composition difference in them (morphine was detected in the WT, however, almost no morphine in the mutant [8]).

Kinetic studies on recombinant T6ODM and CODM from *P. somniferum* [10] have revealed that oripavine is the most preferred substrate of T6ODM, followed by thebaine, while codeine is not accepted as a substrate. On the other hand, CODM showed a higher preference for codeine than thebaine. Considering the substrate preference of these two demethylases, thebaine can be accumulated solely only under the condition that the expression of both *T6ODM* and *CODM* is suppressed, and the suppression of *CODM* may result in accumulation of codeine. In actuality, however, a large amount of thebaine with a smaller amount of codeine is accumulated in the latex from mature plants of PsM1-2 mutants. This pattern of compounds detected in the mutant is similar to that of the *T6ODM*-silenced transformant by virus-induced gene silencing [10]. In contrast, the *CODM*-silenced transformant accumulates mainly codeine, together with smaller amounts of thebaine and morphine [10]. Although the alkaloid productivities of those transformants cannot be simply compared with PsM1-2, as the alkaloid composition varies highly even among the cultivars [14], it is assumed that suppression of CODM did not simply lead to the thebaine accumulation in PsM1-2. And it is also possible that in a Japanese cultivar that does not have the pathway from thebaine to morphine via oripavine [11,12], the substrate preferences of T6ODM and CODM differ from those of oripavine-producing cultivars.

As the regulation of opium alkaloid production in *P. somniferum* is highly complicated and varies among cultivars—and even among the developmental stages [15,16] or individual parts of a single plant [17]—further detailed studies on the molecular regulation of alkaloid production, such as expression analyses of *T6ODM* and *CODM* in the latex-producing capsule of PsM1-2, are required.

3. Experimental Section

3.1. Plant Materials

The wild type (WT) plant of *P. somniferum* L. used was the Japanese cultivar "Ikkanshu", and the *A. rhizogenes* strain MAFF03-01724 T-DNA insertion mutant line was PsM1-2 [8]. The *in vitro* culture of PsM1-2 used in this experiment was previously subjected to a single round of cryopreservation and regenerated to plantlet on Murashige-Skoog (MS) solid media [18] by the method described previously [19,20] with slight modifications.

3.2. Maintenance and Cultivation of Plant Materials

The WT plant seeds were obtained from the field-grown plants at the Research Center for Medicinal Plant Resources, Division of Tsukuba.

The PsM1-2 T_0 *in vitro* shoot culture was maintained on MS solid media at 20 °C under a 14 h light/10 h dark condition and then transplanted in soil in a 9 cm diameter pot and acclimatized in a phytotron in 60% relative humidity under a cycle of 16 h light at 20 °C and 8 h dark at 17 °C.

Seeds of T_1 plant obtained from the soil-cultivated plant of the PsM1-2 T_0 primary mutant were sown on the soil in a 15 cm diameter pot and cultivated in a greenhouse under a 16 h light/8 h dark cycle at 20 °C and 60% relative humidity. Plants were fertilized with 500-fold diluted Hyponex® (Hyponex Japan, Osaka, Japan) once a week.

T_2 seeds from the two lines of T_1 plants that showed high thebaine content and had abundant mature seeds were selected for cultivation of T_2 progeny. The cultivation conditions were the same as for T_1 plants.

T_3 seeds from two lines of T_2 plants with high thebaine content were germinated on rock wool with fertilization with 2,000-fold diluted Hyponex® in a greenhouse under a 16 h light/8 h dark cycle at 20 °C and 60% relative humidity. After one month, seedlings were transplanted onto the soil in a 9 cm diameter pot, and grown in the growth chamber under a 12 h light/12 h dark condition (short day condition) at 20 °C and 60% relative humidity. *Ca.* 80 days after sowing, the lighting was changed to a long day condition of 16 h light/8 h dark at 20 °C and 60% humidity for flowering. After transplanting, plants were fertilized with 500-fold diluted Hyponex® once a week.

For each experiment, WT plants were grown together as an experimental control. All self-pollination events were performed manually.

3.3. Phenotypic Observation of the PsM1-2 Mutants

Phenotypic parameters such as days to flowering, number of petals, appearance of splitting on the boundary of the petal, and the height of the aerial part at the seed-filling stage, were observed on each plant.

3.4. HPLC Analysis of Alkaloid Content in the Latex

The opium alkaloid content in the latex was analyzed by HPLC. Latex was collected from the capsule of either the WT or mutant *P. somniferum ca.* two weeks after flowering, by incising the capsule surface. Collected latex was dried at 50 °C. Approximately 5 mg of dried latex was measured accurately and subjected to alkaloid extraction by adding 5 mL of methanol followed by 30 min of sonication and mixing thoroughly using a tube mixer. After centrifugation at 20,000× *g* for 1 min, supernatant was applied to an Ultrafree-MC spin column (Millipore, Bedford, MA, USA) and centrifuged at 20,000× *g* for 1 min, and then 5 μL of the flow through was injected into an HPLC column. The HPLC conditions were as follows. HPLC instruments: Waters Alliance PDA System (separation module: 2795; photodiode array detector: 2996) (Waters, Milford, MA, USA). Column: TSK-GEL ODS100V (pore size 5 μm, ϕ4.6×250 mm) (Tosoh, Tokyo, Japan). Solvent system: CH_3CN (A), 10 mM sodium 1-heptanesulphonate (pH 3.5) (B). Solvent gradient (A%): 0 min 28%, 15 min 34%, 25 to 39 min 40%, 40 min 28%. Detection: UV 200 to 400 nm (spectrometric identification of compounds), UV 284 nm (quantitative analysis). Column temperature: 30 °C. Flow rate: 0.7 mL/min. HPLC data were collected and analyzed by an Empower system (Waters).

Alkaloid components were identified by the comparison of retention time and the UV spectra with authentic standards. Morphine hydrochloride and codeine phosphate were purchased from Takeda Pharmaceutical Company Limited (Osaka, Japan). Oripavine was a gift from Einar Brochmann-Hanssen (University of California, San Francisco, CA, USA). Magnoflorine iodide and jateorrhizine were gifts from Akira Ikuta (Science University of Tokyo, Japan). Reticuline and columbamine were gifts from Fumihiko Sato (Kyoto University, Japan). Isothebaine was isolated from *Papaver pseudo-orientale* (Fedde) Medw. by our group. Thebaine was a gift from Ruri Kikura-Hanajiri (National Institute of Health Sciences, Japan). Papaverine hydrochloride, noscapine hydrochloride, coptisine chloride, sanguinarine chloride, and berberine chloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). Alkaloid contents were calculated as a weight percent of the dried latex (opium).

3.5. Genomic DNA Preparation from P. somniferum

Genomic DNA was prepared from *ca.* 100 μg of fresh leaves of selfed plants grown in the growth chamber, or from *ca.* 100 μg of whole *in vitro* plantlet of the PsM1-2 T_0 mutant, which mainly consisted of leaves and stems, by using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

3.6. Analysis of T-DNA Insertion Loci by IPCR and AL-PCR

The inverse-PCR (IPCR) method [21,22] and adaptor ligation PCR (AL-PCR) method were used for the analysis of the flanking unknown genome DNA sequence, adjacent to the inserted T-DNA. In this study, the Vectorette PCR method [23-25], an improved method of AL-PCR, was employed to reduce non-specific amplicons.

The genomic DNA library for each PCR method was constructed by digestion of genomic DNA by the appropriate restriction enzymes and self-ligation to form a circular DNA library, or an adaptor linker attached genome DNA library.

3.7. Genomic Library Construction for IPCR

Genomic DNA was digested with the restriction enzymes *Bam*HI, *Eco*RV, *Hae*III, *Kpn*I, *Pvu*II, *Ssp*I, or *Stu*I. Completely digested DNA was ligated by using a Fastlink® DNA Ligation Kit (AR Brown, Tokyo, Japan) to form a circular genome DNA library.

3.8. Genomic Library Construction for AL-PCR

The sequences of the adaptor oligo DNA and adaptor specific primers used in this study are listed in Supplementary Table 2. Two complementary oligo DNAs, AP-LS and AP-SS, were annealed to form an adaptor unit. Genomic DNA was digested with the restriction enzymes *Eco*RV, *Hae*III, *Pvu*II, *Ssp*I, or *Stu*I, which produce blunted ends. The completely digested DNA was ligated with adaptor units by using a Fastlink® DNA Ligation Kit to form an adaptor ligated genome DNA library.

3.9. IPCR and AL-PCR

Amplification of the target region was performed by the nested PCR method using TaKaRa Ex Taq™ DNA polymerase (Takara Bio, Shiga, Japan) under the following conditions. The combinations of PCR methods, template genome DNA libraries and primer sets are listed in Supplementary Table 1. T-DNA-specific primers were designed based on the DNA sequence of the T-DNA region of the *A. rhizogenes* plasmid pRi1724 (DDBJ/EMBL/GenBank accession no. AP002086). The first PCR conditions were as follows: primary denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 1 min, 42 °C for 2 min, and 72 °C for 3 min; with a final extension at 72 °C for 10 min. After PCR, the solution was held at 4 °C. The first PCR reaction solution was applied to a SUPREC™-02 filter (Takara Bio) to eliminate the primers and then used as a template for the second PCR. Second PCR conditions were as follows: primary denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 1 min, 48 °C for 2 min, and 72 °C for 3 min; with a final extension at 72 °C for 10 min. After PCR,

the solution was held at 4 °C. The product of the second PCR was gel purified and cloned into the sequencing vector pT7-Blue® (Novagen, Madison, WI). Propagated plasmid DNA was subjected to DNA sequencing using a BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI PRISM® 3100—Avant Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan). Homology search was performed on T-DNA flanking genome DNA sequences with the BLAST tool at NCBI.

3.10. Direct Amplification of T-DNA Borders Connected in Tandem

PCR was performed on uncut genome DNA of T_0 to amplify the border region of T-DNAs connected in tandem. The primers used are listed in Supplementary Table 1. The PCR conditions were the same as for the IPCR.

