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Plant Callus as a Source of Biochemicals

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ABSTRACT

This article presents a review of the recent literature describing the use of plant callus for the production of biochemicals, as well as specific examples of work done in our laboratory to analyze the production of ribosome inactivating proteins from *Trichosanthes kirilowii* callus. The article discusses research advances in the development, characterization, and improvement of plant callus cell lines, including new cell lines and potentially useful products, influence of media composition, and environmental conditions on growth and product distribution, cell line selection strategies, and long-term stability.

Index Entries: Plant callus; plant cell culture; ribosome inactivating proteins.

INTRODUCTION

Plant callus (dedifferentiated, unorganized, rapidly growing cell mass) can be produced in almost all plant species in response to wounding or endogenous or externally supplied hormones (*see 1* for a description of callus initiation and maintenance procedures). Callus that is regularly subcultured onto fresh nutrient media can be maintained in culture indefinitely. Plant callus tissue cultures (callus grown aseptically on semisolid agar medium with hormones) and callus suspension cultures (callus grown aseptically in liquid medium in test tubes, shake flasks, or bioreactors) are finding increasing use in plant biotechnology as a tool for genetic manipulation of plants, micropropagation, studies of plant metabolism and cellular development, and more recently, for the commercial production of natural products.

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SECONDARY METABOLITE PRODUCTION FROM CALLUS CULTURES

The most common application of plant callus cultures for the production of biochemicals has been the production of known or established, naturally occurring secondary metabolites as an alternative to direct tissue extraction for products that cannot be chemically synthesized. There are a number of cases where plant callus cultures offer advantages owing either to inherent properties of the plant callus system (e.g., higher product levels in callus compared with plant, ability to control and optimize culture conditions, ease of product recovery) or difficulties in whole plant production (e.g., slow growth, geographical limitations, disease and/or climate problems). In addition, a number of medically important plants are becoming severely limited or in danger of extinction (e.g., *Taxus brevefolia, Coleus forskohlii*), and plant callus offers an alternative route for pharmaceutical production, plant micropropagation, and germ-plasm preservation (*2,3*).

A thorough review of the extensive body of literature on secondary metabolite production from plant cultures is beyond the scope of this article. However, interested readers are referred to reviews and books on the subject (*see*, for example *4–8*). Although there are cases in which secondary metabolites are produced in higher concentrations in callus than in differentiated tissue, it is more commonly observed that callus tissue is a poorer producer of secondary metabolites found in the plant (*9*). Second, phenotypical and somoclonal variation in callus cultures often leads to poor stability of product levels over time, requiring repeated cell selection for improvement and maintenance of highly productive callus lines.

A variety of bioprocessing techniques, such as immobilized cell systems, fed-batch cultures, and two-stage bioreactor systems, have been effective in dramatically improving productivity of secondary metabolites in plant cell cultures. The production of secondary metabolites is generally maximized in cells with very low growth rates. This characteristic has led to the development of two-stage fermentation strategies (growth followed by product formation) or producing secondary metabolites. Other techniques, such as the use of biosynthetic pathway analysis and/or modification (*7,10*), hairy root cultures (*11*), fungal elicitors, continuous *in situ* product removal, and novel bioreactor designs, have proven to be effective in increasing secondary metabolite product yields (*12*).

In addition to its use as an alternative production strategy for established natural compounds, plant callus is also a rich source of new compounds that we have only begun to explore. Phillipson (*13*) reports that 85 novel secondary metabolites have been isolated from plant callus cultures; most of these compounds have been discovered in the past 10 years presumably because of the increasing analysis of compounds produced in callus and callus suspension cultures. Mangold (*14*) reviews the unique lipids found in plant callus cultures.

PRODUCTION OF PROTEINS FROM PLANT CULTURES

Although the vast majority of work in the plant culture area has focused on secondary metabolite production, plant cell cultures may be an attractive approach for discovering new proteins and/or producing proteins found in plants. There have been several comparative studies of the production of plant proteins by callus and hairy root cultures. Parkinson et al. (*15*) studied peroxidase production in shake flasks by hairy root cultures and callus suspension cultures of horseradish. They found that suspension cultures of callus cells produced peroxidase at almost twice the rate of hairy root cultures. Both systems secreted some of the peroxidase product into the medium, but the suspension culture secreted more. They found that peroxidase production was closely associated with growth in both systems and that the oxygen transfer rate was an important determinant of growth rate. For plant protein production, it is generally believed that conditions that maximize cell growth also maximize plant protein production.

