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PRODUCTION OF SECONDARY METABOLITES BY HAIRY ROOTS AND REGENERATED PLANTS TRANSFORMED WITH Ri PLASMIDS[1)]

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Transformation of plants belonging to several families was investigated with Agrobacterium rhizogenes which harbours Ri plasmids and transfers T-DNA into the plant genome. Transformed Nicotiana tabacum had typical hairy roots, whereas Cassia torosa, C. occidentalis and C. obtusifolia had rather thick hairy roots lacking fine lateral branching. The hairy roots of N. tabacum regenerated plants with typical phenotypes of Ri plasmid transformants. The nicotine content in the leaves of the regenerants were the highest in the plant that showed a heavily transformed phenotype. The hairy roots of Cassia plants produced anthraquinones and a xanthone, whose production profiles basically reflected those of the parent plant roots.

KEYWORDS —— Agrobacterium rhizogenes; Nicotiana tabacum; nicotine; regenerant; Cassia torosa; Cassia obtusifolia; Cassia occidentalis; anthraquinone; germichrysone; pinselin

Agrobacterium tumefaciens and A. rhizogenes harbour plasmids which transform the host plant by integrating T-DNAs into the plant genome and generate crown-gall and hairy roots. The plasmids are respectively called Ti and Ri plasmids and are useful tools for genetic engineering in higher plants.[2)] Vectors have been developed from Ti plasmid and extensively used to transfer foreign genes into higher plants.[3)] Ti plasmids generate crown-gall which grows in the absence of phytohormones as an undifferentiated lump of cells. In contrast, Ri plasmids yield hairy roots which are differentiated root tissue growing spontaneously without phytohormones. Recently, many workers have reported the production of alkaloids and other secondary metabolites by transformed hairy root cultures.[4,5)] In the present paper we report investigations to establish transformed hairy root cultures and to clarify their potential in secondary metabolite production.

Transformed hairy roots were obtained by infection of aseptically grown plantlets with A. rhizogenes 15834 and A4. Nicotiana tabacum (bright yellow), Atropa belladonna and Datura innoxia (Solanaceae) generated typical hairy roots at the points of infection. Plants belonging to other families were also tested for transformation by Ri plasmids. In contrast, upon infection with A. rhizogenes, Heliantus annus (Compositae),[4a)] Beta vulgaris (Chenopodiaceae),[5a)] Daucus carrota (Umbelliferae),[6)] Abrus precatorius (Leguminosae), Cassia torosa, C. obtusifolia

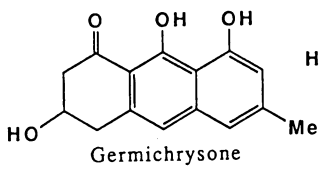
and C. occidentalis (Leguminosae) developed rather thick hairy roots. The transformed hairy roots of carrot and leguminoceous plants lacked fine lateral branching. The hairy root cultures so obtained could be maintained without phytohormones on solid Murashige-Skoog's medium (MS) and in some cases in liquid MS medium.[7] The hairy root cultures were subcultured every three to six weeks. Integration of T-DNA into the plant genomes was confirmed by paper electrophorasis showing opines, agropine and mannopine, in the transformed hairy roots.[8] It has been reported that the opine-producing potential is easily lost by repeated subcultureing,[9] but opines appeared in the transformed hairy roots of Cassia torosa that had been maintained for six months, and in a leaf of a tobacco regenerant. This indicates that genetic transformation in hairy roots are stable in these cases as far as opine production is concerned. The hairy root cultures of solanaceous plants produced alkaloids at levels comparable to parent plant roots.[4,10] This is in contrast to callus and cell cultures where alkaloid synthesis are often very low.[11] The hairy root cultures of N. tabacum spontaneously regenerated plantlets, which were then cultivated in a illuminating chamber to give mature plants. The regenerants showed various phenotypes of Ri transformation and the alkaloid content in their leaves varied by phenotypes (Table I). The highest nicotine content in the leaves was observed in the most heavily transformed plant (No. 7), which had heavily wrinkled leaves and short internode distances.[9] Tobacco plants are usually decapitated before harvest to increase nicotine content in the leaves.[12] A high nicotine content in the leaves of No. 7 regenerant may be attributed to suppression of stem elongation which affected the plant similarly to decapitation. This was the first time that nicotine content was determined in the mature regenerants of Ri-transformed hairy roots.

