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Original Paper

Regeneration of transformed *Ophiorrhiza pumila* plants producing camptothecin

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Abstract Camptothecin derivatives are clinically used as anti-tumor alkaloids that are currently obtained by extraction from intact plants. Seeking for the alternative sources for commercial production and for fundamental study, cell and tissue cultures have been investigated. In the present study, we developed a method for regeneration of *Ophiorrhiza pumila* plant from hairy roots transformed with *Agrobacterium rhizogenes*. The regeneration frequency was over 83%. Integration of a *rol B* gene from T-DNA of *A. rhizogenes* was confirmed by polymerase chain reaction in both of the hairy roots and the regenerated plants. The transformed plants accumulated camptothecin in amounts of 66–111% compared with that in the wild-type plants.

Key words: Camptothecin, indole alkaloids, *Agrobacterium rhizogenes.*

Camptothecin (CPT) is one of the modified monoterpenoid indole alkaloids, originally identified in the extracts of *Camptotheca acuminata* (Nyssaceae) during a screening project for anti-tumor agents by the National Cancer Institute in the United States (Wall et al. 1966). The effectiveness of CPT toward a reduction in mammalian cancer was first reported in 1974 (Schaeppi et al.). However, CPT had not been used immediately in practice due to its severe side effects, until semi synthetic CPT analogues, irinotecan and topotecan, were developed for clinical use (Postmesil 1994) (Figure 1). Since then, these CPT derivatives have been used clinically for cancer chemotherapy. In addition, a number of reports are available announcing the therapeutic values of CPT derivatives against colon cancer (Giovanella et al. 1989), AIDS (Priel et al. 1991), uterine cervical cancer and ovarian cancer (Takeuchi et al. 1991), and falciparum malaria (Bodley et al. 1998). The ever-increasing worldwide market of irinotecan and topotecan has currently reached one thousand million US dollars, which represents approximately one ton of CPT in terms of raw material. Despite its great demand as the only commercially available source of these medicines, CPT is still supplied exclusively from intact plants, mainly *C. acuminata* and *Nothapodytes foetida* (Olacaceae). In addition to the difficulties of the practical total synthesis of these natural compounds, the unpredictable problems of nature such as erratic weather and pests have raised a strong need for the establishment of more stable and profitable methods of producing CPT.

Cell and tissue cultures of several CPT-producing plants have been investigated as alternative sources. Sakato et al. (1974) reported the first establishment of cell suspension cultures of *C. acuminata*, although the CPT productivity was practically insufficient [2.5 μg g^{-1} dry weight (DW)]. Callus cultures of *C. acuminata* established by Wiedenfeld et al. (1997) produced comparatively adequate amounts of CPT (2 mg g^{-1} DW). Calli of *N. foetida* were found to accumulate small amounts of CPT (Roja et al. 1994; Ciddi et al. 2000). The first feasible *in vitro* CPT-production system was developed by transformed hairy roots of *Ophiorrhiza pumila* (Rubiaceae) with *Agrobacterium rhizogenes* (Saito et al. 2001). This hairy root culture produced a high level of CPT ($\leq$1 mg g^{-1} DW) and excreted CPT into the culture medium in a relatively large quantity sustained in a scaled-up 3 L bioreactor (Sudo et al. 2002). Similarly, hairy roots of *C. acuminata*, producing an equal level of CPT, were recently reported (Lorence et al. 2004). For better production of CPT and further potential genetic engineering, a regeneration protocol of transformed plants of those CPT-producing species is needed.

Here, we report a method of plant regeneration from hairy roots of *O. pumila*. This first achievement has a profound significance as one of the CPT-producing plants in which any practical regeneration has not been reported yet.

Abbreviations: CPT, Camptothecin; DW, Dry weight; HPLC, High-performance liquid chromatography; PCR, Polymerase chain reaction.