Plant cells may also be useful as host cells for the expression of foreign proteins (or overexpression of a natural protein), particularly those that undergo processing (e.g., glycosylation), modification, or assembly in vivo, and/or are difficult to express or efficiently excrete in recombinant microbial or mammalian systems. The use of genetically modified plant cell cultures for the production of foreign proteins has been studied by Hogue et al. (*16*), Gao et al. (*17*), and Gao and Lee (*18*).

Ribosome-inactivating proteins (RIPs), commonly occurring plant toxins, are particularly interesting plant proteins; they are currently being studied for their antiviral, antitumor, and abortifacient properties (*see* the recent review article by Stirpe et al. [*19*]). To date, all RIPs that have been tested show antiviral activity in plant and animal cells. Several RIPs, α-trichosanthin, TAP-29, bryodin, GAP-31, DAP-30, DAP-32, and MAP-30, selectively inhibit growth and viral replication in HIV-1 infected human cells (*20–23*), and are being considered as possible AIDS drugs. RIPs have also been conjugated to carrier molecules, such as antibodies, hormones, growth factors, and lectins, to create cell-specific toxins (e.g., immunotoxins or hormonotoxins) (*24,25*). RIP expression in transgenic tobacco has led to increased fungal protection, demonstrating possible usefulness in the agricultural sector (*26*).

RIBOSOME INACTIVATING PROTEINS

RIPs are found throughout the plant kingdom (27). Two distinct types of RIPs have been identified. Type I RIPs are single-chain proteins with mol wt between 26 and 32 kDa, alkaline isoelectric points >9, and are often

glycosylated. Many of them are reported to be very stable with resistance to denaturing agents and proteolytic degradation (*28*). Type II RIPs, which include ricin, abrin, and modeccin, have a two-chain structure with one chain (the A chain) conferring the RIP activity similar to the Type I RIP and the other (the B chain) a galactose-binding lectin, which facilitates cell-surface binding and internalization of the two-chain molecule. Type II RIPs have mol wt between 60 and 65 kDa. The Type II RIPs are generally much more toxic than the Type I RIPs; it is estimated that a single ricin molecule is sufficient to kill a cell (*29*).

Approximately 35 different plant species have been surveyed for RIPs, and a number of other plants have demonstrated RIP activities, although the active proteins have not yet been characterized. Furthermore, information on the types and quantities of RIPs in different parts of an RIP-producing plant is very limited. There have been even fewer studies of RIP production from plant callus, callus suspension, and/or hairy root cultures. One recurring observation that researchers have made is that different, but related, RIPs (isoforms or isozymes) have been found in the same plant tissue, in different parts of a given plant, and under different environmental conditions. For example, two RIPs, colcin 1 and colcin 2, have been isolated from the seeds of *Citrullus colocynthis*. These RIPs are indistinguishable with respect to molecular weight and isoelectric point, differing only slightly with respect to their purification behavior, sugar composition, and biological activities (*30*). Two RIP isoforms purified from seeds of *Bryonia cretica*, bryodin-1 and bryodin-2, differ only slightly in molecular weight (29,000 vs 28,000), but have quite different biological activities against HIV-1-infected human cells (*23*). Different RIPs from the same plant or from plants belonging to the same family have been shown to crossreact with specific antisera raised against a particular RIP (*31*). Reports also show extensive homology around the proposed active site and conformational similarities among the various RIPs isolated from different plant species (*32–34*). Complete amino acid sequences have been reported for a number of RIPs, and N-terminal sequences are available for many others.

In vitro, cell-free experiments have shown that RIPs inhibit protein synthesis by mammalian, plant, protozoal, insect (*35*), fungal, and bacterial (*36*) ribosomes. Although it was initially believed that prokaryotic ribosomes were insensitive to plant RIPs (based primarily on data obtained for ricin and abrin A chains), recent results (*37*) on in vitro studies to determine RIP activity on *E. coli* ribosomes using single-chain RIPs showed significant RIP activity for all of the Type I RIPs studied (MAP, PAP, PAP-S, dianthin 30, and dianthin 32). The mechanism of action on eukaryotic ribosomes has been well studied and appears to be the same for both the Type I and the A chain of Type II RIPs. The glycosidic bond of adenine-4324 of 28S rRNA is cleaved, rendering the RNA susceptible to aniline-catalyzed hydrolysis, which results in an inability to bind elongation fac-

tors 1 or 2 (*29,38–40*). RIPs have also been shown to depurinate the adenine-2660 site in the 23S rRNA of *E. coli* (*37,41*). The affected region of the rRNA has been highly conserved throughout evolution, so it is not surprising that RIPs have the same type of activity on rRNA isolated from bacterial, mammalian, and synthetic oligonucleotides. However, the activity of RIPs on intact ribosomes isolated from cells and ribosomes inside cells varies greatly (*19*).

