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'-GATATCTCCACTCCACTGACGTAAGG-3') and that for *Cj4'OMT* (GenBank accession No. D29812; *Cj4'OMT*-723A: 5'-AAACATGTCCGCCACCAATTCG-3') or a primer pair designed to detect the hygromycin phosphotransferase (*hpt*) gene (*hpt*-220S: 5'-CGGAAGTGCTTGACATTGG-3' and *hpt*-703A: 5'-AGAAGAAGATGTTGGCGACC-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

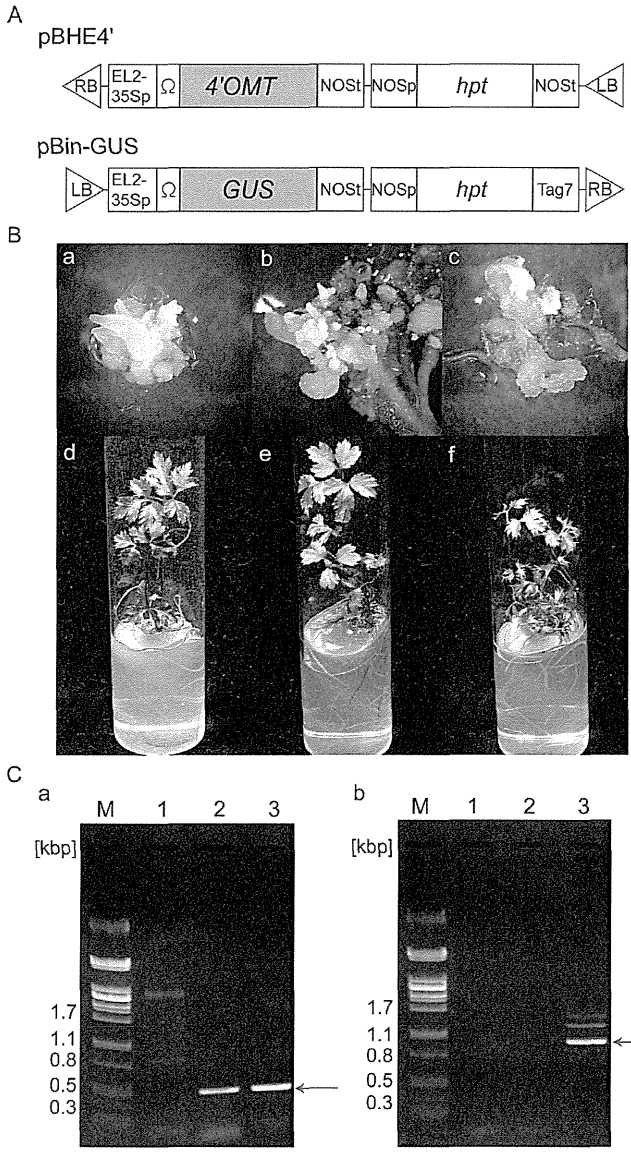

Fig. 2. *Cj* Transformed with *Cj4'OMT* or *GUS* Overexpression Vectors

(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'.

