *ca.* 20 kb length from Ti plasmid to the chromosomes of the plant cell. At least four oncogenes have been identified inside T-DNA, which encode genes for the enzymes responsible for biosynthesis of plant hormones, auxin and cytokinin, in transformed plant cells[10,11].

Non-differentiated crown gall tissue and cell suspension culture induced by wild Ti plasmids have been used for production of secondary metabolites. However, endogenous phytohormones produced by the action of oncogenes in T-DNA can affect the secondary pathway as well as cause morphological changes leading to a lack of differentiation. In some cases, production of differentiating shoot-like teratoma tissues have occurred as a result of transformation with either wild type Ti plasmids[12] or by using mutants which cause the over-expression of the cytokinin biosynthetic gene[13,14]. Analysis of these shooty teratomas indicates that they can produce typical shoot derived metabolites such as terpenoid essential oils[12] and/or biotransform root derived metabolites such as nicotine[13] and hyoscyamine[14] if these are fed to the teratoma cultures (**Table 1**). Like the transformed root cultures, the process of shoot organ differentiation leads to the correct balance of specific cell types and coordination of expression of biosynthetic steps required which results in significant levels of secondary products being produced.

Model gene integration

Model genes can be efficiently used for the investigation of the expression patterns of engineered foreign genes in transgenic plants of interest. The genes, *kan*, encoding neomycin phosphotransferase II (NPT-II) and, *uidA*, encoding β-glucuronidase (GUS) from Tn5 and *Escherichia coli*, respectively, are widely used as the model reporter genes for plant transformation. Because the translational protein products of these prokaryotic genes are rather stable in most plant cells and their

Table 1. Examples of transgenic medicinal plant tissues.

Plant species	Gene	Vector	Effects on development and metabolism	Ref.
Nicotiana tabacum	Mutated T-DNA (*aux*-)	pGV3845	Shooty teratoma, biotransformation of nicotine to nornicotine	13
	Mutated T-DNA (*cyt*-)	pGV3304	Rooty teratoma, production of nicotine and nornicotine	13
	kan, uidA Ri T-DNA	pGSGluC1	GUS and NPT-II	15
	Liver P-450	pSN002	2-Propenylpyrrolidine	16
	fabA	pfab21 pfab22	β-Hydroxydecanoylthioester dehydrase	*a*
	Cysteine synthase	pCSK3F pCSK3R pCSK4F	Cysteine synthase activity	*b*
Atropa belladonna	Mutated T-DNA (*aux*-)	pGV2215	Shooty teratoma, biotransformation of hyoscyamine to scoplamine	14
	kan, uidA Ri T-DNA	pGSGluC1	GUS and NPT-II	*c*
	bar Ri T-DNA	pARK5	Herbicide(PPT) resistance	17
Solanum tuberosum	Mutated T-DNA (*aux*-)	pGV3132	Shooty teratoma, solanine, chaconine	14
Glycyrrhiza uralensis	*kan, uidA* Ri T-DNA	pGSGluC1	GUS and NPT-II	15, 18
Digitalis purpurea	*kan, uidA* Ri T-DNA	pGSGluC1 pBI121	GUS and NPT-II cardiac glycosides	15, 19
Scoparia dulcis	*bar* Ri T-DNA	pARK5	Herbicide(PPT) resistance	*d*

a: K. Saito *et al.*, submitted. *b*: K. Saito *et al.*, unpublished. *c*: M. Yamazaki *et al.*, unpublished. *d*: M. Yamazaki *et al.*, in preparation.

enzymatic activities can be simply 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. A binary vector system, in conjunction with the use of the Ri plasmid has been widely used for integration of foreign genes into medicinal plants. This technique has the several advantages: (1) One can easily obtain transgenic tissues integrated with any desirable foreign genes on a binary vector without selection of transformed cells with growth inhibitorts, *e. g.* antibiotics and herbicides. This is due to the high probability of double transformation with both an Ri plasmid and a binary vector; (2) This technique is suitable for genetic manipulation of secondary metabolism of rapidly growing hairy roots which produce secondary metabolites in high yield; (3) Some plant species can regenerate shoots from transgenic hairy roots to give mature plants and set seeds. The production of specific metabolites of each plant species were proved in some transgenic tissues integrated with the engineered chimeric model genes.

