TRANSGENIC SHOOTS AND PLANTS AS A SOURCE OF NATURAL PHYTOCHEMICAL PRODUCTS

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ABSTRACT

Genetic engineering has allowed the production of plants and in vitro cultures with an altered content of secondary metabolites. In the present work it is hoped to give some detailed background information on obtaining bioactive compounds based on the use of genetically transformed shoots and the whole plants. *Agrobacterium tumefaciens*-mediated shoots have recently been a matter of great interest as a source of chemicals synthesized in the aerial parts of plants. The possibilities for the future exploitation of *Agrobacterium tumefaciens* transformation techniques are enormous. However, we need more knowledge of genes and enzymes controlling secondary metabolic synthesis.

KEY WORDS: *Agrobacterium tumefaciens*, genetic transformation, shooty teratomas, secondary products.

INTRODUCTION

Many higher plant species have been well-known for synthesizing and accumulating a wide range of bioactive compounds which are used in the food, cosmetic and pharmaceutical industries. Plant cell culture, which is currently being investigated as an alternative means for the production of pharmaceuticals, allows to avoid many of the agricultural and geographical limitations, like seasons and climatic conditions.

Cell cultures of many plants are known to produce high yields of secondary products, e.g. *Coptis japonica* cell lines producing more berberine alkaloids (above 18%) than the original plants (Sato and Yamada 1984; Yamada and Sato 1981), *Papaver bracteatum* cell cultures producing alkaloid sanguinarine, which appears to be a trace constituent (10 mg/g fresh weight) in seedlings and mature plants (Cline and Coscia 1988), rosmarinic acid obtained from *Coleus blumei* cell cultures (Petersen 1994) and, above all, industrial scale production of shikonin from cultures of *Lithospermum erythrorhizon* (Fujita 1990; reviewed by Iha 1995) used in Japan in the cosmetic industry. This substance is produced on a large scale from the suspension cultures in a two-stage process with an initial growth stage and final production stage. Not all compounds, however, can be produced in dedifferentiated plant cells and the yield of those which can is often relatively low.

Many researches have pointed out the great importance of some morphological differentiation for enhancing productivity of cell cultures. Synthesis of some important chemicals (e.g. cardenolides, morphinan alkaloids) is promoted by differentiation of shoots. Shoot cultures have been known for some time now. Several reports have been published on their

capacity for secondary metabolic synthesis; these include compounds produced by *Rauwolfia serpentina*, *Catharanthus roseus*, *Papaver bracteatum*, *Chrysanthemum cinerariaefolium*, *Cinchona* sp., *Digitalis* sp. and *Discorea composita* (reviewed in Heble 1985), as well as terpenes in *Mentha spicata* (Hirata et al. 1990) and artemisinin in *Artemisia annua* (Woerdenbag et al. 1993). Like every research method, that of shoot culture has its shortcomings, i.e. low growth-rate in the culture and often low regeneration percentages, perhaps because of strong genotypic effects. For instance mints have been suffering from the lack of the general and reliable shoot regeneration system. Recent literature (Faure et al. 1998) indicates that the leaf disc regeneration method seems to be the most suitable one for peppermint. The other disadvantage of shoot culture is the necessity to use exogenous plant hormones which cause regulatory problems.

A great progress in producing the secondary metabolites was achieved in the late 80's, since genetically transformed organ cultures were used as a model system. From 1985 to 1991, continuously growing hairy root cultures were incited by *Agrobacterium rhizogenes* infection of dicots (Tepfer 1990), most of them medicinal plants.

Agrobacterium tumefaciens is a causative agent of the so called crown-gall disease, which is also induced especially on dicotyledonous plants. There has been a long standing interest in the developing genetically transformed shoots as a source of chemicals synthesized in the aerial parts of plants. Depending on the strain of *A. tumefaciens* used, the morphology of crown-gall tumors is typified either by the production of amorphous, unorganized callus or by teratomas containing aberrantly organized stem and leaf-like structures (the so-called "shooty teratomas").

