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Growth and terpenoid indole alkaloid production in *Catharanthus roseus* hairy root clones in relation to left- and right-termini-linked Ri T-DNA gene integration

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Abstract Hairy root cultures of *Catharanthus roseus* var. Prabal were established by infecting the leaves with *Agrobacterium rhizogenes* agropine-type A4 strain. Two hundred and fifty independent root clones were evaluated for growth, morphology, number of integration of Ri T-DNA genes and alkaloid contents. On the basis of growth pattern, type of branching and number of lateral roots we were able to separate the hairy root clones into four categories. However based on the integration of the Ri T_L-DNA and T_R-DNA genes, there were only three differ- ent categories of independent hairy root clones–C1 (*rolA&B*$^+$/*ags*$^+$), C2 (*rolA&B*$^-$/*ags*$^+$) and C3 (*rolA&B*$^+$/*ags*$^-$). Southern hybridization analysis revealed both single and multiple copies of T-DNA integration in the root clones. The accumulation of considerable amounts of the root-specific alkaloids ajmalicine and serpentine was observed in the presence of both the T_L-DNA and T_R-DNA genes (C1) and the T_L-DNA gene (C3) alone. Two *rolA&B*$^-$ but *ags*$^+$ clones (C2) accumulated much less or only very negligible amounts of ajmalicine. The possible role of the T_L-DNA and T_R-DNA genes on growth and alkaloid accumulation in these root clones is discussed.

Keywords Hairy root culture · *Catharanthus roseus* · Ri T-DNA genes · Morphological evaluation · Alkaloid production

Abbreviations *ags:* Agropine synthase · *Ri:* Root-inducing · *T_L-DNA:* Left-terminus DNA · *T_R-DNA:* Right-terminus DNA · TIAs: Terpenoid indole alkaloids

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Introduction

Agrobacterium rhizogenes, a gram-negative soil bacterium, is well known for having the ability to transfer its T-DNA from the root-inducing (Ri) plasmid to the host genome and thereby causing hairy root disease of plants (Chilton et al. 1982; Brillanceau et al. 1989). *A. rhizogenes* Ri plasmids have been assigned to three classes—mannopine, cucumopine and agropine—depending on the opine synthesized and degraded. In the first two classes, T-DNA consists of a single region, whereas in the third class two T-DNA regions are present, T_L-DNA and T_R-DNA. Transformed roots arise as a result of the integration of T_L-DNA and T_R-DNA into the plant genome by the agropine strains, and its expression induces root differentiation and subsequent growth (Christey 2001). Many variations in the morphology of transformed hairy roots have been reported, and correlative studies on the integration of the genes of the T_L-DNA and T_R-DNA regions with the morphology of hairy roots and regenerated plants have been carried out in a few plant species (Hanisch ten Cate et al. 1990; Limami et al. 1998; Christey 2001).

Root cultures established by *A. rhizogenes*-mediated transformation are widely used as sources of useful compounds due to their rapid growth and relatively high production of secondary metabolites. *Catharanthus roseus* (L.) G. Don, commonly known as Madagascar periwinkle, is a tropical plant of the Apocyanaceae family that produces a number of biologically active terpenoid indole alkaloids (TIAs), including antihypertensive drugs, ajmalicine and serpentine in the roots and the extremely valuable anticancer agents, vinblastine and vincristine, in the leaves. The potential of hairy roots of *C. roseus* has been investigated many times with respect to the production of root-specific indole alkaloids (Toivonen et al. 1989; Verpoorte et al. 1991; Jung et al. 1992; Bhadra and Shanks 1997; Shanks and Bhadra 1997). An analysis of T-DNA integration in the transformed roots of *C. roseus* confirmed the presence of T_L-DNA and T_R-DNA, both as distinct or single inserts and as a continuous one, in-