3.11. Analyses of T-DNA Insertion Loci and Heredity Manner by PCR

The PCR method was employed to confirm the T-DNA integration loci on *P. somniferum* genome DNA and to analyze the heredity manner in the selfed progenies.

To find out the tally of the paired genomic regions found adjacent to the T-DNA left borders (LBs) and right borders (RBs) revealed by IPCR and AL-PCR analyses, PCR amplification was performed with the pair of genomic region-specific LB and RB (e.g., LB1g *vs.* RB2g) primers listed in Supplementary Table 3, under the following PCR conditions: primary denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. After PCR, the solution was held at 4 °C. TaKaRa Ex Taq™ was used as the PCR polymerase. The PCR product was separated on agarose gel. The paired genomic regions, which gave a PCR product was judged as the single T-DNA integrated locus.

To judge whether or not the T-DNA integration loci were present in the selfed progenies, PCR amplification was performed between the genome region-specific primers listed in Supplementary Table 3 and T-DNA LB- or RB-specific primers (MAFF-226A or MAFF-14963S). PCR was performed under the following PCR conditions: primary denaturation at 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. After PCR, the solution was held at 4 °C. GoTaq® Green Master Mix (Promega, Madison, WI, USA) was used as the PCR polymerase. The PCR product was separated on agarose gel.

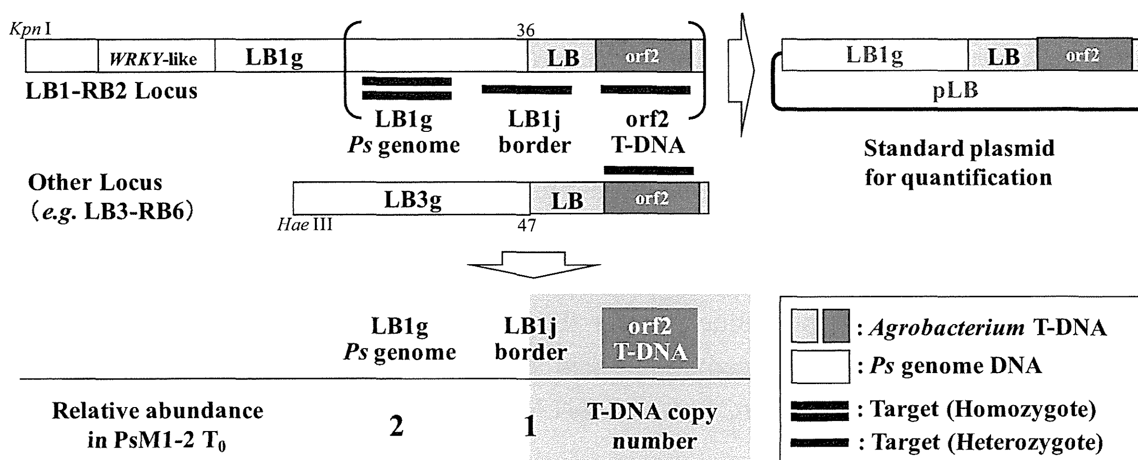
3.12. T-DNA Copy Number Analysis by Real-time PCR

The T-DNA copy number was analyzed by the quantitative real-time PCR method [26,27]. The strategy used for estimating the T-DNA copy number is as follows. When information of one of the integrated T-DNA sites was provided by T-DNA integrated loci analysis, and also, when the T-DNA integrated site was a single copy, the copy number of the integrated T-DNA could be calculated as a multiple of the relative abundance of standard DNA fragments, as shown in Figure 9.

This estimation method can be enacted under the hypotheses that (1) the genome DNA of PsM1-2 is diploid (2n = 22) [28], (2) all of the T-DNA is integrated into the host genome DNA in a heterozygous manner, and (3) one of the integrated T-DNAs for which both the LB and RB borders are known (e.g., the LB1-RB2 locus) is a single copy.

Under these hypotheses, by comparing the relative abundance of the T-DNA internal region (in this case, orf2), the T-DNA—*P. somniferum* genome junction region (LB1j), and the *P. somniferum* genome region (LB1g), we can calculate the inserted T-DNA copy number by fixing the abundance of LB1g as two.

Figure 9. Schematic diagram of the strategy of T-DNA copy number analysis by real-time PCR.



The LB1-RB2 locus whose LB and RB ends are confirmed was used as a standard. By setting the abundance of the genome DNA region (LB1g) of LB1 at a standard value of 2, the relative abundance of LB1j could be calculated as 1. The relative abundance of the orf2 region in the sample corresponded to the integrated T-DNA copy number. For the quantification, a standard plasmid pLB was constructed. "*Ps* genome" indicates the genome DNA of *P. somniferum*.

In our experiment, one of the T-DNA-integrated sites, LB1-RB2, which will be described in the Results section, was set as a standard. And for the quantification standard plasmid DNA, we constructed pLB1, which included, the LB1g, LB1j, and orf2 regions of the T-DNA. A DNA fragment with these three regions was amplified by PCR with the primers LB1-orf2-S (5'-CTC ATA AGC AGT GGT ATT GCT C-3') and LB1-orf2-A (5'-CGC ATT CAT GCG GTT ATG GAG-3') and KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan) under the following PCR conditions: primary denaturation of 94 °C for 2 min; followed by 35 cycles of 94 °C for 15 s, 62 °C for 30 s, 68 °C for 90 s. After PCR, the solution was held at 4 °C. The amplified product was cloned into the pT7-Blue® vector (Novagen) and then propagated in *E. coli*. The quantitative standard plasmid DNA pLB1 and genome DNA prepared from the primary T_0 mutant and selected T_1, T_2, and T_3 progenies of the PsM1-2 mutant were diluted serially with the dilution buffer supplied with the real-time PCR reagent SYBR® Premix Ex Taq™ II (Perfect Real Time; Takara Bio). Real-time PCR was run using the target region-specific primers listed in Supplementary Table 4 with the real-time PCR reagent on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems Japan). The obtained data were analyzed using the supplied software (Applied Biosystems Japan) and the relative abundance of each target region was deduced from each Delta Rn value using standard curves. Standard curves for each target region were plotted with the plasmid concentration (fg/μL) on the x-axis and the Delta Rn on the y-axis. The curves showed good correlations. The relative abundances of each target region were calculated so that the abundance of the LB1g region was 2, and then rounded off to a whole number.

3.13. Actin Gene Amplification from P. somniferum

A fragment of actin cDNA was amplified by degenerate PCR using the forward primer 5'-AAR GCN AAY MGN GAR AAR ATG AC, and the reverse primer 5'-CCR TAN ARR TCY TTN CKD ATR TC, which were designed from the completely conserved regions of the amino acid sequences of other actins, such as *Arabidopsis thaliana* (*actin-1*: DDBJ/EMBL/GenBank accession No. M20016), *Nicotiana tabacum* (*actin*: X63603), and *Zea mays* (*Maz56*: U60514). cDNA synthesized from the total RNA of young seedlings of *P. somniferum* was used as a template for PCR. The manual hot-start procedure was used for the amplification. TaKaRa Ex Taq^{TM} DNA polymerase was added after primary denaturation at 94 °C for 5 min, and then the following protocol was carried out in a GeneAmp2400 thermal cycler (Applied Biosystems Japan): 30 cycles of 94 °C for 1 min, 48 °C for 2 min, and 72 °C for 3 min; with a final extension at 72 °C for 10 min. After PCR, the solution was held at 4 °C. The amplified fragment was cloned into the pT7-Blue® vector followed by DNA sequencing. Two representative actin cDNA sequences, whose deduced amino acid sequences showed 92% and 95% identity to the *Arabidopsis actin-1*, were named *PsACT1* (AB574417) and *PsACT2* (AB574418), respectively.

3.14. Expression Analysis of the Morphine Biosynthetic Genes

The expression levels of selected morphine biosynthetic genes downstream of (*S*)-*N*-methylcoclaurine, *CYP80B3* (DDBJ/EMBL/GenBank accession no. AF134590 [29]), (*R,S*)-3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (*4'OMT*; AY217333 [15]), *SalAT* (AF339913 [30]), *T6ODM* (GQ500139 [10]), *COR* (allele *Cor1-1*: AF108432; allele *Cor2-1*: AF108438 [31]), and *CODM* (GQ500141 [10]) in the WT plant and the PsM1-2 mutant were analyzed and compared by the reverse transcription PCR (RT-PCR) method.

Total RNA was prepared from the whole plants of two-week-old seedlings of field-grown WT *P. somniferum*, or from whole *in vitro* plantlet of the PsM1-2 T_0 mutant, which mainly consisted of leaves and stems, by using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA samples was subjected to single-stranded cDNA synthesis by reverse-transcription with oligo-(dT) primer (RACE32: 5'-GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TT-3') [32] using Superscript® II Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Synthesized ss-cDNA was used as a template for PCR with the gene-specific primers listed in Supplementary Table 5. The PCR conditions were as follows: primary denaturation 94 °C for 5 min; followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; with a final extension at 72 °C for 10 min. After PCR, the solution was held at 4 °C. PCR products were separated on 1.0% agarose gel and signal intensities were observed. The actin gene *PsACT1* from *P. somniferum* was used as an experimental control.

3.15. Statistical Analysis

Values were expressed as the mean ± standard deviations (SD) and were analyzed by the Tukey-Kramer multiple comparison test using the statistical analysis system "R" software package [33]; a p value of less than 0.05 was considered significant.

4. Conclusions

By combining genetic and phenotypic analyses of the T-DNA insertional mutant PsM1-2 with selfing, we have succeeded in stabilizing the high thebaine phenotype in coordination with a reduction in the number of inserted T-DNA copies. Although the genetic mode of CODM suppression in *in vitro* plantlet and of the accumulation of thebaine still remain unknown, studies on this mutant and its progenies may provide new insights into the molecular basis of morphine biosynthesis, and could ultimately allow us to manipulate the biosynthesis of this compound at will.

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Conflict of Interest

The authors declare no conflict of interest.

