Secondary Products from Plant Cell Cultures: Early Experiences with *Agrobacterium rhizogenes*-Transformed Hairy Roots

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Adrian J. Parr

Abstract

The enormous range of secondary metabolites produced within the plant kingdom includes many of scientific and commercial interest. There are frequently problems associated both with the study of secondary product biosynthesis in planta, and the reliability of agricultural systems for the production of commercially valuable products. In recent decades there has thus been an interest in developing in vitro cell culture systems that produce high levels of selected secondary metabolites. While callus and cell suspension cultures have been widely developed, their secondary metabolite productive capacities can be rather low and unpredictable. A major step forward came with the development of organ cultures, particularly the so-called hairy roots produced by transformation of plants with the bacterium *Agrobacterium rhizogenes*. The group at the Institute of Food Research, Norwich, was one of the first to exploit the technology, and this article describes their experiences with hairy roots and illustrates the range of approaches that can be taken to maximize their potential. In particular, because hairy root formation already involves a genetic transfer, they are especially good systems in which to study the effects of transgenesis. While some of the techniques described have now been extensively exploited, others have still not reached their full potential, and hopefully this article might serve to throw some light on possible future developments.

Keywords

Agrobacterium rhizogenes • Biotransformation • *Datura* • Hairy roots • Secondary products • Transgenesis • Tropane alkaloids

A.J. Parr (✉)
Institute of Food Research, Norwich Research Park, Colney, Norwich, UK
e-mail: adrian.parr@btinternet.com

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Abbreviations

ADC	Arginine decarboxylase
HCHL	*p*-hydroxycinnamoyl-CoA hydratase/lyase
ODC	Ornithine decarboxylase
T-DNA	Transferred DNA

Contents

1 Introduction

The production of large numbers of bioactive secondary metabolites by plants has long been of interest to man. Commercially, trade in herbs, spices, pigments, and fragrances which owe their utility to their secondary metabolite content has existed for millennia. More recently, there has also been growing interest in the science of secondary metabolite production. Understanding secondary product formation at both the biochemical and genetic levels thus gives insights into not only the metabolic pathways involved but also their control [1–3] and evolution [2, 4, 5].

Given the interest in secondary metabolites, there has been a growing movement over recent decades to develop in vitro production systems. Such systems mean that, from a scientific perspective, secondary metabolism can be more easily studied and manipulated, and from a commercial perspective, production can be freed from some of the geographical and political constrains that operate "in the field."

One of the first developments in the in vitro study of secondary metabolism was the establishment of cell culture systems, either callus material growing on a solid substrate in the presence of added phytohormones [6] or small cell aggregates (and more rarely free cells) growing in liquid suspension [7]. Such systems do, however, have a number of fundamental problems. Expression of the relevant biosynthetic pathway, if it occurs at all, is thus frequently rather variable and unpredictable [8, 9], and cell cultures are often phenotypically and genetically unstable [9–11]. Much work has gone on to exert some sort of empirical control over these problems – e.g. manipulation of nutrient and hormonal conditions to stimulate metabolite biosynthesis [12] and the application of elicitors to induce the production of metabolites that serve as phytoalexins [9, 12, 13]. Cell culture systems have thus now found a wide range of applications in modern-day science and biotechnology, but underlying problems are potentially never far away.