cluding the presence of the region between the T_L-DNA and T_R-DNA (Brillanceau et al. 1989). However, correlative studies with respect to the presence or absence of T_L-DNA and/or T_R-DNA in the hairy roots of *C. roseus*, their copy number and morphological variations and/or product formation have not yet been carried out. Establishment of the full Ri syndrome in hairy roots is correlated with the expression of the *rolA*, *-B*, *-C* and *-D* loci located on the T_L-DNA (Taylor et al. 1985). The products of these loci have been reported to show synergistic activities and are involved in rhizogenesis (Cardarelli et al. 1987; Spena et al. 1987). Recently, a putative *rolB* gene homologue that has a morphogenic activity has been identified in the T_R-DNA of *A. rhizogenes*. However, this was not the functional homologue of the $rolB_{TL}$ gene (Lemcke and Schmulling 1998). In the investigation reported here we studied the presence or absence of specific parts of the T_L-DNA and T_R-DNA genes in hairy root cultures of *C. roseus*, the copy number of the integrated genes and their effect, if any, on both morphological parameters and alkaloid accumulation.

Materials and methods

Plant material and hairy root induction

Leaves from 1-month-old in vitro-grown plants of *Catharanthus roseus* var. Prabal were pre-incubated on half-strength Gamborg's B_5 medium (Gamborg et al. 1968) for 24 h and inoculated with one of four different strains of *Agrobacterium rhizogenes* (A4, R1022, K599 and SA79) for hairy root induction. Single colonies of *A. rhizogenes* were propagated in liquid yeast extract-peptone medium for 48 h at 28°C. Pre-incubated leaves were infected with the bacterial culture ($O.D._{600}$ = 0.8–1.0) by multiple pricks with a syringe, and the infected leaves were incubated in the dark. Two to three weeks following infection, hairy roots emerged from the infection site. Single transformed roots were excised after one subculture and maintained separately as independent clones. Axenic root cultures thus derived were grown at 23±1°C under a 16/8-h (light/dark) photoperiod and transferred routinely to fresh half-strength Gamborg's B_5 medium every 5–6 weeks.

Morphological characterization of root clones

Root clones were characterized into different morphological categories based on their growth habit (slow, fast and moderately growing) and branching patterns (less or profusely branched and callusing types). A few root clones were selected from these groups and further studied for their growth index (GI = increase in dry weight/Initial dry weight × 100) based on dry weight.

Genomic DNA extraction and PCR amplification

Total genomic DNA was extracted from transformed root tissue using the CTAB DNA isolation method (Khanuja et al. 1999) with some modifications. In order to show the integration of T_L-DNA and T_R-DNA of *A. rhizogenes* pRi A4 in the transformed roots, segments from both the T_L-DNA and T_R-DNA regions were amplified using the gene-specific primers 5′-ATGGAATTAGCCGGACTAAACG-3′ and 5′-ATGGATCCCAAATTGCTATTCC-3′, which amplify a segment complimentary to the 5′ coding sequence of *rolA* to the 3′ coding sequence of *rolB* on the T_L-DNA region, and 5′-CGGAAATTGTGGCTCGTTGTGGAC-3′ and 5′-AATCGTTCAGAGAGCGTCCGAAGTT-3′, which amplify the *ags* gene of the T_R-DNA region (Aoki et al. 1997). Non-transformed *C. roseus* root DNA was used as a negative control for PCR analysis. Amplification involved 35 cycles of PCR with 50 ng of DNA template. The cycling conditions consisted of a 1-min denaturation at 94°C, a 2-min annealing at 55°C followed by a 3-min extension at 72°C and a final extension at 72°C for 7 min. PCR products were analysed by electrophoretic separation on 1% agarose gels (w/v) in 1× TAE buffer and staining with ethidium bromide.

Southern hybridization analysis

Southern blot analysis was performed as per Sambrook et al. (1989). Ten-microgram aliquots of root DNA were digested with *Xba*I, *Hin*dIII and *Eco*RI and fractionated on a 0.8% (w/v) agarose gel. The DNA was then transferred to Hybond nylon membrane (Amersham Pharmacia, UK) by capillary blotting. The blots were hybridized with random primed α-[^{32}P]-labelled *rol*/*ags* genes generated by PCR using the method described above. Hybridization was carried out at 68°C for 16 h followed by two washes in 2× SSC, 0.1% (w/v) sodium dodecyl sulphate (SDS) at room temperature for 10 min and then one wash in 0.2× SSC, 0.1% (w/v) SDS at 50°C for 5–10 min. The blots were exposed to Kodak X-Omat film with intensifying screen (Amersham Pharmacia) at −70°C for 1–3 days.

