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 CjHE4′) by introducing one copy of Cj4′OMT by Agrobacterium-mediated transformation. The successful overexpression of 4′OMT was confirmed in all tissues of CjHE4′ by real-time polymerase chain reaction (PCR) analysis. HPLC analysis revealed that the berberine content of CjHE4′ leaves and roots cultivated for 4 months was increased to 2.7- and 2.0-fold, respectively, compared with non-transgenic wild-type (CjWT), and these inductions of alkaloids were stable for at least 20 months. Furthermore, in CjHE4′ 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 CjHE4′ resulted in the improvement of berberine yields (1.5-fold), whereas CjHE4′ showed slower growth than CjWT. 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.5 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,4 anti-hypertension effects via induction of mobilization of circulating endothelial progenitor cells3,5 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 pharmacologically important compounds have the same steps in their early biosynthetic pathways as benzylisoquinoline alkaloids, such as the alkalgesic morphone, 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:co lumbarine 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 and overexpression of the Ps berberine bridge enzyme

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(BBE) in *Eschscholzia californica* (*Ec*) root culture, resulting in increased levels of the end products, benzophenanthridine alkaloids. Overexpression of *Cj6OMT* in *Ec*-cultured cells also resulted in an effective increase of benzophenanthridine alkaloids. 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. Antisense *PsCYP80B3*-expressing plants showed remarkable reduction of the end products, benzophenanthridine alkaloids, whereas RNAi suppression of *EcBBE* resulted in reduced levels of alkaloids in *Ec*-cultured cells and no accumulation of intermediates, whereas RNAi suppression of *EcBBE* resulted in reduced end products and accumulation of important intermediate reticuline and its derivatives. Similar accumulation of reticuline and its derivatives was obtained in the latex of antisense *PsBBE* transgenic *Ps*, but not in its roots. RNAi of *PsSalAT* also induced the accumulation of salutaridine, which is 2 enzymatic steps upstream of the intermediate. Overexpression of *CjSMT* in *Cj*-cultured 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. In this study, *Cj4/uni2032OMT* 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 medium22) containing 10mg/L l-glutamine, 1mg/L 1-naphthalenacetic acid, 2mg/L kinetin (WPWNIK2), 3% sucrose and 0.25% gelrite (San-Ei Gen F.E.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 Cj’4’OMT23) for 2d in WPWNIK2 liquid medium containing 2% sucrose; then bacteria was eliminated on WPWNIK2 medium containing 500mg/L aclorfan (Sanofi Aventis, Tokyo, Japan). After 1 to 2 months, the segments which formed calli and adventitious roots were cultured on WPWNIK2 medium containing 25mg/L hygromycin at 20°C in the dark. The hygromycin-resistant calli were subcultured on the WPWNIK2 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/GUS21) (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 for the primer with the cauliflower mosaic virus 35S promoter (CaMV35Sp: 5′-GATATCTCCACTCTCGACGTAAGG-3′) and that for Cj’4’OMT (GenBank accession No. D29812; Cj’4’OMT-723A: 5′-AACATGTCCGCACACCAATTCG-3′) or a primer pair designed to detect the hygromycin phosphotransferase (hpt) gene (hpt-220s: 5′-CGGAAGTGC-TTGACATTGG-3′ and hpt-703A: 5′-AGGAAGAGATTTGGCGACC-3′23)). After 5 min of denaturation at 94°C, 30 cycles of PCR were performed at 94°C for 30s, 58°C for 30s and 72°C for 1min, followed by incubation at 72°C for 10min. The presence of PCR products was confirmed by agarose gel electrophoresis.

**Genomic DNA Blot Analysis** Genomic DNA was prepared from 2g fresh leaves of Cj/WT and Cj/HE4’ by the modified cetyl trimethyl ammonium bromide method, as previously described,24) and digested with either EcoRI, EcoRV, HindIII, NdeI or XbaI. 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 90min at 55°C in an AlkPhos Direct hybridization buffer, followed by hybridization with alkaline phosphatase-labeled probes at 55°C for 14h. The blot was washed twice at 55°C for 10min with 50mM phosphate buffer (pH 7.0), 2m umea, 0.1% sodium dodecyl sulfate (SDS), 150mM NaCl, and 10mM MgCl2, then twice at room temperature for 5min with 2m NaCl and 1m 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. 20mg) under 30min reflux at 70°C using 1.5mL HCl-acidified methanol (10% HCl:methanol=1:100), and the combined extract solution was diluted to 5mL 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 (4.6mm i.d.<250mm, 5µm, TOSOH, Tokyo, Japan). The mobile phase consisted of acetonitrile (solvent A) and 10mM 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 0.8mL/min at 40°C (0–15min 27–29% solvent A, 15–25min 29–39% solvent A, 25–31min 39–51% solvent A, 31–34min 51% solvent A, 34–35min 51–27% solvent A). The elution of benzylisoquinoline alkaloids was monitored at 200–400nm, and alkaloid contents were quantified by the peak area obtained at 284nm 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 RNAsasy 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 865ng 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
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'-TTGGTGCCGACATGTATAAC-3' and Rv: 5'-CTTGATCGATCTGTCTATTCC-3'. Those for Cj6OMT (GenBank accession No. D29811) were Fw: 5'-CTGGCTTTCTATCTCATTCTTG-3' and Rv: 5'-GACATTCTTCAGTGGGCTTC-3'. Those for CjNCS (GenBank accession No. AB267398) were Fw: 5'-ATGCTAAGACCTTTGCTGGATTG-3' and Rv: 5'-CGTGGTAACTTCTCTTGTGTTAAGAGG-3'. Those for CjACT1 were Fw: 5'-TCGGCTTACCGAATCTTCTCT-3' and Rv: 5'-ATTCTCAGTCTGCTGTGCTGGTG-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...
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 clorofuran, 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 (CjHE4) by PCR analysis. The transgenic plantlets were spontaneously regenerated from this hygromycin-resistant callus by subculture on phytohormone-free medium. Non-transformed CjWT plantlets and CjGUS 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 CjHE4. 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 CjHE4 (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 (EcoRV, EcoRI, HindIII and XbaI) resulted in one specific band in CjHE4 (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 (NdeI), resulted in two specific bands in CjHE4 (ca. 7.3, 1.1 kbp). Conversely, no hybridizable band was detected in untransformed CjWT (Fig. 3). These results suggested that CjHE4 had a single copy of the integrated T-DNA region.

