Plant Cell Reports (1997) 17: 150–154

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Agrobacterium-mediated transformation of the terpenoid indole alkaloid-producing plant species *Tabernaemontana pandacaqui*

Received: 16 June 1997 / Revision received: 12 July 1997 / Accepted: 13 July 1997

Abstract Plants of the Apocynaceae family produce a wide range of terpenoid indole alkaloids (TIAs) which have important pharmaceutical applications. Studies of the molecular mechanisms controlling TIA biosynthesis may eventually provide possibilities to improve product yield by genetic modification of plants or cell cultures. However, these studies suffer from the lack of transformation/regeneration protocols for Apocynaceae plants. We chose to study the feasibility of *Agrobacterium tumefaciens*-mediated transformation of *Tabernaemontana pandacaqui*, because of the availability of an efficient regeneration procedure for this member of the Apocynaceae family. A procedure to produce transgenic *T. pandacaqui* plants was established, albeit with low efficiency. Transgenic expression was demonstrated of an intron-containing β-glucuronidase reporter gene and of a gene coding for the TIA biosynthetic enzyme strictosidine synthase from *Catharanthus roseus*, another Apocynaceae species.

Key words *Agrobacterium*-mediated transformation · *Tabernaemontana pandacaqui* · *Catharanthus roseus* · Terpenoid indole alkaloids · Strictosidine synthase

Abbreviations *GUS* β-glucuronidase · *MS* Murashige and Skoog · *SSS* Strictosidine synthase · *TIA* Terpenoid indole alkaloid

Communicated by N. Amrhein

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Introduction

Plants of the Apocynaceae family produce a wide range of terpenoid indole alkaloids (TIAs) as part of their secondary metabolism. Within the intact plant, these metabolites have possible functions in inhibiting predator feeding and protection against pathogens or UV radiation. Several TIAs are extremely valuable for their pharmacological activities. This is especially true for the dimeric TIAs, such as vincristine and vinblastine, which occur in trace amounts in some members of the Apocynaceae family and represent powerful antitumour drugs.

In recent decades, efforts have been made to elucidate the biosynthetic pathways leading to the production of TIAs and to characterize the enzymes involved. This work has focussed mainly on two Apocynaceae family members, *Catharanthus roseus* and *Rauwolfia serpentina* (reviewed by Hashimoto and Yamada 1994; Kutchan 1995; Meijer et al. 1993). Some genes encoding key enzymes in TIA biosynthesis have been isolated and the molecular mechanisms that control their expression are being studied. Progress in understanding the regulation of this pathway may eventually provide ways to improve product yield by genetic modification of plants or cell cultures.

A serious problem encountered by groups wishing to study the regulatory mechanisms of TIA production in plants is the lack of transformation/regeneration protocols for plants of the Apocynaceae family. Reports of successful transformation of members of this family concern only the production of tumours or hairy roots using oncogenic *Agrobacterium tumefaciens* or *A. rhizogenes* strains, respectively (Benjamin et al. 1993; Shanks and Bhadra 1997; Trémouillaux-Guiller et al. 1994; and references therein). In a few cases, regeneration of plants from hairy roots has been described, among others for *C. roseus* (Brillanceau et al. 1989), but the regenerated plants from this report could not survive greenhouse conditions. A clear disadvantage of the use of tumours or hairy roots in studies of gene regulation mechanisms is that the results may be influenced by the expression of the plant hormone biosynthetic genes

of the *Agrobacterium* T-DNA and, in the case of *A. rhizogenes*, by the expression of the *rol* genes. Therefore, such experiments may lead to conclusions that are true only for a specific transgenic cell or root culture and which are not generally applicable. Hence, there is a need for protocols for the production of transgenic Apocynaceae plants by means of non-oncogenic (disarmed) *Agrobacterium* strains.