Total alkaloid extraction and high-performance liquid chromatography analysis

Roots dried at room temperature in the shade were homogenized and total alkaloids extracted following the protocol of Singh et al. (2000). The HPLC separation of major indole alkaloids of *C. roseus* was carried out according to Tikhomiroff and Jolicoeur (2002). Alkaloids were separated using a reversed-phase C18 column with binary gradient mobile phase profile (Singh et al. 2000; Tikhomiroff and Jolicoeur 2002). The compounds were identified on the basis of their retention time and the comparison of their UV spectra with those of authentic standards. Solutions of the authentic samples, ajmalicine and serpentine, were prepared in methanol (0.25 mg/ml) and used at different concentrations for the preparation of the calibration graphs, which were linear in the range of 0.25–25 μg. Quantification was repeated three times for each root line.

Result and discussion

Development of heterotrophic root clones

Each of the four different strains of *A. rhizogenes*—A4, R1022, K599 and SA79—showed different levels of infection in the leaves of 1-month-old in vitro-grown plants of *C. roseus* var. Prabal. The agropine strain A4 was the most virulent and induced roots at the infection site in 52% of explants within 2–3 weeks. Independent root clones derived from primary root tips were established in vitro after one subculture, and approximately 250 hairy root clones have been successfully maintained for the past 2 years. These root clones were further evaluated for growth, morphology, type and number of integrations of Ri T-DNA genes and for alkaloid content.