The dual bidirectional promoter, TR1′-2′, was derived from the genes for mannopine synthases on the TR-DNA of an octopine-type Ti plasmid, pTiAch5. This promoter is rather widely used for the expression of chimeric genes in transgenic plans. We have obtained transgenic plants of *Nicotiana tabacum, Glycyrrhiza uralensis* and *Digitalis purpurea* integrated with TR1′-*kan* and TR2′-*uidA* from a binary vector pGSGluc1[15]. Using these transgenic plants, the mode of expression controlled by the TR promoters was analyzed by a histochemical method and by *in vitro* enzymatic assay. We found that phloem was the site for specific expression by the TR2′ promoter and the expression by the bidirectional TR1′-2′ promoters was coordinately enhanced by wounding and the addition of phytohormones.

Specific gene integration

Specific genes that confer useful genetic traits to medicinal plants should be transferred and expressed by means of transgenic technology. Several examples studied in our laboratory in the past few years are summarized in **Table 1**.

1. *Herbicide resistance*

Herbicide-resistant trait is a successful target of genetic engineering of medicinal plants as well as the crop plants such as potato, tomato, *Brassica* and maize. In particular, the *bar* gene encoding phosphinothricin (PPT) acetyltransferase from *Streptomyces hygroscopicus* has been used to confer the resistant trait towards PPT and bialaphos, the tripeptide containing PPT moiety. The chimeric expression gene containing *bar* was efficiently transferred and expressed in the medicinal plants, *Atropa belladonna*[17] and *Scoparia dulcis*, by means of Ri plasmid based binary vector system. Transgenic regenerated plantlets of *A. belladonna* and *S. dulcis* were obtained from PPT-resistant hairy roots and showed resistance towards PPT and bialaphos (**Fig. 1**). The progenies also showed resistance towards the herbicides. Some progenies only showed the trait of herbicide resistance but not the undesirable property of so-called hairy root syndrome, indicating the segregation of the foreign genetic traits and the T-DNAs derived from the mini Ti plasmid and the Ri plasmid. These progenies can be suitable for molecular breeding of herbicide-resistant medicinal plants.

2. *Mammalian Cytochrome P-450*

In the general context of genetic manipulation of plant secondary metabolism by expressing foreign genes, mammalian cytochrome P-450 is interesting for the following reasons; (1) In mammalian liver cells, some xenobiotics and drugs, including plant products, are oxidized to more highly bioactive metabolites by the acition of P-450; (2) Toxic compounds are detoxified by oxidative reactions of P-450; (3) The general broad-substrate-specificity electron transfer activity

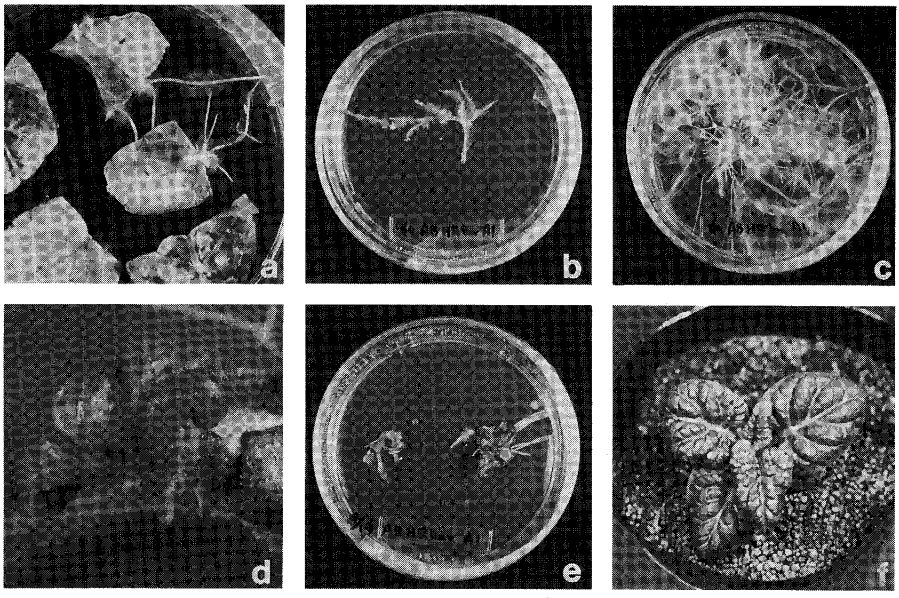

Fig. 1 Regeneration of transgenic plant of *A. belladonna* from bialaphos-resistant hairy roots transformed with *A. rhizogenes* (pRi15834; pARK5).
a: Induction of hairy roots on leaf discs. b: Selection on B5 agar medium supplemented with 5 mg/*l* bialaphos. c, d: Formation of adventitious shoots on B5 agar medium. e: Rooting of regenerated shoots on B5 medium. f: Regenerated plant on culture soil.

