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Original Paper

Establishment of *Rhizobium*-mediated transformation of *Coptis japonica* and molecular analyses of transgenic plants

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Abstract *Coptis japonica* is a perennial medicinal plant grown in Asia, and its rhizome is used as crude drug, in which berberine is highly accumulated as the main alkaloid. In this study, a transformation method for *C. japonica* using *Rhizobium* has been established. Young petioles were infected with *Rhizobium radiobactor* Gv3101 (pMP90) harboring a plasmid with *β-glucuronidase* (*gus*) driven by cauliflower mosaic virus 35S promoter and *hygromycin phosphotransferase* (*hpt*) gene as the selection marker. GUS assay showed that 46% of the calli grown on hygromycin plates clearly expressed the *gus* gene. As an application of the transformation method, an endogenous cDNA encoding an ABC protein, CjMDR1, which is involved in berberine transport, was introduced in sense orientation. *Cjmdr1* transgenic plant (Sense-2) regenerated from hygromycin resistant callus line had a single copy of integrated *hpt* gene in its genomic DNA. Northern analyses of the Sense-2 plant showed that *Cjmdr1* mRNA levels were lower in all organs examined than those of wild-type plant, which suggested that co-suppression of *Cjmdr1* expression occurred. Indeed, the berberine content in Sense-2 was also suppressed. This is the first report of stable transformation of *Coptis* spp. and also of the alteration of a secondary metabolite by transport engineering.

Key words: Berberine, *Coptis japonica*, GUS expression, *Rhizobium radiobacter*.

An important medicinal plant, *Coptis japonica* (Ranunculaceae), is a representative source of berberine, a yellow benzylisoquinoline alkaloid. Its rhizomes are the medicinal part, because it highly accumulates berberine as the main alkaloid, and is used as a valuable crude drug in Asian countries. Berberine shows strong antimicrobial activity toward both Gram-positive and -negative bacteria as well as other microorganisms (Iwasa et al. 1998). It is used as a bitter stomachic and an anti-diarrhetic. Its biosynthesis has been one of the most intensively studied topics among various alkaloids in higher plants (Zenk 1995; Sato et al. 2001). Although berberine is highly accumulated in the rhizome of *C. japonica*, genes for its biosynthesis are specifically expressed in the roots, where only a low level of berberine is detected (Fujiwara et al. 1993). This suggests that berberine is transported from the root tissue to the rhizome after the biosynthesis, and then unloaded to be highly accumulated. As a berberine transporter which is responsible for the translocation, we recently isolated a cDNA encoding a multidrug-resistant protein (MDR)-type ATP-binding cassette (ABC) transporter (*Cjmdr1*) from berberine-producing cultured *C. japonica* cells. In the intact plant, highest expression was observed in the rhizome (Yazaki et al. 2001). Functional analysis of *Cjmdr1* by using a *Xenopus* oocyte expression system showed that CjMDR1 transported berberine in an inward direction (Shitan et al. 2003). *In situ* hybridization indicated that *Cjmdr1* mRNA was expressed preferentially in xylem tissues of the rhizome. These findings strongly suggested that CjMDR1 was involved in the translocation of berberine from the root to the rhizome.

To further clarify the physiological function of CjMDR1 *in planta*, biochemical and genetical analyses using transgenic plants are effective approaches. Transformation of *C. japonica* by *Rhizobium* has been so far reported for cultured *C. japonica* cells (Sato et al. 2001), but transformation method to obtain stable transformant plants of *C. japonica* was not yet established. In this paper, we report the effective transformation procedure of *Coptis* using its petiole and

Abbreviation: ABC, ATP binding cassette; CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase; MDR, multidrug-resistant protein; NAA, 1-naphthaleneacetic acid; PCR, polymerase chain reaction; UTR, untranslated region; WP, Woody Plant; X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.

Rhizobium radiobacter harboring a binary plasmid.

Materials and methods

Plant materials

Petioles were excised from a *Coptis japonica* Makino var. *dissecta* (Yamabe) Nakai plant that is cultivated at the Research Center for Medicinal Plant Resources, NiBio, Japan. The explants were dipped in 75% ethanol for 10 seconds followed by rinsing in sterilized water, and were surface-sterilized for 10 min in 2% sodium hypochlorite containing Tween 20 (1 drop per 40 ml). Explants were then washed thrice with sterilized water. The petiole explants (*ca.* 5 mm in length) were used for transformation.