2 Hairy Root Systems

A significant development in the study and exploitation of secondary metabolism in vitro came with the development of organ cultures, most notably "hairy" root cultures produced by infection of plant tissues with the bacterium *Agrobacterium rhizogenes* [14–16]. In this process the bacterium, which carries a large root-inducing Ri-plasmid, interacts with host cells, and sections of plasmid DNA, the T-DNA, are then transferred to the host and integrated into its genome. By a complex process that is still incompletely understood, but which likely involves altered phytohormone production and changes in hormone perception [17], infected cells subsequently develop into roots (often with prominent root hairs, hence the term "hairy roots"). These can then be excised and grown indefinitely in vitro using a culture medium based simply on sucrose and mineral salts. Such hairy roots frequently possess many lateral branches and can thus grow and produce biomass at high rates. More importantly, they have been shown to stably express the general biosynthetic capacities characteristic of roots from the parent plants [15, 18]. This is seemingly true across a wide range of plant families and potentially in general, though in recent years some subtle differences have been reported [e.g., 19, 20], perhaps as a result of developmental effects or as biochemical consequences of the introduced T-DNA [20]. Hairy root systems thus offer a number of very considerable advantages for the study and exploitation of plant secondary metabolism. From a practical point of view, it should however be noted that the induction of hairy roots in certain genera is not always facile and indeed doesn't readily occur with monocots, though there are reports of success with *Zea mays* [21] and a few other species. Clearly hairy root systems are also only useful tools for the study of secondary products that are made in roots, but the number of such metabolites has been found to be rather greater than originally anticipated from early phytochemical work. Within intact plants, certain products may thus actually be synthesized in roots but be transported and stored elsewhere.

Many examples of detailed fundamental biochemical work on secondary metabolite biosynthesis in hairy roots now exist. Robins et al. [22], for instance, studied (i) the levels of enzyme activities present, (ii) the pool sizes of metabolic intermediates and end products, and (iii) the effects of feeding metabolic intermediates, in order to understand what limits flux into tropane alkaloids in root cultures of a *Datura candida* x *D. aurea* hybrid. They identified levels of ODC activity and the supply of activated acids for condensation with tropine as potentially rate-limiting steps [22]. A few years later, Robins et al. [23] fed 13C-labeled tropane alkaloid precursors to transformed roots of *Datura stramonium* and looked at incorporation of label into alkaloids using NMR. In this way they were able to deduce the likely mechanism by which littorine, a putative intermediate in the pathway, rearranges to hyoscyamine, one of the major end products in tropane alkaloid biosynthesis. In recent years, the use of hairy root systems for fundamental biochemical work has become even more widespread [e.g., 24], as has their use in genetic approaches to understand biosynthetic pathways.

Table 1 Examples of species used for the production of hairy roots during early work at IFR, Norwich, with typical secondary metabolites investigated either then or later

Species	Metabolite	Reference
Nicotiana rustica	Pyridine alkaloids, e.g., nicotine	[14]
N. umbratica		[11]
N. africana		[11]
N. hesperis		[28]
Datura stramonium	Tropane alkaloids, e.g., hyoscyamine	[29–31]
D. ferox	Tropane alkaloids, e.g., hyoscyamine	[29]
D. inoxia	Tropane alkaloids, e.g., hyoscyamine	[29]
D. wrightii	Tropane alkaloids, e.g., hyoscyamine	[29]
D. fastuosa	Tropane alkaloids, e.g., hyoscyamine	[29]
D. quercifolia	Tropane alkaloids, e.g., hyoscyamine	[29]
D. sanguinea	Tropane alkaloids, e.g., hyoscyamine	[29]
Hyoscyamus albus	Tropane alkaloids, e.g., hyoscyamine	[29]
H. desertorum	Tropane alkaloids, e.g., hyoscyamine	[29]
H. muticus	Tropane alkaloids, e.g., hyoscyamine	[29]
Scopolia stramonifolia	Tropane alkaloids, e.g., hyoscyamine	[29]
Nicandra physaloides	Hygrine	[32]
Beta vulgaris	Betacyanins, phenylpropanoids	[14, 33]
Catharanthus roseus	Indole alkaloids, e.g., ajmalicine	[34]
Cinchona ledgeriana		[11]
Phaseolus vulgaris		[11]