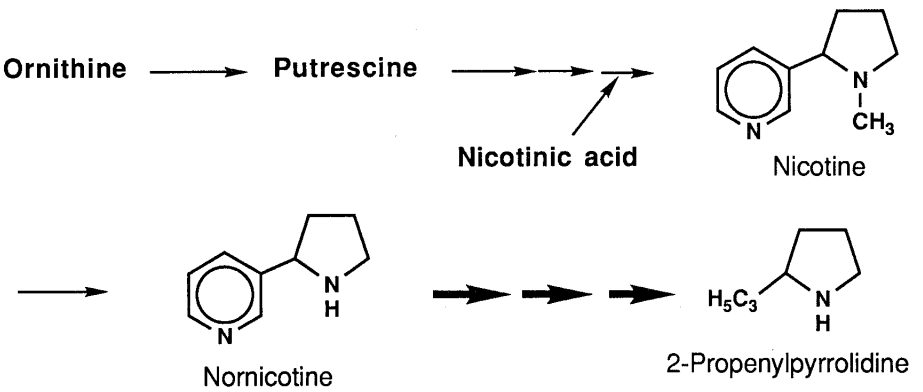

Fig. 2 Metabolism of nicotine alkaloids in transgenic tobacco integrated with the cDNA for a rabbit liver cytochrome P-450.
Pathway from nornicotine to 2-propenylpyrrolidine is enhanced concomitantly with senescence.

of P-450 in the microsomal membrane may initiate unpredictable metabolic changes with the expression of foreign P-450. This may, in turn, induce some phenotypic changes and subsequent changes in metabolic pattern of transgenic plants. The cDNA of a form of cytochrome P-450 isolated from phenobarbital-treated rabbit liver was integrated and expressed in tobacco under the transcriptional control of the TR2′ promoter by using *Agrobacterium*-mediated transformation[16]. The transformants in which the P-450 protein was expressed showed marked phenotypic changes, notably a tendency to senesce rapidly. A degradative metabolite of nicotine alkaloids, 2-propenylpyrrolidine, was accumulated in transgenic tobacco showing this pronounced phenotypic change (**Fig. 2**). Such metabolic change in secondary products is likely to be due to the effect of senescence. However, this study is an example of successful modification of plant secondary metabolism by expression of the foreign gene for animal drug-metabolizing enzymes.

3. *Cysteine synthase*

Cysteine synthase (CSase) [*O*-acetyl-L-serine acetate-lyase (adding hydrogen sulfide), EC 4. 2. 99. 8] plays a central role in sulfur assimilation in plant cells. This pyridoxal phosphate-dependent enzyme catalyzes the formation of L-cysteine from *O*-acetyl-L-serine and hydrogen sulfide. This

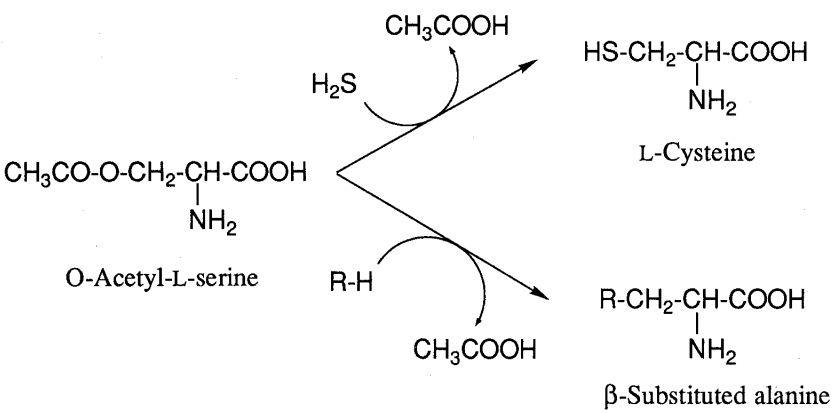

Fig. 3 Biosynthesis of cysteine and non-protein β-substituted alanines by cysteine synthase (EC 4. 2. 99. 8) in plants.

enzyme is also responsible for the biosynthesis of some heterocyclic β-substituted alanines, some of the important secondary plant products. These nonprotein amino acids, *e.g.*, mimosine and quisqualic acids, are formed through the actions of particular isoforms of CSase from heterocyclic compounds instead of hydrogen sulfide as shown in **Fig. 3.**