The biological function of RIPs is currently in debate. Some researchers believe that RIPs play a role as a suicide agent in higher plants by shutting down protein synthesis in tissue infected by viruses or parasitic fungi. Prestle et al. (*41*) have shown that even ribosomes in an RIP-producing plant are susceptible to inactivation by their own RIP during extraction and purification of RIPs, suggesting that plant ribosomes are generally susceptible to RIP attack and that compartmentalization of the functional RIP may prevent ribosome inactivation in vivo.

Thus, given the potential importance of this class of compounds, there have been remarkably few studies on the production of these proteins. Most of the studies have concentrated on purification, physical, and biological characterization of RIPs from plant sources (usually seeds) with very little quantitative information on achievable yields, distribution of RIPs in different plant tissues, influence of environmental conditions on RIP production, variability of RIP type and concentration, and/or selection of high-RIP-producing plants. There are even fewer studies that consider alternative methods for RIP production, such as production of RIPs from callus, plant cell suspension cultures, hairy root cultures, or expression in heterologous systems.

Attempts to clone RIP genes and express them in heterologous systems have been somewhat successful, although very few systems have been tried. Ricin A-chain has been expressed intracellularly in *E. coli* at levels up to 10% of cell protein, resulting in yields of 15–25 mg/L, and the B-chain has been expressed as a secreted protein (to the periplasm) at levels of 1 mg/L. Type I RIPs, such as MAP and α-trichosanthin, have proven more difficult to express in *E. coli* with relatively low yields of intracellular product (.1 mg/L for MAP production in *E. coli* [*42*]). Shaw and coworkers (*43*) expressed α-trichosanthin as an intracellular product at low levels, only 0.01% of total cellular protein, using a α-trichosanthin cDNA clone under control of a modified *tac* promoter, but were able to improve expression significantly using a T7 RNA polymerase promotor, although most of the protein was produced as insoluble inclusion bodies (*44*). Biologically active α-trichosanthin was also expressed in tobacco by transfection with an RNA viral vector (*45*). Expression of RIPs in yeast, mammalian, and insect cell cultures so far has resulted in extremely low levels, presumably because of the effect of the protein and/or preproprotein on protein synthesis and cell growth. RIP production using transgenic plant cell systems is a research area that has been completely unexplored to date.

PLANT CELL CULTURE PRODUCTION OF RIPS

There are several reports in the literature of the production of RIPs in plant callus suspension cultures. Ikeda et al. (*46*) reported the intracellular production of *Mirablis* antiviral protein (MAP) in suspension cultures of *Mirablis jalapa* and reported that MAP production was growth-associated. In a subsequent paper (*47*), they demonstrated that MAP productivity could be increased by a factor of 2 by optimizing the medium composition. They also reported significant decreases in the intracellular MAP levels with extended subculturing (over 30 subcultures), indicating potential problems with the long-term stability of these cultures. Variation in MAP levels among callus cell lines obtained from the same plant source was also high (of 108 cell lines studied, the average MAP content was 125 μg/g, but the standard deviation was 104 μg/g). Repeated selection of high-MAP-producing callus led to significant improvements in MAP yields (about three times the MAP levels found in the root) with stable production reported over 25 subculturings (*48*). Barbieri et al. (*27*) presented results of studies aimed at producing RIPs by using several different plant cell cultures, *Phytolacca americana* (pokeweed), *Saponaria officinalis* (soapwort), and *Zea mays* (corn). They found high levels of RIPs only in the pokeweed cells in suspension and much lower levels in the other two cell lines, despite the fact that the cell cultures were derived from plants containing comparable amounts of RIPs in their leaves or seeds. They also found that phytohormone levels in the media and culture conditions had a very strong effect on the levels of RIP produced in culture (as much as two orders of magnitude variation in RIP activity) and that optimal culture conditions were different for the different cell lines. Thomsen et al. (*49*) have studied RIP production in callus and suspension cultures of *Phytolacca dodecandra.* One of the most interesting results of their study was that the suspension callus produced two new RIPs with slightly higher molecular weights, which were not previously found in intact plant tissue. In contrast, the well-known RIP, dodecandrin, found in leaf tissue was not found in their cultures. Bonness and Mabry (*31*) also studied RIP production in *P. dodecandra* callus and suspension culture, noting the variability of RIP production from different callus cell lines, the absence of dodecandrin in most of the cultures, and the presence of other immunoreactive RIP proteins in callus and suspension cells.