TYPES OF *AGROBACTERIUM TUMEFACIENS* STRAINS INDUCING SHOOT FORMATION

There are some types of *A. tumefaciens* strains which have been shown to induce shoot formation:

- octopine and nopaline strains bearing mutations in either of the auxin genes affecting balance in the auxin/cytokinin ratio (Ooms et al. 1981; Garfinkel et al. 1981),
- disarmed octopine and nopaline strains which carry the isopentenyl transferase (*ipt*) gene also affecting the response of plant cells to auxin and cytokinin (Schmulling et al. 1988, reviewed in Spencer et al. 1993),
- disarmed octopine or nopaline strains containing the *ipt* coding sequence under the control of the CaMV35S promoter (Spencer et al. 1993; Subroto et al. 1996a),
- wild-type nopaline strains C58 and C37; the reason for shooty teratoma production is unclear (Spencer et al. 1990; Alvarez et al. 1994).

THE MOLECULAR MECHANISM OF THE TRANSFORMED ORGAN FORMATION

The causative agents of both crown-gall and hairy root diseases are Ti (tumor inducing) or Ri (root inducing) plasmids, respectively. There are numerous classes of them but they share certain characteristics. They are large (from 200 to bigger than 800 kbp) and contain two regions necessary for tumorigenesis. These include T (transferred)-DNA region, delimited by 24 directly repeated sequences, destined to be transferred into the chromosomal DNA of the plant and the *vir* /virulence/ region, active in trans-position, which is involved in the T-DNA transfer (Gelvin 1990). In spite of their procaryotic origin, the T-DNA genes are expressed in the plant cells; their 5' and 3' flanking region contains eucaryotic regulation sequences such as "TATA" and "CAAT" boxes, and polyadenylation signals; they are transcribed by host RNA polymerase II, recognised by plant trans-acting factors, at least partially translated in plant cell (Willmitzer et al. 1982) and in consequence stably but randomly integrated into the plant nuclear genome.

The T-DNA causes the plant cells to exhibit two new properties: the cell lose their requirement for exogenously added phytohormones and produce tumor-specific amino acid derivatives, called opines, such as octopine, nopaline and agropine (Garfinkel et al. 1981; Gelvin et al. 1981). The compounds can be utilized by the *Agrobacterium* strains as a source of carbon, and, in some instances, nitrogen. In addition, some opines can induce the conjugal transfer of Ti or Ri-plasmids between bacterial cells (Gelvin 1990).

SHOOTY TERATOMA PRODUCTION OF SECONDARY METABOLITES

Although *A. tumefaciens* mediated transformants have recently been a matter of great interest, there has been a limited number of plants developing shooty teratomas (Table 1). Saito et al. (1991) developed genetically transformed shoots of some solanaceous plants in order to study their abilities to synthesize and biotransform some alkaloids. The shooty teratomas have the abilities to bioconvert hyoscyamine to scopolamine and nicotine to nornicotine in *Atropa belladonna* and *Nicotiana tabacum*, respectively. Subroto et al. (1996b) established shooty teratoma cultures of *Atropa belladonna* and *Du-*