From a more biotechnological point of view (Table 1), the biosynthetic capacity of hairy roots has now also been exploited by a large number of workers for the bulk production of secondary products. This can be achieved by growing roots in appropriate culture vessels and either harvesting the roots after maximal biomass/metabolite content has been reached, or else continuously extracting products from the growth medium, should they be found to be excreted [25]. Because in planta the root plays a major role in conditioning the rhizosphere and controlling allelopathic or symbiotic interactions with other organisms, a considerable range of metabolites are indeed excreted by roots. Although on a laboratory scale, culture vessels are typically no more than Erlenmeyer flasks, scale up can be achieved and quasi-industrial scale vessels are possible. Quite early in the development of hairy root technology, the group in Norwich thus described the operation of a 500 l fermenter for trial studies with hairy roots of *Datura stramonium* [26], allowing the production of 40 kg (fresh weight) of tropane alkaloid-containing roots per run. More recent developments with fermenters, though rarely on such a large scale, are described by Ruffoni et al. [27].

In many ways the stability of hairy root cultures offers considerable practical advantages, but there is a potential downside in that levels of metabolite production are, to a large extent, predetermined. A low-producing plant will thus give low-producing hairy roots after transformation. Should levels of secondary metabolite production be insufficient for one's needs, some strategies do however

exist for improving productivity. At their simplest, these involve searching for higher-producing parent plants. One example from early work at the Institute of Food Research involved the screening of 1000+ individual plants within the genera *Datura, Scopolia*, and *Hyoscyamus*, which allowed parent material possessing a range of potentially interesting traits in tropane alkaloid biosynthesis to be identified [29]. Many of the techniques of media manipulation that have been applied to cell cultures can, in addition, also be applied to hairy roots. The production of sesquiterpenoid phytoalexins could thus be induced in roots of *Datura stramonium* by the application of abiotic elicitors [30]. Another potential approach to improving the productivity of hairy roots is to combine the techniques of cell and organ culture more directly. Furze et al. [35] were able to produce suspension cultures from hairy roots of *Nicotiana rustica* by treatment with exogenous phytohormones. Protoplasts were then isolated and used as a source of single-cell-derived callus lines where a degree of genetic and epigenetic variability had ensued. At this point, hormones were removed and fully organized hairy roots allowed to reform as the calli grew on. Although individual regenerated roots appeared to have regained their stability, clear differences in the behavior of lines produced from different roots were now apparent [35], in contrast to the predictability of the parent root material. Some lines indeed showed improved alkaloid production characteristics (e.g., total levels of nicotine, and/or the proportion released into the culture medium) compared to the parent material [35].

3 Hairy Roots and Biotransformation

Early work on hairy roots concentrated initially on simply generating cultures producing useful levels of secondary metabolites, be they model compounds of scientific importance or specific metabolites of direct commercial relevance. Another approach to exploiting the roots' biosynthetic capacities is however possible – namely, their use in biotransformation. Selected metabolites are thus added to the culture medium, and the roots are used essentially as a biocatalyst to convert these compounds into others. The nature of the reactions that occur need to be determined analytically, which is in itself frequently of considerable scientific interest. Many biotransformations carried out by roots are typical xenobiotic detoxification processes (though of course they are frequently easier to carry out and to study than the equivalent reactions occurring in planta). Reactions such as hydroxylation and glycosylation are thus frequent [36, 37]. If the fed metabolites are, however, close in structure to metabolic intermediates found within the roots, then provided there is a degree of plasticity in the substrate specificity of the enzymes involved, they can be more extensively metabolized by the endogenous secondary metabolite biosynthetic pathways. At the Institute of Food Research, Boswell et al. thus fed *N*-ethyl putrescine to transformed root cultures of *Nicotiana rustica* and showed the extensive formation of N-ethyl nornicotine – the higher homologue of nicotine [38]. N-modified versions of hygrine and tropane alkaloids could similarly be

produced by feeding appropriate substrate analogues to *Brugmansia* (=*Datura*) hairy roots [39]. The differing levels of incorporation of various analogues into the different alkaloids present also gave considerable insight into the substrate specificities of many of the enzymes involved in the biosynthetic pathways [39].