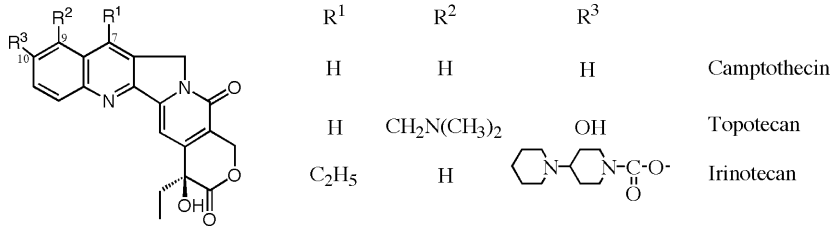

Figure 1. Chemical structures of CPT and its clinically used derivatives.

Materials and methods

Induction and cultivation of hairy roots

Sterile *O. pumila* plants were cut off their upper portions and infected with *A. rhizogenes* strain 15834 onto the sections of the stems according to the procedure detailed by Saito (2001). The hairy roots which appeared from the infected spots of the explants were excised and transferred individually to B5 (Gamborg et al. 1968) gellan gum-solidified medium with 2% sucrosesupplemented with cefotaxime (Claforan®, 200 mg l^{-1}) under dark conditions. The established hairy roots were sub-cultured to fresh plates every ten days for one month, then transferred to the same medium as described above without cefotaxime.

Polymerase chain reaction (PCR) analysis of rol B gene

Hairy roots and plantlets of up to 300 mg were ground with 1.5 ml of extraction buffer [100 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 μM 2-mercaptoethanol, 350 mM D-sorbitol] in mortars and pestles. The slurry was centrifuged for 5 min at 4 °C and 6,000 rpm in a 1.5-ml tube. The supernatant was discarded and the pellet was re-suspended in 300 μl of the extraction buffer and 300 μl pre-heated (65°C) lysis buffer [55 mM hexadecyltrimethyl ammonium bromide, 250 mM Tris-HCl (pH 7.5), 50 mM EDTA, 2 M NaCl], and 120 μl 5% (w/v) sarkosyl were added. After incubation at 65°C for 1 h, the samples were extracted with 600 μl of chloroform-isoamyl alcohol (24 : 1). The solution was inverted to mix for 15 min and centrifuged for 10 min at 4 °C and 15,000 rpm. The upper phase was decanted to a new tube, precipitated with 600 μl of isopropanol and centrifuged for 10 min at 4°C and 15,000 rpm. The supernatant was discarded, and the pellet was washed once with 700 μl of 70% cold ethanol before drying by aspiration for 3 min. The pellet was dissolved in 2000 μl water and stored at −20 °C until use. The hairy root of *O. pumila* which had established by Saito et al. (2001) was served as a positive control.

For the amplification of the inserted *rol B* gene homologous sequences, the following primers were used, *rol B* (+) primer: CCTCTAGAGTAACTATCCAACTCACATCACAAG and *B* (−) primer: TTGAATTCGTGGCTGGCGGTCTTGGATTCATTTC (Minlong et al. 2000). PCR reactions were carried out in a PCR Thermal Cycler (TAKARA Co. Ltd.). The program consisted of 40 cycles with the following conditions: (initial 95°C for 3 min) 93°C for 1 min, 40°C for 2 min,72°C for 2 min; and the final 5 min extension at 72°C, followed at the end by 4°C hold. PCR products were analyzed by 1% agarose gel electrophoresis followed by staining with ethidium bromide and UV detection.

Plant regeneration from hairy roots and acclimatization

Following one month of the culture under dark conditions, the hairy roots were exposed to the light (a 16-h/day, 2,000 lux). Shoot buds appeared were transferred to half-strength MS (Murashige et al. 1962) gellan gum-solidified medium containing 1% sucrose for rooting.

Extraction of CPT and high-performance liquid chromatography (HPLC) analysis

The hairy roots were maintained in 100-ml Erlenmeyer flasks containing 50 ml of B5 medium and 2% sucrose at 25°C on a rotary shaker (60 rpm) in the dark for three weeks. Following incubation for three weeks, they were harvested to be freeze-dried. The protocols of extraction and quantification of CPT using HPLC were based on the procedure by Saito et al. (2001). In the same way, two-month-old regenerated plants ranging from one to 15 cm in height were extracted and examined for their chemical constituents.