boisia leichhardtii × *D. myoporoides* hybrid. *De novo* synthesis of hyoscyamine and scopolamine in these cultures was limited, but shooty teratomas had the ability to convert hyoscyamine to scopolamine. It was also found that the development of roots significantly improved hyoscyamine synthesis by *A. belladonna* shooty teratomas. Coculture of shooty teratomas and hairy roots of *A. belladonna* in the same hormone-free medium was therefore investigated by Subroto et al. (1996b), as a means of providing a continuous source of hyoscyamine for conversion to scopolamine. Hyoscyamine produced by the roots was transported through the medium to the shoots, where H6H (hyoscyamine 6 β -hydroxylase) enzyme converted it to scopolamine. The maximum scopolamine concentration in coculture of shooty teratomas was 0.84 mg/g dry weight (DW), 3-11 times the average levels reported for leaves of the whole plant. Some attempts to enhance scopolamine production were also made by application of scopolamine-rich plant, *Duboisia* spp. However, this coculture was not useful due to vitrification of *Duboisia*'s shooty teratomas. For bioreactor-scale applications, the shoots must remain fully differentiated and callus-free, and must be capable of growing in liquid culture without browning and vitrification. Further studies are still required. Transformed organ cultures of *Solanum eleaqualifolium* were established as a system for *in vitro* solasodine production (Alvarez et al. 1994). Solasodine yield in shoots transformed by *A. tumefaciens* (1.90 ± 0.08 mg/g DW) was higher than those found in shoots and leaves of the wild-grown plant (0.35 ± 0.09 mg/g DW). The concentration of steroidal alkaloids in shooty teratomas of *Solanum aviculare* was between 3 and 30% of the levels found in the intact plant (Bradley et al. 1978; reported by Subroto et al. 1996a). Ehmke et al. (1995) reported the establishment of tumor and shooty teratoma cultures of *Solanum dulcamara* by transformation with *Agrobacterium tumefaciens* and compared the alkaloid profiles of transformed and non-transformed *in vitro* systems. The shooty teratoma cultures showed a total glycoalkaloid content of 1% DW, which is about fivefold higher than in the source plant. It is commonly known that shoot differentiation is required for synthesis of many flavours and fragrances occurring as essential oils in glands and glandular hair of leaves and stems of some plants. According to Spencer et al. (1990, 1993) shooty teratomas of *Mentha citrata* and *Mentha piperita* possess oil glands, and accumulate the typical spectrum of terpenes as the parent plant. Salem and Charlwood (1995) reported that transformed shoots of *Pimpinella anisum* accumulate the monoterpenoid compounds of the oil associated with the original plant. However, the yield was nine-fold lower than that in the leaves of the parent plant. On the other hand synthesis of artemisinin was slightly higher in shooty teratoma cultures of *Artemisia annua* than in non-transformed shoots or plantlets (Ghosh et al. 1997). Authors suggested that it might be a consequence of the alterations in the *A. annua* genome which modified secondary metabolism in the teratoma cells. Ten percent of the galls of *Withania somnifera* spontaneously developed shooty teratomas (Ray and Jha 1999). Withanolide synthesis in the transformed shoots was higher (0.07-0.1% DW withaferin A and 0.025-0.085% DW withanolide D) than in non-transformed shoots growing in the presence of 1 mg/l BAP (0.04% DW withaferin A and 0.06% DW withanolide D). The difference between transformed and non-transformed shoot productivity may be due to the insertion and/or position effects of Ti plasmid in transformed or to exogenous cytokinin in non-transformed shoots. Too little attempts have been made to investigate if there is any relationship between the

TABLE 1. Secondary metabolite production by transgenic shooty teratomas induced with Ti plasmids.

Plant species	<i>A. tumefaciens</i> strain Ti plasmid	Specific metabolites	References
<i>Atropa belladonna</i>	p Ti C58 (aux-) with p GV 3845 p Ti B6S3 (aux-) with p GV 2215	biotransformation of hyoscyamine to scopolamine biotransformation of hyoscyamine to scopolamine	Saito et. al., 1991
<i>Atropa belladonna</i>	p Ti T37 p Ti C58 p FIH 10 ipt	biotransformation of hyoscyamine to scopolamine	Subroto et. al., 1996a; 1996b
<i>Duboisia leichhardtii</i> × <i>D. myoporoides</i> hybrid	p Ti T37	biotransformation of hyoscyamine to scopolamine	Subroto et. al., 1996a; 1996b
<i>Nicotiana tabacum</i>	p Ti C58 (aux-) with p GV 3845 p Ti C58 C1 with p GV2260	biotransformation of nicotine to normicotine	Saito et. al., 1991
<i>Solanum aviculare</i>	p Ti T37	steroidal alkaloids	Subroto et. al., 1996a
<i>Solanum eleagnifolium</i>	p Ti T37	solasodine	Alvarez et. al., 1994
<i>Solanum dulcamara</i>	p Ti C58	glycoalkaloids (soladulcidine-tetraoside, solamargine, solasonine)	Ehmke et al., 1995
<i>Mentha citrata</i>	p Ti T37	mint oil terpenes	Spencer et. al., 1990; 1993
<i>Mentha piperita</i>	p Ti T37	mint oil terpenes	Spencer et. al., 1993
<i>Pimpinella anisum</i>	p Ti T37	monoterpenoid compounds	Salem and Charlwood, 1995
<i>Artemisia annua</i>	p Ti C58 p Ti N273	artemisinin	Ghosh et. al., 1997
<i>Artemisia annua</i>	p Ti C58 C1 with p GV2260 p Ti C58C1 with php 90	artemisinin	Vergauve et. al., 1996
<i>Withania somnifera</i>	p Ti N2/73	withanolides (withaferin A, withanolide D)	Ray and Jha, 1999

