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RELATIONSHIP BETWEEN *AGROBACTERIUM RHIZOGENES* TRANSFORMED HAIRY ROOTS AND INTACT, UNINFECTED *NICOTIANA* PLANTS

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Key Work Index—*Nicotiana*; Solanaceae; tobacco; *Agrobacterium rhizogenes*; hairy roots; biosynthetic capacity; transport; pyridine alkaloids.

Abstract—Hairy root cultures were obtained following infection of a range of *Nicotiana* species with *Agrobacterium rhizogenes*. Such cultures synthesized alkaloids in amounts which closely reflected, in both qualitative and quantitative terms, the biosynthetic capacity of roots from the uninfected parent species or variety. Cultures also released alkaloids from the roots into the growth medium. Such release was not however correlated with the ability of intact plants to mobilize alkaloids from the roots to aerial parts. The predictable nature of many aspects of secondary product synthesis in hairy roots should be advantageous to the development of biotechnological processes.

INTRODUCTION

Infection of dicotyledonous plants with the bacterium *Agrobacterium rhizogenes* leads to the production of characteristic outgrowths of 'hairy roots' at the points of infection [1]. Such roots arise from cells which have been genetically transformed by the acquisition of DNA (the transferred DNA, or T-DNA) from a plasmid (the R_i plasmid) in the infecting bacterium [2, 3]. This DNA codes for auxin synthesis, and other rhizogenic functions [4, 5], such that the normal differentiated state of cells is altered in favour of root formation. Recent results [6] have shown that, when excised from the parent plant, the transformed hairy roots can be successfully cultured on a large scale in fermenter systems, with a growth rate similar to that for conventional cell suspensions. It has also been shown [7–10] that hairy roots are able to synthesize secondary products characteristic of the roots of the original plant. Transformed hairy root cultures thus offer a novel approach to the biotechnological production of valuable plant secondary metabolites.

Preliminary indications [7, 8] suggested that one feature of transformed root systems is that their biosynthetic capacity might closely reflect that of normal roots from the original plants. These system would thus offer advantages over conventional cell culture systems, where secondary product synthesis is often at much lower levels than in the parent plant [11], and where the spectrum of products produced is also often different to that seen in plants [12]. In the present paper we report on investigations to determine to what extent the analysis of whole plants can be used to predict the biosynthetic potential of transformed hairy root cultures. In plants of the genus *Nicotiana*, pyridine alkaloids are known to be synthesized in the roots, and then to some extent transported to the aerial parts [13]. Tansformed hairy roots can similarly synthesize alkaloids, and release a proportion into the growth medium [7]. We chose to examine a range of *Nicotiana* species of differing alkaloid content, composition and distribution (e.g. [14]) in order to test whether plant root alkaloid content might be a useful indicator of alkaloid levels in transformed hairy roots, and whether shoot alkaloid content might indicate to what extent alkaloids are secreted by hairy root cultures.

RESULTS

Alkaloid synthesis

As anticipated, substantial differences were observed between different *Nicotiana* species with regard to the alkaloid spectrum and content of intact plants (Table 1). Results broadly resembled those of ref. [14]. In some species however, plantlets grown aseptically in agar showed a lower alkaloid content and/or an increased proportion of the nicotine demethylation product, nornicotine [15]. This may indicate that conditions in sterile culture are sub-optimal for alkaloid synthesis in certain species of the genus *Nicotiana*, as has also been found for tropane alkaloid synthesis in *Atropa belladonna* [9].

Hairy roots cultures were easily obtained following infection of aseptic plantlets with *Agrobacterium rhizogenes*. Southern blotting showed all lines to possess T_L T-DNA (Table 2), at levels ranging from one integration event in *N. cavicola* and *N. africana* to five or six integration events in *N. hesperis* and *N. rustica*. The majority of lines also possessed T_R T-DNA (Table 2), although *N. africana* apparently lacked sequences homologous to the T_R T-DNA probe used (covering *ca* 50%+ of the normal T_R T-DNA region [17]). This is in line with observations that in some species T_L T-DNA is apparently more important in determining the hairy root morphology [4, 5].

Table 1. Alkaloid content of *Nicotiana* spp. plants and hairy root cultures.