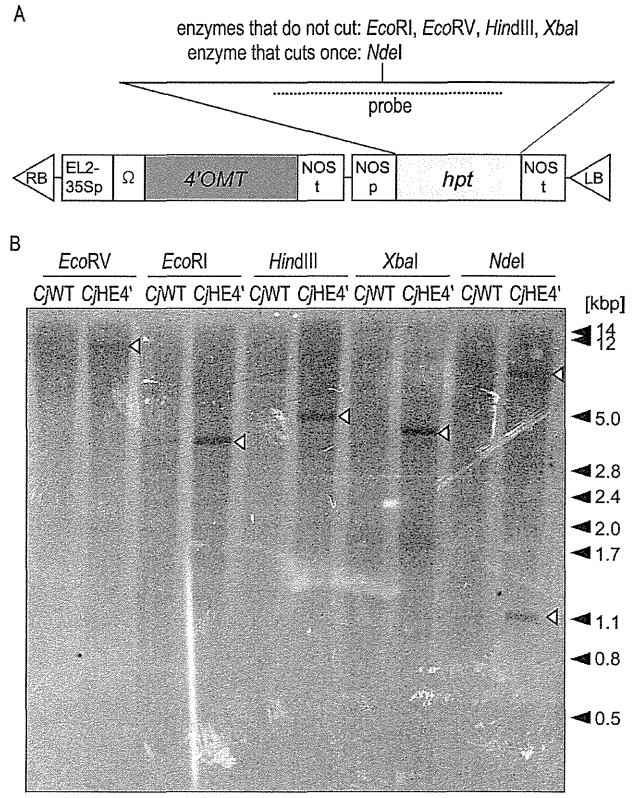

Fig. 3. Genomic DNA Gel Blot Analysis Using *hpt* Gene Fragment as a Probe

(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

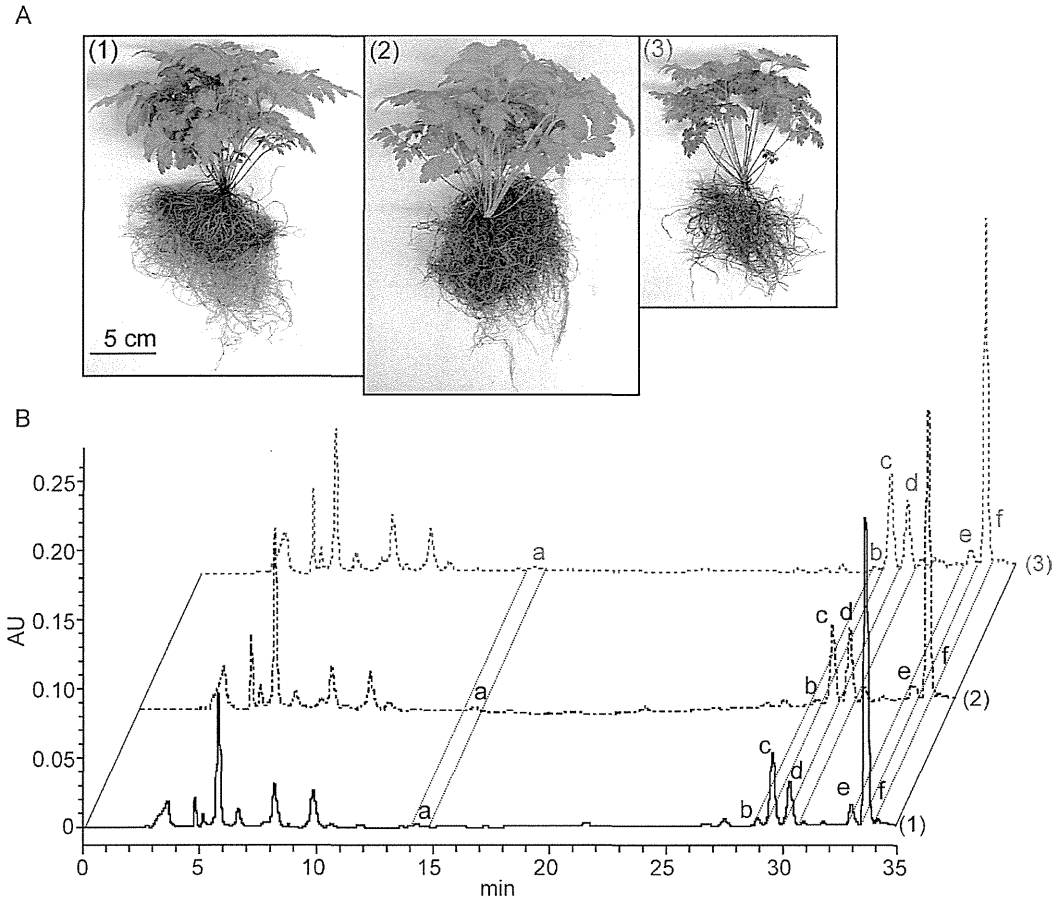

Fig. 4. Non-transgenic and Transgenic *Cj* Plants and Their Alkaloid Profiles

(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 284nm. (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 500mg/L claforan, the transgenic calli were selected by 25mg/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.* 480bp and a CaMV35Sp-*Cj4′OMT* fragment band at *ca.* 850bp (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, 4.1kbp, 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, 1.1kbp). 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