Table I. Nicotine Content in N.tabacum Transformed by A.rhizogenes A4

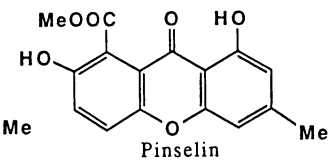
Transformed plant Regenerant No.	Phenotype	Part	Nicotine(% dry wt.)[12]
No. 2	Light	Leaves	0.45
		Roots	0.22
No. 5	Medium	Leaves	0.63
		Roots	0.54
No. 7	Heavy	Leaves	1.50
Normal plant[12]		Leaves	0.99
		Roots	0.84
Decapitated normal plant[12]		Leaves	2.34
		Roots	0.84

A heavy pigmentation appeared in C. torosa hairy roots, which looked black in culture flasks. The main pigment of a hairy root culture line in liquid MS medium was germichrysone which was also produced by cell culture.[13] A preliminary incorporation experiment with [1-^{13}C]-acetate revealed that ^{13}C enrichment at each labelled carbon was ca. 10 %.[14] Although the hairy root culture is not so proliferative as cell culture, secondary metabolism has proved to be fairly active. This indicates that hairy root culture would provide a useful method to produce secondary metabolites for biosynthetic studies.

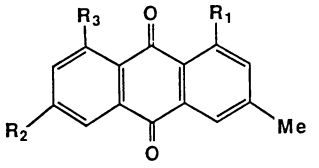
The hairy roots of C. occidentalis were black and produced germichrysone and pinselin which had been isolated from parent plant roots.[15] The effects of bacterial strains and media on pigment production was investigated by HPLC in a culture line of transformed hairy roots on solid MS medium. The results so far (Table II) show no definitive alteration in the ratios of the two pigments. Germichrysone remained as the main pigment, though the ratios varied in each experiment. The difference in bacterial strains did not significantly affect pigment ratios and contents. This indicates that pigment production is a phenomenon associated with rhizogenesis caused by T-DNA integration. Production was increased in cultures on Nitsch and Nitsch (NN) medium containing 3% sucrose. This is well in accord with reports on the production of secondary metabolites in cell culture enhanced with high-concentration carbon sources.[16]



Germichrysone



Pinselin



1,8-Di-*O*-methylchrysophanol R_1, R_3=OMe R_2=H
8-*O*-Methylchrysophanol R_1=OH, R_2=H, R_3=OMe
Chrysophanol R_1, R_3=OH R_2=H
Emodin R_1, R_2, R_3=OH
Physcion R_1, R_3=OH R_2=OMe

Table II. Content of Pigments in The Hairy Root Culture of C.occidentalis

Strain	Medium	Sucrose (%)	Germichrysone (μg/g fresh weight)	Pinselin
15834	NN	1	437	39
A4	NN	1	450	96
15834	MS	1	348	35
A4	MS	1	289	54
15834	NN	3	1275	111
A4	NN	3	1250	145
15834	MS	3	742	66
A4	MS	3	666	62

Table III. Pigment Content in The Hairy Root Culture of C.obtusifolia on MS Medium

Strain	1,8-Di-O-methyl-chrysophanol	8-O-Methyl-chrysophanol	Chrysophanol	Emodin*	Physcion
		(μg/g fresh weight)			
15834	69	Trace	14	19	trace
A4	14	Trace	24	22	trace
Normal roots	23	21	15	21	12

*Due to overlap with the peak of an unidentified compound, values are not accurate.

The cultured hairy roots of C. obtusifolia were light brown and contained anthraquinones (Table III). A large number of pigments have been isolated from the roots of normal plants grown in soil.[17] However 1,8-di-O-methylchrysophanol was found first time from nature.[18] Pigment spectra were different among samples

tested, however they are not so significant as to indicate any direct effect of genetic transformation on pigment spectra. Transformed hairy root culture capable of producing secondary metabolites can be established much more easily than cell culture if the desired compound is contained in the plant roots. Further studies of Ri plasmid-transformed hairy roots, in particular application to biosynthetic studies, are in progress in our laboratory.

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