For *C. roseus*, the main reason for the lack of reports of transgenic plant production seems to be the difficulty setting up efficient regeneration systems. Only one report of high-frequency *C. roseus* regeneration has appeared in the literature, but it requires the establishment and maintenance of embryogenic suspension cultures initiated from tissue-cultured anthers (Kim et al. 1994). In contrast, high-frequency regeneration from leaf, stem and root explants has been described for *Tabernaemontana pandacaqui*, another member of the Apocynaceae family, which can also easily be micropropagated in tissue culture (Sierra et al. 1991). *T. pandacaqui* produces a variety of TIAs, including dimeric forms closely related to those found in *C. roseus*. Because of the availability of an efficient regeneration protocol, transgenic plant production may prove to be more feasible for *T. pandacaqui* than for other Apocynaceae species. Strictosidine, a compound composed of an indole moiety (provided by the amino acid derivative tryptamine) and a terpenoid moiety (provided by the secoiridoid secologanin) is the universal precursor of TIAs (Stöckigt and Zenk 1977) and in different plant species, the same key enzymes catalyse the biosynthetic conversions leading to the formation of this compound. Therefore, *T. pandacaqui* can be considered as a suitable plant for studying mechanisms controlling early steps in TIA biosynthesis, leading to knowledge that will be very likely applicable to other members of the Apocynaceae family.

Here we describe a procedure for transformation of *T. pandacaqui* making use of a non-oncogenic, supervirulent *A. tumefaciens* strain (LBA 1119), which has previously been shown to be capable of gene transfer to many recalcitrant plant species (reviewed by Van Wordragen and Dons 1992). Although the efficiency of the method is still low, regenerated transgenic *T. pandacaqui* plants could be obtained. Transgenic expression of an intron-containing β-glucuronidase (GUS) reporter gene and of the *C. roseus* gene coding for the enzyme strictosidine synthase (SSS), which catalyses the formation of the TIA precursor strictosidine, was demonstrated.

Materials and methods

Plasmid constructions

An intron-containing GUS (*gusA*int) reporter gene linked to promoter and terminator sequences from the CaMV 35S gene (Vancanneyt et al. 1990) was subcloned as a *Hin*dIII fragment in pIC19H (Marsh et al. 1984). Subsequently, the *Xba*I, *Bam*HI and *Sma*I restriction sites between the 35S promoter and *gusA* start codon, as well as the *Sst*I and *Kpn*I sites between the *gusA* stop codon and 35S terminator were deleted using the exonuclease and polymerase activities, respectively, of the Klenow fragment of DNA polymerase I. This altered *gusA*int gene was cloned as a *Pst*I fragment in pIC20R (Marsh et al. 1984) and subsequently cloned as an *Xba*I fragment into the *Xba* I site of binary vector pMOG22 (Mogen International, Leiden, The Netherlands), which contains a T-DNA region with multiple cloning sites and a hygromycin (*hpt*) selection gene driven by CaMV 35S promoter and *nos* terminator sequences. In the resulting construct, pMOG22-*gusA*int, the orientation of the *gusA*int gene was the same as that of the *hpt* gene.

An *Sss* cDNA clone, harbouring the complete coding region for the SSS enzyme from *C. roseus*, was generated by replacing a 135-bp *Sma*I-*Bsm*I fragment from the partial *Sss* cDNA clone pCCR38 (Pasquali et al. 1992) with the corresponding 150-bp *Hin*cII-*Bsm*I fragment from the genomic *C. roseus Sss* clone pGCR38 (Pasquali 1994). Subsequently, the *Sss* cDNA clone was cloned as a *Bam*HI-*Xho*I fragment in pIC19R (Marsh et al. 1984), and then inserted in sense orientation as a *Bam*HI-*Bgl*II fragment in the *Bam*HI site of pMOG463, an expression vector with CaMV 35S promoter and *nos* terminator sequences (Mogen International). The resulting *Sss* gene construct was cloned as an *Sst*I fragment in the *Sst*I site of pMOG22-*gusA*int, resulting in construct pMOG22-*Sss-gusA*int, in which the *Sss*, *gusA*int and *hpt* gene constructs have identical orientations.