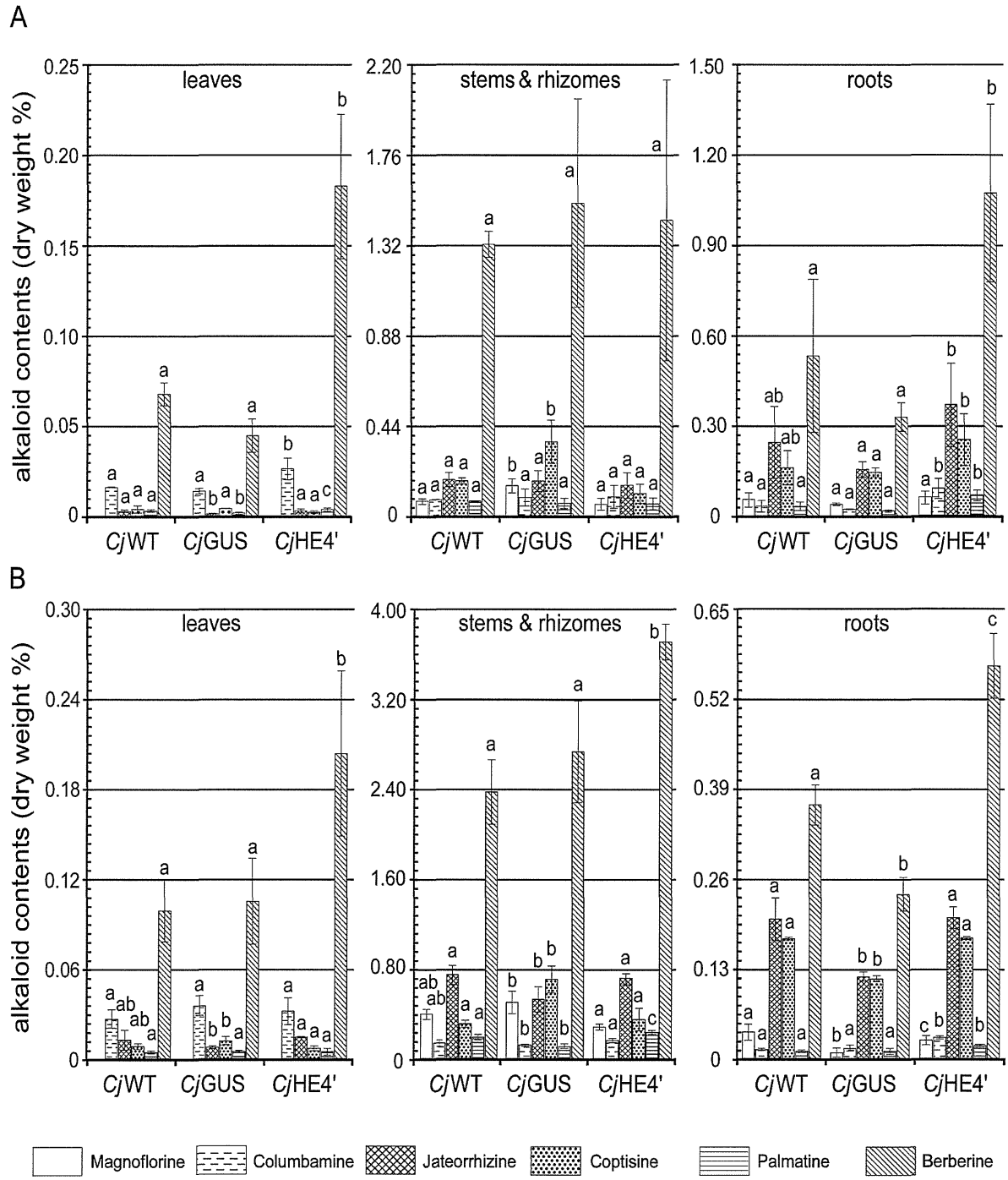

Fig. 5. Alkaloid Contents in Various Tissues (Leaves, Stems and Rhizomes, and Roots) of *Cj*WT (n=2), *Cj*GUS (n=4) and *Cj*HE4′ (n=2)

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).