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Improvement of Benzylisoquinoline Alkaloid Productivity by Overexpression of 3′-Hydroxy-*N*-methylcoclaurine 4′-*O*-Methyltransferase in Transgenic *Coptis japonica* Plants

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Coptis japonica (_Cj_) rhizomes are used as a crude drug for gastroenteritis, since they accumulate antimicrobial berberine. Berberine also shows various useful bioactivities, including cholesterol-lowering activity. Unfortunately, _Cj_ is a slow-growing plant and more than 5 years are required to obtain a crude drug suitable for the Japanese Pharmacopoeia. To improve alkaloid productivity, we overexpressed the 3′-hydroxy-_N_-methylcoclaurine 4′-_O_-methyltransferase (_4′OMT_) gene in _Cj_. We established the transgenic plant (named _Cj_HE4′) by introducing one copy of _Cj_4′OMT by _Agrobacterium_-mediated transformation. The successful overexpression of _4′OMT_ was confirmed in all tissues of _Cj_HE4′ by real-time polymerase chain reaction (PCR) analysis. HPLC analysis revealed that the berberine content of _Cj_HE4′ leaves and roots cultivated for 4 months was increased to 2.7- and 2.0-fold, respectively, compared with non-transgenic wild-type (_Cj_WT), and these inductions of alkaloids were stable for at least 20 months. Furthermore, in _Cj_HE4′ cultivated for 20 months, the berberine content in medicinal parts, stems and rhizomes was significantly increased (1.6-fold). As a consequence, increased amounts of alkaloids in _Cj_HE4′ resulted in the improvement of berberine yields (1.5-fold), whereas _Cj_HE4′ showed slower growth than _Cj_WT. These results indicated that 4′OMT is one of the key-step enzymes in berberine biosynthesis and is useful for metabolic engineering in _Cj_.

Key words benzylisoquinoline alkaloid; *Coptis japonica*; metabolic engineering; transgenic plant; 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase; berberine

Coptis japonica MAKINO var. *dissecta* NAKAI (*Cj*) is an important medicinal plant. Its rhizome is a useful crude drug for gastroenteritis, since they highly accumulate bioactive benzylisoquinoline alkaloids. The main alkaloid of *Cj*, berberine, shows significant antimicrobial activity against both bacteria and fungi.[1] In addition, it has recently been reported that berberine has beneficial effects on metabolic syndromes, for example, blood cholesterol-lowering activity *via* up-regulation of low-density lipoprotein receptor expression,[2] anti-hypertension effects *via* induction of mobilization of circulating endothelial progenitor cells,[3] and lowering of blood glucose level through increasing insulin receptor expression.[4] Accordingly, various clinical trials of berberine have been conducted.[2–4] Unfortunately, *Cj* is a slow-growing plant, and more than 5 years are required to obtain a crude drug suitable for the Japanese Pharmacopoeia. Therefore, it would be helpful if alkaloid productivity were improved and the cultivation period shortened.

The biosynthetic pathway of benzylisoquinoline alkaloids has been well studied, and cDNA sequences of biosynthetic enzyme genes have been cloned, because various classes of pharmaceutically important compounds have the same steps in their early biosynthetic pathways as benzylisoquinoline alkaloids, such as the analgesic morphine, the antitussive drugs codeine and noscapine and the antibacterial agent sanguinarine from *Papaver somniferum* (*Ps*). In *Cj*, almost all the biosynthetic genes from tyrosine to berberine and some biosynthetic genes of related compounds have been isolated and characterized using high berberine-producing cultured cells, including norcoclaurine synthase (NCS), *S*-adenosyl-L-methionine : norcoclaurine 6-*O*-methyltransferase (6OMT), *S*-adenosyl-L-methionine : coclaurine *N*-methyltransferase (CNMT), *N*-methylcoclaurine hydroxylase (NMCH, cytochrome P450 (CYP)80B2), *S*-adenosyl-L-methionine : 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT), *S*-adenosyl-L-methionine : scoulerine 9-*O*-methyltransferase (SMT), canadine synthase (CAS, CYP719A1), *S*-adenosyl-L-methionine : columbamine *O*-methyltransferase (CoOMT), and corytuberine synthase (COS, CYP80G2) (Fig. 1).[5–7] Using these genes, we have examined the effects of overexpression of biosynthetic genes on the improvement of alkaloid productivity.

Genetic modification of the biosynthetic pathway enables us to alter the productivity and composition of useful compounds. Overexpression of rate-limiting enzyme genes can increase the end products. Antisense or RNA interference (RNAi)-mediated suppression of the pathway can decrease undesired compounds or help accumulate variable intermediates, and the expression of new genes from other species can create the pathway for new products. In the biosynthesis of benzylisoquinoline alkaloids, several papers have reported successful alteration of productivity and unexpected accumulation of alkaloids. Overexpression of *Ps* codeinone reductase (*COR*), *CYP80B3*, and salutaridinol 7-*O*-acetyltransferase (*SalAT*) resulted in an increase of the morphinan alkaloid in the latex of *Ps*[8–10] and overexpression of the *Ps* berberine bridge enzyme

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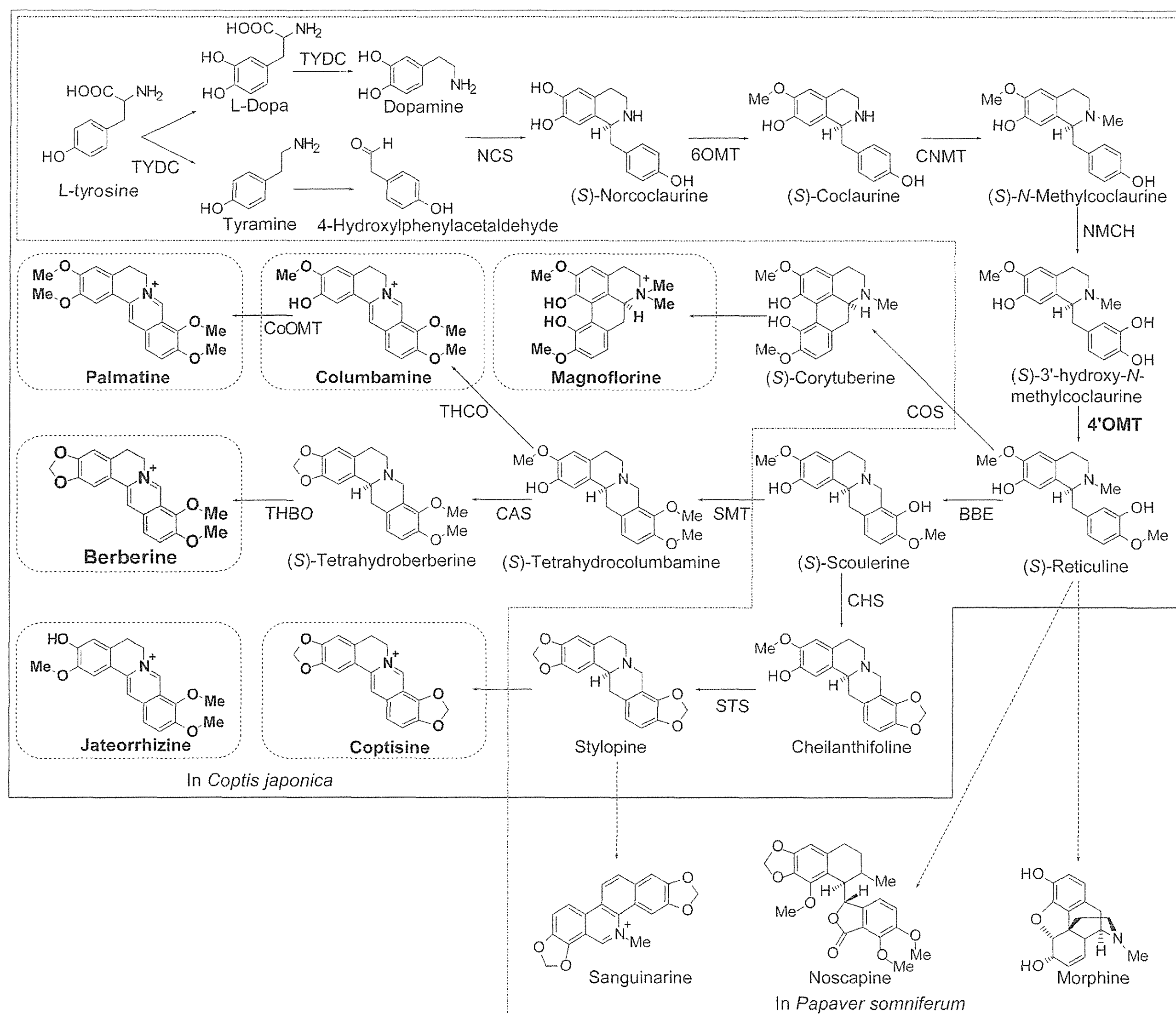

Fig. 1. A Schematic Biosynthetic Pathway for Various Isoquinoline Alkaloids in *Cj* and *Ps*

The boxes with dotted lines indicate alkaloids detected in this study. The dotted arrows indicate more than one step conversion. TYDC, L-tyrosine decarboxylase; NCS, (*S*)-norcoclaurine synthase; 6OMT, *S*-adenosyl-L-methionine:norcoclaurine 6-*O*-methyltransferase; CNMT, *S*-adenosyl-L-methionine:coclaurine *N*-methyltransferase; NMCH, *N*-methylcoclaurine 3′-hydroxylase (CYP80B2); 4′OMT, *S*-adenosyl-L-methionine:3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase; BBE, berberine bridge enzyme; SMT, *S*-adenosyl-L-methionine:scoulerine 9-*O*-methyltransferase; CAS, canadine synthase (CYP719A1); THBO, tetrahydroberberine oxidase; THCO, tetrahydrocolumbamine oxidase; CHS, cheilanthifoline synthase (CYP719A5); STS, stylopine synthase (CYP719A2/A3); CoOMT, *S*-adenosyl-L-methionine:columbamine *O*-methyltransferase; COS, corytuberine synthase (CYP80G2).