Construction of GUS, Cjmdr1-sense and -antisense expression cassettes

The *gus* gene or *Cjmdr1* cDNA was subcloned via *Sac*I/*Xba*I restriction sites, into a binary vector (pBiHyg-HSE), a pBin19 derivative (Gatz et al. 1992), downstream the cauliflower mosaic virus (CaMV) 35S promoter. The resulting three plasmids, pBin-GUS, pBin-*Cjmdr1*-sense and pBin-*Cjmdr1*-antisense were introduced in *Rhizobium radiobacter* Gv3101 (pMP90) by a standard electroporation method.

Rhizobium-mediated transformation

Three infection methods were used for transformation with *Rhizobium radiobacter* Gv3101 (pMP90) harboring either pBin-GUS, pBin-*Cjmdr1*-sense or pBin-*Cjmdr1*-antisense. General infection procedure was preculture of the petiole explants on WP (Lloyd and McCown 1980) solid medium containing 10 mg l^{-1} L-glutamine (WPG), infection with *Rhizobium* and then elimination of bacteria on WPG solid medium containing 500 mg l^{-1} Claforan® (Hoechst Japan Ltd.). The WPG solid medium contained 3% sucrose and 0.25% Gelrite (San ei gen FFI) unless otherwise stated. The *Rhizobium* cultured overnight in Yeast Extract Broth (YEB) (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM $MgSO_4$, pH 7.2) liquid medium (20 ml medium in a 100 ml Erlenmeyer flask) at 25°C on a rotary shaker (100 rpm) was used for infection. Method 1: The petiole explants were precultured on phytohormone-free (HF) WPG solid medium (WPGHF) for 9 days at 20°C in the dark. These explants were transferred into WPG liquid medium containing 2% sucrose, 1 mg l^{-1} 1-naphthaleneacetic acid (NAA) and 2 mg l^{-1} kinetin (20 ml medium in a 100 ml Erlenmeyer flask). A portion (0.2 ml) of the *Rhizobium* culture (approximately 10^6 bacteria) was added to the flask with petiole segments. They were co-cultured for 3 days at 25°C in the dark on a rotary shaker (100 rpm). Method II: The petiole explants were precultured on WPG solid medium containing 1 mg l^{-1} NAA and 2 mg l^{-1} kinetin (WPGNK) for 16 days at 20°C in the dark. These explants were co-cultured with the *Rhizobium* for 2 days as mentioned above. Method III: The sterilized petiole explants were immersed in the *Rhizobium* culture for over 60 min. The segments were washed with sterilized water for over 30 min. The infected segments were then placed onto WPGNK solid medium containing 500 mg l^{-1} Claforan®, and kept at 20°C in the dark. The medium was exchanged when the bacteria proliferation was observed. After 1 to 2 month, the segments were transferred onto the WPGNK solid medium containing 25 mg l^{-1} hygromycin for the selection, and cultured at 20°C in the dark. Segments, which turned brown in this selection procedure, were evacuated to the WPGNK solid medium (-hygromycin). The calli generated in the absence of hygromycin were again transferred onto the WPGNK solid medium (+hygromycin) and this procedure was repeated until callus stably grew in the presence of hygromycin. The hygromycin-resistant calli were excised, and subcultured on the WPGNK solid medium under the selection pressure at 20°C in the dark with the interval of 1–2 months for 6 months. The calli stably grown on the WPGNK solid medium (+hygromycin) were used for further experiments.

GUS assay

The calli generated from transformation with *Rhizobium* harboring pBin-GUS were assayed for expression of GUS after incubation with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc). The calli were directly incubated in 100 mM phosphate buffer (pH 7.3) containing 1 mM X-gluc overnight at 37°C.