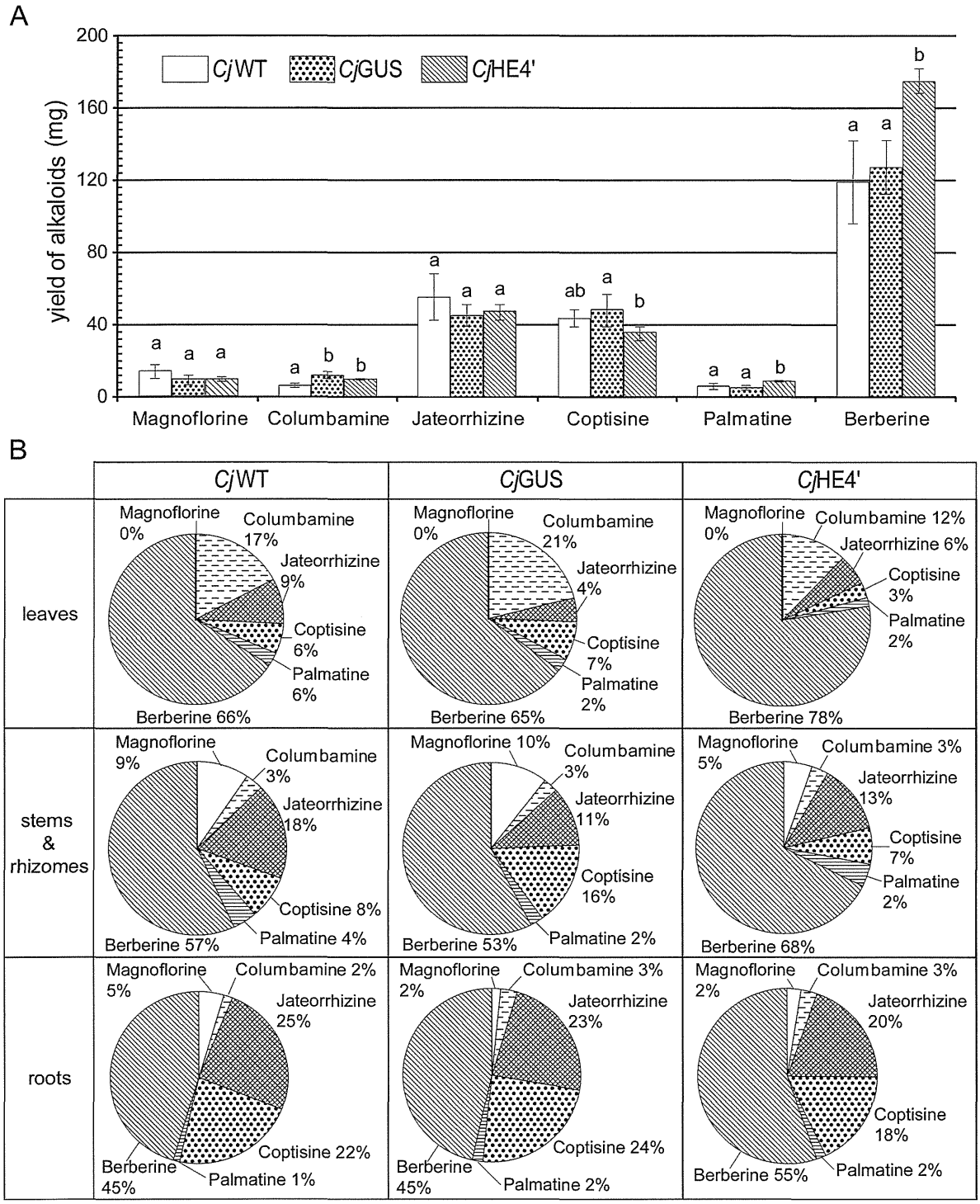

Fig. 6. Comparison of Alkaloid Productivity of Non-transgenic and Transgenic *Cj* Plants Cultivated for *ca.* 20 Months in a Containment Greenhouse