Species	System	Roots				Shoots/Medium			
		Nornicotine	Nicotine	Anatabine	Anabasine	Nornicotine	Nicotine	Anatabine	Anabasine
		(μg/g fr. wt)				(μg/g fr. wt/μg/ml)			
N. rustica	Plant: (aseptic)	(3)	(41)	(5)	(1)	(0.25)	(5.3)	(tr.)	(tr.)
var. V12	soil-grown	3	129	7	3	11*	48*	1*	tr.*
	Hairy root								
	6 day	4	167	12	2	tr.	8.8	0.4	tr.
	12 day	6	217	49	7	0.15	8.1	1.7	0.1
	19 day	8	293	70	12	0.15	7.4	1.6	0.2
N. tabacum	Plant: (aseptic)	(1)	(6)	(1)	(tr.)	(1.2)	(25)	(1.6)	(0.2)
var. Gatersleben	soil-grown	10	144	23	3	10*	118*	12*	tr.*
	Hairy root								
	6 day	7	366	40	3	tr.	8.5	0.7	tr.
	12 day	8	353	37	5	tr.	15.2	1.5	tr.
	19 day	†	†	†	†	0.15	5.5	0.9	tr.
N. tabacum	Plant: soil-grown	5	132	35	8	1	73	7	tr.
var. LAFC	Hairy root								
	6 day	9	151	22	3	tr.	0.6	0.1	tr.
	12 day	14	123	21	4	tr.	0.7	0.1	tr.
	19 day	†	†	†	†	tr.	0.5	0.1	tr.
N. tabacum	Plant: soil-grown	17	405	51	8	5	305	22	tr.
var. SC58									
	Hairy root								
	6 day	36	583	39	5	0.2	21.6	1.0	0.1
	12 day	46	1048	58	10	0.5	27.8	1.4	tr.
	19 day	55	950	68	10	0.6	38.0	2.3	tr.
N. hesperis	Plant: (aseptic)	(19)	(11)	(8)	(20)	(5.5)	(12)	(3.0)	(3.2)
	soil-grown	18	72	34	72	2	95	4	5
	Hairy root								
	5 day	34	604	77	163	tr.	6.3	0.3	0.45
	12 day	36	433	61	104	0.1	2.3	0.2	0.2
	18 day	59	325	35	63	tr.	0.75	tr.	tr.
N. africana	Plant: (aseptic)	(81)	(131)	(35)	(5)	(210)	(310)	(tr.)	(tr.)
	soil-grown	226	88	29	18	52	187	21	5
	Hairy root								
	5 day	285	804	117	38	0.5	1.75	0.3	0.1
	12 day	173	709	133	40	tr.	0.3	0.1	tr.
	18 day	132	585	125	32	0.15	0.65	0.2	tr.
N. umbratica	Plant: (aseptic)	(50)	(41)	(1)	(7)	(0.7)	(0.5)	(tr.)	(tr.)
	Hairy root								
	5 day	177	547	8	110	0.5	9.7	0.2	0.7
	12 day	174	556	10	138	0.9	18.9	0.4	2.0
	18 day	342	503	20	147	0.35	0.75	0.05	0.2
N. velutina	Plant: (aseptic)	(208)	(16)	(6)	(49)	(11)	(0.5)	(tr.)	(0.5)
	soil-grown	85	97	8	112	57	1	1	5
	Hairy root								
	5 day	270	446	12	73	0.55	5.5	0.15	0.4
	12 day	498	478	14	110	0.75	3.5	0.15	0.35
	18 day	733	467	25	129	0.4	0.7	0.1	0.15
N. cavicola	Plant: (aseptic)	(16)	(6)	(4)	(2)	(1.2)	(tr)	(0.2)	(0.3)
	soil-grown	15	147	14	32	26	3	0.5	1
	Hairy root								
	5 day	152	239	40	54	0.5	1.15	0.3	0.25
	12 day	183	254	47	54	0.25	0.6	0.15	0.2
	18 day	265	210	49	59	0.15	0.1	tr.	tr.

*Leaves only analysed.
†Roots starting to callus.