Plant regeneration

The Sense-2 callus, which was generated from transformation with *Rhizobium* harboring pBin-*Cjmdr1*-sense, was maintained on the WPGNK solid medium containing 25 mg l^{-1} hygromycin at 20°C in the dark. Plantlet formation from the transformed callus was spontaneously observed when the callus was transferred onto WPGHF solid medium and cultured at 20°C in the dark. The plantlets were transplanted onto the WPGHF solid medium containing 25 mg l^{-1} hygromycin (30 ml in 30 mm i.d.×150 mm test tube) and cultured at 22°C under 14 h light/10 h dark for further development of plantlets. The plantlets thus obtained were transplanted into pots (12 cm i.d., soil-leaf mold-Kureha compost= 3 : 1 : 1), acclimatized with covering transparent plastic cups for 1 week and cultivated in a phytotron at 20°C/16 h light and 17°C/8 h dark with 60% relative humidity.

Culture of wild-type C. japonica

Embryogenic callus cultures of *C. japonica* were induced

from the field-grown plants in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin and maintained on HF half macro salts strength (1/2) Murashige and Skoog (Murashige and Skoog 1962) (MS) solid medium containing 3% sucrose and 0.25% Gelrite at 20°C in the dark. The plantlets spontaneously regenerated on the 1/2 MS solid medium in the dark and were transferred onto the WPGHF solid medium (30 ml in 30 mm i.d.×150 mm test tube) and cultured at 22°C under 14 h light/10 h dark for further development of plantlets. The developed plantlets were transplanted into pots and cultivated in a phytotron as previously mentioned.

Detection of transgene by PCR

The polymerase chain reaction (PCR) was used to detect the foreign gene integrated into the plant genome. Genomic DNA was prepared from calli or leaves of *C. japonica* transformants (100 mg fresh weight each sample) using Nucleon phytopure (Amersham). In the PCR, a set of primer pair, which was designed to detect the *hpt* gene, was used to amplify the 450 bp fragment (Yazaki et al. 1998). The PCR mixture (total volume 50 μl) contained 100 ng of genomic DNA as the template, 1× PCR buffer, 1.2 mM $MgSO_4$, 15 pmoles of each primer, 0.2 mM deoxyribonucleotide triphosphate, 5% dimethyl sulfoxide and 1 unit of KOD-plus DNA polymerase (TOYOBO). After PCR amplification, 5 μl of the reaction mixture was loaded on an agarose gel (1.5%) for electrophoresis.

Northern and Southern hybridization

Total RNA was prepared from *C. japonica* transformant callus with *Cjmdr1* sense and antisense using RNeasy Plant Mini Kit (Qiagen). Aliquots of 7.5 μg of RNA were loaded on formamide-containing agarose gel, electrophoresised, blotted onto a nylon membrane and hybridized with *hpt* gene fragment as the probe according to a standard procedure. Ribosomal RNA (rRNA) was used as the load control. The genomic DNA digested with either *Eco*RI, *Eco*RV or *Hin*dIII was separated by electrophoresis, blotted onto a nylon membrane and hybridized with *hpt* gene fragment as the probe.

Organ-specific expression of Cjmdr1 of Cjmdr1-sense transformant

Total RNA was isolated from roots, petioles, and leaves of stable transformants of *C. japonica* as well as the wild-type plant. As the hybridization probe, 3′-untranslated region (UTR) of *Cjmdr1* (200 bp) was used. The rRNA was used as the load control.

Quantification of berberine content

For HPLC analysis, alkaloids were extracted from each freeze-dried organ using HCl-acidified methanol until the plant material lost their yellow color. The methanol extract was subjected to HPLC analysis: mobile phase, 50 mM tartaric acid solution containing 10 mM SDS–acetonitrile–methanol (100 : 100 : 25); column, TSK-GEL ODS-80TM (TOSOH, 4.6 i.d.×250 mm); temperature, 40°C; flow rate, 1.2 ml/min; detection, absorbance measured at 260 nm.