Results and discussion

Induction and culture of hairy roots

Over 20 independent adventitious roots emerged from the wounded sites on each *O. pumila* plant one week after the infection. When these roots grew to about one cm in length, after ten days, they were individually cultured on plates. Approximately 60% of them were assumed to be hairy roots judged from the rapid growth on hormone-free medium. The hairy roots were sterilized while transferred to fresh medium containing 200 mg l^{-1} cefotaxime. There was considerable morphological variability and differences in growth rates among the

hairy root lines, and we selected 40 lines at random and maintained them for further analysis.

Plant regeneration from hairy roots

Hairy roots of about one-month-old spontaneously developed shoot buds in the dark. After transferred on half-strength MS medium under the light (a 16-h photoperiod) for two weeks, these buds grew to greenish shoots. The amount of the hairy roots on plates and light conditions were found to significantly influence the plant regeneration. When young hairy roots were individually cultured under light conditions, they apparently stopped growing. In contrast, a cluster (about the size of one cm) of the hairy roots, which had been sub-cultured in the dark for one month, was abundantly regenerated to greenish shoots, under the light within three weeks (Figure 2), much faster than those in the dark.

The regeneration of shoot buds was observed in the restricted region of the hairy root cluster (Figure 2). Most of shoot buds were formed on relatively old parts of the hairy roots. Wardell et al. (1969) reported that flower formation on tobacco stem segment *in vitro* depended on their original positions of a plant, presumably due to the presence of hormonal gradient in plant body. Our result suggested that the shoot bud formation might require a physiological gradient such of hormone in the hairy root. It is generally accepted that photosynthetic activity is not very important factor during initial phases of culture *in vitro*, but at later stages the culture materials are induced to become photoautotrophic to a certain degree (Rout et al. 2000). Also for our hairy roots, during the prolonged cultures in dark the regenerated shoot buds gradually died. These indicate that two following conditions are favorable to initiate the shoot regeneration from *O. pumila* hairy roots; sizable mass of the hairy roots and the light exposure after being cultured in the dark. The regeneration frequency was over 83%; 33 lines out of 40 randomly-selected hairy roots finally. Regenerated plants showed various, and typical characteristic features of hairy root-derived plants, such as shortened internodes and malformed leaves.

Confirmation of transformed state by PCR analysis

To confirm integration events of *rol* genes into the *O. pumila* genome, we performed PCR using *rol B* primers on 20 putative hairy root lines and 7 regenerated plants. The amplified band of expected size (approximatory 1.3 kb) appeared in the hairy roots and the regenerated plants (Figure 3). On the other hand, the amplification of wild type *O. pumila* plant as a negative control gave no band. The result of the PCR analysis provided 60% of the adventitious roots which appeared from the infected spots were transformed hairy roots. We also analyzed one shoot derived from the rests of the adventitious roots, which have stopped growing during subculture. Because they differentiated into shoots

Figure 2. Regeneration of transgenic *O. pumila* plants from hairy roots. Regenerated shoots emerged from hairy roots after five weeks of culture under light conditions.