These studies, as well as numerous other investigations of plant cell culture systems, indicate the potential use of plant callus cultures for production of novel compounds, and the importance of cell line selection and culture conditions in the optimization of plant cell culture processes.

RIP PRODUCTION IN *T. kirilowii* PLANTS AND CULTURES

In our laboratory, we are studying the production of the Type I RIPs, which are found in *Trichosanthes kirilowii*, a Chinese medicinal plant in the

Cucurbitaceae family (*28,50*) in plant callus, callus suspension cultures, and hairy root cultures. Ng et al. (*51*) presented a recent review of the pharmacology, characterization, and purification of RIPs found in the Cucurbitaceae family. The RIPs found in *T. kirilowii* plant tissues include α-trichosanthin and TAP-29. α-Trichosanthin is the active ingredient in a very promising potential AIDS drug known as GLQ223 or "Compound Q" that is currently being studied as a treatment for AIDS. In vitro experiments on infected cells showed α-trichosanthin to have potent activity against HIV-1-infected human T-cells and macrophages (*20*). GLQ223 has recently completed Phase I clinical trial (*52,53*) and is now in Phase II clinical trials. Lee-Huang et al. (*21*) have identified TAP-29, a 29-kDa RIP that is also found in *T. kirilowii* root tuber. They report that TAP-29 has anti-HIV activity similar to α-trichosanthin, but that its toxicity is much lower than trichosanthin, suggesting that is may be safer than α-trichosanthin for the treatment of AIDS.

α-Trichosanthin, an RIP obtained from the root tubers of *T. kirilowii*, is a single-chain, nonglycosylated protein (247 amino acids) with M_r27,000. The complete primary sequence for α-trichosanthin has been published by Collins et al. (*34*). The gene encoding α-trichosanthin has been isolated, and the DNA sequence has been reported by Chow et al. (*54*). They suggest that α-trichosanthin is produced as a preproprotein with 23 amino acids at the N-terminus that resemble a consensus secretory signal peptide and a sequence of 19 amino acids following the C-terminus of the mature protein that may function to keep the protein inactive presumably until it is sequestered within the secretory pathway. Other slightly different forms of α-trichosanthin (i.e., isoforms) are obtained from different parts of the plant (*55*) or from different varieties (*56*).

In our laboratory, we have studied the production of RIPs in *T. kirilowii* callus grown on semisolid media, callus grown in suspension, and callus resulting from infection with *Agrobacterium rhizogenes* A4 grown in suspension on hormone-free media (details of experimental and analytical procedures can be found in [*57*]).

Roots tubers of *T. kirilowii* were extracted and analyzed to provide a basis for comparison with the proteins found in the callus cultures. An SDS-PAGE gel of proteins extracted from the root tuber showed approximately nine proteins ranging in mol wt from 15.7 to 60.9 kDa (data not shown). A Western blot of elution fractions from the purified root tuber extract confirmed the presence of α-trichosanthin in the root tuber. The root tuber also contained a strong band that ran about 2 kDa above the α-trichosanthin band (most likely TAP-29). An alcian blue glycoprotein stain showed that α-trichosanthin was not glycosylated, whereby the 29-kDa was a glycosylated protein. The yield of α-trichosanthin obtained from the root tuber is around 1.3–1.8% of the dry weight of the root tuber, whereas the yield of the 29-kDa protein is estimated to be 0.4–1.0% of the dry weight of the root tuber.

Analysis of intracellular and extracellular extracts from callus grown on semisolid media and in suspension (in the presence of hormones, 5

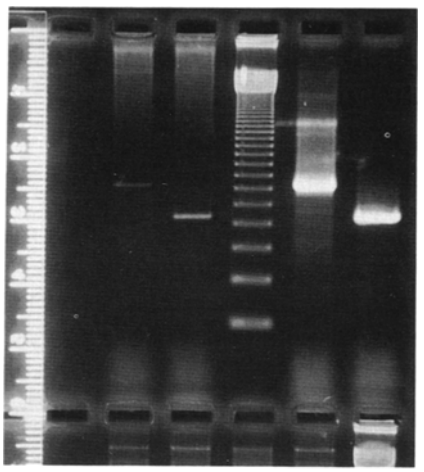

Fig.1. PCR analysis of *rol*b and *rol*c genes. Lane descriptions: (1) blank, (2) *Agrobacterium*-transformed callus PCR for *rol*b, (3) *Agrobacterium*-transformed callus PCR for *rol*c, (4) 123-bp ladder, (5) *E. coli* 113 PCR for *rol*b, (6) *E. coli* 113 PCR for *rol*c.