(*BBE*) in *Eschscholzia californica* (*Ec*) root culture, resulting in increased levels of the end products, benzophenanthridine alkaloids.[11] Overexpression of *Cj6OMT* in *Ec*-cultured cells also resulted in an effective increase of benzophenanthridine alkaloids.[12] On the other hand, suppression of the biosynthetic pathway showed more prominent and complicated results. RNAi suppression of *PsCOR* resulted in the surprising accumulation of reticuline, which is 7 enzymatic steps upstream of COR.[13] Antisense *PsCYP80B3*-expressing plants showed remarkable reduction of alkaloids in latex with an alteration of the alkaloid ratio.[9] Antisense *EcBBE* and *EcCYP80B1* resulted in reduced levels of alkaloids in *Ec*-cultured cells and no accumulation of intermediates,[14] whereas RNAi suppression of *EcBBE* resulted in reduced end products and accumulation of important intermediate reticuline and its derivatives.[15] Similar accumulation of reticuline and its derivatives was obtained in the latex of antisense *PsBBE* transgenic *Ps*, but not in its roots.[16] RNAi of *PsSalAT* also induced the accumulation of salutaridine, which is 2 enzymatic steps upstream of the intermediate.[10,17] These results indicated that benzylisoquinoline alkaloid biosynthesis could be intricately regulated. A new pathway was created in transgenic *Ec*-cultured cells. Overexpression of *CjSMT* in *Ec* cells resulted in the production of columbamine, which was not detected in non-transformed wild-type (WT) cells, accompanying lower levels of benzophenanthridine alkaloids as end products.[18]

In *Cj*-cultured cells, we previously reported that the ectopic expression of the branch point enzyme *Cj*SMT increased the metabolic flow to berberine and columbamine (*ca.* 15%), whereas it had little influence on the total alkaloid content.[18] In this study, *Cj4′OMT*[19] was selected as a target gene for improving the productivity of alkaloids in *Cj*, since the corresponding enzyme seemed to be one of the key rate-limiting factors in benzylisoquinoline alkaloid biosynthesis in *Cj*.

Cultured *Cj* cells showed relatively low enzyme activities in the early steps from norcoclaurine to scoulerine compared to the later steps, and in high berberine-producing cell lines, enzyme activities of the early steps, especially 4'OMT activity, were greatly induced compared to non-selected low berberine-producing cell lines.[20] We report here the establishment of transgenic *Cj* plants overexpressing *4'OMT* and the stable increase of benzylisoquinoline alkaloids in *4'OMT* transformants. This is the first report of genetically modified *Cj* plants with increased alkaloid production.

MATERIALS AND METHODS

Transformation and Plant Regeneration The *Agrobacterium*-mediated transformation method of *Cj* has been detailed elsewhere.[21] In brief, sterilized petiole segments were precultured on Woody Plant solid medium[22] containing 10 mg/L L-glutamine, 1 mg/L 1-naphthaleneacetic acid, 2 mg/L kinetin (WPGN1K2), 3% sucrose and 0.25% gelrite (San-Ei Gen F.F.I. Inc., Osaka, Japan) at 20°C in the dark. After preculture, the segments were co-cultured with the *A. tumefaciens* strain LBA4404 harboring pBHE4′ (vector for overexpression of *Cj4′OMT*[12]) for 2 d in WPGN1K2 liquid medium containing 2% sucrose; then bacteria was eliminated on WPGN1K2 medium containing 500 mg/L claforan (Sanofi Aventis, Tokyo, Japan). After 1 to 2 months, the segments which formed calli and adventitious roots were cultured on WPGN1K2 medium containing 25 mg/L hygromycin at 20°C in the dark. The hygromycin-resistant calli were subcultured on the WPGN1K2 medium at 20°C in the dark at an interval of 1 to 2 months. After elimination of bacteria and hygromycin-selection, the calli were subjected to polymerase chain reaction (PCR) analysis to confirm genetic transformation. One positive callus clone derived from a single root, which showed spontaneous plant regeneration, was selected and used for further study. The regenerated plantlets were transferred into pots (soil : Kureha compost : leaf mold=3 : 1 : 1) and cultivated in a containment greenhouse at 20°C with 16-h light in 60% relative humidity.

Detection of Transgene by PCR The integration of foreign genes into the plant genome was confirmed by PCR. Genomic DNA was prepared from *Cj*WT, *Cj*GUS[21] (transgenic plants into which the *β-glucuronidase* (*GUS*) gene had been introduced) and *Cj*HE4′ (transgenic plants into which *4′OMT* gene had been introduced) using a DNeasy Plant Mini Kit (Qiagen Sciences, Germantown, Maryland, U.S.A.), following the manufacturer's instructions. The insert was amplified with the primer for the cauliflower mosaic virus 35S promoter (CaMV35Sp: 5′-GATATCTCCACTCCACTG-ACGTAAGG-3′) and that for *Cj4′OMT* (GenBank accession No. D29812; *Cj4′OMT*-723A: 5′-AAACATGTCCGCCACCAA-TTCG-3′) or a primer pair designed to detect the hygromycin phosphotransferase (*hpt*) gene (*hpt*-220S: 5′-CGGAAGTGC-TTGACATTGG-3′ and *hpt*-703A: 5′-AGAAGAAGATGTTGG-CGACC-3′[23]). After 5 min of denaturation at 94°C, 30 cycles of PCR were performed at 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, followed by incubation at 72°C for 10 min. The presence of PCR products was confirmed by agarose gel electrophoresis.

Genomic DNA Blot Analysis Genomic DNA was prepared from 2 g fresh leaves of *Cj*WT and *Cj*HE4′ by the modified cetyl trimethyl ammonium bromide method, as previously described,[24] and digested with either *Eco*RI, *Eco*RV, *Hin*dIII, *Nde*I or *Xba*I. Thirty micrograms of digested genomic DNA was separated by 1% agarose gel electrophoresis and then blotted onto a Hybond N+ membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.). DNA fragments including integrated genes were detected with an *hpt* cDNA fragment synthesized by PCR using a set of primers mentioned above and labeled with alkaline phosphatase using the AlkPhos Direct Labeling and Detection System (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). Hybridization and signal detection were performed according to the manufacturer's instructions. In brief, the membrane was prehybridized for 90 min at 55°C in an AlkPhos Direct hybridization buffer, followed by hybridization with alkaline phosphatase-labeled probes at 55°C for 14 h. The blot was washed twice at 55°C for 10 min with 50 mM phosphate buffer (pH 7.0), 2 M urea, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, and 10 mM $MgCl_2$, then twice at room temperature for 5 min with 2 M NaCl and 1 M Tris(hydroxymethyl) aminomethane (pH 10.0). Hybridization signals were detected by CDP-*Star* chemiluminescent detection reagent. Ethidium bromide-stained bands of digested genomic DNA were used as the load control.

Quantification of Benzylisoquinoline Alkaloids Contents Benzylisoquinoline alkaloids were extracted twice from an accurately weighed powder of freeze-dried tissue (*ca.* 20 mg) under 30 min reflux at 70°C using 1.5 mL HCl-acidified methanol (10% HCl : methanol=1 : 100), and the combined extract solution was diluted to 5 mL with HCl-acidified methanol. The benzylisoquinoline alkaloid content in diluted extract solution was determined by HPLC analysis using a Waters Alliance HPLC system (Waters, Milford, Massachusetts, U.S.A.). The HPLC system consisted of the 2795 separation module and the 2996 photodiode array detector. The HPLC column was a TSKgel ODS-100V column (4.6 mm i.d.×250 mm, 5 μm, TOSOH, Tokyo, Japan). The mobile phase consisted of acetonitrile (solvent A) and 10 mM sodium 1-heptanesulphonate in water (adjusted to pH 3.5 with phosphoric acid; solvent B). Benzylisoquinoline alkaloids were resolved using the following gradient condition with a flow rate of 0.8 mL/min at 40°C (0–15 min 27–29% solvent A, 15–25 min 29–39% solvent A, 25–31 min 39–51% solvent A, 31–34 min 51% solvent A, 34–35 min 51–27% solvent A). The elution of benzylisoquinoline alkaloids was monitored at 200–400 nm, and alkaloid contents were quantified by the peak area obtained at 284 nm using standard curves.

Quantitative Real-Time PCR Total RNAs were prepared from leaf blades, petioles, stems and rhizomes, and root of *Cj*WT, *Cj*GUS and *Cj*HE4′ plants using RNeasy Plant Mini Kit (Qiagen Sciences), according to the manufacturer's instructions. The extracted total RNAs were subsequently treated with a TURBO DNA-free kit (Ambion Inc., Austin, Texas, U.S.A.) to avoid contamination by genomic DNA. cDNA was prepared from 865 ng of total RNA using a Prime Script RT reagent Kit (TaKaRa Bio Inc., Shiga, Japan) following the manufacturer's instructions. Transcript levels of biosynthetic genes were determined by the relative standard curve method using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, California, U.S.A.). Two-microliter aliquots of the 50-μL product of