Results

To establish a stable transformation method for *C. japonica*, a *R. radiobacter* Gv3101 (pMP90) harboring the binary vector pBin-GUS was used. The *Rhizobium* vector carried the *gus* gene driven by CaMV 35S promoter as well as the *hpt* gene as the selection marker (Figure 1). Young petioles of *C. japonica* plant were sterilized and infected with the *R. radiobacter* using three different methods (Table 1). For callus generation from the infected petioles, culturing in the absence of hygromycin after the infection was necessary. This is because the application of a continuous selection pressure by hygromycin directly after the *R. radiobacter* infection caused browning of the petioles. No callus generation was observed from petioles that turned brown. Among the three infection methods, method II was the most effective for the induction of GUS-positive transformants showing 33.3% efficiency as explant bases. Since the calli was formed on the both cut ends of the petiole explants and on the wounded sites (2 to 3 callus clones per one explant), in total 7 clones of GUS transformants were obtained by the method II. Among the total 28 hygromycin-resistant callus clones obtained by method I and II, 13 clones showed GUS activity (46.4%). Thus *C. japonica* stable transformants were successfully obtained with high efficiency.

As an application of the transformation method established, we transformed *C. japonica* with the cDNA of *Cjmdr1* in the sense and antisense orientation (Figure 1) to alter its endogenous expression. We obtained 2 hygromycin-resistant callus lines from the

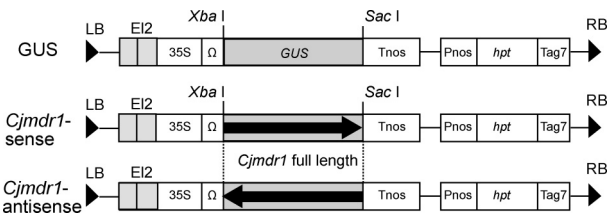

Figure 1. T-DNA regions of binary constructs introduced into *C. japonica*, pBin-GUS, pBin-*Cjmdr1*-sense and pBin-*Cjmdr1*-antisense. Each gene was driven by El2 promoter that had two enhancer sequences in tandem upstream of the CaMV 35S promoter. Full-length *Cjmdr1* cDNA was used to construct both sense and antisense vectors. *hpt*, hygromycin phosphotransferase; RB, right border; LB, left border; Pnos, nopaline synthase promoter; Tag7, agropine terminator 7.

Table 1. Transformation efficiency of *C. japonica* petiole explants by use of *R. radiobacter* Gv3101 harboring a plasmid pBin-GUS.

Method	Preculture		Infection treatment	No. of segs. infected	Callus formation		Transformation		
	Medium	Duration			No. of segs.	Efficiency %	No. of segs.	Efficiency %	No. of clones
I	WPGHF	9 days	Co-culture for 3 days	28	8	28.6	2	7.1	6
II	WPGNK	16 days	Co-culture for 2 days	15	13	86.6	5	33.3	7
III			Immersion for 60 min	12	11	91.7	0	0	0

Transformation was confirmed by GUS assay.
WPGHF: Phytohormone-free WP solid medium containing 10 mg l^{-1} L-glutamine
WPGNK: WP solid medium containing 10 mg l^{-1} L-glutamine, 1 mg l^{-1} NAA and 2 mg l^{-1} kinetin

