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Alkaloid production by a *Cinchona officinalis* 'Ledgeriana' hairy root culture containing constitutive expression constructs of tryptophan decarboxylase and strictosidine synthase cDNAs from *Catharanthus roseus*

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Abstract *Cinchona officinalis* 'Ledgeriana', former called *Cinchona ledgeriana*, hairy roots were initiated containing constitutive-expression constructs of cDNAs encoding the enzymes tryptophan decarboxylase (TDC) and strictosidine synthase (STR) from *Catharanthus roseus*, two key enzymes in terpenoid indole and quinoline alkaloid biosynthesis. The successful integration of these genes and the reporter gene *gus*-int was demonstrated using Southern blotting and the polymerase chain reaction. The products of TDC and STR, tryptamine and strictosidine, were found in high amounts, 1200 and 1950 $\mu g\ g^{-1}$ dry weight, respectively. Quinine and quinidine levels were found to rise up to 500 and 1000 $\mu g\ g^{-1}$ dry weight, respectively. The results show that genetic engineering with multiple genes is well possible in hairy roots of *C. officinalis*. However, 1 year after analyzing the hairy roots for the first time, they had completely lost their capacity to accumulate alkaloids.

Key words Quinoline alkaloids · Genetic engineering · *Catharanthus roseus* · *Cinchona officinalis* 'Ledgeriana' · Terpenoid indole alkaloids

Abbreviations *DW*: Dry weight · *FW*: Fresh weight · *GUS*: β-Glucuronidase · *SGD*: Strictosidine β-D-glucosidase · *STR*: Strictosidine synthase · *TDC*: Tryptophan decarboxylase

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Introduction

Alkaloids constitute a diverse group of plant secondary metabolites, many of which are of pharmaceutical importance. The monomeric and dimeric terpenoid indole alkaloids produced by *Catharanthus roseus* serve as an example. The monomeric compounds ajmalicine and serpentine are used in the treatment of cardiac and circulatory diseases, while the dimeric compounds vincristine and vinblastine are used in the treatment of cancer. The quinoline alkaloids quinine and quinidine, produced by the *Cinchona* species, are other examples. These compounds are used as drugs in the therapy of malaria and cardiac arrhythmias, respectively.

The biosynthesis of both terpenoid indole and quinoline alkaloids proceeds via the formation of strictosidine, a glucoalkaloid assembled from tryptamine and secologanin. Somewhere beyond strictosidine formation, however, the biosynthetic pathway diverges, giving rise to the different alkaloids (Fig. 1).

The biosynthesis of alkaloids in *Catharanthus* and *Cinchona* species has been well studied (for reviews see Verpoorte et al. 1991, Meijer et al. 1993). These studies resulted in the purification of tryptophan decarboxylase (TDC, E.C. 4.1.1.28) and strictosidine synthase (STR, E.C. 4.3.3.2) from *C. roseus*, both of which are key enzymes in alkaloid biosynthesis (Fig. 1). Subsequently, cDNA clones encoding these enzymes were isolated from *C. roseus* (De Luca et al. 1989; McKnight et al. 1990), which have now been used in studies on the regulatory mechanisms controlling the expression of the corresponding genes. These cDNA clones have also been expressed in *Nicotiana tabacum* plants and cell cultures (Goddijn et al . 1993; Poulsen et al. 1994; Hallard et al. 1997).

Plants are able to accumulate high levels of alkaloids. For example, the bark of *Cinchona* trees may contain quinoline alkaloids accounting for up to 15% of its dry weight (DW). Cell suspension cultures of *Cinchona officinalis* 'Ledgeriana' have been established