4 Hairy Roots and Transgenesis

Since the formation of hairy roots by necessity involves a genetic transfer, such cultures are also excellent systems for targeted genetic modification of secondary metabolite biosynthesis. This can be achieved either by inserting genes of interest into the T-DNA of the Ri-plasmid of the *A. rhizogenes* strain used for inducing hairy roots or, more conveniently, by introducing a second plasmid carrying the desired genes in mobilizable form into *A. rhizogenes* alongside the Ri-plasmid itself, using a binary strategy [40].

One approach to the use of genetic modification for manipulating secondary metabolite production is to modify the overall flux through preexisting pathways. This may involve either targeted upregulation or downregulation of specific routes. Fundamentally, the upregulation route is perhaps the easiest and can most simply be achieved by insertion and expression of genes related to rate-limiting steps within the relevant biosynthetic pathway. Prior biochemical work to identify such flux-controlling enzymatic steps is thus useful, though to some extent a trial-and-error approach is not without merit, as it may potentially generate information on flux control within the relevant biosynthetic pathways even if end-product overaccumulation is not achieved. The introduced genes can be either additional copies of host genes, orthologs from other species, or functionally equivalent genes from other pathways. "Foreign" genes need not necessarily come from a closely related species. Indeed the overexpression of genes of yeast or bacterial origin may sometimes offer technical advantages as well as potentially allowing the expression of enzymes with different control mechanisms (e.g., feedback control) to those normally operating in planta and thus perhaps producing stronger effects.

One of the first demonstrations of the potential for upregulation of pathways in hairy roots came from Hamill et al. [41]. Here, hairy roots of *Nicotiana rustica* were produced that also expressed a yeast (*Saccharomyces cerevisiae*) gene for ornithine decarboxylase (ODC), the key enzyme in the production of putrescine. Putrescine is the precursor of the characteristic tobacco pyridine alkaloids such as nicotine and nornicotine, and by potentially increasing substrate supply, Hamill et al. [41] hoped to increase alkaloid levels within the hairy roots. This they achieved quite readily, though concentrations of nicotine were generally only twice that of control roots produced by infection of the same parent plants with *A. rhizogenes* carrying a biosynthetically neutral gene. This was so despite a very strong promoter (the Cauliflower Mosaic Virus CaMV 35S promoter with an upstream duplicated enhancer sequence [42]) being used to drive expression of the introduced ODC gene. Analysis of enzyme activities in root lines showed only a relatively modest

increase in peak ODC activity following introduction of the yeast gene, though activity was maintained at proportionally higher levels later in the growth cycle, when activity from the endogenous *Nicotiana* ornithine decarboxylase decreased sharply. Levels of OCD enzyme activity also did not always correlate closely with the increased nicotine levels produced. These facts illustrate two of the potential problems with genetic manipulation of biosynthetic pathways. Firstly, it is possible that either, or both, the expression of the introduced gene or its enzymatic activity may be subject to strong control within the plant, so that additional copies of a gene do not necessarily feed through into an equivalent increased enzyme activity. This issue is perhaps less important in the final stages of biosynthesis of secondary metabolites, which have little physiological impact on the plant. In the case discussed above, putrescine, in addition to being the precursor for nicotine, is however also the precursor for spermidine and spermine. These polyamines, along with putrescine itself, have important functions in plants [43], so it is perhaps unsurprising that the formation of putrescine seems to be strongly controlled in hairy roots. Secondly, as mentioned above, the rate-limiting steps in secondary metabolic pathways need to be taken into account. Primary substrate supply is often likely to have at least some input into product generation, but there may come a stage when increasing the activity of one key step simply shifts flux limitation elsewhere. Moreover the physical or enzymatic compartmentation of biosynthesis also needs to be born in mind. It is unlikely that the overexpression of a gene leading to, for instance, increased enzyme activity within the cytoplasm will have an effective action on biosynthesis that takes place within organelles. In the case of the biosynthesis of nicotine, it is also theoretically possible that the putrescine that acts as a precursor is derived from arginine, via arginine decarboxylase (ADC), and not from ornithine via ODC. Effects of overexpressing ODC might thus be due to a "bleed-through" into an ADC-dependent pathway perhaps involving multienzyme complexes, which could quite easily limit the effectiveness of any manipulation. Recent research by DeBoer et al. [44] in transformed roots of *N. tabacum* has provided evidence that this ADC route is, in fact, not very likely, but the concept still remains as a salutary warning.