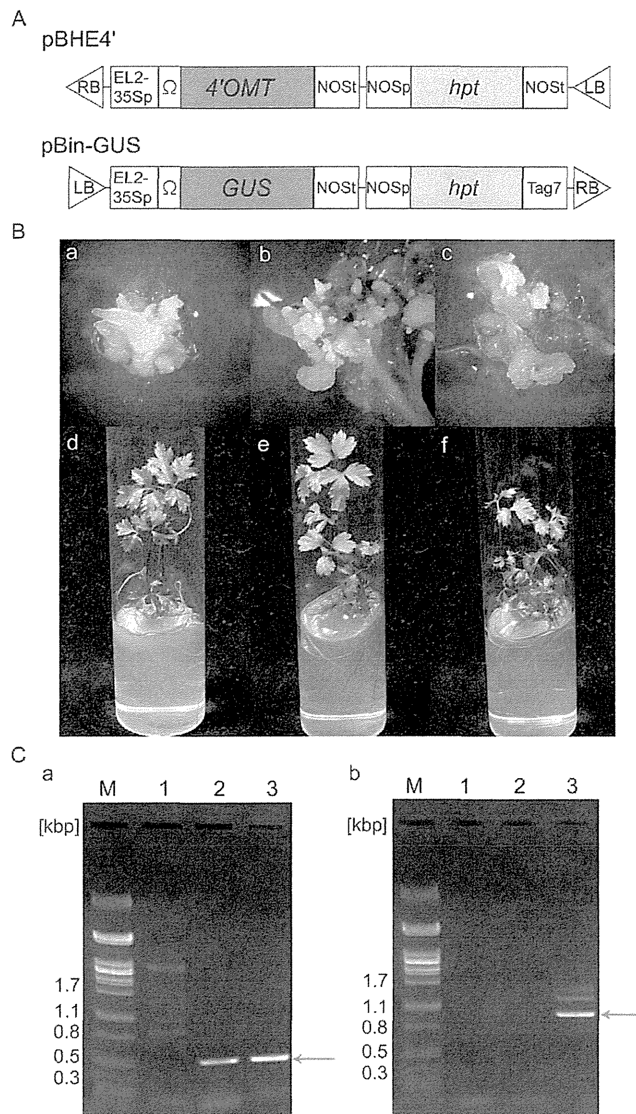

Fig. 2. *Cj* Transformed with *Cj4'OMT* or *GUS* Overexpression Vectors

(A) T-DNA regions of binary vectors introduced into *Cj* (pBHE4' and pBin-GUS). *4'OMT* and *GUS* genes were driven by modified cauliflower mosaic virus 35S promoter (EL2-CaMV35Sp). RB, right border of T-DNA; NOSt, nopaline synthase terminator; NOSp, nopaline synthase promoter; *hpt*, hygromycin phosphotransferase; Tag7, agropine terminator 7; LB, left border. (B) *Cj* cells (a–c) and regenerated plantlets (d–f). a and d, non-transformed *Cj*WT; b and e, *GUS* transformants (*Cj*GUS); c and f, *4'OMT* transformants (*Cj*HE4'). (C) Amplification of introduced genes from genomic DNA of non-transformed and transformed *Cj* plantlets. The arrow indicates the size of the expected amplified segment. (a) *hpt* primer pairs (b) CaMV35Sp and *4'OMT* primer pairs. Lane M, DNA size marker (λDNA digested with *Pst*I); Lane 1, amplification from genomic DNA of *Cj*WT; Lane 2, that of *Cj*GUS; Lane 3, that of *Cj*HE4'.

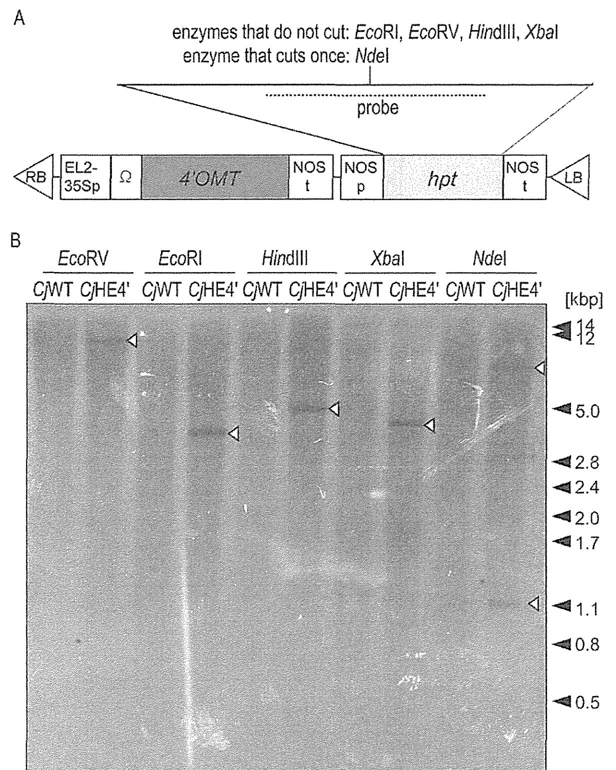

Fig. 3. Genomic DNA Gel Blot Analysis Using *hpt* Gene Fragment as a Probe

(A) A schematic representation of the transformation vector pBHE4'. The broken bar represents the hybridization probe. This region contains one *Nde*I restriction site, whereas there is no restriction site for *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I in this region. (B) Genomic DNA extracted from leaves of *Cj*WT and *Cj*HE4' was digested with either *Eco*RV, *Eco*RI, *Hin*dIII, *Nde*I or *Xba*I. Thirty micrograms of digested genomic DNA was separated by agarose gel electrophoresis and blotted onto a membrane. The transferred DNA fragment was hybridized with alkaline phosphatase-labeled *hpt* probes. Open arrows indicate the positive bands.

reverse transcription were used as templates for quantitative real-time PCR in 25 μL volumes, consisting of 9 μL sterile water, 12.5 μL SYBR premix Ex Taq (TaKaRa Bio Inc.), 0.5 μL ROX reference dye, 0.5 μL each of 10 μM Fw and Rv primers. After 10 s of denaturation at 95°C, 40 cycles of PCR were performed at 95°C for 5 s and 60°C for 31 s. The specific amplification was confirmed by a melting curve program of heating samples from 60 to 95°C at the end of cycles, and by an agarose gel electrophoresis of PCR products. To normalize the results, the *β-actin* gene (*CjACT1*; GenBank accession No. AB587096) was used as an internal control. This analysis was performed in triplicate. The primers used for amplification of *Cj4'OMT* were Fw: 5'-TTGGTGGCGACATGTTTAAATC-3' and Rv: 5'-CTTGATCGAGTCTTCGTCATTCC-3'. Those for *Cj6OMT* (GenBank accession No. D29811) were Fw: 5'-CTGGCTTTCATTCCTCTTTGTG-3' and Rv: 5'-GCACTTTCTCAGTTGGGCTTC-3'. Those for *CjNCS* (GenBank accession No. AB267398) were Fw: 5'-ATGGCTAAGAACTTGGGACTTG-3' and Rv: 5'-GCATGAGGACATGGAGGGTAG-3'. Those for *CjSMT* (GenBank accession No. D29809) were Fw: 5'-CGTTGAATCCTGGTGGTAAAGAG-3' and Rv: 5'-ATGAAGCCCTTGTGAAATTGG-3'. Those for *CjACT1* were Fw: 5'-TCGTTTGGACCTTGCTGGTAG-3' and Rv: 5'-ATTTCTCGCTCTGCTGCTGTGGTG-3'.

Statistical Analysis The mean±standard deviations are shown in the figures, and statistical differences in means were determined by Tukey–Kramer multiple comparison test using the statistical analysis System "R" software package (http://www.R-project.org/). Different letters over the tops of the columns in the figures indicate significant differences ($p<0.05$) by Tukey–Kramer's test.

RESULTS AND DISCUSSION

Establishment of *Cj* Plants Transformed with the *Cj4'*OMT Gene Sterilized segments of *Cj* petioles were transformed with *Agrobacterium tumefaciens* strain LBA4404 harboring pBHE4', which induced high constitutive expression

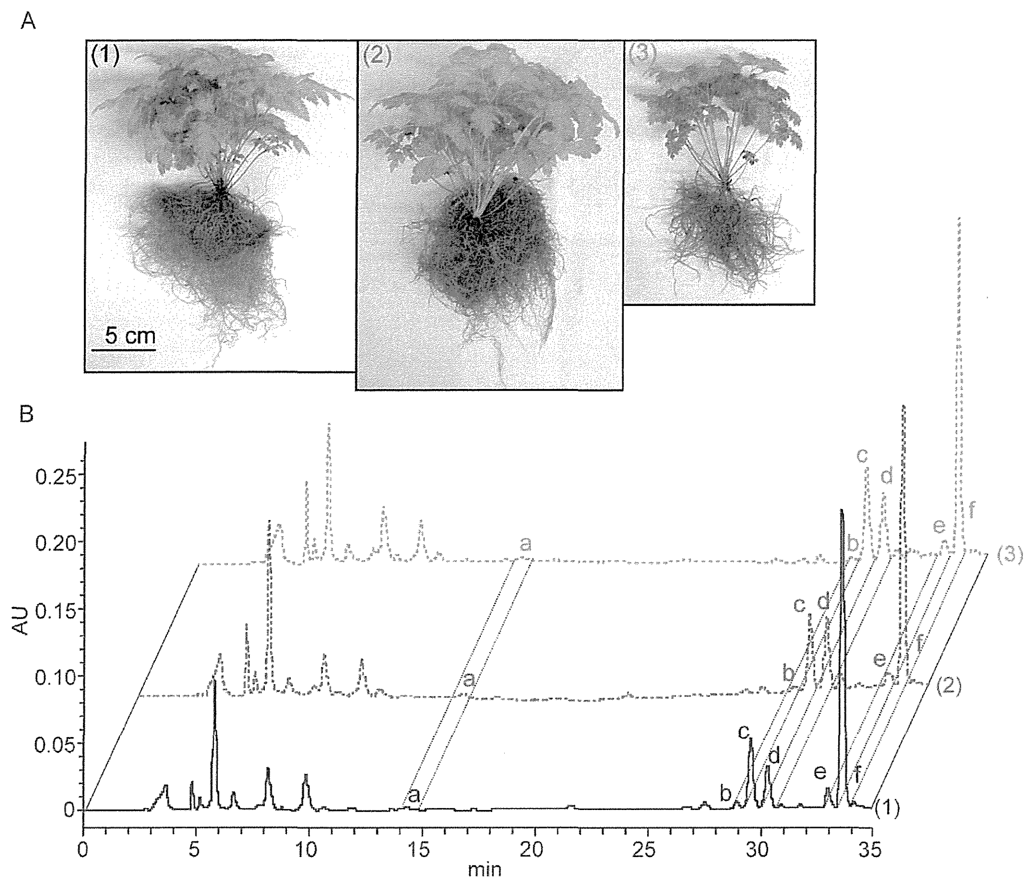

Fig. 4. Non-transgenic and Transgenic *Cj* Plants and Their Alkaloid Profiles

(A) *Cj* plants cultivated in a containment greenhouse for *ca.* 4 months. (1) *Cj*WT; (2) *Cj*GUS; (3) *Cj*HE4′. (B) HPLC profiles of *Cj* roots monitored at 284 nm. (1) solid line, *Cj*WT; (2) dash-dotted line, *Cj*GUS; (3) dotted line, *Cj*HE4′. a, magnoflorine; b, columbamine; c, jateorrhizine; d, coptisine; e, palmatine; f, berberine.