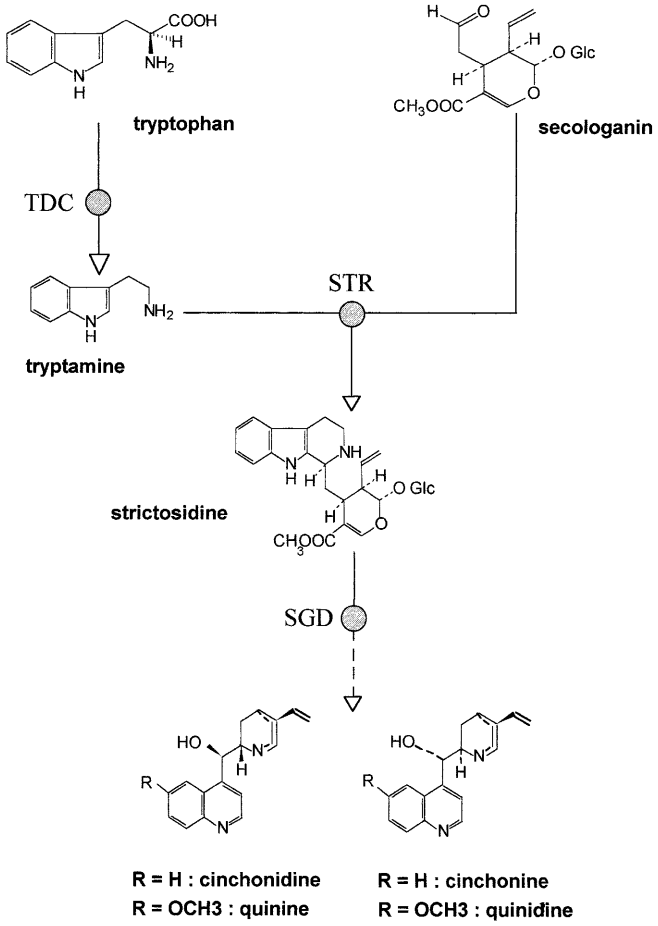

Fig. 1 Biosynthesis of quinoline alkaloids in *Cinchona* species. *Dashed arrow* indicates that the biosynthetic route(s) involved are not exactly known (*TDC* tryptophan decarboxylase, *STR* strictosidine synthase, *SGD* strictosidine β-D-glucosidase)

but proved to be poor producers of quinoline alkaloids (less than 1 μg g^{-1} DW). For such cultures, it was found that accumulation of these compounds is correlated with their extent of cellular differentiation (Verpoorte et al. 1987). In view of their cellular differentiation, hairy root cultures provide an interesting alternative production system. Hairy roots of *C. officinalis* have been reported to produce up to 50 μg g^{-1} fresh weight (FW) quinoline alkaloids (Hamill et al. 1989). This is still much lower than found in the bark of trees, but as a production system the plant has the disadvantage that it takes 7–10 years before its bark can be harvested. Here we report the successful integration of two genes (*tdc* and *str* from *C. roseus*) from the indole alkaloid biosynthesis pathway into hairy roots of *C. officinalis*, as a first step toward using metabolic engineering for increasing the production of alkaloids.

Materials and methods

Binary vector T-DNA and establishment of hairy root culture

A binary plant vector was constructed whose T-DNA region contains constitutive-expression versions (CaMV35S promoter with double enhancer and *nos* terminator) of *tdc* and *str* cDNA clones from *C. roseus*, together with an intron possessing the β-glucuronidase (*gus*-int) reporter gene and a hygromycin phosphotransferase (*hpt*) selection gene (Fig. 2). This binary vector construct was used in conjunction with *Agrobacterium rhizogenes* strain LBA 9402 to obtain *tdc*+*str*-gene-transformed hairy roots of *C. officinalis* L. These hairy root cultures were produced by infecting the leaves of axenically grown seedlings with *A. rhizogenes* (strain LBA 9402) containing the binary vector using forceps. Infected seedlings were placed in light with a photoperiod of 12 h at 28 °C. After about 6 weeks, roots emerging from the leaves were transferred to hormone-free solid 1/4 Gamborg B5 medium (1/4 macrosalts, 50 mg l^{-1} L-cysteine, 30 g l^{-1} sucrose) containing 100 μg ml^{-1} Augmentin. At this point, the introduction of the binary vector T-DNA into these *C. officinalis* roots was tested by a β-glucuronidase (GUS) assay (uniform blue staining after incubation with 1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl glucuronide at 37 °C). After growth on solid medium, the hairy roots which were positive in the GUS assay were transferred to 25 ml hormone-free liquid 1/4 Gamborg B5 medium and grown in the dark at 25 °C under continuous shaking; the medium was refreshed every week.