Given that initial efforts to genetically manipulate biosynthetic pathways may be only partially successful, even if very strong promoters are used and lines derived from the most favorable transformation events are selected, various techniques can be used to increase the observed effects. On an empirical level, because hairy roots share several features with in vitro cell cultures, one way to try and maximize the effects of transgenesis would be simply to manipulate culture conditions, as, for example, has been done by Singh et al. [45] who looked at phenylpropanoid production in lines of *Beta vulgaris* carrying a gene which affected *p*-hydroxycinnamoyl-CoA metabolism. More fundamentally, it may also be worth investigating the manipulation of other steps in the biosynthetic pathway, which may show greater flux control. It may also be far more effective to try and influence the expression not so much of a single biosynthetic gene but rather of a regulatory gene such as one of the MYB family [46] which may then go on to influence the expression of the desired biosynthetic pathway as a whole. Much work has been

done, in particular, on the genetic regulation of flavonoid biosynthesis, and this is just starting to be applied to hairy roots [47].

Another approach to genetic modification of secondary product production in hairy root cultures involves downregulation of biosynthetic routes. Even this "negative" regulation could be useful in increasing the production of certain compounds, by acting to reduce the flow of carbon away from products of interest. A number of genetic techniques for producing downregulation have been described over recent decades, with RNA-mediated interference, or RNAi, now typically being the most convenient option [48]. Over recent years there have been many examples of the use of downregulation in hairy roots to identify potential physiological functions of secondary metabolites [e.g., 49], and to examine flux control in pathways [e.g., 50], but as yet rather few more practical applications toward controlling the production of specific desired products have been reported.

Finally, it should be noted that hairy root cultures are, in practice, not totally divorced from the intact plant, and it is possible to regenerate plants from transformed roots of many species [28, 51] should the need arise to express the introduced genetic manipulation in soil-grown plants. Such regeneration is, however, not always facile [51]. Interestingly, in the genus *Nicotiana*, where regeneration can sometimes be so efficient as to even be spontaneous [28], there are signs that an ancient exposure to *Agrobacterium* may have occurred in the genus's evolutionary past [52]. Preexisting partial morphogenic compensation mechanisms may thus already exist in this and other(?) genera.

5 Hairy Roots and the Production of Unusual Metabolite Patterns or Novel Metabolites

Introduction of genes to modify the spectrum of products produced, or to create entirely novel products, is also a potential target for genetic manipulation in hairy roots. At its simplest, this may involve the introduction of, e.g., hydroxylases or methylases to "fine tune" the broad array of related secondary products that are typically produced by any one species. More fundamental changes in metabolism can however also be brought about, and one of the first demonstrations of the potential for such an approach came from Mitra et al. [31], who expressed a pseudomonad gene for *p*-Hydroxycinnamoyl-CoA hydratase/lyase (HCHL) in hairy root cultures of *Datura stramonium*. This enzyme catalyzes the side chain cleavage of a range of substituted 4-hydroxycinnamoyl-CoAs to produce the equivalent 4-hydroxybenzaldehyde derivatives, of which vanillin (3-methoxy, 4-hydroxybenzaldehyde) is a notable example of commercial importance.