μM 2,4-D and 1 μM BAP) showed the presence of a 29-kDa protein in all of the culture samples. α-Trichosanthin, however, was not detected in any of these samples. Elution fractions from extracellular extracts had fewer proteins, although these proteins were present at much higher levels.

Infection of *T. kirilowii* leaf, stem, and petiole with *A. rhizogenes* A4 resulted in callus rather than hairy root growth, although the growth rate of the tranformed callus on hormone-free medium was similar to that obtained for the nontransformed callus on hormone medium. An agropine assay was performed on the callus tissue generated from the carrot disks and *T. kirilowii* leaf, petiole, and stem. Agropine was not detected in any of the samples (data not shown). A PCR analysis was used to detect the *rol*b and *rol*c genes in tenth generation *Agrobacterium*-transformed callus growing on hormone-free Linsmaier-Skoog media without antibiotics. The results are shown in Fig. 1; lanes 2 and 3 show the expected 775- and 539-bp sequences for *rol*b and *rol*c genes, respectively, for the callus, lane 3 contains a 123-bp ladder marker, and lanes 4 and 5 are the positive controls for *rol*b and *rol*c.

Figures 2 and 3 show the ion-exchange elution profiles using an S-Sepharose column and the RIP activity (measured using a cell-free in vitro protein translation assay based on rabbit reticulocyte lysate) of the purified cell extract and broth from suspension cultures of the *Agrobacterium*-transformed callus, respectively. Silver-stained SDS/PAGE gels of representative elution fractions of the purified intracellular and extracellular samples are presented in Figs. 4 and 5. Figures 6 and 7 show Western blots (for α-trichosanthin), for the intracellular and extracellular proteins, respectively.

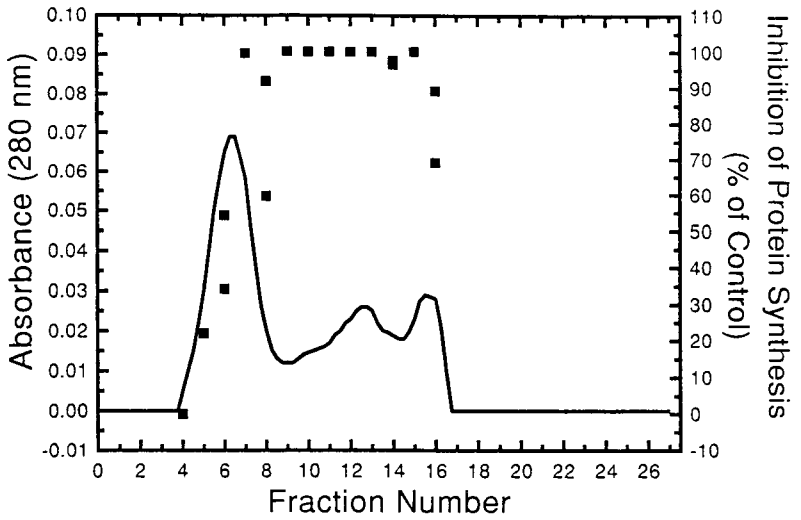

Fig. 2. Elution profile from ion-exchange purification of the intracellular proteins from the *Agrobacterium*-transformed *T. kirilowii* cells grown in shake flasks (solid line) and RIP activity of elution fractions reported in terms of percent inhibition of protein synthesis using a rabbit reticulocyte system (symbols).

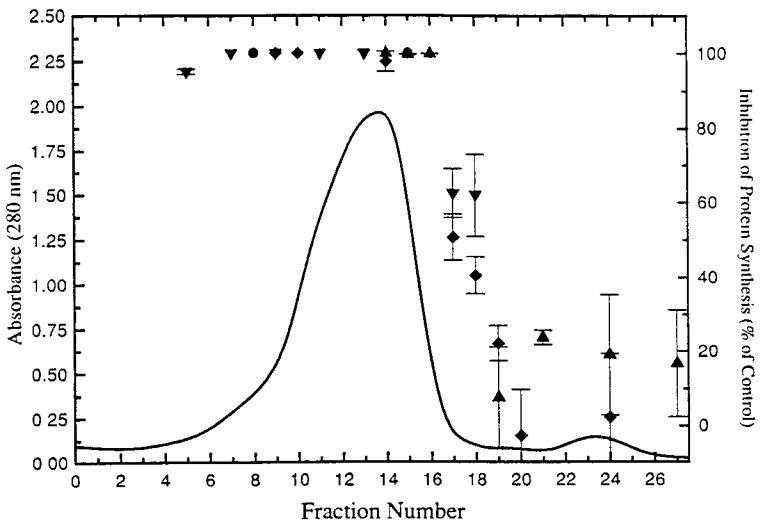

Fig. 3. Elution profile from ion-exchange purification of the shake-flask broth from the *Agrobacterium*-transformed *T. kirilowii* cultures (solid line) and RIP activity of elution fractions reported in terms of percent inhibition of protein synthesis using a rabbit reticulocyte system (symbols).