of the *Cj4′OMT* gene under the control of a modified CaMV35S promoter with duplicated enhancer (El2-CaMV35Sp) (Fig. 2A). After elimination of *Agrobacterium* by 500 mg/L claforan, the transgenic calli were selected by 25 mg/L hygromycin. Among the three hygromycin-resistant calli induced from 16 infected petiole segments, the existence of *hpt* gene was confirmed in one clone derived from a single adventitious root (*Cj*HE4′) by PCR analysis. The transgenic plantlets were spontaneously regenerated from this hygromycin-resistant callus by subculture on phytohormone-free medium. Non-transformed *Cj*WT plantlets and *Cj*GUS were regenerated by the same method and used as control plants (Fig. 2B). The integration of the *hpt* gene and CaMV35Sp-*Cj4′OMT* gene fragment into the genome of the *Cj* plantlets was confirmed by PCR. As the result of genomic PCR, the integration of both genes was confirmed at *Cj*HE4′. A clear *hpt* fragment band at *ca.* 480 bp and a CaMV35Sp-*Cj4′OMT* fragment band at *ca.* 850 bp (indicated by arrows) were specifically amplified from the genomic DNA of *Cj*HE4′ (Fig. 2C). To examine the copy number of the integrated gene, we performed genomic DNA blot analysis using the *hpt* gene fragment as the probe. Genomic DNA digested with restriction enzymes that do not cut the internal sequence of the probe template (*Eco*RV, *Eco*RI, *Hin*dIII and *Xba*I) resulted in one specific band in *Cj*HE4′ (*ca.* 11, 3.7, 5.0, 4.1 kbp, respectively). Genomic DNA digested with restriction enzymes that cut the internal sequence of the probe once (*Nde*I), resulted in two specific bands in *Cj*HE4′ (*ca.* 7.3, 1.1 kbp). Conversely, no hybridizable band was detected in untransformed *Cj*WT (Fig. 3). These results suggested that *Cj*HE4′ had a single copy of the integrated T-DNA region.

Regenerated *Cj*HE4′ plants were transplanted into pots, as were *Cj*WT and *Cj*GUS plants, and these were grown in a containment greenhouse at 20°C with 60% relative humidity under a 16-hour photoperiod for *ca.* 4 or 20 months. *Cj*HE4′ plants showed no morphologic abnormality, but did show a tendency toward being a slower-growing phenotype than *Cj*WT and *Cj*GUS (Fig. 4A). Secondary metabolites play important roles in protecting plants against pathogens and herbivores by their intense bioactivities.[25,26] Indeed, berberine is highly toxic to plants that do not produce it.[27] On the other hand, *Cj* detoxifies it by multiple methods; as an example, berberine is exclusively localized to the vacuole *via* H^+/berberine anti-porter,[28] and galactinol synthase is postulated to be involved in berberine tolerance due to the protein-protection effect of galactinol.[29] It is not clear why *Cj*HE4′ had a slower-growing phenotype, but alteration of alkaloid productivity (mentioned below) would cause cytotoxicity in *Cj*HE4′, or *Cj*HE4′ would consume more intermediates and energy for biosynthesis and detoxification of alkaloids than for growth.

Benzylisoquinoline Alkaloid Contents of *Cj* Transgenic Plants To evaluate the alkaloid concentration of *Cj* transgenic (*Cj*HE4′: 2 plants and *Cj*GUS: 4 plants) and non-transgenic (*Cj*WT: 2 plants) plants growing for *ca.* 4 months, HCl-acidified methanol extracts of leaves, stems and rhizomes, and

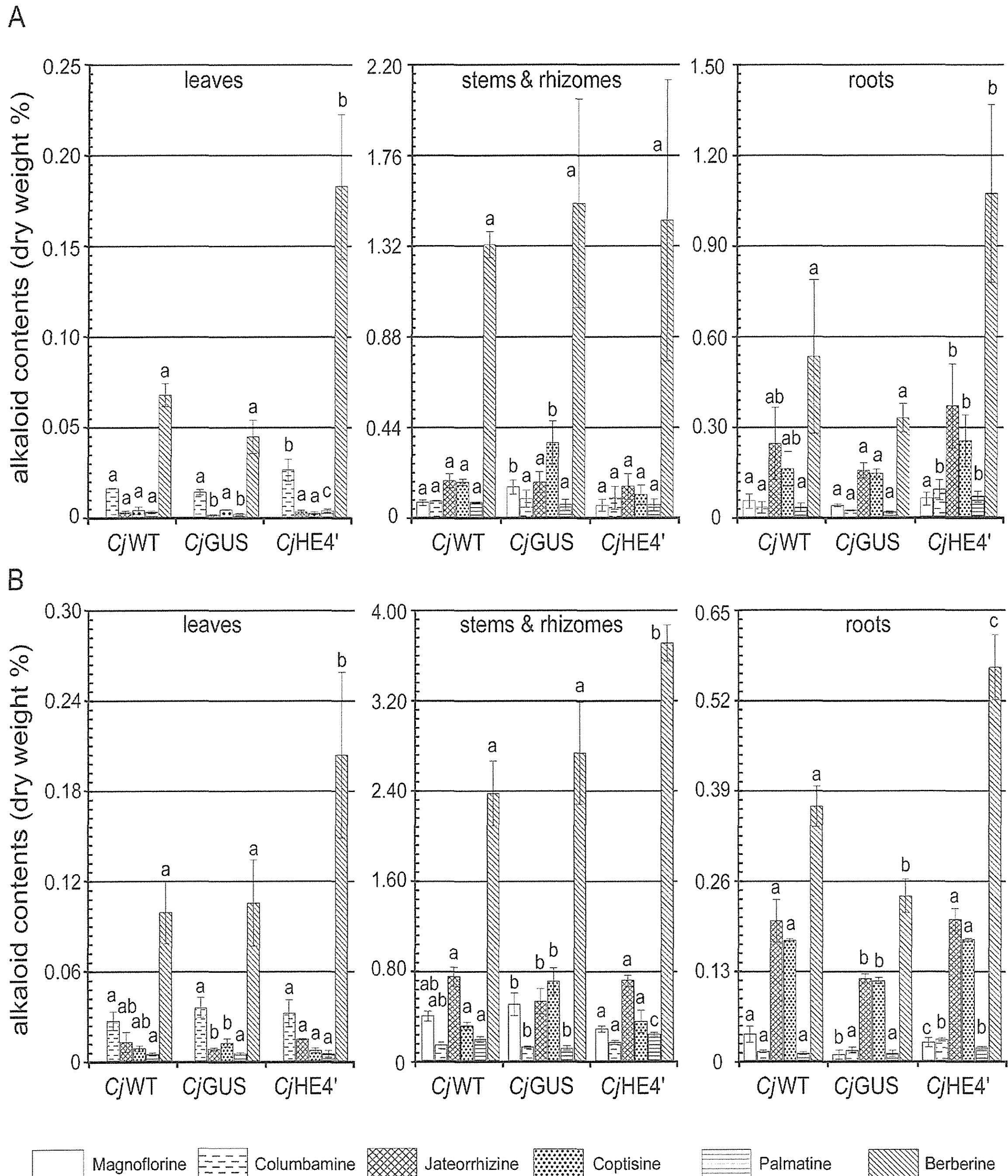

Fig. 5. Alkaloid Contents in Various Tissues (Leaves, Stems and Rhizomes, and Roots) of *Cj*WT (n=2), *Cj*GUS (n=4) and *Cj*HE4′ (n=2)

Plants were cultivated for *ca.* 4 (A) or 20 months (B) in a containment greenhouse. From left to right: magnoflorine, columbamine, jateorrhizine, coptisine, palmatine, berberine.

roots were analyzed by HPLC. All the plants had similar alkaloid profiles and no new peaks were detected on HPLC chromatograms, including reticuline, which is the product of the introduced 4′OMT (Fig. 4B). This result indicated that the enzyme activities of a step later than the 4′-*O*-methylation step in berberine biosynthesis were sufficient to convert reticuline into further metabolites. Quantitative analysis reveals that the berberine contents in the leaves and roots of *Cj*HE4′ were significantly increased by 2.7-fold (*Cj*HE4′, 0.183±0.040% dry weight (DW); *Cj*WT, 0.068±0.006% DW; $p<0.001$ by Tukey–Kramer's multiple comparisons test) and 2.0-fold (*Cj*HE4′, 1.071±0.294% DW; *Cj*WT, 0.531±0.254% DW; $p<0.01$), respectively, compared with that of *Cj*WT. Similar results were obtained for palmatine and columbamine contents. Palmatine contents of *Cj*HE4′ leaves and roots were increased up to 1.6-fold (*Cj*HE4′, 0.0040±0.0010% DW; *Cj*WT, 0.0025±0.0007% DW; $p<0.05$) and 2.1-fold (*Cj*HE4′, 0.067±0.018% DW; *Cj*WT, 0.032±0.015% DW; $p<0.005$). Columbamine contents of *Cj*HE4′ leaves and roots were increased up to 1.7-fold (*Cj*HE4′, 0.027±0.006% DW; *Cj*WT, 0.016±0.000% DW; $p<0.005$) and 2.9-fold (*Cj*HE4′, 0.090±0.034% DW; *Cj*WT, 0.031±0.019% DW; $p<0.005$) (Fig. 5A).