(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%

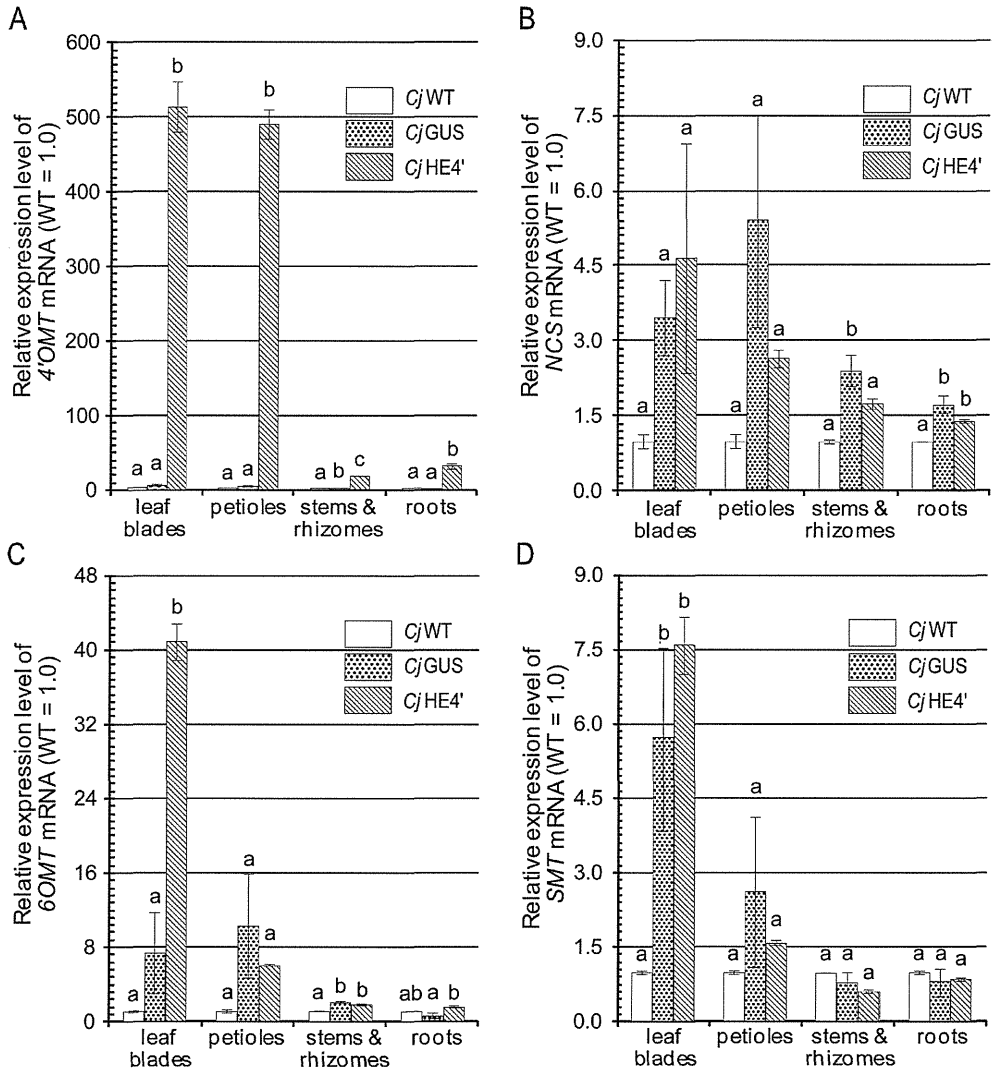

Fig. 7. Quantitative Real-Time PCR Analysis of Berberine Biosynthetic Genes

(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*.

Acknowledgements This work was supported in part by a Grant-in-Aid, "Characterization of gene network in isoquinoline alkaloid biosynthesis and its application," from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by the project "Development of Fundamental Technologies for the Production of High-Value Materials Using Transgenic Plants" from the Ministry of Economy, Trade and Industry of Japan.

REFERENCES

1) Iwasa K, Nanba H, Lee DU, Kang SI. Structure–activity relationships of protoberberines having antimicrobial activity. *Planta Med.*, **64**, 748–751 (1998).
2) Kong W, Wei J, Abidi P, Lin M, Inaba S, Li C, Wang Y, Wang Z, Si S, Pan H, Wang S, Wu J, Wang Y, Li Z, Liu J, Jiang JD. Berberine is a novel cholesterol-lowering drug working through a unique mechanism distinct from statins. *Nat. Med.*, **10**, 1344–1351 (2004).
3) Xu MG, Wang JM, Chen L, Wang Y, Yang Z, Tao J. Berberine-induced mobilization of circulating endothelial progenitor cells improves human small artery elasticity. *J. Hum. Hypertens.*, **22**, 389–393 (2008).
4) Zhang H, Wei J, Xue R, Wu JD, Zhao W, Wang ZZ, Wang SK, Zhou ZX, Song DQ, Wang YM, Pan HN, Kong WJ, Jiang JD. Berberine lowers blood glucose in type 2 diabetes mellitus patients through increasing insulin receptor expression. *Metabolism*, **59**, 285–292 (2010).
5) Sato F, Inui T, Takemura T. Metabolic engineering in isoquinoline alkaloid biosynthesis. *Curr. Pharm. Biotechnol.*, **8**, 211–218 (2007).
6) Morishige T, Dubouzet E, Choi KB, Yazaki K, Sato F. Molecular cloning of columbamine *O*-methyltransferase from cultured *Coptis japonica* cells. *Eur. J. Biochem.*, **269**, 5659–5667 (2002).