During the gradient elution of the intracellular proteins, three somewhat distinct peaks were observed (Fig. 2). The first peak (elution fractions 4-8) was comprised of two proteins with mol wt of 21.5 and 33.5 kDa (lanes 2 and 3, Fig. 4). As the second peak is beginning around elution fraction 10, we start to see a band just above the α-trichosanthin standard (lane 4,

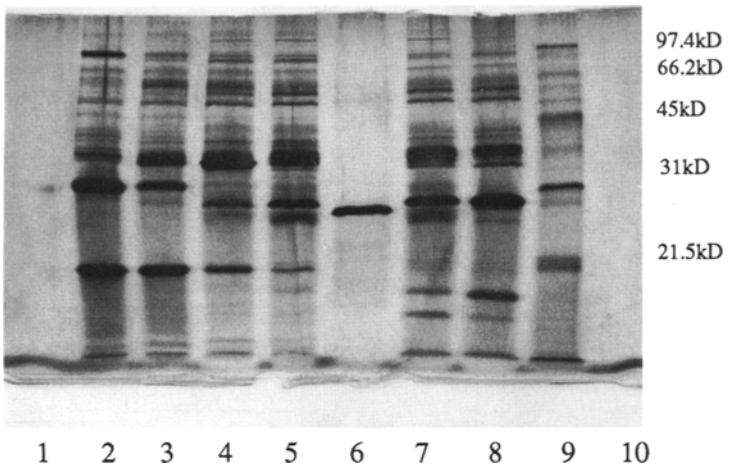

Fig. 4. Silver-stained SDS-PAGE gel of intracellular proteins extracted and purified from *Agrobacterium*-transformed *T. kirilowii* cells grown in suspension. Lane descriptions (amounts shown are actual volumes loaded and estimated protein mass based on an extinction coefficient of 0.75 AU/[mg/mL]): (1) blank, (2) fraction 6 (19 μL; ~750 ng), (3) fraction 8 (19 μL; ~1500 ng), (4) fraction 10 (19 μL; ~300 ng), (5) fraction 12 (19 μL; ~1300 ng), (6) α-trichosanthin standard (200 ng), (7) fraction 14 (19 μL; ~600 ng), (8) fraction 16 (19 μL; ~600 ng), (9) Bio-Rad silver-stained low-mol wt standards™: 97.4, 66.2, 45.0, 31.0, 21.5 kDa (10 μL), (10) blank.