Enzyme activity measurements

Every 4 or 5 days, two hairy root cultures were harvested and used for protein and alkaloid extractions starting at day 1 after subculturing. After harvesting, the roots were immediately frozen in liquid nitrogen and stored at –80 °C. Protein extractions were done by grinding the frozen hairy root material in a mortar and pestle. Per gram of frozen hairy root material, 5% polyvinylpolypyrrolidone and 1 ml extraction buffer (0.1 M sodium phosphate buffer pH 7, 2 mM EDTA, 4 mM 1,4-dithiothreitol) were added. After thawing, the homogenate was centrifuged at 8000 *g* for 5 min. The supernatant was desalted on Sephadex G-25 (PD-10 column, Pharmacia, Uppsala, Sweden). Protein concentrations were measured by the method of Peterson (1977). The enzyme activities of TDC, STR, and strictosidine β-D-glucosidase (SGD, E.C. 3.2.1.105) were measured in the protein extracts in triplicate, as described elsewhere (Pennings et al. 1987; Pennings et al. 1989; Stevens et al. 1992). For TDC measurements, L-tryptophan or 5-methoxy-tryptophan, and for STR measurements, tryptamine

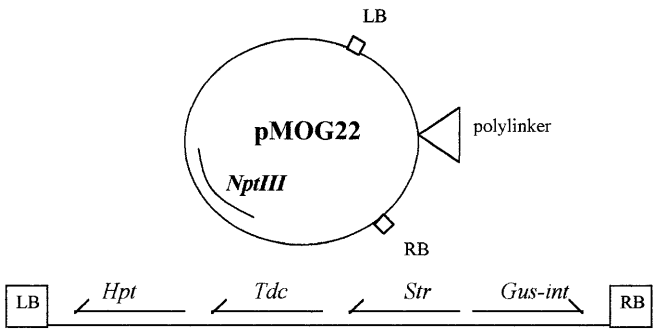

Fig. 2 Schematic representation of the pMOG22 plasmid used for transformations (*Hpt* hygromycin resistance gene, *Tdc* tryptophan decarboxylase gene, *Str* strictosidine synthase gene, *Gus-int* β-glucuronidase-intron reporter gene, *LB* left border, *RB* right border)

or 5-methoxy-tryptamine together with secologanin were used as substrates.

Alkaloid determinations

Alkaloids were extracted by adding 2 ml methanol:0.1 M hydrochloric acid (1:1) to 50 mg freeze-dried cells. After centrifugation (15 min at 8,000 *g*), the supernatant was collected and the pellet was extracted once more with 2 ml methanol:0.1 M hydrochloric acid (1:1). The combined supernatants were lyophilized and subsequently resuspended in 1 ml HPLC eluent (100 mM ammonium formate:formic acid:acetonitrile, 88:4:8). The alkaloid contents in these extractions were measured by HPLC as described before (Giroud et al. 1991). Tryptamine concentrations from the same alkaloid extracts were determined using the same HPLC system as used for measuring TDC activities (Pennings et al. 1987).

Southern and Northern blot analysis

Genomic DNA was extracted from 1 g of *C. officinalis* hairy roots according to the method of Webb and Knapp (1990). Genomic DNA was also extracted form a cell culture of *C. officinalis* without the *Tdc* and *Str* genes from *C. roseus*. The genomic DNA was restricted with *EcoRI*. Total RNA extraction was done using Tri Reagent from Sigma. Southern and Northern blot analyses using ^{32}P-labelled probes of *Tdc* and *Str* from *C. roseus* were performed according to Memelink et al. (1994). For the Northern blot, a ^{32}P-labelled probe of *Gus*-int was also used.

PCR reaction

PCR was performed using two primers for the *gus*-int gene: primer 1 (forward, 5′-GCAACGTCTGGTATCAGC-3′) and primer 2 (reverse, 5′-CACTGACCGGATGCCGAC-3′) giving rise to a theoretical PCR product of 819 bp. Forty cycles were performed using an annealing temperature of 50 °C.