4-Hydroxycinnamoyl-CoA derivatives are major metabolic intermediates in essentially all plant species, being involved in the biosynthesis of both simple phenylpropanoids and of lignin [53–55]; they also act as activated substrates for the conjugation of phenylpropanoids with other secondary metabolites or with cell

wall carbohydrates [53, 56]. The substrates for *p*-hydroxycinnamoyl-CoA hydratase/lyase are thus likely to be readily available following introduction of the HCHL gene, which would then act to divert carbon flow into formation of chain-shortened products. Although a limited amount of phenylpropanoid chain-shortening occurs naturally in many plants, it is normally a very minor reaction in most species (except of course in the vanilla orchid *Vanilla planifolia* and related species). The introduction of HCHL activity thus has the potential to produce major shifts in metabolism. Although *D. stramonium* normally accumulates very little chain-shortened phenylpropanoids, hairy roots co-transformed with, and actively expressing, the HCHL gene were however found to contain up to 0.5% of the dry weight of 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol glucosides [31]. A major shift in secondary metabolite partitioning had thus been introduced. The accumulation not of free hydroxybenzaldehydes but rather of glucosides of the related acids and alcohols is an interesting if not entirely unexpected result. Oxidation/reduction and conjugate formation are very typical ways in which plants deal with exposure to unexpected chemicals [36, 37]. Given that vanillin is more commercially interesting than the 4-hydroxyacids/alcohols, the initial results were in some respects disappointing from a practical, if not from a scientific, perspective. In recent years further research has gone on to try and develop improved systems. Hairy roots of *Beta vulgaris*, a species that incorporates a lot of ferulic acid into cell walls [57] and thus is likely to have a ready supply of feruloyl-CoA (from which vanillin can be produced by HCHL action), were found to accumulate high levels of chain shortened products [33]. Levels reached an impressive 14% of the dry weight in one line [33], rather implying that continuous removal of phenylpropanoids had increased total flux through the pathway, possibly by affecting feedback inhibition systems. Even in this species, products were however still predominantly 4-hydroxy derivatives produced from coumaroyl-CoA rather than 3-methoxy, 4-hydroxy derivatives from feruloyl-CoA, perhaps as a consequence of selective substrate availability to the introduced HCHL enzyme. Accumulation of aldehydes, while detectable, was also still low [33].

In a slightly more speculative context, one of the potentially most exciting uses of GM technology and hairy roots would be to introduce several genes for sequentially related enzymes into an easily cultivated host species to try and create entire new biosynthetic routes for that species. This type of approach currently remains very much in its infancy. There are examples of where genes for "foreign" biosynthesis have been inserted into hairy roots, and in the absence of any metabolic connection, suitable precursors have then been fed exogenously [58]. Conceptually, this is however rather different to the creation of entire new biosynthetic pathways. Since the metabolic outcome of inserting foreign pathways into plants is to some extent unpredictable, not only do the gene isolation issues have to be resolved but it is perhaps not until the recent advances in the powerful unfocused analytical techniques of metabolomics [59] that such an approach has even become fully feasible from the analytical side. The application of metabolomics to understand the broader implications of the simple gene modifications that have so far been carried out with

hairy roots indeed also has much to recommend it, and research in this area is now starting to appear [50].

6 Conclusions

The use of hairy roots for the study of secondary metabolite biosynthesis, and for the (attempted) production of commercially important target compounds, has now been in existence for nearly thirty years. The development of genetically engineered roots, containing not just the T-DNA involved in root formation but also specifically chosen metabolic or regulatory genes, has led to a great number of advances in recent years. At the same time, some of the concepts developed in the early years still remain very relevant, and a number of issues (e.g., optimal large-scale cultivation) remain only partially solved. It is hoped that the present article, focusing both on where the field started and where progress has taken it, might throw some light on potential future developments. In particular, continued research using transgenic root material, most notably systems with either suppressed or overexpressed regulatory gene function, would seem to offer many advantages.

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