T. pandacaqui transformation

T. pandacaqui plants were grown in tissue culture at 28°C and 3000 lux (Philips TL95), with a 16-h light/8-h dark regime, on agar-solidified Murashige and Skoog (1962) (MS) medium containing 30 g/l sucrose (MS-0 medium). For transformation, leaves were excised and wounded by making six to eight incisions with a sterile surgical knife at regular intervals perpendicular to the main vein or by cutting the leaves in two. After wounding, the leaves were incubated for 30 min in 90 ml liquid MS medium containing 0.1 mg/l indoleacetic acid, 5 mg/l benzylaminopurine and 30 g/l sucrose (MS-22 medium) to which 10 ml of an overnight culture of *A. tumefaciens* strain LBA 1119 containing construct pMOG22-*Sss-gusA*int was added. Subsequently, the infected leaves were blotted dry between sterile filter paper and transferred to MS-22 agar plates. In the finally established transformation procedure, the leaves were transferred 4 days after cocultivation to MS-22 agar plates supplemented with 0.4 mg/ml of cefotaxime and 0.1 mg/ml of vancomycin to counterselect *Agrobacterium* and with 10 µg/ml of hygromycin to select for regeneration of transgenic shoots. Three weeks later, regenerated shoots were transferred to jars containing agar-solidified MS-0 medium supplemented with 0.4 mg/ml of cefotaxime and 0.1 mg/ml of vancomycin. In all experiments tissue culture media were refreshed every 2 weeks.

Determination of SSS activity

Liquid-nitrogen-frozen green plant parts were homogenized with a mortar and pestle in the presence of polyvinylpyrrolidone (50 mg/g fresh weight). To the frozen material one volume of extraction buffer containing 0.1 M Tris-HCl (pH 7.5), 4 mM dithiothreitol and 2 mM EDTA was added. The material was allowed to thaw and the extracts were clarified by centrifugation for 30 min at 10 000 rpm. The supernatant was desalted on Sephadex G-25 (PD-10 columns, Pharmacia, Uppsala, Sweden) equilibrated with the extraction buffer. Incubation mixtures for SSS activity determination contained 25 µl of desalted protein extract and 62.5 µl of a cocktail containing 1.6 mM tryptamine-HCl and 8.0 mM secologanin in 0.1 M sodium phosphate buffer pH 6.8, together with 12.5 µl of a freshly prepared solution of 0.8 M D(+)-gluconic acid-δ-lactone in 0.8 M Tris. After incubation at 30°C for 60 min, the reaction was stopped by adding 75 µl of 5% trichloroacetic acid. Blanks were made by adding trichloroacetic acid prior to the 60-min incubation period. After addition of 14.8 µl of internal standard (8.0 mM codeine-HCl) and centrifugation, samples were analysed by HPLC (Pennings et al. 1989). All measurements were performed in duplicate. Protein extracts from

C. roseus and tobacco plants were always used as positive and negative controls, respectively.

Alkaloid analysis

Freeze-dried cell material (50-mg portions) was extracted with 15 ml ethanol. After centrifugation, a 10-ml aliquot was removed and evaporated. The residue was dissolved in 0.5 ml of 1 M phosphoric acid, centrifuged again and subsequently analysed by HPLC using photodiode array detection (Moreno et al. 1993).

Histochemical GUS assay

Histochemical localization of GUS activity was performed essentially as described by Jefferson (1987), using X-gluc (Sigma) as substrate. Staining was performed overnight at 37°C. Chlorofyl, which interfered with the visibility of blue staining, was removed by incubation in 70% ethanol.