Results and discussion

Initiation of *Cinchona* hairy roots

In previous studies in our laboratories, *Cinchona* species have been shown to be very recalcitrant to transformation by *A. rhizogenes* (Hoekstra 1993). To date, only one paper was dealing with a hairy root culture of *Cinchona* has been published (Hamill et al . 1989). Infection of axenic leaves from *C. officinalis* seedlings also resulted in our studies in a pronounced stress reaction visible as red pigmentation. To reduce this stress reaction, axenic seedlings were infected by wounding their leaves and stems with forceps contaminated with *A. rhizogenes*. About 6 weeks after infection, six roots emerged from the leaves of the seedlings infected with the strain carrying the binary vector containing the *Tdc* and *Str* genes. One root was shown to be transformed by the binary vector T-DNA since a GUS assay revealed expression of the *gus*-int gene. After excision and subculturing on solid 1/4 B5 medium, where it exhibited the hairy root phenotype (branched, phagiotropic growth on hormone-free medium), it was transferred to liquid 1/4 B5 medium. In this medium, the *C. officinalis* hairy roots developed into a very compact structure with many short root tips on the outside and a rusty-colored tissue mass on the inside. The rusty color of the hairy root line was probably due to the accumulation of anthraquinones. It is noteworthy that this particular phenotype of *C. officinalis* hairy roots has also been reported by other authors (Hamill et al. 1989).

Growth and enzyme activities

C. officinalis hairy roots were characterized 6 months after the initiation of the hairy roots, by dividing four subcultures of this line, each containing about 12 g of FW into 16 equally sized portions and transferring each to 25 ml of fresh medium. Every 4 or 5 days, two cultures were taken to determine their growth, enzyme activities and alkaloid contents. A doubling time of about 15 days indicated that the growth of the cultures was relatively slow (Fig. 3). The very compact structure of the culture might have played a role in this.

Compared to *C. officinalis* cell suspension cultures (Skinner et al. 1987) and seedlings (Aerts et al. 1990), high TDC and STR activities (about tenfold higher for both enzymes) were measured (Fig. 3). The highest enzyme activities were detected 15 days after subculturing. For TDC and STR, activities of up to 4.1 and 450 pkat mg^{-1} protein, respectively, were recorded. The SGD activity detected amounted to up to 300 pKat mg^{-1} protein at day 15 after subculturing.

The STR activity extracted from the *C. officinalis* hairy roots did not accept 5-methoxytryptamine as a substrate. This finding indicates that the STR activity, at least in part, results from the expression of the introduced *Str* gene, since, in contrast to STR from *Cinchona* species, STR from *C. roseus* is unable to catalyze the formation of 10-methoxystrictosidine (Stevens et al. 1993b).

Alkaloid contents

High levels of tryptamine and strictosidine were measured of up to 1200 and 1950 μg g^{-1} DW, respectively (Fig. 3). These results indicate that the availability of secologanin and the conversion of strictosidine might limit the production of quinoline alkaloids in the *C. officinalis* hairy root line. Hairy roots of *C. roseus* already established in our laboratories also showed a rate-limiting step in the biosynthesis of secologanin (Hoekstra 1993). The presence of both SGD activity and its substrate strictosidine could be explained by compartmentation (Stevens et al 1993a).

Despite its high enzyme activities, this *C. officinalis* hairy root line was found to accumulate amounts of quinoline alkaloids that were similar to a *C. officinalis* hairy root line described by Hamill et al. (1989). For quinine, levels up to 500 μg g^{-1} DW were recorded.