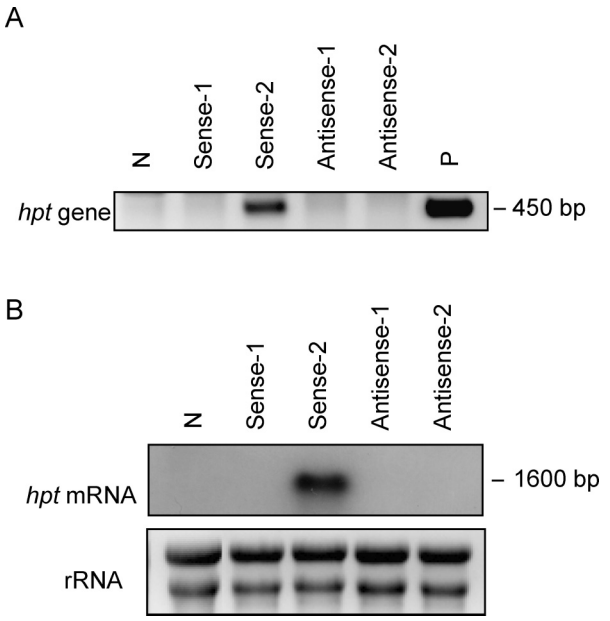

Figure 2. Integration of *hpt* gene in the genome of *C. japonica* transformants and its expression. (A) Genomic PCR to detect *hpt* gene integrated in plant genome. An internal fragment of *hpt* gene (450 bp) was amplified. N, negative control using genomic DNA of wild-type *C. japonica* cultured cell; P, positive control in which plasmid DNA (pBin-*Cjmdr1*-sense) was used as the template. (B) Northern analysis of *hpt* gene in transgenic *C. japonica* plant. Total RNA (7.5 μg) was isolated from each transgenic *C. japonica* callus line, which was separated on formamide-containing agarose gel, blotted and hybridized with *hpt* gene fragment as the probe. rRNA was used as the load control, which was stained with ethidium bromide. N, negative control using total RNA of *C. japonica* cultured cell.

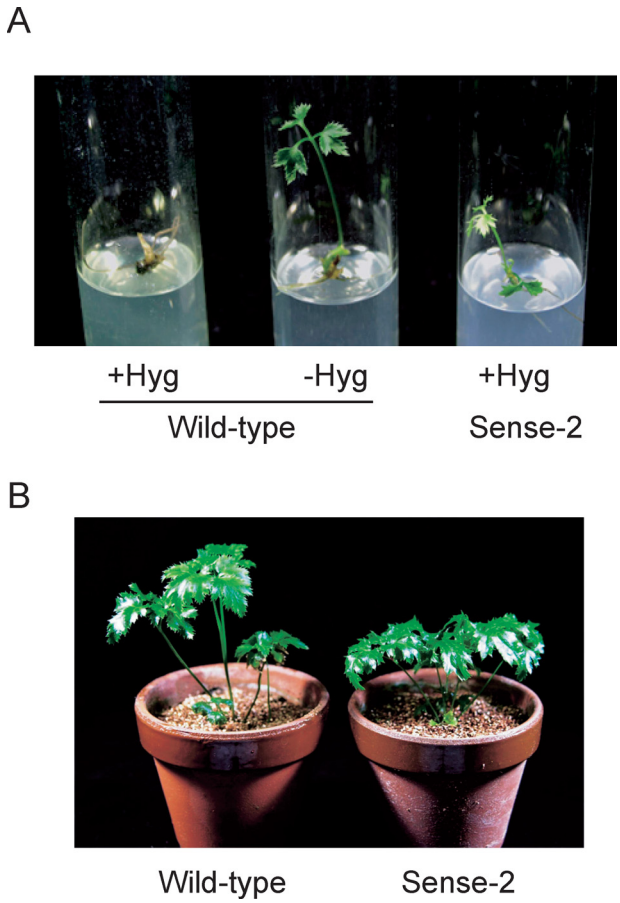

Figure 3. Stable transformants of *C. japonica*. (A) Regenerated plantlets of wild-type and transgenic *C. japonica* grown on selection medium with/without hygromycin. Left, wild-type with hygromycin; middle, wild-type without hygromycin; right, transformant Sense-2 with hygromycin. These plants were cultured on WPG solid medium at 22°C under 14 h light/10 h dark. (B) Wild-type (left) and transformant Sense-2 (right) plants grown on the soil for 3 months in a phytotron at 20°C/16 h light and 17°C/8 h dark.

transformation with sense construct (designated Sense-1 and Sense-2) and also 2 lines from antisense transformation (designated Antisense-1 and Antisense-2). The integration of the *hpt* gene in the genome and its expression in these callus lines were confirmed by genomic PCR and Northern hybridization, respectively. As shown in Figure 2A, a clear band at about 450 bp was amplified from the genomic DNA of Sense-2 by the PCR. Northern hybridization also showed that *hpt* gene was expressed in the Sense-2 callus (Figure 2B), whereas neither genomic PCR nor Northern analyses gave the PCR product for *hpt* in other clones, suggesting that only Sense-2 clone was a stable transformant.