Regenerated CjHE4 plants were transplanted into pots, as were CjWT and CjGUS 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. CjHE4 plants showed no morphologic abnormality, but did show a tendency toward being a slower-growing phenotype than CjWT and CjGUS (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 CjHE4 had a slower-growing phenotype, but alteration of alkaloid productivity (mentioned below) would cause cytotoxicity in CjHE4’, or CjHE4’ 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 (CjHE4’: 2 plants and CjGUS: 4 plants) and non-transgenic (CjWT: 2 plants) plants growing for ca. 4 months, HCl-acidified methanol extracts of leaves, stems and rhizomes, and
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 CjHE4' were significantly increased by 2.7-fold (CjHE4', 0.183±0.040% dry weight (DW); CjWT, 0.068±0.006% DW; p<0.001 by Tukey-Kramer’s multiple comparisons test) and 2.0-fold (CjHE4', 1.071±0.294% DW; CjWT, 0.531±0.254% DW; p<0.01), respectively, compared with that of CjWT. Similar results were obtained for palmatine and columbamine contents. Palmatine contents of CjHE4' leaves and roots were increased up to 1.6-fold (CjHE4', 0.0040±0.0010% DW; CjWT, 0.0025±0.0007% DW; p<0.05) and 2.1-fold (CjHE4', 0.067±0.018% DW; CjWT, 0.032±0.015% DW; p<0.005). Columbamine contents of CjHE4' leaves and roots were increased up to 1.7-fold (CjHE4', 0.027±0.006% DW; CjWT, 0.016±0.000% DW; p<0.005) and 2.9-fold (CjHE4', 0.090±0.034% DW; CjWT, 0.031±0.019% DW; p<0.005) (Fig. 5A).
To examine the stability of the high productivity of benzylisoquinoline alkaloids in CjHE4', we performed further analysis of the alkaloid content of Cj plants growing for ca. 20 months (CjWT: 2 plants, CjGUS: 4 plants and CjHE4'/uni2032: 2 plants). HPLC analysis revealed that CjHE4'/uni2032 plants cultivated for 20 months, as well as 4 months, accumulated benzylisoquinoline alkaloids at greater levels than its counterparts, CjWT and CjGUS. The berberine content in the leaves and roots of CjHE4' was significantly increased by 2.1-fold (CjHE4', 0.204±0.055% DW; CjWT, 0.098±0.021% DW; p<0.005) and 1.5-fold (CjHE4', 0.568±0.047% DW; CjWT, 0.367±0.029% DW; p<0.001), respectively, compared with CjWT. Columbamine and palmatine contents in CjHE4'/uni2032 roots were also markedly increased up to 2.3-fold (CjHE4', 0.030±0.002% DW; CjWT, 0.013±0.002% DW; p<0.001) and 1.8-fold (CjHE4', 0.018±0.002% DW; CjWT, 0.010±0.002% DW).
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\(^{29,31}\) and CAS\(^{32}\)) might have more available capacities than branch pathway enzymes (COS\(^{6}\)).

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

(A) 4’OMT, (B) NCS, (C) 6’OMT 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 \( \beta \)-actin gene as an internal control. Real-time PCR analysis was performed in triplicate and similar results were obtained from duplicate experiments.
CHS: chelanthifoline synthase and THCO: tetrahydrocolumbamine oxidase, respectively). Although the enzymatic properties of BBE, chelanthifoline 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 berbysinoquinoline alkaloid content was induced by the overexpression of Cj4OMT, the transcript levels of 4′OMT in Cj plants (CjWT: 2 plants; CjGUS: 4 plants and CjHE4′: 2 plants) were determined. Quantitative real-time PCR revealed that in CjHE4′, 4′OMT significantly increased to not less than 490 times in leaves and 19 times in stems and rhizomes and roots compared with CjWT (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 CjHE4′ (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 oxi-doreductase were upregulated in a coordinated manner.39) On the other hand, no significant differences in NCS and SMT expression levels in all tissues were shown between CjHE4′ 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.40) Overexpression of PsBBE in Ec also resulted in a 5-fold increase of end products, accompanied by a decrease in the amino-acid pool.41) Overexpression of PsCYP80B3 in Ps showed 4.5-fold greater alkaloid content of latex without changing the ratio of the individual alkaloids.42) Therefore, introducing these enzymes into CjHE4′ 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.43) Further problems are cytotoxicity and the capacity for alkaloid accumulation. In this study, CjHE4′ showed slower growth, possibly due to the cytotoxicity of alkaloids. Frick et al.49) 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 CjHE4′ 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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