7) Ikezawa N, Iwasa K, Sato F. Molecular cloning and characterization of CYP80G2, a cytochrome P450 that catalyzes an intramolecular C–C phenol coupling of (*S*)-reticuline in magnoflorine biosynthesis, from cultured *Coptis japonica* cells. *J. Biol. Chem.*, **283**, 8810–8821 (2008).
8) Larkin PJ, Miller JA, Allen RS, Chitty JA, Gerlach WL, Frick S, Kutchan TM, Fist AJ. Increasing morphinan alkaloid production by over-expressing codeinone reductase in transgenic *Papaver somniferum*. *Plant Biotechnol. J.*, **5**, 26–37 (2007).
9) Frick S, Kramell R, Kutchan TM. Metabolic engineering with a morphine biosynthetic P450 in opium poppy surpasses breeding. *Metab. Eng.*, **9**, 169–176 (2007).
10) Allen RS, Miller JA, Chitty JA, Fist AJ, Gerlach WL, Larkin PJ. Metabolic engineering of morphinan alkaloids by over-expression and RNAi suppression of salutaridinol 7-*O*-acetyltransferase in opium poppy. *Plant Biotechnol. J.*, **6**, 22–30 (2008).
11) Park SU, Yu M, Facchini PJ. Modulation of berberine bridge enzyme levels in transgenic root cultures of California poppy alters the accumulation of benzophenanthridine alkaloids. *Plant Mol. Biol.*, **51**, 153–164 (2003).
12) Inui T, Tamura K, Fujii N, Morishige T, Sato F. Overexpression of *Coptis japonica* norcoclaurine 6-*O*-methyltransferase overcomes the rate-limiting step in benzylisoquinoline alkaloid biosynthesis in cultured *Eschscholzia californica*. *Plant Cell Physiol.*, **48**, 252–262 (2007).
13) Allen RS, Millgate AG, Chitty JA, Thisleton J, Miller JA, Fist AJ, Gerlach WL, Larkin PJ. RNAi-mediated replacement of morphine with the nonnarcotic alkaloid reticuline in opium poppy. *Nat. Biotechnol.*, **22**, 1559–1566 (2004).
14) Park SU, Yu M, Facchini PJ. Antisense RNA-mediated suppression of benzophenanthridine alkaloid biosynthesis in transgenic cell cultures of California poppy. *Plant Physiol.*, **128**, 696–706 (2002).
15) Fujii N, Inui T, Iwasa K, Morishige T, Sato F. Knockdown of berberine bridge enzyme by RNAi accumulates (*S*)-reticuline and activates a silent pathway in cultured California poppy cells. *Transgenic Res.*, **16**, 363–375 (2007).
16) Frick S, Chitty JA, Kramell R, Schmidt J, Allen RS, Larkin PJ, Kutchan TM. Transformation of opium poppy (*Papaver somniferum* L.) with antisense berberine bridge enzyme gene (*anti-bbe*) *via* somatic embryogenesis results in an altered ratio of alkaloids in latex but not in roots. *Transgenic Res.*, **13**, 607–613 (2004).
17) Kempe K, Higashi Y, Frick S, Sabarna K, Kutchan TM. RNA*i* suppression of the morphine biosynthetic gene *salAT* and evidence of association of pathway enzymes. *Phytochemistry*, **70**, 579–589 (2009).
18) Sato F, Hashimoto T, Hachiya A, Tamura K, Choi KB, Morishige T, Fujimoto H, Yamada Y. Metabolic engineering of plant alkaloid biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 367–372 (2001).
19) Morishige T, Tsujita T, Yamada Y, Sato F. Molecular characterization of the *S*-adenosyl-L-methionine:3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase involved in isoquinoline alkaloid biosynthesis in *Coptis japonica*. *J. Biol. Chem.*, **275**, 23398–23405 (2000).