process of transformation and shooty teratomas productivity. Further experiments are necessary to explain this phenomenon. Tanaka et al. (1995) conducted experiments on *Vinca minor* to examine vincamine production in multiple-shoot cultures regenerated from hairy roots. The results were compared with productivity of non-transformed shoot growing in the culture. Vincamine is an indole alkaloid which is normally synthesized mainly in the leaves of *Vinca minor*. Both transformed and non-transformed cultures were growing without phytohormones. The vincamine content of pRi transformed shoot culture was different among lines (0.18-0.44% DW). Successful selection of one superior line was achieved. The line produced almost double the amount of vincamine ($0.44 \pm 0.10\%$ DW) than untransformed cultured shoots ($0.24 \pm 0.06\%$ DW). The results demonstrate the importance of selection of high producing lines in enhancing transgenic shoot production of some secondary metabolites. Transgenic shoots production of natural phytochemical products is being investigated further.

TRANSGENIC MEDICINAL PLANTS USING AGROBACTERIUM – MEDIATED TRANSFORMATION

In some cases a whole plant is required in order to realise the ability for the full potential for biosynthesis of some phar-

maceuticals. Results reported by Malarz et al. (1993) confirm this statement. The quantitative chemical analysis of the plant material from *Arnica montana* showed a 4 times higher content of sesquiterpene lactones in the leaves of the plantlets (0.073% DW) than in proliferated shoots (0.016% DW). The metabolites are detected in green organs only. The similar findings were presented for *Artemisia annua* (Ferreira and Janick 1996). Shoots with roots in liquid medium produced about twice as much artemisinin on a percentage of shoot (0.075 vs. 0.035% DW) or total production per explant (6.64 vs. 3.48 mg) as shoots from which the roots were excised. The results indicate that the expression of artemisinin biosynthesis in green organs is influenced by root formation. It is possible that roots may provide a precursor or promotor of artemisinin production that is translocated to shoots.

There are two ways of obtaining transgenic plants by the use of *Agrobacteria*: Ti and Ri *Agrobacterium* systems. The first one is commonly used for plants for which regeneration methods have been established, e.g. tobacco, potato. However, a number of medicinal plants are difficult to regenerate from callus to mature plants and need very special conditions. Therefore, there is a limited number of reports in the literature concerning Ti transformation of medicinal important plants. Such transformation was established for *Artemisia annua* by Vergauve et al. (1996) in order to over-produce ar-

temisinin. The content of artemisinin in *Artemisia annua* transgenic plants was higher in comparison with normal grown plants. The authors have undertaken several additional experiments to establish the best procedure for *A. annua* *Agrobacterium*-mediated transformation (1998). Several binary vectors were useful for the production of transgenic callus, whereby transgenic plants were obtained only with one of them (pTJK 136). The *A. tumefaciens* wild type strain has been reported to induce shooty teratoma formation of *Coleus forskohlii*, an important plant in Indian medicine (Mukherjee et al. 1996). 1- or 2-month-old rooted plantlets were subjected to HPLC analysis; forskolin could not have been detected. Niu et al. (1998) obtained transgenic peppermint (*Mentha piperita*) plants by *A. tumefaciens*-mediated transformation. The production of secondary metabolites was not examined. Cardoso et al. (1997) established a procedure to produce transgenic *Tabernaemontana pandacqui* plants which have important pharmaceutical applications because of terpenoid indole alkaloid (TIAS) content.