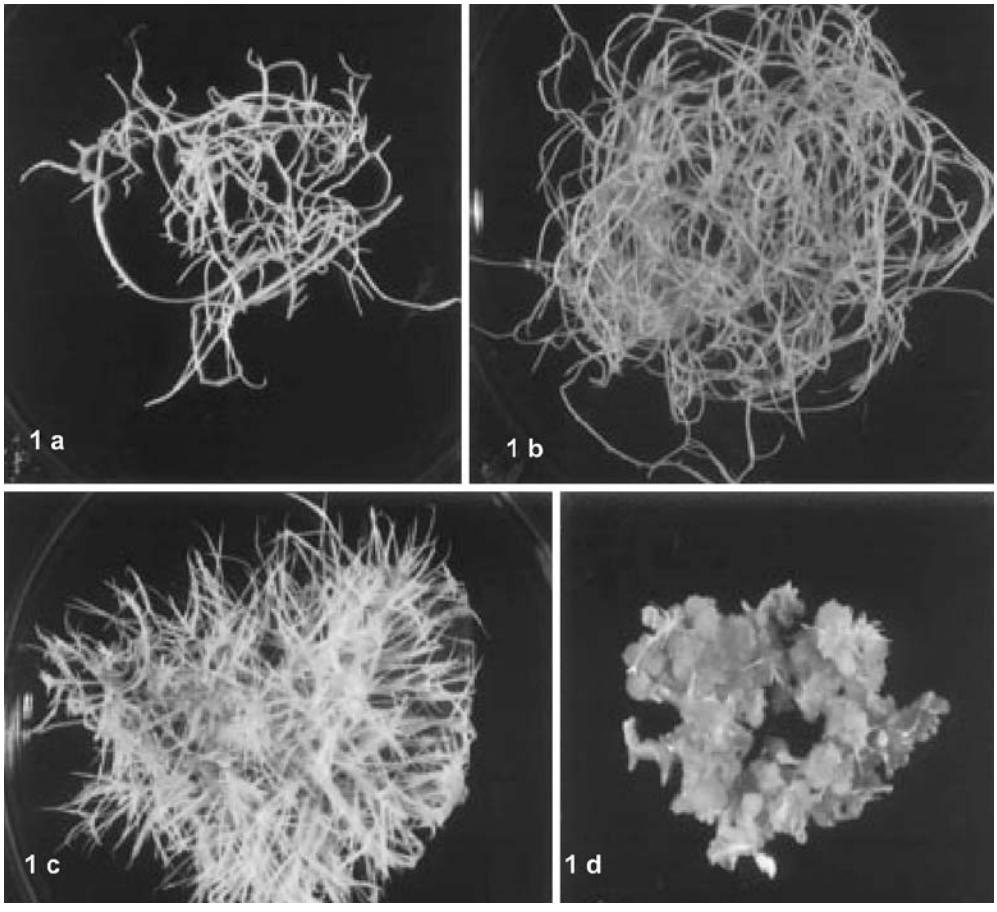

Fig. 1a–d Different morphological categories of 3-month-old hairy root clones of *Catharanthus roseus* var. Prabal induced after infecting leaves with *Agrobacterium rhizogenes* strain A4. **a** Less branched and slow growing (LBS), **b** highly branched and moderately growing (HBM), **c** profusely branched and fast growing (PBF), **d** callusing type and slow growing (CSG)

Morphological and growth characteristics of hairy root clones

There was enormous variability in the morphology and growth patterns among individual root clones, which can be accounted for by the fact that each root clone arose from a separate transformation event. Based on growth pattern, type of branching and number of lateral roots the hairy root clones could be classified into four categories: (1) less-branched, slow-growing type (LBS); (2) highly branched, moderately growing type (HBM); (3) profusely branched, fast-growing type (PBF); (4) callusing and slow-growing type (CSG) (Figs. 1a–d, 2). There were also differences in root-hair formation between the hairy root clones.

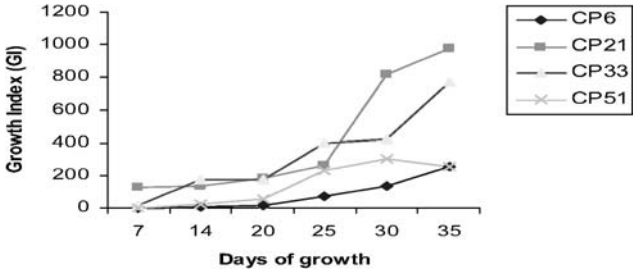

Fig. 2 Growth index (GI) patterns of the four morphological categories of hairy root clones of *C. roseus* var. Prabal (clone nos. CP 6, 21, 33, 51). GI = Increase in dry weight/Initial dry weight × 100

Ri T-DNA analysis in hairy root clones

In agropine Ri plasmids the T-DNA region consists of two parts—T_L-DNA and T_R-DNA—which are physically separated by a non-transferred DNA sequence of approximately 18 kb (Jouanin 1984; De Paolis et al. 1985). Upon integration, the size of the T_L-DNA has been reported to be almost constant (19–20 kb) except in *Nicotiana tabacum*, where it is shorter (Durand-Tardif et al. 1985; Taylor et al. 1985). In contrast, T_R-DNA has been found to vary between 8 kb and 20 kb (Slightom et al. 1986).

We were able to demonstrate the presence of Ri T-DNA in 250 transformed hairy root clones of different morphological categories by showing the presence of the *rolA* and *-B* genes (1.6 kb) from T_L-DNA segments and/or the agropine synthase (*ags*) gene (1.6 kb) from T_R-DNA segments (Fig. 3). Based on the combination of integrated Ri T-DNA genes, we identified three different categories of independent hairy root clones: C1, presence of T_L-DNA (*rolA&B)* and T_R-DNA (*ags*) segments; C2, presence of only T_R-DNA (*ags*) segments; C3, presence of only T_L-DNA (*rolA&B*) segments (Fig. 4a,b). In the first