20) Sato F, Takeshita N, Fujiwara H, Katagiri Y, Huan L, Yamada Y. Characterization of *Coptis japonica* cells with different alkaloid productivities. *Plant Cell Tissue Organ Cult.*, **38**, 249–256 (1994).
21) Shitan N, Kiuchi F, Sato F, Yazaki K, Yoshimatsu K. Establishment of rhizobium-mediated transformation of *Coptis japonica* and molecular analyses of transgenic plants. *Plant Biotechnol.*, **22**, 113–118 (2005).
22) Lloyd G, McCown B. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Pro. Int. Plant Prop. Soc.*, **30**, 421–427 (1980).
23) Yazaki K, Tanaka S, Matsuoka H, Sato F. Stable transformation of *Lithospermum erythrorhizon* by *Agrobacterium rhizogenes* and shikonin production of the transformants. *Plant Cell Rep.*, **18**, 214–219 (1998).
24) Sato F, Kitajima S, Koyama T, Yamada Y. Ethylene-induced gene expression of osmotin-like protein, a neutral isoform of tobacco PR-5, is mediated by the AGCCGCC *cis*-sequence. *Plant Cell Physiol.*, **37**, 249–255 (1996).
25) Dixon RA. Natural products and plant disease resistance. *Nature*, **411**, 843–847 (2001).
26) Howe GA, Jander G. Plant immunity to insect herbivores. *Annu. Rev. Plant Biol.*, **59**, 41–66 (2008).
27) Sakai K, Shitan N, Sato F, Ueda K, Yazaki K. Characterization of berberine transport into *Coptis japonica* cells and the involvement of ABC protein. *J. Exp. Bot.*, **53**, 1879–1886 (2002).
28) Otani M, Shitan N, Sakai K, Martinoia E, Sato F, Yazaki K. Characterization of vacuolar transport of the endogenous alkaloid berberine in *Coptis japonica*. *Plant Physiol.*, **138**, 1939–1946 (2005).
29) Takanashi K, Shitan N, Sugiyama A, Kamimoto Y, Hamamoto M, Iwaki T, Takegawa K, Yazaki K. Galactinol synthase gene of *Coptis japonica* is involved in berberine tolerance. *Biosci. Biotechnol. Biochem.*, **72**, 398–405 (2008).
30) Sato F, Takeshita N, Fitchen JH, Fujiwara H, Yamada Y. *S*-Adenosyl-L-methionine: scoulerin-9-*O*-methyltransferase from cultured *Coptis japonica* cells. *Phytochemistry*, **32**, 659–664 (1993).
31) Takeshita N, Fujiwara H, Mimura H, Fitchen JH, Yamada Y, Sato F. Molecular cloning and characterization of *S*-adenosyl-L-methionine:scoulerine-9-*O*-methyltransferase from cultured cells of *Coptis japonica*. *Plant Cell Physiol.*, **36**, 29–36 (1995).
32) Ikezawa N, Tanaka M, Nagayoshi M, Shinkyo R, Sakaki T, Inouye K, Sato F. Molecular cloning and characterization of CYP719, a methylenedioxy bridge-forming enzyme that belongs to a novel P450 family, from cultured *Coptis japonica* cells. *J. Biol. Chem.*, **278**, 38557–38565 (2003).
33) Kato N, Dubouzet E, Kokabu Y, Yoshida S, Taniguchi Y, Dubouzet JG, Yazaki K, Sato F. Identification of a WRKY protein as a transcriptional regulator of benzylisoquinoline alkaloid biosynthesis in *Coptis japonica*. *Plant Cell Physiol.*, **48**, 8–18 (2007).
34) Apuya NR, Park JH, Zhang L, Ahyow M, Davidow P, Van Fleet J, Rarang JC, Hippley M, Johnson TW, Yoo HD, Trieu A, Krueger S, Wu CY, Lu YP, Flavell RB, Bobzin SC. Enhancement of alkaloid production in opium and California poppy by transactivation using heterologous regulatory factors. *Plant Biotechnol. J.*, **6**, 160–175 (2008).