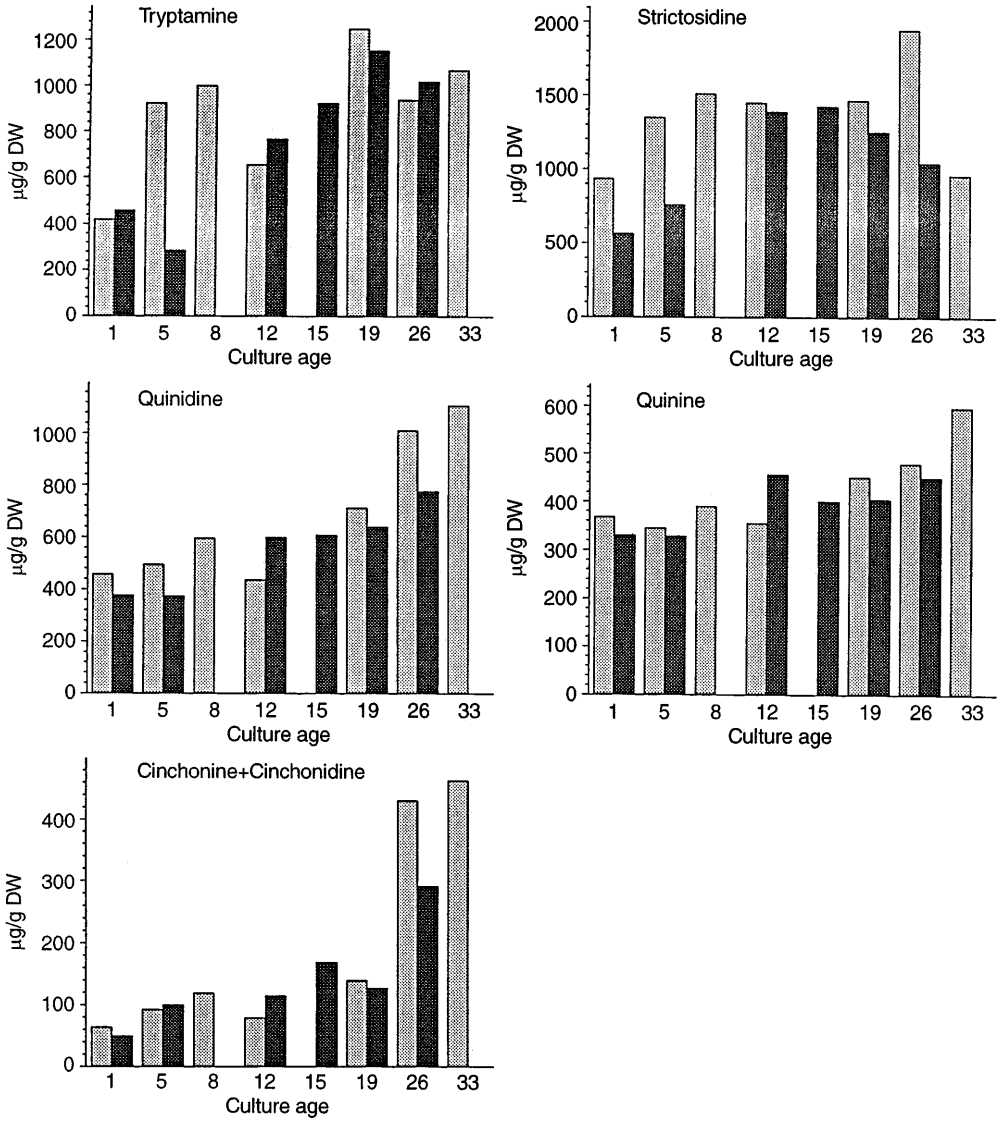

Fig. 3 Increase in dry biomass (*Growth*), activities of the enzymes tryptophan decarboxylase (*TDC*), strictosidine synthase (*STR*) and strictosidine β-D-glucosidase (*SGD*), and accumulation of *tryptamine*, *strictosidine*, *quinidine*, *quinine* and *cinchonine+cinchonidine* in *Cinchona officinalis* 'Ledgeriana' hairy root cultures. The graphs have the same layout, bars of the *same shading* each same timepoint are derived from the same culture, whereas *different shadings* represent different cultures

Cinchonine and cinchonidine could not be detected separately: a total of 400 µg g^{-1} DW was measured. Quinidine was the only compound found with levels three- to fivefold higher (up to 1000 µg g^{-1} DW) than in hairy roots of *C. officinalis* not transformed with the *Tdc* and *Str* genes from *C. roseus* (Hamill et al. 1989). Because we did not succeed in establishing our own control culture, only a comparison with results in the literature could be made. However, our results show that genetic engineering using multiple genes is feasible in hairy roots of *C. officinalis*.

Stability of the hairy roots

One year after the analysis of the transgenic hairy root line of *C. officinalis*, they were analyzed again at different timepoints (3, 5, 10, 15, and 20 days after subculture). Although the growth and morphology of the roots were still the same, they had completely lost their ability to accumulate alkaloids. No alkaloids could be detected, nor were strictosidine and tryptamine present. Enzyme measurements showed that the TDC activity had completely vanished. STR activity was still present (169 pkat mg^{-1} protein). The absence of alkaloids, strictosidine, and tryptamine can thus be explained by the absence of TDC activity. To check if the hairy roots were still transgenic, a GUS assay was performed on them together with a PCR reaction using genomic DNA of the hairy roots in combination with two primers designed for the *gus*-int gene. Both methods showed that the hairy roots still contained the *gus*-int gene (data not shown). A Southern blot was also made using *Tdc* and *Str* probes from *C. roseus* to check if these genes from *C. roseus* were also present in the *C. officinalis* hairy roots. they were. Northern blot analysis showed that the *Tdc* gene from *C. roseus* was not being expressed, since, except for the GUS probe, no signal could be detected on the blot (data not shown). Attempts to regain *Tdc* expression, using the