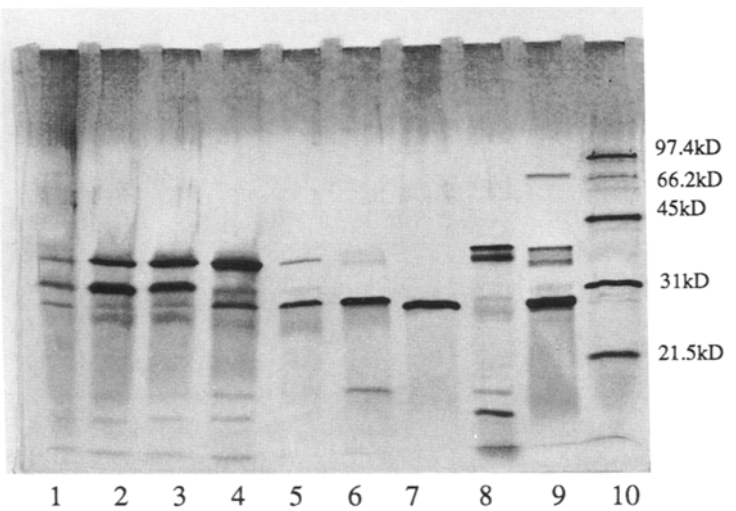

Fig. 5. Silver-stained SDS-PAGE gel of extracellular proteins purified from suspension broth of *Agrobacterium*-transformed *T. kirilowii* cultures. Lane descriptions (for elution fractions, the amounts shown are actual volumes loaded and estimated protein mass based on an extinction coefficient of 0.75 AU/[mg/mL]): (1) wash fraction 1 (19 μL), (2) wash fraction 9 (19 μL), (3) fraction 4 (19 μL; ~2900 ng), (4) fraction 8 (4 μL; ~3500 ng), (5) fraction 11 (0.4 μL; ~600 ng), (6) fraction 15 (0.2 μL; ~400 ng), (7) α-trichosanthin standard (200 ng), (8) fraction 19 (19 μL; ~2000 ng), (9) fraction 24 (8 μL; ~1500 ng), (10) Bio-Rad silver-stained low-mol wt standards™: 97.4, 66.2, 45.0, 31.0, 21.5 kDa (10 μL).

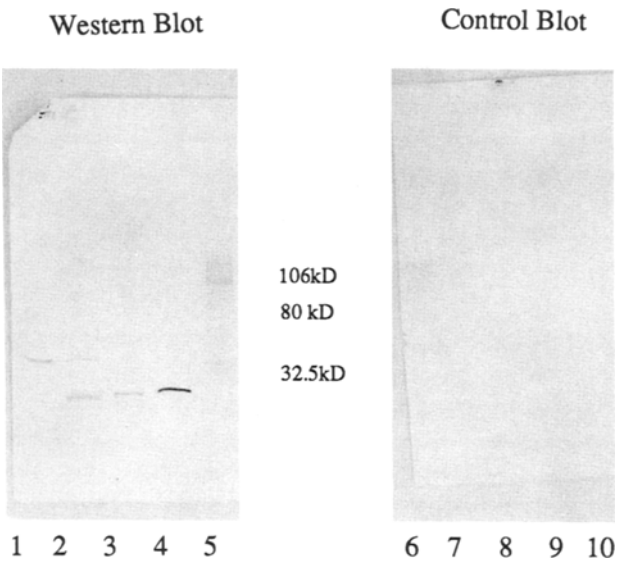

Fig. 6. Trichosanthin Western blot and control blot of intracellular proteins extracted and purified from *Agrobacterium*-transformed *T. kirilowii* cells grown in suspension. Lane descriptions (amounts shown are actual volumes loaded and estimated protein mass based on an extinction coefficient of 0.75 AU/[mg/mL]): (1) fraction 10 (11 μL; ~150 ng), (2) fraction 12 (11 μL; ~700 ng), (3) fraction 14 (11 μL; ~350 ng), (4) α-trichosanthin standard (20 ng), (5) Bio-Rad prestained mol wt standards™: 106.0, 80.0, 49.5, 32.5, 27.5, 18.5 kDa (7 μL), 6–10 reverse order of lanes 1–5.

Fig. 4) at about 29 kDa and a somewhat more diffuse band with the same relative mobility as the α-trichosanthin standard. The 29-kDa protein continues to elute during the second and third peaks (lanes 5, 7, and 8, Fig. 4) with the highest levels seen at peak #3 (lane 8, Fig. 4). The band at the same relative mobility as α-trichosanthin appears to be the strongest in elution fractions 12–14, which correspond to the tail of peak #2 (lanes 5 and 7, Fig. 4). The Western blot of elution fractions 12 and 14 confirms the presence of α-trichosanthin (lanes 2 and 3, Fig. 6). Figure 2 shows significant RIP activity for most of the elution fraction in the peaks, particularly fractions 9–15. However, inhibition assays of diluted samples are needed to discern the true shape of the RIP activity profile. These results indicate that there is another RIP present in some of these elution fractions, since α-trichosanthin was not detected in Western blots of fraction 10, for example.

The gradient elution of the extracellular proteins shows a much larger amount of protein with one major peak between elution fractions 6 and 18 and a much smaller peak at the end of the elution profile (Fig. 3). The 29-kDa protein elutes throughout most of the large peak; it is first seen in fraction 8 and extends at least through fraction 15 (lanes 4–6, Fig. 5). The 29-kDa protein is also observed to be a major component in the smaller peak. Although a slight diffuse band at the same molecular weight as α-trichosanthin was observed on the SDS/PAGE gels, α-trichosanthin was not