PCR analysis

T. pandacaqui DNA was extracted as described by Lassner et al. (1989). PCR reactions contained 2.5 µl of plant DNA extract or 10 ng plasmid DNA, 0.2 units of SuperTaq (HT Biotechnology, Cambridge, UK), 20 pmol of each primer and 100 µM of each dNTP in 50 µl of SuperTaq reaction buffer. For detection of the *Sss* gene construct, the upper primer (5′CCACTGACGTAAGGGATGAC3′) was in the CaMV 35S promoter and the lower primer (5′CTGCCATCAT GG3′) was in the *Sss* cDNA sequence (positions 86–97; Pasquali et al. 1992). Primers for *hpt* detection (5′CGCACAATCCCACTAT CCTTCGCAA3′ and 5′GGCAGTTCGGTTTCAGGCAGGTCTT3′) were both in the *hpt* coding region. Amplification was performed by 40 cycles of denaturation at 94°C, annealing at 45°C and extension at 72°C.

Results and discussion

To set up a method for generation of transgenic *T. pandacaqui* plants by *Agrobacterium*-mediated transformation we used the supervirulent *A. tumefaciens* strain LBA 1119 (C58 pTiBo542 ΔT-DNA), carrying the binary vector pMOG22-*Sss-gusA*int, with a T-DNA region containing an *hpt* gene construct to allow hygromycin selection, an *Sss* gene construct encoding the enzyme SSS from *C. roseus* and a reporter gene construct encoding GUS. This reporter gene construct (*gusA*int) carried an intron which cannot be processed in *Agrobacterium*, thereby making it possible to rapidly identify transformed plant tissues by histochemical staining for GUS activity.

In agreement with previous studies (Sierra et al. 1991), wounded leaf material of *T. pandacaqui* gave rise to abundant shoot formation from wound callus when placed on regeneration medium containing a low concentration of the natural auxin indoleacetic acid and a high concentration of the synthetic cytokinin benzylaminopurine. The first shoots appeared within 3 weeks and after that shoots continued to form for a number of weeks. Shoot formation was completely abolished when regeneration medium was supplemented with 10 µg/ml hygromycin, suggesting that application of this concentration of hygromycin in regeneration medium may allow recovery of transformed shoots from leaf material infected with the described *Agrobacterium* strain.

To test if *Agrobacterium* itself had any effect on the regeneration frequency under non-selective conditions, an experiment was performed in which wounded leaves were cocultivated with *Agrobacterium* for 4 days on regeneration medium and subsequently transferred to regeneration medium supplemented with the antibiotics cefotaxime and vancomycin to conterselect for *Agrobacterium*. Regeneration still occurred after this treatment, although shoots appeared somewhat later and with an approximately two- to threefold lower frequency. These effects could be attributed to *Agrobacterium*, since cefotaxime and vancomycin alone had no appreciable influence on shoot formation.

Since the regeneration frequency of *Agrobacterium*-treated *T. pandacaqui* material was still relatively high, transformation experiments were started. In the first experiment, 10 µg/ml hygromycin was chosen. Wounded leaf material was cocultivated for 4 days with LBA1119 carrying the binary vector pMOG22-*Sss-gusA*int. Subsequently, the leaf material was divided into three groups: the first was exposed immediately to hygromycin selection, the second was allowed to recover for 1 week before application of selective conditions, and the third was allowed to regenerate under non-selective conditions. Although a concentration of 10 µg/ml hygromycin efficiently blocked regeneration of untransformed material, in this experiment both the time and frequency of shoot appearance were almost the same in the presence of hygromycin as under non-selective conditions. Therefore, we decided to raise the hygromycin concentration from 10 to 25 µg/ml at day 55 following the start of cocultivation. After this, the shoots originating from the group of leaves that was subjected to hygromycin selection immediately after cocultivation stayed green, whereas all shoots that originated from the groups that were placed under non-selective conditions or allowed to recover for 1 week before application of hygromycin selection turned brown. This finding led to the choice of immediate selection in further experiments. The condition of the shoots that had survived the transfer to 25 µg/ml hygromycin started to decline after some weeks. Therefore, we decided to discontinue the experiment and subject the remaining green shoots to histochemical analysis with the chromogenic dye X-gluc to test for expression of the *gusA*int reporter gene. Two out of six shoots turned completely blue, indicating that gene transfer had indeed occurred in this experiment.