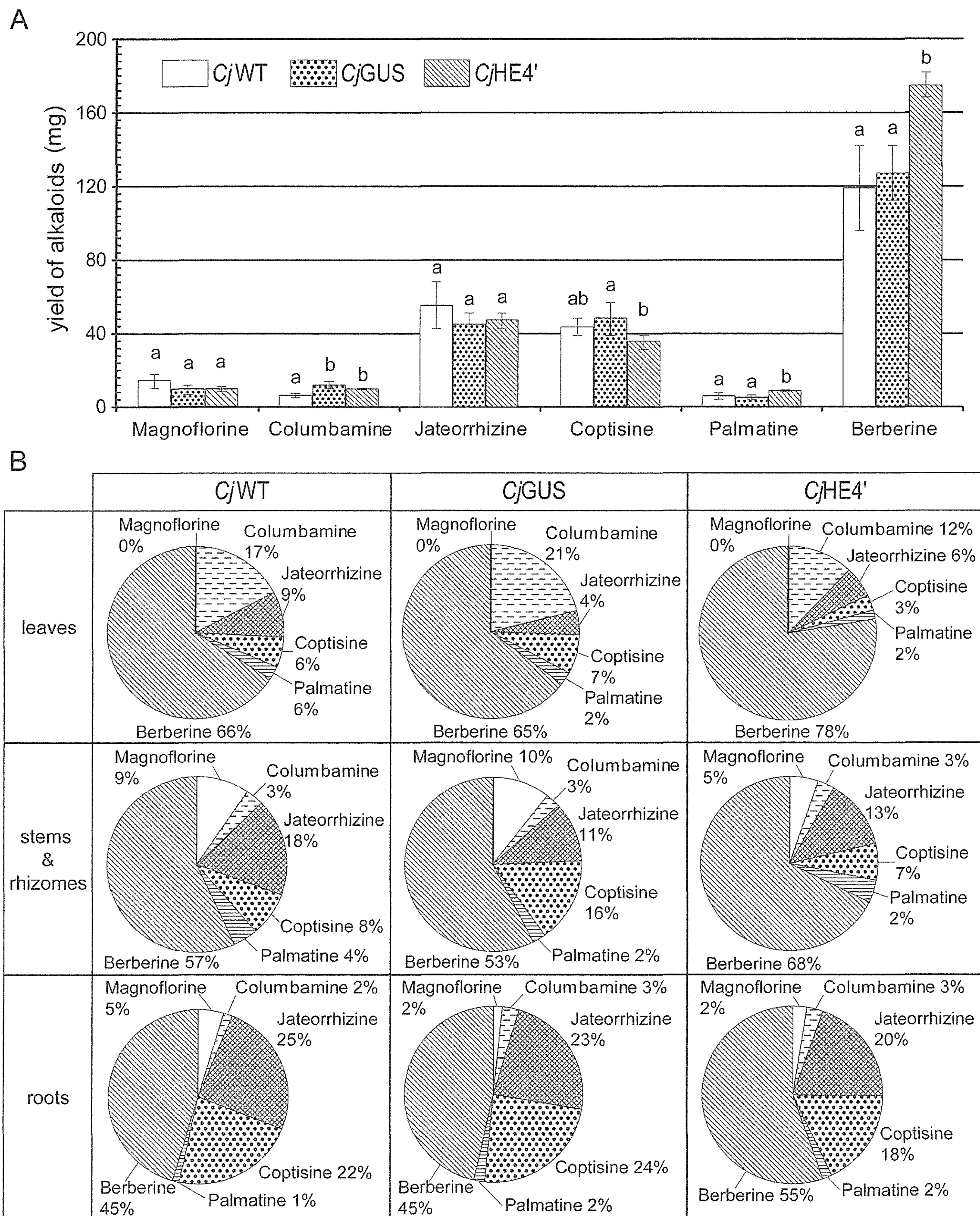

Fig. 6. Comparison of Alkaloid Productivity of Non-transgenic and Transgenic *Cj* Plants Cultivated for *ca.* 20 Months in a Containment Greenhouse

(A) Yield of benzylisoquinoline alkaloids (sum of leaf blades, petioles, stems and rhizomes, and roots) in *Cj*WT (n=2), *Cj*GUS (n=4) and *Cj*HE4′ (n=2) plants. (B) Ratios of individual benzylisoquinoline alkaloids in leaves, stems and rhizomes, and roots of *Cj*WT (n=2), *Cj*GUS (n=4) and *Cj*HE4′ (n=2) plants. Total amount of detected alkaloids was set to 100% and each substance ratio was calculated.

To examine the stability of the high productivity of benzylisoquinoline alkaloids in *Cj*HE4′, we performed further analysis of the alkaloid content of *Cj* plants growing for *ca.* 20 months (*Cj*WT: 2 plants, *Cj*GUS: 4 plants and *Cj*HE4′: 2 plants). HPLC analysis revealed that *Cj*HE4′ plants cultivated for 20 months, as well as 4 months, accumulated benzylisoquinoline alkaloids at greater levels than its counterparts, *Cj*WT and *Cj*GUS. The berberine content in the leaves and roots of *Cj*HE4′ was significantly increased by 2.1-fold (*Cj*HE4′, 0.204±0.055% DW; *Cj*WT, 0.098±0.021% DW; p<0.005) and 1.5-fold (*Cj*HE4′, 0.568±0.047% DW; *Cj*WT, 0.367±0.029% DW; p<0.001), respectively, compared with *Cj*WT. Columbamine and palmatine contents in *Cj*HE4′ roots were also markedly increased up to 2.3-fold (*Cj*HE4′, 0.030±0.002% DW; *Cj*WT, 0.013±0.002% DW; p<0.001) and 1.8-fold (*Cj*HE4′, 0.018±0.002% DW; *Cj*WT, 0.010±0.002%

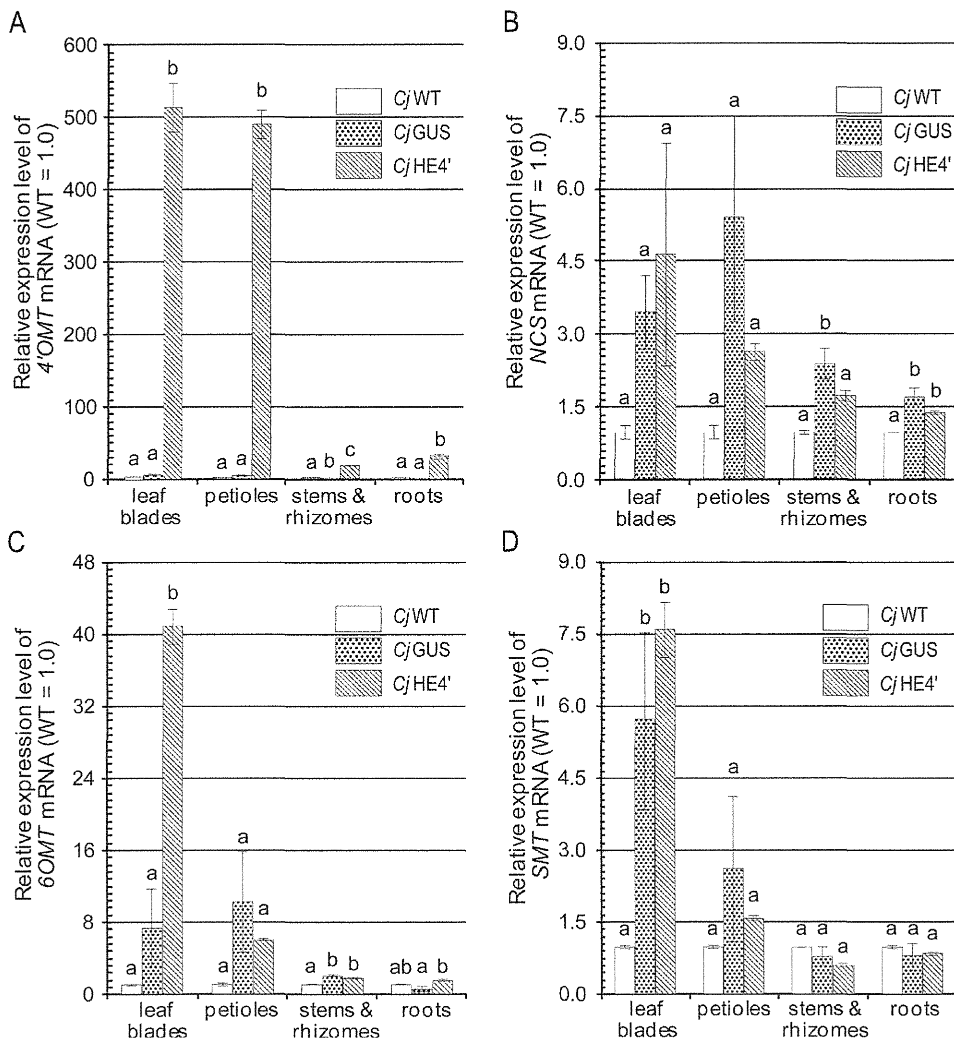

Fig. 7. Quantitative Real-Time PCR Analysis of Berberine Biosynthetic Genes

(A) *4'OMT*, (B) *NCS*, (C) *6OMT* and (D) *SMT* in various tissues (leaf blades, petioles, stems and rhizomes, and roots) of *Cj*WT (n=2), *Cj*GUS (n=4) and *Cj*HE4′ (n=2) cultivated for *ca.* 20 months. The relative transcript level referred to *Cj*WT was calculated using the *β-actin* gene as an internal control. Real-time PCR analysis was performed in triplicate and similar results were obtained from duplicate experiments.

DW; $p<0.05$), respectively, compared with those of *Cj*WT. Furthermore, after 20 months' cultivation, berberine and palmatine contents of *Cj*HE4′ medicinal parts, stems and rhizomes were significantly increased to 1.6-fold (*Cj*HE4′, 3.721±0.160% DW; *Cj*WT, 2.379±0.288% DW; $p<0.005$) and 1.2-fold (*Cj*HE4′, 0.244±0.018% DW; *Cj*WT, 0.196±0.025% DW; $p<0.05$), respectively, compared with *Cj*WT (Fig. 5B), though alkaloid contents cultivated for 4 months were not markedly increased. This might be caused by the transport of alkaloids from roots to rhizomes and their accumulation in rhizomes, associated with the growth of rhizomes. These results revealed that *Cj*HE4′ could stably accumulate greater levels of benzylisoquinoline alkaloids than *Cj*WT and *Cj*GUS. In addition, this enhancement of the alkaloid content resulted in significant increases in berberine and palmatine yields per individual plant (1.5-fold greater than *Cj*WT; $p<0.005$; berberine yield: *Cj*HE4′=174.98±6.52 mg/plant, *Cj*WT=118.88±22.99 mg/plant; palmatine yield: *Cj*HE4′=7.88±0.32 mg/plant, *Cj*WT=5.15±1.53 mg/plant) (Fig. 6A), whereas *Cj*HE4′ exhibited slower-growth phenotype and lower biomass than *Cj*WT and *Cj*GUS. Similar enhancement of alkaloid contents in *Cj*HE4′ was stably observed in other independent cultivations.