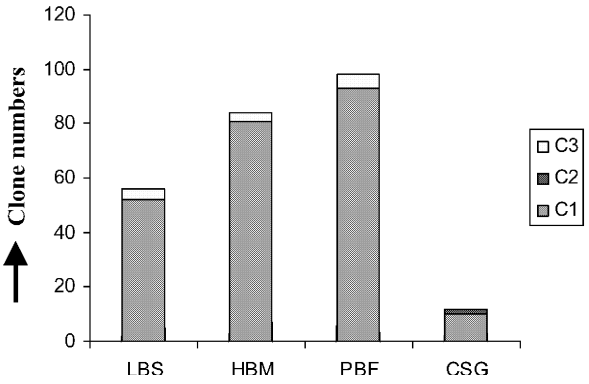

Fig. 3 Bar graph showing four morphological categories of hairy root clones of *C. roseus* var. Prabal: *LBS* less branched, slow-growth type, *HBM* highly branched, moderately growing type, *PBF* profusely branched, fast-growing type, *CSG* callusing and slow-growing type. The *shaded portions* indicate the occurrence of categories C1 (*rolA&B* $^{+}$*/ags* $^{+}$), C2 (*rolA&B* $^{-}$*/ags* $^{+}$) and C3 (*rolA&B* $^{+}$*/ags* $^{-}$) in each morphological type

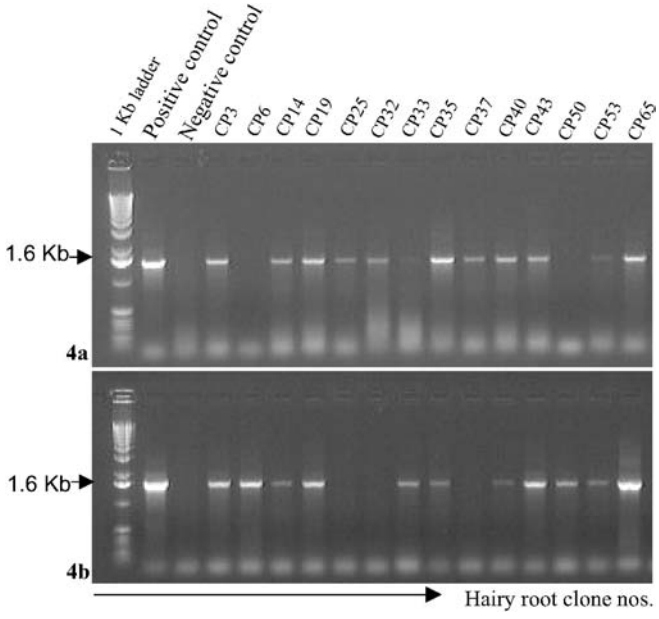

Fig. 4 PCR analysis showing the presence or absence of *rol* (**a**) and *ags* (**b**) genes in selected hairy root clones of *C. roseus* var. Prabal (*CP3–CP65*)

category (C1) of 236 transformed hairy root clones we found both the *rolA&B* and *ags* genes, thereby constituting a collection of *rolA&B*$^{+}$*/ags*$^{+}$ clones. These clones revealed diverse morphologies and growth patterns and were distributed throughout the four different morphological categories of hairy root clones (LBS, HBM, PBF, CSG; Fig. 3). Two independent clones of the C2 category showed the absence of *rolA&B* gene amplification but the presence of the *ags* gene of T_R-DNA, thereby constitut-ing *rolA&B*$^{-}$*/ags*$^{+}$ clones. These belonged only to the callusing and slow-growing morphological category (CSG) (Figs. 3, 4a). Due to their extreme slow growth, these root clones did not increase in mass compared to other clones and were maintained for a period of about 10–12 months. This decreased growth (mass and rate) indicates the importance of *rolA&B* in both the induction and sustained growth of hairy roots. The remaining 12 clones belonged in C3 category and did not show *ags* amplification but did show the presence of the *rolA&B* genes; thus they constituted *rolA&B*$^{+}$*/ags*$^{-}$ clones (Fig. 4b). These clones were distributed among three different morphological categories (LBS, HBM, PBF) and did not show the callusing type of growth (Fig. 3).

Results from the PCR analysis indicated that the transformed hairy root clones of *C. roseus* had both complete and partial insertion of Ri T-DNA. White et al. (1985) identified the *rol* loci on T_L-DNA to be the most important virulent factors and indicated that *rolB* has a role in pathogenicity. In our investigation only very few hairy root clones had the *rolA&B*$^{-}$*/ags*$^{+}$ constitution (C2). These clones showed the callusing-type morphology with slow growth, indicating that the transfer of the two T-DNA fragments may be such a highly efficient process as well as being important in the *A. rhizogenes* Ri T-DNA-mediated induction of hairy roots that only rarely do clones exist or grow properly in the absence of their transfer. This is in contrast to the more frequent occurrence of hairy root clones showing the absence of *ags* gene integration (C3 category; *rolA&B*$^{+}$*/ags*$^{-}$). These clones showed a root phenotype with slow, moderate and profuse growth patterns and belonged to the morphological categories of LBS, HBM and PBF, indicating that the loss of the *ags* gene from the T_R-DNA did not affect the developmental fate of the infected cell and further growth of the transformed roots in *C. roseus*.