In the following transformation experiment, two concentrations of hygromycin (10 and 25 µg/ml) for immediate selection were tested. A concentration of 25 µg/ml hygromycin appeared to be too high to allow shoot regeneration, although in the first experiment shoots that had already emerged on the leaves at 10 µg/ml hygromycin could survive this higher concentration. As in the previous experiment, regeneration frequencies were almost equal on 10 µg/ml hygromycin as under non-selective conditions. Shoots coming from immediate selection at 10 µg/ml hygromycin were excised and transferred to hormone-free medium containing 0, 10 or 25 µg/ml hygromycin. Only

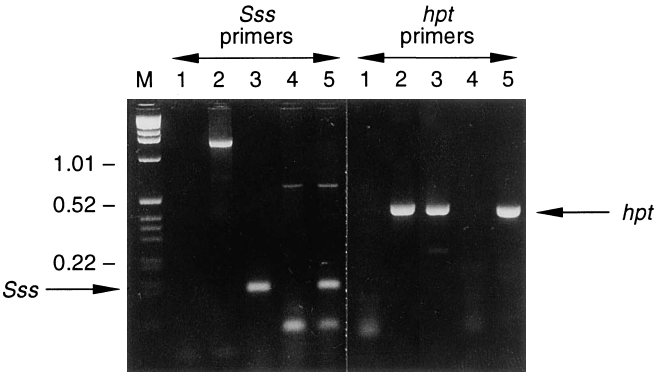

Fig. 1 PCR analysis of a transgenic *Tabernaemontana pandacaqui* plant containing a T-DNA construct with *Sss*, *hpt* and *gusA*int genes. *Lane M* contains 1 μg of 1-kb DNA ladder (Gibco) for which the sizes of some bands are indicated on the left of the figure. PCR reactions were performed with primers to detect the *Sss* or *hpt* gene constructs as indicated above the figure. The *Sss* and *hpt* PCR products are indicated with *arrows*. Templates used in the PCR reactions were: *lane 1* H_2O control; *lane 2* 10 ng of binary vector DNA containing *hpt* and *gusA*int gene constructs (pMOG22-*gusA*int); *lane 3* 10 ng of binary vector DNA containing *Sss*, *hpt* and *gusA*int gene constructs (pMOG22-*Sss*-*gusA*int); *lane 4* extract from an untransformed control plant; *lane 5* extract from the transgenic *T. pandacaqui* plant

shoots placed on medium lacking hygromycin were able to grow, suggesting that the excised shoots were either not transformed or transformed but, for unknown reasons, more sensitive to hygromycin than when still attached to the leaf material from which they had emerged. To clarify this point, leaves of the plantlets developed from the shoots on hygromycin-lacking medium were tested for expression of the *gusA*int reporter gene. Histochemical analysis revealed that 2 out of 50 plants tested expressed the *gusA*int gene. Hence, only 4% were true transformants despite the fact that all shoots had emerged under selective conditions that do not allow shoot regeneration from *T. pandacaqui* leaves which had not been cocultivated with *Agrobacterium*. Of the two transgenic *T. pandacaqui* plants obtained, one was lost to an infection. The other transgenic plant was micropropagated in tissue culture and analysed further. Leaves of the micropropagated plant became uniformly blue when stained for GUS activity, indicating that the plant was not chimaeric. Furthermore, PCR analysis confirmed that the *hpt* and *Sss* gene constructs were present in the transgenic plant but not in a control plant (Fig. 1). Subsequently, SSS activities were determined in protein extracts made from leaves of the transgenic plant and of seven control plants that were obtained from the same transformation experiment but were untransformed. The SSS activity of the transgenic plant (206 pkatal/mg protein) was two- to threefold higher than the activities detected in the series of untransformed plants, which indicated proper expression of the introduced *Sss* gene.