Table 2. Presence of T-DNA in transformed hairy root cultures (absent from untransformed tissue) as detected by Southern blotting

	T_L		T_R
Restriction Nuclease	Bam HI	Eco RI	Bam HI
Probe	pM P66 Bam8a	pM P66 Eco3a	pM P137 Bam1a
N. rustica var. V12	+	+ (6)	+ (2)
N. tabacum var. Gatersleben	+	+ (4)	?*
N. hesperis	+	+ (5)	+ (1, faint)
N. africana	+	+ (1)	-
N. umbratica	+	+	+ (2)
N. velutina	+	+ (3)	+ (6)
N. cavicola	+	+ (1)	+ (2)

Figures (in parentheses) indicate the number of separate integration sites, corresponding to bands of different M_rs seen when probing for T-DNA/host DNA boundary fragments.

*Contains DNA homologous to the T-DNA probe, but a similar band is also seen in DNA from control, untransformed, tissue. Some species of *Nicotiana* are known to contain such DNA as a result of a probable transformation during the evolution of the genus [16].

As with intact plants, hairy root cultures were found to show substantial differences between species with respect to the total levels and pattern of alkaloids present (Table 1). These differences were not correlated with variation in the number of T-DNA integration events in different cultures. Rather, much of the variation in root alkaloid content could be traced back to differences in the biosynthetic capacities of the parent plants. For instance comparisons between soil-grown normal roots and hairy roots, grown *in vitro*, showed both types of roots of *N. rustica* and *N. tabacum* to contain principally nicotine and anatabine. Those of *N. hesperis* contained principally nicotine and anabasine and those of *N. africana* contained principally nicotine and nornicotine (Table 1). On a quantitative basis the anatabine and anabasine content of hairy roots of all species closely approximated to, or was slightly above, that of soil grown normal roots. The same was true for nornicotine with the exception of *N. velutina*, where nornicotine levels increased markedly with age, and *N. cavicola*. In the case of nicotine the situation appeared subtly different, with hairy roots cultures of several species containing significantly higher concentrations of nicotine than roots from intact plants. Despite the lack of precise quantitative agreement in this case, nicotine production in hairy roots was however still related to the biosynthetic capacity of intact plants. Thus, for example, in the inbred *N. tabacum* varieties studied there was a close correlation between the relative production of nicotine in hairy roots and in normal roots of the different varieties. Variety LAFC always had slightly less nicotine than var. Gatersleben, while var. SC58 had *ca* three-fold more nicotine than var. Gatersleben in both normal and hairy roots (Table 1).

Our results indicate that the biosynthetic capacity of hairy root cultures closely mirrors that of the roots of intact plants. The explanation for the quantitative differences in accumulation sometimes seen between systems is uncertain, but there are at least two possibilities. Nicotine and other alkaloids are known to be transported from roots to shoots in intact *Nicotiana* plants [18]. Although hairy root cultures can release alkaloid into the growth medium this may provide a weaker sink than shoot tissue in intact plants and the transport processes also have different characteristics (see below). Thus hairy roots might accumulate alkaloids which would otherwise have been transported to the shoot in intact plants. The second possible explanation for at least part of the excess of some alkaloids seen in hairy root cultures is related to the different growth of hairy roots and normal roots. Hairy roots are more highly branched and rapidly growing, and thus there is no strict equivalence between the two systems. It has been suggested that in *N. glauca* nicotine is synthesized in younger tissue than is anabasine [19]. Changes in the alkaloid composition of hairy root cultures seen in the present study as the cultures age (Table 1) would also be in line with an effect of physiological age on patterns of alkaloid synthesis in other *Nicotiona* species. Some of the differences in alkaloid content of hairy roots and normal roots might thus relate simply to differences in physiological age structure within the two roots sytems.

Alkaloid transport

Plants of many of the species studied transported large amounts of alkaloid from the roots to the aerial parts. Average alkaloid concentrations in green tissue ranged up to 350% of concentrations in the root, with typically 40–90% of the total alkaloid being found in the shoots in the case of agar-grown plants and 65–95% in the case of soil-grown plants (Table 3). *Nicotiana umbratica* however achieved an aerial alkaloid concentration of only 1.2% of root alkaloid levels and a figure of only 7% of total alkaloid in the shoots, in line with previous reports of low shoot alkaloid levels in the species [14]. *Nicotiana cavicola* showed a similar, though less pronounced, tendency to retain alkaloids within the roots (Table 3).