In the plant cells there are at least two isoforms of CSases. CSaseA is found in cytoplasm; whereas CSaseB is localized in chloroplasts. We have cloned for the first time cDNAs encoding plant CSaseA from spinach by means of a strategy involving the microsequencing of peptide fragments followed by synthetic oligonucleotide screening[20]. The expression of plant CSaseA cDNA in *E. coli* lacking CSase loci could functionally complement the cysteine requirement of the auxotrophic *E. coli*. The experiments of site-directed mutagenesis replacing 12 conserved Lys residues into Ala identified a functional residue, Lys-49, responsible for the binding of pyridoxal phosphate cofactor. The cDNA clone for CSaseB was also recently isolated and proved to contain the putative transit-peptide sequence for transport of pre-CSaseB protein to chloroplasts. The predicted amino acid sequence of mature CSaseB showed *ca.* 75% homology to that of CSaseA. *Cys 1* encoding CSaseA expressed in leaves and roots; whereas *cys2* encoding CSaseB expressed primarily in leaves.

We made three expression vectors for plants containing cDNA sequence of CSaseA. The vector pCSK3F contained the promoter for CaMV 35S RNA fused to the cDNA of CSaseA by sense orientation. A second construct pCSK3R contained CaMV35S promoter - the cDNA by antisense orientation. A third vector pCSK4F was made by chimeric fusion of CaMV35S promoter - the transit peptide (TP) of pea for targeting to chloroplasts - the sense cDNA. Transgenic tobacco integrated with these constructs was obtained by the transformation system of *A. tumefaciens*-pGV2260. Analysis of transgnic plants showed the several-folds enhanced CSase activities in the plants with the TP fused construct pCSK4F and the two-folds enhanced activities in the plants with pCSK3F. The activities in the plants by antisense pCSK3R were decreased to *ca.* 80% of those of the control plants. Further detailed analyses will be necessary on the effects of modified CSase activities to the metabolic flux of sulfur-containing amino acids. The cDNAs for other enzymes involved in the assimilation of inorganic sulfur into cysteine are being cloned by means of genetic complementation of *E. coli* mutants lacking each step of sulfur assimilation.

Future prospects and conclusions

In the last several years, many efforts have been made in the area of genetic manipulation of secondary metabolism in medicinal plants. Transgenic techniques have definitely been offering promising possibilities and indications for future research. However, we need more detailed knowledge, in particular, on basic plant biology. The genetic manipulation of the flavonoid pathway aimed at the change of floral color has been successful in past years.The reasons for this success can be ascribed to the long-term accumulation of basic knowledge of chemistry, biochemistry and molecular biology of flavonoid biosynthesis. The following guidelines could assist this area of research future.

(1) Isolation and characterization of enzymes and genes for regulatory steps of each secondary pathway such as those which exist for the anthocyanin biosynthetic pathway. These genes have been isolated and are providing a valuable insight into the regulation of secondary metabolism at the molecular level.

(2) Clarification of cell-type specific secondary metabolite expression with reference to the developmental stage where these genes are expressed. Identification of *cis* and *trans* acting

factors that regulate the temporal and spatial gene expression of each secondary pathway.

(3) For highly precise genetic manipulation, an extremely specific but powerful promoter is preferable. Otherwise, some gene products under non-specific promoter may show adverse effects on the normal physiological homeostasis in plant cells. It may be possible to use enhancers from strong promoters such as CaMV35S in conjunction with DNA motifs from the tissue specific promoter to achieve these goals.

(4) Reproducible methods for regeneration of whole plants of medicinal plant species is necessary. *Agrobacterium*-based transformation is suitable for some but not all medicinally important species. This problem might be overcome by application of newly developed physical gene delivery techniques, *i. e.* microprojectile bombardment and other innovative methods, which allow genes to be introduced into plant tissues without the need for *Agrobacterium* transformation.

(5) Exploitation of the novel biosynthetic potential of modified medicinal plants may be carried out using regenerated whole plants grown in soil or possibly using novel bioreactor procedures. This will depend, in the final analysis, upon the commercial viability of either production method.

In conclusion, although we need more detailed information of biochemistry, cell biology and molecular biology of plant secondary pathway, the first trend of genetic manipulation of secondary metabolism in medicinal plants has been already made in the last couple of years.

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