In conclusion, our results demonstrate that transgenic *T. pandacaqui* plants can be generated by application of the following conditions. First, *Agrobacterium*-infected leaf explants are placed on regeneration medium containing the selective agent hygromycin at a concentration of 10 μg/ml. Subsequently, regenerated shoots are transferred to hormone-free medium lacking hygromycin. Finally, transformants must be identified amongst the regenerated plants by PCR analysis or by detection of the expression of a convenient reporter gene construct, such as the intron-containing *gusA* gene used in our experiments. Obviously, further studies will be required to attain higher transformation efficiencies. In particular, improvement of the selection system is desirable to guarantee that most of the regenerating shoots are true transformants. A complicating factor in our experiments was that the sensitivity of *T. pandacaqui* to hygromycin showed large variability between the various types of plant tissues that occur in the experimental procedure. Thus, for application of strict hygromycin selection, detailed studies of the optimal concentration of this antibiotic during the different stages of transformation and regeneration will be necessary. In addition, other selective agents could be considered. We found that *T. pandacaqui* is rather insensitive to the frequently used antibiotic kanamycin, but the *bar* gene of *Streptomyces hygroscopicus* which provides resistance to the herbicide phosphinothricine may be a useful alternative for the *hpt* selectable marker gene. Possible further improvements may result from testing *A. tumefaciens* strains other than the LBA 1119 strain used here for their capacity to transfer T-DNA to *T. pandacaqui*.

Although the efficiency of our method is still low, the establishment of a transformation/regeneration procedure for *T. pandacaqui* represents a noteworthy achievement as it is the first report of non-oncogenic transgenic plant generation for a member of the Apocynaceae family. It is clear that the possibility of transgenic plant production will make a positive contribution to studies of the molecular mechanisms controlling biosynthesis of the important group of TIAs that are produced by members of this plant family. Moreover, it may lead to biotechnological applications, such as the creation of *T. pandacaqui* plants with modified TIA contents. Transgenic expression of genes coding for TIA biosynthetic enzymes may result in the identification of rate-limiting steps in the TIA pathway. As a first step in this direction we determined whether TIA production was altered in our transgenic *T. pandacaqui* plant which showed an up to threefold increased SSS activity compared to a range of control plants. Apart from strictosidine, the main TIAs found in the leaves of *T. pandacaqui* plants were apparicine, 3-hydroxyvoacangine and voacangine. Despite the higher SSS activity of the transgenic plant, neither qualitative nor significant quantitative differences were observed in comparsion with the control group. Although preliminary, our data are in agreement with the postulation put forward by several other authors that SSS is not a rate-limiting enzyme in TIA biosynthesis, despite the fact that it occupies a key position in the TIA pathway by catalysing the condensation of the two TIA precursors tryptamine and secologanin. Earlier support for this postulation has come from observations that increased TIA accumulation in cell cultures transferred to certain TIA production media was accompanied by a rapid increase in the activities of sev-

eral enzymes involved in the formation of strictosidine precursors, whereas SSS activity remained relatively constant (Knobloch et al. 1981; Schiel et al. 1987). During *C. roseus* seedling development also (De Luca et al. 1988) and during the growth cycles of cell cultures of several Apocynaceae species (Stevens et al. 1992) SSS activity was not under the strict regulation observed for some of the other enzymes in TIA biosynthesis. Moreover, we recently found that overexpression of *Sss* in *C. roseus* callus lines did not lead to enhanced TIA production (unpublished results). Future generation of a larger number of transgenic *T. pandacaqui* plants expressing *Sss* or other TIA biosynthetic genes should provide further insight into the regulatory mechanisms governing TIA production.

Acknowledgements The authors wish to thank Dr. Paulo Moreno for help given with the enzyme assays and alkaloid determinations and Paula van der Duyn-Schouten for initial testing of the *T. pandacaqui* regeneration procedure. M. Inês Lopes Cardoso was supported by a grant from Junta Nacional de Investigação Científica e Tecnológica, Portugal.

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