Plantlets were regenerated from the Sense-2 callus by culturing on the WPGHF solid medium. The regenerated plant of Sense-2 transformant showed clear hygromycin resistance, whereas the regenerated plant from wild-type callus could not survive on the hygromycin medium (Figure 3A). The *in vitro* culture of Sense-2 transformant

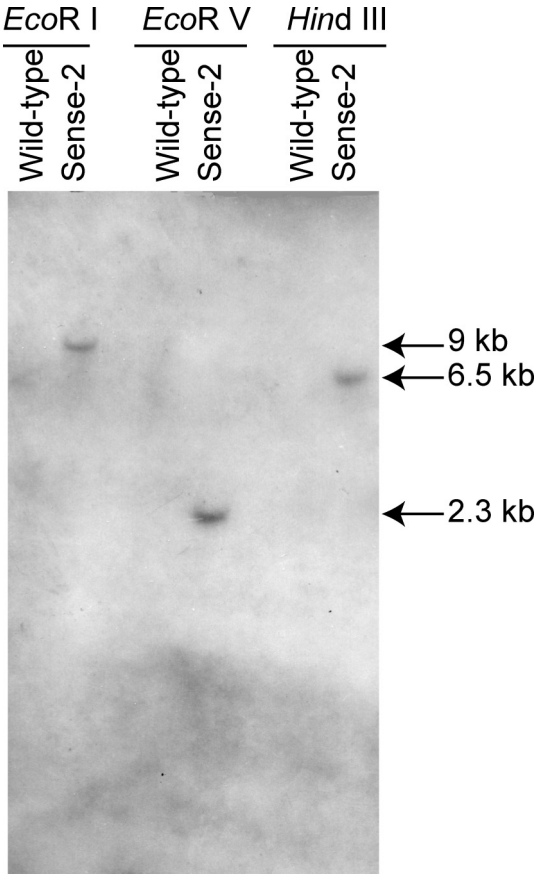

Figure 4. Southern blot analysis of the *hpt* gene. Genomic DNA (10 μg) prepared from regenerated leaves of the wild-type and Sense-2 clone was digested either with *Eco*RI, *Eco*RV or *Hin*dIII, followed by separation in an agarose gel, which was transferred to a nylon membrane and hybridized with *hpt* gene fragment as the probe.

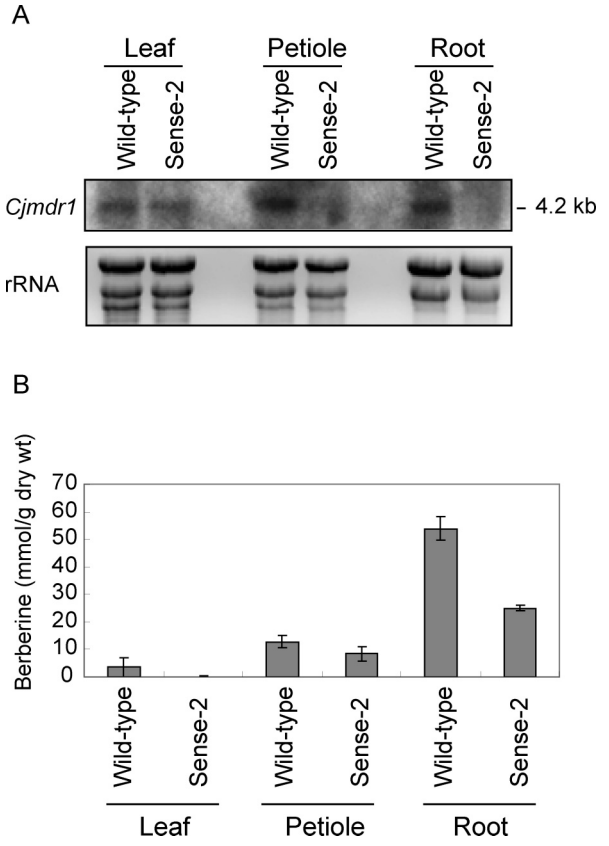

Figure 5. Northern hybridization of *Cjmdr1* (A) and berberine accumulation in the transformant Sense-2 (B). (A) Organ-specific expression of *Cjmdr1* mRNA in the wild-type and transgenic Sense-2 plant. Total RNA (7.5 μg) was isolated from each organ, with which Northern hybridization was carried out as described in Materials and methods with the *Cjmdr1* 3′-UTR as the hybridization probe. rRNA detected with ethidium bromide was used as the load control. (B) Berberine accumulation in wild-type and transgenic Sense-2 plants. Berberine was extracted from each freeze-dried material and quantified by HPLC analysis.

and the wild-type were transplanted onto the soil, and grown in a phytotron for 3 months. Sense-2 did not show morphological aberration when compared with wild-type plant (Figure 3B). In the genomic Southern hybridization using *hpt* gene fragment as the probe, only one band was detected in Sense-2 transformant, which suggested that Sense-2 plant had a single copy of the integrated T-DNA (Figure 4).