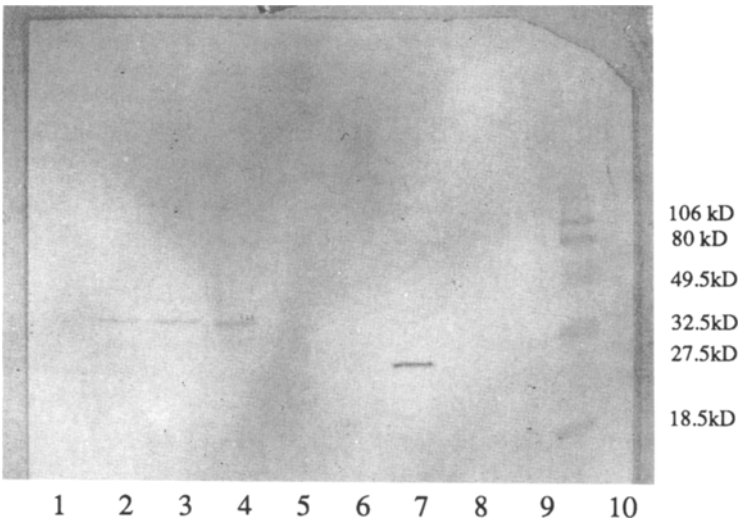

Fig. 7. Trichosanthin Western blot of extracellular proteins purified from broth of *Agrobacterium*-transformed *T. kirilowii* cultures. Lane descriptions (amounts shown are actual volumes loaded and estimated protein mass based on an extinction coefficient of 0.75 AU/[mg/mL]): (1) wash fraction 1 (19 μL), (2) wash fraction 9 (19 μL), (3) fraction 4 (19 μL; ~3000 ng), (4) fraction 8 (4 μL; ~1800 ng), (5) fraction 11 (0.4 μL; ~600 ng), (6) fraction 15 (0.2 μL; ~500 ng), (7) α-trichosanthin standard (20 ng), (8) fraction 19 (19 μL; ~2000 ng), (9) fraction 24 (8 μL; ~1600 ng), (10) Bio-Rad prestained mol wt standards™: 106.0, 80.0, 49.5, 32.5, 27.5, 18.5 kDa (7 μL).

detected in the Western blot of these broth elution fractions (Fig. 7). The amount of the 29-kDa protein obtained from the broth of the *Agrobacterium*-transformed cultures is approx 1.1–2.0% of the dry weight of the callus.

Although α-trichosanthin was not detected in Western blots of the extracellular samples, there appears to be an RIP present. Since several of the elution fractions are fairly pure, containing predominantly the 29-kDa protein (lanes 5 and 6, Fig. 5), and since TAP-29, a 29-kDa protein obtained from the root tuber, is known to be an RIP, the 29-kDa protein is most likely an RIP. It is also interesting to note, however, that a strong 29-kDa band is observed in elution fraction 24 (lane 9, Fig. 5), although essentially no RIP activity was observed for this fraction. It may be that these proteins, although very close in molecular weight, differ enough in composition to confer very different purification characteristics and biological activity. We are currently performing additional experiments to characterize further the proteins obtained from the intracellular and extracellular samples. Although there is evidence that the callus has been transformed, it is unclear whether the difference in protein production (i.e., presence of α-trichosanthin) is a result of the genetic transfer or the result of other factors resulting from the infection process.

SUMMARY AND FUTURE WORK

In addition to their use in the production of known secondary metabolic products, plant callus and callus suspension cultures are proving to be a valuable approach for the production of novel protein products, such as RIPs. Although other biological and bioprocessing strategies are being investigated to increase product yields from plant cell cultures, such as hairy root, organ, and immobilized cell systems, callus cultures offer a number of inherent advantages. Compared with hairy root cultures, callus is much easier to work with in a bioreactor allowing for easier inoculation, sampling, and mixing. The transformed callus cell line offers additional advantages. Although the exact nature of the genetic transformation is not yet known, it appears that the transformation has altered the regulation of RIP gene expression, perhaps because of altered levels of endogenous hormones. Such systems may also be useful as a mechanism of *de novo* synthesis of proteins or RIP isoforms that are not expressed in the intact plant. From a bioprocessing standpoint, the tumorous cell line offers a number of potential advantages over alternative methods of RIP production. Efficient excretion of RIPs into the broth simplifies purification and allows the possibility for *in situ* product removal. Furthermore, our preliminary studies indicate that purification of the 29-kDa protein from the broth would be relatively straightforward. The tumorous callus grows well on hormone-free medium, thereby eliminating the costs of synthetic hormones and the tedious experimentation required to optimize hormone types and levels if exogenously supplied. Further work is necessary to improve purification of similar RIPs, further characterize the physical and biological activities of new RIPs, determine the dynamics of RIP production in culture, and to assess the relative advantages of callus, hairy root, and transformed callus with respect to growth rates, genetic stability, and stability of production rates over time.

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