Earlier reports of T-DNA analysis in hairy root clones of *C. roseus* revealed the presence of either distinct inserts or a single and continuous insert of both T_L-DNA and T_R-DNA including the region between T_L-DNA and T_R-DNA of *A. rhizogenes* pRi 15834 (Brillanceau et al. 1989). However, frequent spontaneous deletions of the Ri T-DNA of *A. rhizogene*s agropine strains during prolonged root culture and in regenerated shoots have been reported in potato (Hanisch ten Cate et al. 1990). Whether these frequent changes in T_L-DNA or T_R-DNA are due to the properties of the Ri T-DNA integration or to the tissue culture method used is not clear. In the case of *A. tumefaciens*-mediated transformation, characterization of transgene loci indicates a complex structure arising as a result of the involvement of several DNA break-repair mechanisms (Kumar and Fladung 2002). Our results showing the absence of T_L-DNA or T_R-DNA segments of pRi A4 in a few independent hairy root clones of *C. roseus* is indicative of fragmented integration of Ri T-DNA. However, detailed analysis of the integration process is necessary to understand the mechanism of fragmented Ri T-DNA integration in hairy root clones.

Integration of Ri T-DNA into the genome of *C. roseus* was further confirmed by Southern hybridization analy-sis using the *rolA&B* and *ags* genes of T_L-DNA and T_R-DNA, respectively, as probes. Strong hybridization signals were detected in *Hin*dIII-digested genomic DNA with the *ags*

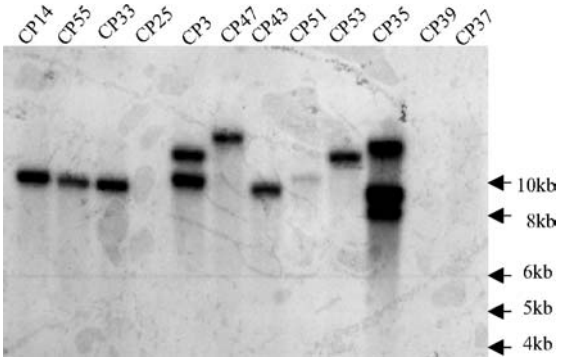

Fig. 5 Southern blot analysis of *Hin*dIII-digested genomic DNA of selected hairy root clones of *C. roseus* var. Prabal (*CP3–CP55*) using α-[^{32}P]-radiolabelled 1.6-kb *ags* gene fragment of T_R-DNA of pRiA4

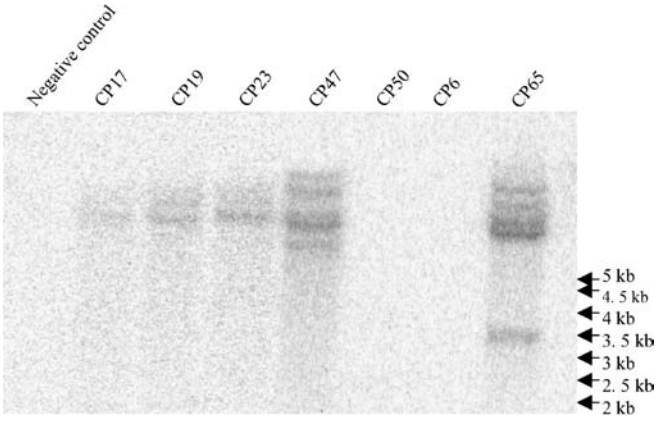

Fig. 6 Southern blot analysis of *Eco*RI-digested genomic DNA of selected hairy root clones of *C. roseus* var. Prabal (*CP17–CP65*) using α-[^{32}P]-radiolabelled 1.6-kb *rolA&B* gene fragment of T_L-DNA of pRiA4