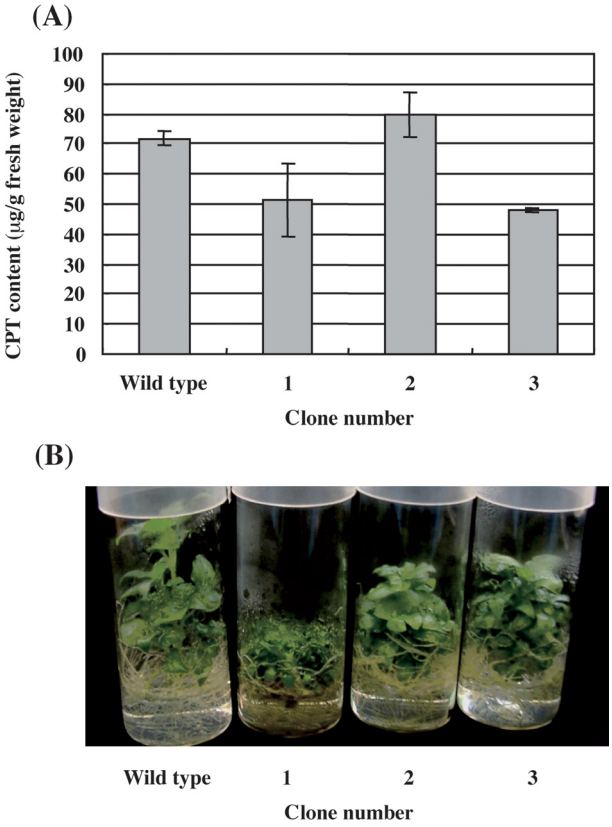

Figure 5. CPT content of transgenic regenerated plants from hairy roots. (A) CPT accumulation in regenerated plants. (B) The shapes of regenerated plants. Clone numbers correspond to those in (A).

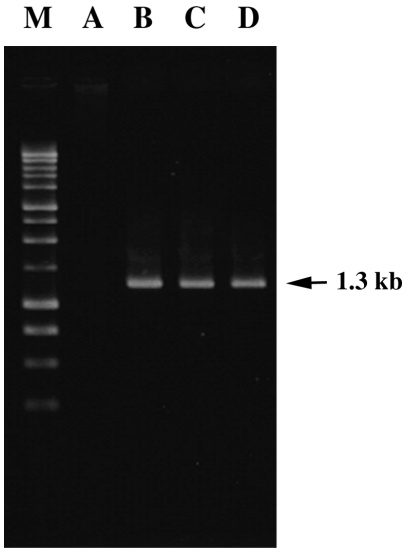

Figure 3. Detection of *rol B* gene in transgenic *O. pumila* by PCR. (M) Size marker DNA. (A) Non-transgenic *O. pumila* plant. (B) Hairy root induced by *A. rhizogenes*. (C) Regenerated plant derived from hairy roots. (D) Positive control hairy root reported previously (Saito et al. 2001).

without rooting after being sub-cultured several times, the shoot was sampled for PCR analysis and consequently proved to be negative (data not shown).

Production of CPT in hairy roots and regenerated plants

Nineteen hairy root lines were cultured in the B5 liquid medium for three weeks. The hairy roots contained $0.79 \pm 0.05\,mg\,g^{-1}$ DW camptothecin on average, the growth rates widely ranged from 5.6 to 11.8-fold in the culture period of three weeks. As shown in Figure 4, there is a trend that the better growth rates, the less alkaloid concentrations.

We also examined the regenerated plants derived from the hairy roots for CPT contents and metabolite patterns by HPLC/photodiode array detection. They accumulated 66–111% of CPT compared with that in the wild-type *O. pumila* plant (Figure 5). The levels of CPT in the regenerated plants from the hairy roots varied more intensively than those in the hairy roots. The transformed regenerated plant had the ability to produce the same pattern of compounds as aseptic wild-type *O. pumila* plant (Figure 6). Since *O. pumila* produces a variety of CPT-related alkaloids compared to *C. acuminata* and *N. foetida* (Yamazaki et al. 2003a), transformed *O. pumila* plant would be a desirable experimental material for biosynthetic studies such as tracer experiments (Yamazaki et al. 2004) and further functional genomics approach.

Although the final skeleton of CPT belongs to the quinoline alkaloid groups, CPT is synthesized via strictosidine, a universal biosynthetic intermediate from

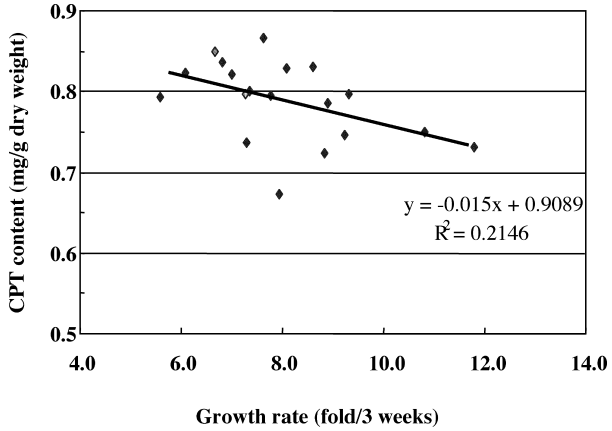

Figure 4. Negative correlation between CPT content and growth rate of hairy root. After being cultured for three weeks in liquid medium, CPT contents of 19 hairy root lines were measured by HPLC.