There have been some reports on utilizing Ri-transformation system for regeneration of medicinal plants. Hairy roots of some species are capable of regenerating whole plants from adventitious buds. Such buds can be spontaneously regenerated on the surface of roots, e.g. *Nicotiana glauca* (Hamil and Rhodes 1988) and *Hyoscyamus muticus* (Oksman-Caldentey et al. 1991), after transferring them to the light (*Armoracia lapathifolia* and *Convolvulus arvensis*) (Noda et al. 1987; Tepfer 1984) or after supplementing the medium with growth regulators (*Astragalus membranaceus*) (Ionkova 1995).

The pRi induced plants have an altered phenotype (the so called hairy root syndrome), as an effect of T-DNA introduction. It consists of altered root growth with increased branching and plagiotropism, wrinkled leaves, shortened internodes and a reduced apical dominance. Among the eighteen open reading frames (ORFs) on the T-DNA integrated to the plant genome after infection, four loci, denominated *rol* (root locus) A, B, C and D, acting synergistically, are responsible for the modified phenotype (Di Cola et al. 1996). The coding sequences of *rol* genes do not correspond to any known gene sequences. Each of these genes shows a specific pattern of expression, and different types of regulation. *Rol* genes have been introduced into herbaceous plants, such as tomato (van Altvoorst et al. 1992), tobacco and potato (Schmullig et al. 1993) in order to study a role of each one in the hairy root syndrome development. Wrinkled leaves, reduced and retarded flowering, decreased pollen viability and hyperstilly (Jasik et al. 1997) and increased auxin sensitivity during the flowering stage (Gaudin et al. 1994) are ascribed to the *rol* A. *Rol* B seems to have a role in the promotion of root formation, reduced apical dominance, shortened internodia and leaf senescence (Jasik et al. 1997). The gene is also responsible for an increased sensitivity of cells to auxin. The increased auxin content could explain some of the characteristic phenotypes of the *rol* B transgenic plants. According to Estruch (1991b) it acts as a glucosidase activator. Filippini et al. (1994) has identified the biochemical function of the *rol* B protein as a tyrosine-phosphatase and proposed a role for it in the signal transduction cascade that follows auxin stimulation (reviewed by Di Cola et al. 1996). The *rol* C gene is responsible for dwarfness, reduced flower size, lack of apical dominance, reduction of internode length, male sterility, reduction of chlorophyll content and increased root growth (Jasik et al. 1997). The putative function of this gene has been described by Estruch (1991a). *Rol* C may code for cytokinin β -

glucuronidase, cleaving cytokinin-N-glucosidase, weakly active and metabolically stable conjugated forms, into highly active cytokinin. However, this hypothesis remains to be elucidated: cytokinins are known to inhibit root initiation, whereas root growth is stimulated in *rol* C transgenic plants. In addition to the above morphological modifications the Ri T-DNA is also known for its ability to affect physiological changes concerning osmotic pressure (Ooms et al. 1986) and polyamine metabolism (Mengoli et al. 1992).

A. rhizogenes-mediated transformation offers several advantages over other methods of transformation, including *A. tumefaciens*:

- it can be an extremely rapid method to produce transgenic plants (more rapid than that with the use of *A. tumefaciens*),

- transgenic plants can be obtained without using a selection agent, thus avoiding use of chemicals which may inhibit shoot regeneration,

- spontaneous root regeneration avoids a callus phase and therefore the risk of somaclonal variation in the resulting plants,

- the method should prevent regeneration of chimeric plants (Christey 1997) whereby *A. tumefaciens*-mediated transformation is connected with a risk of obtaining a high frequency of escapes,

- the highly branching root system has been used to improve the rooting of cuttings from some recalcitrant crops, particularly woody species,