probe from independent hairy root clones of the C1 and C2 categories, but none were detected in the control or C3 type (CP25, -37, -39) (Fig. 5). Similar hybridization experiments using *Eco*RI-digested genomic DNA with a radiolabelled *rolA&B* probe revealed strong signals in the root clones of the C1 and C3 categories and not in clones of the C2 category (CP6, -50) (Fig. 6). Hybridization experiments using genomic DNA of a number of independent hairy root clones digested with three different restriction enzymes revealed mostly single-copy integration of both the T_R-DNA-linked *ags* gene and the T_L-DNA-linked *rolA&B* genes although in some cases there were two to three copies of integration (Figs. 5, 6, 7a,b). However, there was no correlation between the numbers of copies of integration and specific morphological types.

HPLC analysis of hairy root clones

The establishment of hairy root cultures of *C. roseus* and the manipulation of media and other growth conditions for the production of different indole alkaloids have been reported (Parr et al. 1988; Toivonen et al. 1992; Shanks and Bhadra 1997; Rijhwani and Shanks 1998; Morgan et al. 2000). In the present study, we used HPLC to analyse 50 independent hairy root clones belonging to the C1–C3 categories for the production of root-specific indole alkaloids. Two major root alkaloids, serpentine and ajmalicine, were found to accumulate in these clones (Fig. 8a,b). Differences in the amounts of accumulated alkaloids at both the intra- and inter-category level were observed. The differences in the amounts of specific alkaloids within the C1 clones (*rolA&B*$^+$/*ags*$^+$) may be due to differences in the integration site of the T-DNA and the presence or absence of other open reading frames (ORFs) of Ri T-DNA, neither of which was studied in the present investigation. Two *rolA&B*$^-$/*ags*$^+$ clones (C2) accumulated less or negligible amounts of ajmalicine. However, the accumulation of serpentine in one C2 clone was equal to or greater than the same content of a few C1 root clones.

The constitutive expression of *rolB* loci is reported to interfere profoundly with plant morphogenesis, thereby resulting in growth alterations, and to play a major role in pathogenicity (White et al. 1985; Spena et al. 1987). In the present study clones with the *rolA&B*$^-$/*ags*$^+$ genotypes showed reduced growth and a lower accumulation of alkaloids. However, this lower accumulation of alkaloids can not be specifically correlated with the absence of the *rolA&B*genes of T_L-DNA as other C1 and C3 clones also revealed similar or lower amounts of same alkaloid (Fig. 8a,b).

Christey (2001) reported that the integration of Ri T_L-DNA and T_R-DNA leads to alterations in hormone metabolism, transport properties and the production of opines in the transformed roots. This altered behaviour of the roots has led to the hypothesis that the regulation of secondary metabolite pathways may be different in transformed and normal roots. Our investigation has shown that the independent root clones with T-DNA left terminus-linked *rol* genes alone (C3) or in combination with the right terminus-linked *ags* gene (C1) accumulate variable amounts of ajmalicine and serpentine. This is due to the fact that the type of T-DNA transferred and the site of integration are different in different clones. *A. tumefaciens*-mediated T-DNA transfer in plants is thought to be a random process unless targeted T-DNA integration into the host genome by various methods is attempted (Chilton and Que 2003). The underlying mechanisms of hairy root and crown gall tumorigenesis are likely to be very similar. However, further investigations on *A. rhizogenes*-mediated Ri T-DNA transfer in the plant genome will reveal a better understanding of the importance of each of the 18 T_L-DNA ORFs (including *rolA*, *-B*, *-C*, *-D*) and T_R-DNA genes in the induction of hairy root clones and their subsequent effects on growth and on the regulation of the secondary metabolite accumulation in independent hairy root clones.