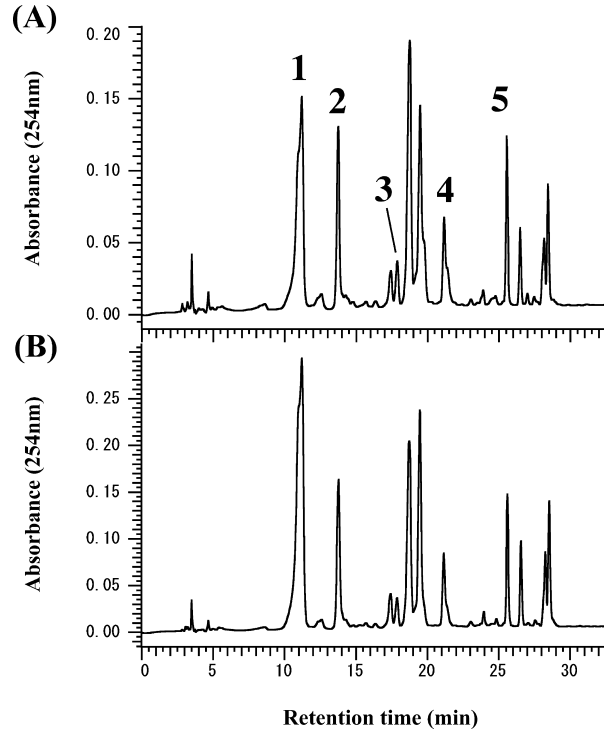

Figure 6. HPLC-photodiode array chromatograms of the extracts of *O. pumila* plants detected at 254 nm. (A) Extracts from wild-type *O. pumila* plant. (B) Extracts from transformed *O. pumila* plant regenerated from the hairy roots. 1, 3-*O*-caffeoylquinic acid; 2, strictosidinic acid; 3, chaboside; 4, 3 (*S*)-pumiloside; 5, camptothecin. The conditions of HPLC are as followings: column, Cica Mightysil RP-18 (4.6×250 mm); gradient, linear gradient from solvent A to solvent B (0–35 min.), isocratic at 100% of solvent B (35–40 min), solvent A (20% methanol, 0.2% acetic acid in H_2O), solvent B (90% methanol, 0.025% acetic acid in H_2O); flow rate, 0.8 ml/min; column temperature, 37°C.

which all monoterpenoid indole alkaloids are derived (Hutchinson 1981). The cDNAs encoding strictosidine synthase and tryptophan decarboxylase, which are responsible for synthesizing strictosidine, were isolated

from *Rauvolfia serpentina* (Kutchan et al. 1988), *C. roseus* (McKnight et al. 1990; De Luca et al. 1989), and *O. pumila* (Yamazaki et al. 2003b). Since cDNAs of key enzymes involved in indole alkaloid biosynthesis have become partly clear, several attempts have been conducted to express those genes in the heterologous plants. The strictosidine synthase gene from *C. roseus* was expressed in tobacco plants (McKnight et al. 1991). The cDNAs of strictosidine synthase and tryptophan decarboxylase together were introduced on a single transforming plasmid into tobacco leaves by particle bombardment, resulting in high expressions of both genes being observed (Leech et al. 1998). However, it is clear that these studies still suffer from the lack of efficient transformation/regeneration systems. For the plant species producing the commercially useful anti-tumor compounds, only a limited information has been available (Fulzele et al. 2003; Lee et al. 2003). Since transformation system using a binary vector for *O. pumila* had previously achieved (Saito et al. 2001), it is possible to have biosynthetic key enzymes express in transgenic plants which would allow further understanding of indole alkaloid biosynthesis and efficient production of alkaloids.

Conclusion

We have established an efficient transformation and regeneration system of *O. pumila* plants accumulating CPT. Because of its importance as a feasible supply source of CPT, this plant regeneration will make a substantial contribution as an essential and useful means not only to further fundamental study of CPT formation but also to the establishment of genetically modified plants feasibly producing CPT.

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