As a preliminary experiment, we examined whether the expression of *Cjmdr1* was affected by the introduction of 35S promoter-driven *Cjmdr1* at the mRNA level. RNA samples were prepared from the leaves, petioles and roots of Sense-2 transformant as well as those from wild-type of the same age. We were unable to analyze the rhizome, the main part of berberine accumulation and *Cjmdr1* expression, because the rhizome grew very slowly and no clear rhizome tissue was observed at this growth stage tested. Figure 5A shows the *Cjmdr1* mRNA levels in each organ of the Sense-2 plant were lower in all sections analyzed than those in wild-type plant, although this transgenic plant should have been the overexpressor of *Cjmdr1*. It is thus presumed that co-suppression occurred in this Sense-2 line. Similarly the berberine production in the Sense-2 plant was also lower than that of wild-type plant (Figure 5B), which is in conformity with the function of CjMDR1 involved in the cellular accumulation of berberine.

Discussion

Plant cells produce a large variety of alkaloids that have diverse chemical structures and biological activities. Some of them are used as medicines, such as anticancer drugs, analgesics and antimicrobiotics. In many cases, the chemical structures of the alkaloids are too complicated to be chemically synthesized on a commercial base (Raskin et al. 2002). Thus, plants are still main resources for the supply of alkaloids in the market, although some species are on the brink of extinction. Therefore, it is getting more difficult to meet the increasing demand for these valuable alkaloids.

Molecular breeding to establish new plant resources that accumulate high amounts of desired pharmaceuticals is an effective approach to solve the current problems (Yazaki 2004). In general, however, the establishment of a reasonable transformation method is a big hurdle, in particular, for such plant species that produce valuable secondary metabolites.

In the present study, we established the transformation method of *C. japonica* plant, a valuable medicinal plant in Asia. The preculture of petiole explants could enhance transformation efficiency especially in the presence of $1\,mg\,l^{-1}$ NAA and $2\,mg\,l^{-1}$ kinetin. After the infection, callus was induced by culturing the petiole in the absence of hygromycin. This was the key process to obtain the stable transformant calli, since continuous selection pressure by hygromycin seemed to cause too much damage to the cells. This phenomenon not to be observed in tobacco or Arabidopsis may be because the *Coptis* plant produces a large amount of alkaloids that could decompose to give toxic catabolites where non-transformed cells are dead on hygromycin medium. A similar report was also made in *Lithospermum erythrorhizon* that also contain high amount of phenolic secondary metabolites (Yazaki et al. 1998).

The transformation efficiency appeared to be different between *gus* and *Cjmdr1*. More than 40% hygromycin-resistant callus lines generated from GUS transformation showed GUS activity, whereas 7.6 and 18.8% stable transformants of sense and antisense clones out of hygromycin resistant callus lines, respectively, were generated from *Cjmdr1* transformation in subsequent transformation (data not shown). These results suggested that this difference of transformation efficiency might depend on the gene, i.e., *Cjmdr1* cDNA might have a difficulty in the integration of T-DNA due to the large insert size. Increasing the transformation efficiency is necessary for further use of this protocol to introduce various useful genes into *C. japonica*.

Analyses of *Cjmdr1* transformant (Sense-2) showed that *Cjmdr1* mRNA levels in the Sense-2 plant were lower than those of wild-type plant in all organs examined probably due to the co-suppression, and accordingly the berberine accumulation was also strongly reduced. This clear correlation between *Cjmdr1* expression and berberine accumulation supports our previous finding that CjMDR1 is involved in berberine uptake by the *C. japonica* cells. This data is the first example of transport engineering to alter the accumulation of secondary metabolites by modification of transporter gene expression. For precise statistic analysis, more transgenic *C. japonica* plants, both suppressor and overexpressor of *Cjmdr1*, need to be characterized.

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