Hairy root cultures also transported alkaloids, in this case secreting them into the growth medium. Alkaloid release *in vitro* however showed little correlation with *in vivo* transport capacities. Thus, while *N. cavicola* hairy roots released little alkaloid, *N. umbratica* cultures achieved the second highest peak levels of alkaloid in the medium for all the species studied, at nearly 20 μg/ml (Table 1). Even after normalizing for total intracellular alkaloid levels, *N. umbratica* still showed the third most efficient release of alkaloid, behind *N. rustica* and *N. tabacum*. In addition *N. africana*, which showed one of the higest relative shoot alkaloid levels of any species, was found to show the least relative release of alkaloid from hairy root tissue.

Although the capacity of plants to accumulate alkaloids in the aerial parts obviously does not correlate with the ability of hairy roots to release alkaloids in culture, it is conceivable that the pattern of alkaloids found in shoots might still usefully predict the pattern of alkaloids released by cultures. However, in practice this was found not to be the case. Irrespective of which alkaloids are normally found in shoots, all alkaloids were secreted by hairy roots to approximately the same extent, so that the pattern of alkaloids found in the culture medium broadly resembled that found in the roots (Table 1). Some species, e.g. *N. umbratica* and *N. velutina*, showed a slight preferential release of nicotine and anatabine, but this was not correlated with an enhanced contribution of these compounds to the total shoot alkaloid. Rather, it was ap-

Table 3. Alkaloid levels in shoots of a range of *Nicotiana* spp. in relation to the total alkaloid content of plants

Species	System	Concentration in shoots	% Amount in shoots
		Concentration in roots	Total alkaloids
N. rustica var. V12	Aseptic	11	42
	Soil-grown	67	76
N. tabacum var. Gatersleben	Aseptic	350	90
N. tabacum var. LAFC	Soil-grown	45	65
N. tabacum var. SC58	Soil-grown	69	80
N. hesperis	Aseptic	41	81
	Soil-grown	54	82
N. africana	Aseptic	206	87
	Soil-grown	73	96
N. umbratica	Aseptic	1.2	7
N. velutina	Aseptic	4.3	37
	Soil-grown	21	86
N. cavicola	Aseptic	6.1	15
	Soil-grown	15	61

parently more associated with the ability of the hairy roots cultures to achieve high extracellular alkaloid concentrations (Table 1).

The lack of correlation between transport *in vitro* and product partitioning *in vivo* is presumably a reflection of different processes being involved. In intact plants long distance transport via the vascular system, specific uptake and accumulation in shoot tissue, and the possibility of biochemical modification during or after transport may all be involved. In hairy root cultures, even if xylem transport may still operate, the dominant factor in product release is likely to be exchange across the root surface. In *N. rustica* the ability of hairy root cultures to release nicotine is very similar to that of undifferentiated suspension cultures (Aird, E. L. H. and Parr, A. J., unpublished). Perhaps a better predictor of product release by hairy roots will be the ability of intact plants to release products from the root into the rhizosphere [20]. The significance of the slight suggestion from Tables 1 and 2 that cultures with only a few T-DNA inserts might show a low release capacity remains uncertain. There is however no exact correlation between T-DNA copy number and alkaloid release over the full range of species studied.

DISCUSSION

Although alkaloid transport in *Nicotiana* spp. hairy root cultures could not be simply predicted from the parameters we examined, our results show that the pyridine alkaloid synthesis capacity of such cultures closely resembled that of roots of the parent plants. In this respect hairy root cultures resemble untransformed root cultures, where close similarities between the biosynthetic capacities of root cultures and intact plants have been reported in a wide variety of species [21–24]. The genetic changes involved in R_i plasmid-mediated transformation apparently have not only modified cell differentiation in favour of root formation, but have also resulted in normal activation of root-specific secondary metabolism. Mano *et al.* [10] have reported variation in tropane alkaloid synthesis between different hairy root clones of *Scopolia japonica* and have suggested that this is a result of differences in the nature of transformation events between clones. In *Nicotiana*, variation in the number of T-DNA insertions does not, however, seem to be associated with any obvious perturbations to the biosynthetic potential of different lines. The synthesis of hyoscyamine and scopolamine is known to be critically dependent on a variety of factors including root maturity and growth rate [10, 23, 25]. Since alkaloid levels in many *S. japonica* lines examined by Mano *et al.* [10] were broadly similar to the parent root, it seems probable that the variation between clones arose from secondary effects of transformation on root growth, morphology or internal hormone balance. As T-DNA can insert at numerous localities in the genome [26], the occurrence of genetic changes as a consequence of transformation ('insertion mutagenesis') might in some cases be a possibility. Such changes are however likely to only very rarely affect the formation of specific secondary products, especially in diploid tissue.