- the characteristically altered phenotype induced by *A. rhizogenes* transformation, usually regarded as undesirable, may be of benefit for ornamental potted plants, for example in snapdragon (*Antirrhinum* sp.) *A. rhizogenes*-transformed plants flower number was dramatically increased due to increased branching (Handa 1992, reviewed in Christey 1997). Other applications of altered development include the rapid formation of increased root mass which gives the potential to increase production of root derived bioactive compounds. Tanaka and Matsumoto (1993) obtained Ri-transformed regenerated plant of *Ajuga reptans*, which produced more than 3-times as much 20-hydroxyecdysone as the original field grown *Ajuga* plant's roots, which are the site of this compound biosynthesis. Pradel et al. (1997) regenerated *Digitalis lanata* plants from hairy roots. Shoots and plants regenerated from transgenic roots showed similar spectra and contents of cardenolides as untransformed ones. The cardenolides were probably formed in the shoots and transported into the roots. According to Pellegrineschi et al. (1994, 1996) transformed scented *Pelargonium* spp. shoots regenerated from hairy roots showed an increased production of essential oils with geraniol, linalool and cineole. These aromatic substances are synthesized in leaves of the plants.

CONCLUSIONS AND PERSPECTIVES

Agrobacterium-mediated transformation system opens a new approach to genetic improvement of medicinal plants. Recently, specific genes coding for the enzymes involved in secondary metabolite pathways have been ligated into binary vector introduced into *Agrobacterium* and thus transferred to and integrated in plants. Thus, switching on specific steps of biochemical pathway to produce a desired product, using over-expression, antisense or cosuppression technologies, intact plants or cell/organ cultures can produce important pharmaceuticals. For example Yun et al. (1992) introduced the

hyoscyamine hydroxylase gene from *Hyoscyamus niger* into *Atropa belladonna* using *A. tumefaciens*-mediated transformation. The resultant transgenic plants contained high levels of medicinally important alkaloid scopolamine. The transgenic tobacco calli containing the stilbene synthase gene from *Arachis hypogea* produced resveratrol, a compound with antifungal and antiplatelet activities. The experiments represent a modest beginning towards the development of more productive plants or cell/organ cultures.

This new approach means that already available genes encoding a wide range of desirable traits for secondary metabolic pathways can be introduced into numerous plants, using either co-integrative or binary vectors. As T-DNA from the second binary vector can be mobilised in trans by *vir* gene products of the Ti/Ri plasmid, co-transformation results in the introduction of both the foreign gene(s) and the Ti/Ri T-DNA. When binary vectors are used, due to the independent insertion of the Ti/Ri T-DNA and binary vector T-DNA, segregation of the T-DNAs at meiosis can occur in subsequent generations.

However, all these approaches require knowledge of the genes and enzymes controlling secondary metabolic synthesis. After identifying them we have to learn how the genes are expressed and which factors are involved in the regulation of the different pathways.

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TRANSGENICZNE PĘDY I ROŚLINY JAKO ŹRÓDŁO NATURALNYCH PRODUKTÓW

STRESZCZENIE

Obserwowany w ostatnich latach rozwój technik genetycznych stwarza nowe możliwości w zakresie biotechnologii roślinnej. Szeroko opisywane w literaturze kultury korzeni transformowanych wykorzystywane są m.in. do biosyntezy metabolitów wtórnych ze względu na genetyczną stabilność i dużą szybkość wzrostu. Znacznie mniej rozpowszechnione są kultury transgenicznych pędów, tzw. teratomy pędowe oraz całe transformowane regeneranty, uzyskiwane w wyniku zakażenia roślin różnymi szczepami *Agrobacterium tumefaciens*. Mogą one stanowić źródło metabolitów wtórnych występujących w nadziemnych częściach roślin. Artykuł przedstawia dotychczasowe osiągnięcia w zakresie ich wytwarzania.

SŁOWA KLUCZOWE: *Agrobacterium tumefaciens*, transformacja genetyczna, teratomy pędowe, metabolity wtórne.