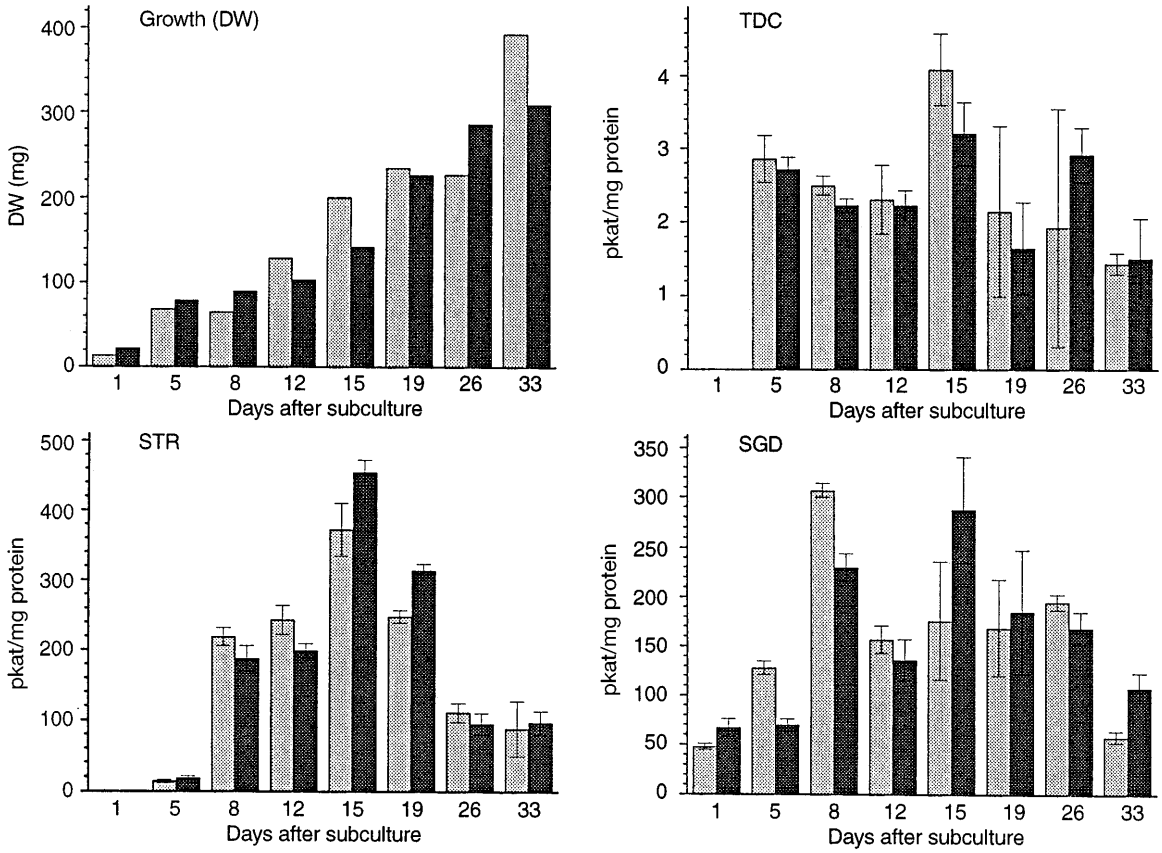

Fig. 3 (Continuation)

demethylating drug, 5-azacytidine, were unsuccessful. Silencing of the *Tdc* gene and transgene is probably not due to methylation problem, but may result from cosuppression, although additional causes of transgene inactivation have been described (for a review see Finnegan and McElroy 1994). The results presented here demonstrate once more that *Cinchona* species are difficult to work with: generating hairy roots with *A. rhizogenes* is not straightforward, and once they have been established they might not be very stable.

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