Hairy roots have a number of features important in the biotechnological production of plant secondary products, including stable high-level production [27] plus fast auxin-independent growth [2, 28] and suitability to fermenter systems [6]. The demonstration that many of the properties of cultures can be predicted in advance further adds to the case that hairy roots may have a major role to play in the coming development of commercial systems.

EXPERIMENTAL

Plant material. Nicotiana cavicola, N. velutina, N. hesperis, N. africana and a limited number of seeds of *N. umbratica* were obtained from Dr G. White, Tobacco Research Laboratory, United States Department of Agriculture. *N. tabacum* var. SC58 and var. LAFC were similarly obtained from the USDA. The origins of the highly inbred lines *N. tabacum* var. Gatersleben and *N. rustica* var. V12 have been published elsewhere [29, 30 respectively]. Plants were either grown in soil under greenhouse conditions, or following surface sterilization of seeds with 10%

(v/v) Domestos for 20 min, in 8 g/l agar plus 30 g/l sucrose and Gamborg's B5 salts [31] in sterile 175 ml glass jars (25°, 8 hr dark: 16 hr light—2000 lx).

Hairy root cultures. Hairy roots were obtained by inoculating 6–8 week old sterile plantlets with *Agrobacterium rhizogenes* strain LBA9402 as described in refs [7, 32]. Roots appearing at the site of infection were transferred to liquid culture in Gamborg's B5 medium (no phytohormones) plus 30 g/l sucrose. Ampicillin at 0.5 g/l was included at this stage to remove residual *Agrobacterium*. Cultures grew rapidly, and were routinely maintained by transferring 0.2 g root tissue to 250 ml flasks containing 50 ml growth medium (as above, but with ampicillin at 0.25 g/l) at 2–5 week intervals depending upon the species and its precise growth rate. Cultures were maintained at 25° with shaking (90 rev/min) and constant dim illumination (200 lx).

Proof of transformation DNA from hairy root cultures and from control uninfected plants was probed with ^{32}P labelled T-DNA fragments isolated from clones pMP66 and pMP137 [33], in order to show the insertion of T-DNA. Bam HI fragment 8a from pMP66 lies internal to the borders of the T_L T-DNA of *A. rhizogenes* [17]. Eco RI fragment 3a from pMP66 and Bam HI fragment 1a from pMP137 are fragments which overlap border regions of T_L and T_R- T-DNA, respectively [17]. Integration of T-DNA at different sites within the plant genome can be detected by digesting total DNA with Eco RI or Bam HI and hybridizing to the ^{32}p labelled probes which overlap border regions of the T-DNA. DNA extraction and Southern blotting procedures were as described in ref. [25].

Analysis of alkaloids. Plants were harvested at 2–3 months, and separated into roots and shoots. Hairy roots cultures were harvested at intervals during the first 3 weeks of growth and separated into roots and medium. Alkaloids were purified from all fractions by a conventional alkaline/acidic double extraction. Roots and shoots were homogenized in 0.2% H_2SO_4 (0.5–3 g per 10 ml), then debris sedimented by brief centrifugation (7000 *g*; 20 sec). Aliquots of tissue extract or medium were made alkaline (*ca* pH 11) with NH_4OH, and extracted with an equal vol. of $CHCl_3$. The $CHCl_3$ phase was re-extracted with 1 vol. dilute acid (H_2SO_4 or HOAc, pH 3), and the aqueous layer used for qualitative and quantitative HPLC analysis as described in ref. [34]. Figures quoted are means of generally two independent replicates.

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