The ratio of individual alkaloids was different in each tissue of *Cj*WT. Namely, in leaves, berberine (66%) and columbamine (17%) were present at relatively high ratios. In stems and rhizomes, berberine (57%) and jateorrhizine (18%) were high. Berberine (45%), jateorrhizine (25%) and coptisine (22%) were prominently accumulated in roots. Similar tendencies were observed in *Cj*GUS. On the other hand, in *Cj*HE4′ the ratio of berberine showed *ca.* 10% increase in all tissues compared with those of the control plants (Fig. 6B). This increase in the berberine ratio indicated that intermediates induced by overexpressed 4′OMT mainly flowed to the berberine pathway. This notion was consistent with the results that alkaloid contents increased only in berberine, palmatine and columbamine, because palmatine and columbamine share a common pathway with berberine until the formation of tetrahydrocolumbamine (Fig. 1). Although jateorrhizine biosynthesis is still unclear, our results indicated that berberine biosynthetic enzymes (BBE, SMT[30,31] and CAS[32]) might have more available capacities than branch pathway enzymes (COS,[6])

CHS: cheilanthifoline synthase and THCO: tetrahydrocolumbamine oxidase, respectively). Although the enzymatic properties of BBE, cheilanthifoline synthase and tetrahydrocolumbamine oxidase from *Cj* have unfortunately not yet been reported, characterization of these enzymes should provide further insight into the regulation mechanism of biosynthesis and accumulation of berberine and related compounds in *Cj*.

Analysis of Expression Levels of Berberine Biosynthetic Genes in *Cj* Transgenic Plants To confirm that the increase in benzylisoquinoline alkaloid content was induced by the overexpression of *Cj4'OMT*, the transcript levels of *4'OMT* in *Cj* plants (*Cj*WT: 2 plants, *Cj*GUS: 4 plants and *Cj*HE4': 2 plants) were determined. Quantitative real-time PCR revealed that in *Cj*HE4', *4'OMT* significantly increased to not less than 490 times in leaves and 19 times in stems and rhizomes and roots compared with *Cj*WT (Fig. 7A). To study the influence of overexpression of *4'OMT* on other genes involved in berberine biosynthesis, the transcript levels of two entry-step enzyme genes of benzylisoquinoline alkaloid biosynthesis, *NCS* and *6OMT*, and that of a downstream biosynthetic gene, *SMT*, were determined. Quantitative real-time PCR analysis using the *β-actin* gene as an internal standard revealed that the unexpected enhancement of *6OMT* gene expression was observed only in the leaf blades of *Cj*HE4' (Fig. 7C). Similar transcriptional upregulation of endogenous genes was reported in *CYP80B3*-overexpressing *Ps*. In this plant, mRNA expression levels of *BBE*, *COR* and NADPH cytochrome P450 oxidoreductase were upregulated in a coordinated manner.[9] On the other hand, no significant differences in *NCS* and *SMT* expression levels in all tissues were shown between *Cj*HE4' and control plants (Figs. 7B, D). Although we need to examine the expression levels of other biosynthetic genes, this result suggested that the early-step OMTs would be specifically regulated by specific transcription factors or by the changing amount of intermediates.

These data also indicated that at least in the root, overexpression of only *Cj4'OMT* was sufficient for a 1.5- to 2.0-fold increase in berberine. However, despite maximum a 500-fold increase in *4'OMT* mRNA expression, the increase of alkaloid content did not exceed 3-fold. These results indicated that second rate-limiting steps might be involved. Recently, a number of successes in the increase of benzylisoquinoline alkaloid content by overexpression of an early-step enzyme gene were reported. Overexpression of *Cj6OMT* in *Ec*-cultured cells induced a 7.5-fold increase in alkaloid content over that of non-transgenic WT, whereas overexpression of *Cj4'OMT* had only a marginal effect, because non-transformed cells might lack specific 6OMT.[12] Overexpression of *PsBBE* in *Ec* also resulted in a 5-fold increase of end products, accompanied by a decrease in the amino-acid pool.[11] Overexpression of *PsCYP80B3* in *Ps* showed 4.5-fold greater alkaloid content of latex without changing the ratio of the individual alkaloids.[9] Therefore, introducing these enzymes into *Cj*HE4' might have the potential to induce a further increase in alkaloid productivity by overcoming the second rate-limiting steps. Another possibility is an overall increase in the expression levels of biosynthetic enzyme genes by a master transcription factor. Despite the importance of transcription factors, our knowledge of transcription factors involved in benzylisoquinoline alkaloid biosynthesis is still limited, and only a few transcription factor genes have been reported; for example, *CjWRKY1* was isolated from the *Cj* Expressed Sequence Tag library, and introduction of its double-stranded RNA into protoplast reduced the expression levels of all genes involved in berberine biosynthesis.[33] Such a restricted knowledge makes it difficult to improve benzylisoquinoline alkaloid productivity using transcription factors. To overcome this limitation of information on transcription factors involved in benzylisoquinoline alkaloid biosynthesis, regulatory factors of *Arabidopsis thaliana*, soybean and corn were heterologously expressed in *Ps* and *Ec*. Several factors increased their levels of biosynthetic genes, and these inductions resulted in enhancement of the alkaloid productivity and production of new alkaloids.[34] Further problems are cytotoxicity and the capacity for alkaloid accumulation. In this study, *Cj*HE4' showed slower growth, possibly due to the cytotoxicity of alkaloids. Frick *et al.*[9] reported that in *Ps*, integration of the overexpression construct was less efficient than that of the antisense construct because the storage capacity of the alkaloids in the latex vesicles could be limited. Hence, other strategies, *e.g.* co-overexpressing genes for detoxification, or enlarging the sink tissues of alkaloids, would also be efficient for improving alkaloid yields.

In conclusion, we have demonstrated that transgenic *Cj* plants overexpressing *4'OMT* showed stable increases in berberine content, and this induction of alkaloid content resulted in an increase in alkaloid yield, despite the fact that *Cj*HE4' showed a slower-growth phenotype. These results suggest that 4'OMT is one of the rate-limiting step enzymes in berberine biosynthesis of *Cj*, and that *4'OMT* will be useful for the metabolic engineering of berberine biosynthesis in *Cj*.

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北海道の生薬　第17回

アルテミシニンの生産を目的としたクソニンジンの栽培

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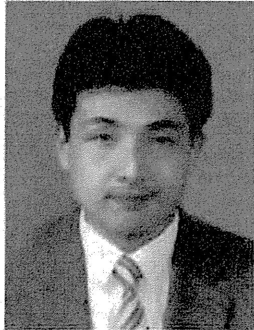

Ⅰ．はじめに

クソニンジン（別名ホソバニンジン）*Artemisia annual* L.は、生薬「青蒿」及び「黄花蒿」の基原植物である[1~3]。青蒿は、クソニンジンの乾燥した地上部で長さが60～100cm。茎は円柱状で縦に稜があり、色はうすい褐色～灰褐色である。質は硬く、断面はあらく、中央に白色の髄がある（写真1）。若い枝には葉が多数あり、もろく砕けやすい。花穂を持った枝はほとんど葉がない。花序は、小球形で黄褐色。特徴的な芳香がある。

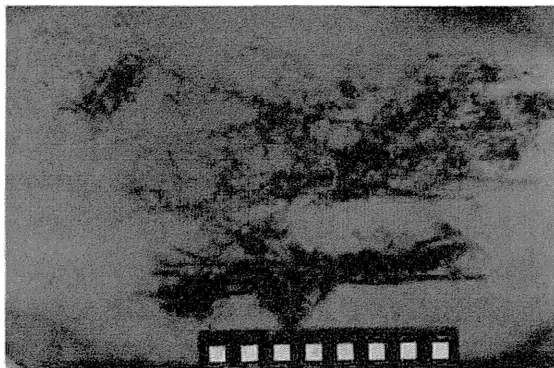

写真1　青蒿（市場品）

日本では、青蒿はほとんど用いられないが、解熱薬として、結核の熱、慢性の間歇熱、産褥熱、黄疸、各種神経性熱症病などの慢性熱病に用いる。また止血薬として、衂血、便血の治療に、殺虫薬として疥瘡、蜂毒に外用する。青蒿を配合した処方として、青蒿亀甲湯、蒿芩清胆湯がある[3]。

一方、クソニンジンの葉（写真2）には、アルテミシニン（artemisinin，図1）が含まれ、アルテミシニンが抗マラリア剤として非常に有効であることから、現在、中国、ケニア、タンザニア及びベトナムでは大規模栽培が行われ、インド、アフリカ諸国、西ヨーロッパ及び南アメリカで小規模で栽培が行われている[4]。

写真2　葉の性状

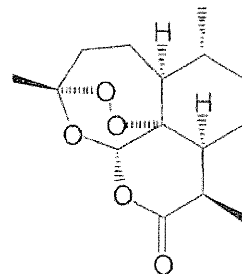

図1　アルテミシニンの構造式

Ⅱ．植物の特徴

クソニンジンは、キク科ヨモギ属の1年生草本植物である。中国原産とされ、中国、朝鮮半島、シベリア、インド、西アジア及び東欧に分布する。日本では帰化植物として北海道、本州、四国及び九州に広く分布する[5]。日本への導入時期は古く、おそらく中国から薬用として渡来したのが野生化したと考えられている[6]。

形態的な特徴[7, 8]は、茎は直立して高さ1.5～