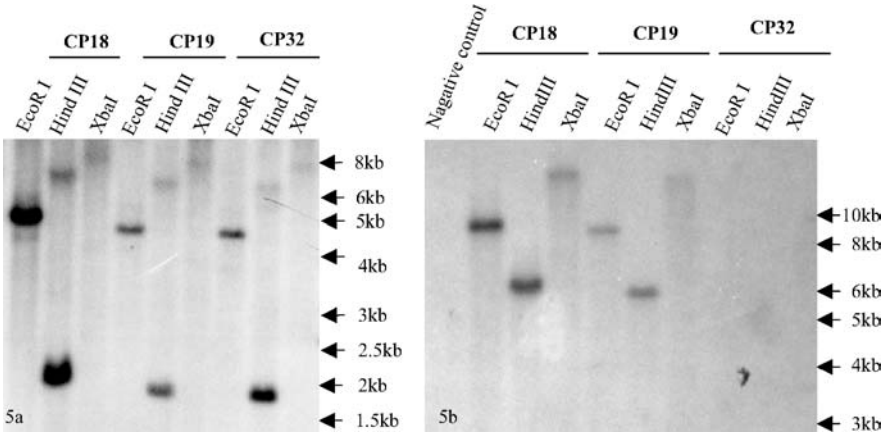

Fig. 7a,b Southern Blot analysis using a 1.6-kb *rolA&B/ags* gene fragment consisting of both T_L-DNA and T_R-DNA of pRiA4. Genomic DNA from different hairy root clones of *C. roseus* var. Prabal was digested separately with *Eco*RI, *Hin*dIII and *Xba*I, electrophoresed and transferred to a nylon membrane. Blots were hybridized with the α-[^{32}P]-radiolabelled *rolA&B* (**a**) and *ags* (**b**) gene-specific probes, revealing mostly single-copy insertions (*rolA&B* contains one internal *Hin*dIII site, thus showing two bands after hybridization)

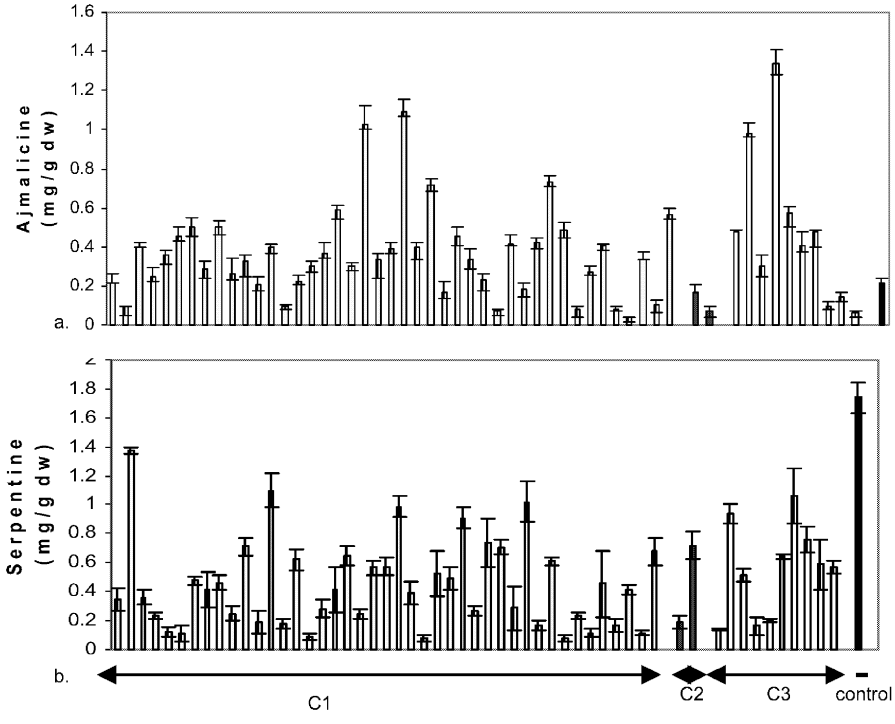

Fig. 8 Ajmalicine (**a**) and serpentine (**b**) production on the basis of milligram per gram dry weight of different hairy root clones of *C. roseus* var. Prabal belonging to three different groups: C1, *rolA&B*$^+$/*ags*$^+$; C2, *rolA&B*$^-$/*ags*+; C3, *rolA&B*$^+$/*ags*$^-$. Non-transformed roots from 3-month-old field-grown plants were used as the control

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