投稿/ノート

医薬品医療機器レギュラトリーサイエンス
Pharmaceutical and Medical Device Regulatory Science
Vol. 43, No. 7, 644~649 (2012)

HPTLCによる生薬「オウゴン」国内流通品の成分比較

天倉 吉章[*1,#], 渕野 裕之[*2], 好村 守生[*1], 山上 沙織[*1],
吉田 隆志[*1], 合田 幸広[*3], 川原 信夫[*2]

(受付：平成24年3月12日, 受理：平成24年5月28日)

High-performance TLC Comparison of Components in the Crude Drug "Scutellariae Radix" Available in Japan

Yoshiaki AMAKURA[*1,#], Hiroyuki FUCHINO[*2], Morio YOSHIMURA[*1],
Saori YAMAKAMI[*1], Takashi YOSHIDA[*1], Yukihiro GODA[*3] and Nobuo KAWAHARA[*2]

Summary

As part of a project to construct a database of official crude drugs, constituents in fifteen samples of "Scutellariae Radix" (the root of *Scutellaria baicalensis*) on the market in Japan were compared by means of high-performance TLC (HPTLC) according to the crude drug identification test in the Japanese Pharmacopoeia. A main spot corresponding to standard baicalin was detected in all of the HPTLC chromatograms, together with spots of wogonin, wogonoside, and chrysin 6-*C*-arabinoside 8-*C*-glucoside. These data provide a characteristic pattern that should be useful as an indicator for quality inspection of crude drugs available on the market. In addition, HPLC analysis was performed, confirming the presence of baicalin as the main component of these crude drugs. Peaks of wogonin, wogonoside, baicalein, and chrysin 6-*C*-arabinoside-8-*C*-glucoside were also detected.

Key words

Crude drug, Scutellaria Root, *Scutellaria baicalensis*, Baicalin, HPTLC

1. 緒言

漢方薬の有効性・安全性を確保するためには，原料の生薬の品質確保が必須である．日本では，医薬品として使用される主要な生薬が日本薬局方（局方）[1]に収載され，同規格を満たすことで，生薬の標準化が行われ，品質確保が図られている．現在，局方では，多くの生薬について薄層クロマトグラフィー（TLC）による確認試験法が規定されている．局方で確認試験は，医薬品又は医薬品中に含有されている主成分などを，その特性に基づいて確認するための試験であると規定されている．TLCによる確認試験は，指標成分を検出することで生薬の基原などの特異性を確認できる．一方でその実施により，指標成分以外の成分も含め，含有成分の傾向を明らかにすることができるため，個々の生薬の多様性の範囲を確認し，不良な生薬を除外するための簡便で有効な手段の一つとも考えられる．

近年，インターネットシステムの発展により，インター

[*1] 松山大学薬学部　愛媛県松山市文京町4-2（〒790-8578）
College of Pharmaceutical Sciences, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan
[*2] 医薬基盤研究所薬用植物資源研究センター筑波研究部　茨城県つくば市八幡台1-2（〒305-0843）
Tsukuba Division, Research Center of Medicinal Plant Resources, National Institute of Biomedical Innovation, 1-2 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan
[*3] 国立医薬品食品衛生研究所生薬部　東京都世田谷区上用賀1-18-1（〒158-8501）
Division of Pharmacognosy, Phytochemistry and Narcotics, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[#] 責任著者 Corresponding author

ネットに直結したデータベースがあれば，誰でもそこに容易にアクセスできることになり，データベース上の情報と自分が持つ情報とを照合できるようになった．特に，言葉で表しにくい画像情報がそのようなデータベース上に収載されていれば，出版物ベースのものより圧倒的に利便性が高いものと考えられる．このような背景のもと，筆者らのグループは，日本で使用量の多い生薬を対象として，生薬流通品を幅広く入手し，局方のTLC確認試験を実施し，得られた結果を，医薬基盤研究所で構築している「薬用植物総合データベース」上に公開し，個々の生薬の多様性の範囲を視覚的に示すことを目標に研究を開始している．本論文では，重要生薬である「オウゴン」の国内流通品15検体について，上記目的に沿って，未だデータのない高性能TLC（HPTLC）による一斉分析を実施した．

2. 実験方法

2.1 試料，試薬及び装置

試料としたオウゴン国内15流通品は，日本漢方製剤協会，日本生薬連合及び東京生薬協会を通じて入手した（Table 1）．HPTLCは，HPTLC Silica gel $60F_{254}$ Glass plate（20 × 10 cm）（Merck社製）を用いた．検出は，紫外線（UV）照射（254，366 nm），塩化鉄（III）・メタノール試液（局方に準拠して調製）[2]により行った．標準品のbaicalin，wogoninは局方生薬試験用（薄層クロマトグラフィー用），baicaleinは生薬試験用（いずれも和光純薬製）を使用した．Wogonoside（wogonin 7-*O*-glucuronide）及びchrysin 6-*C*-arabinoside 8-*C*-glucosideは市販標準品がないため，別途オウゴンから単離，同定したものを用いた．Wogonoside[3]：^{1}H-NMR(500 MHz, DMSO-d_6)：δ 8.09 (2H, m, H-2', 6'), 7.62 (3H, m, H-3'-5'), 7.06 (1H, s, H-3), 6.72 (1H, s, H-6), 5.29 (1H, d, J=7 Hz, glcA H-1), 4.03 (1H, d, J=9.5 Hz, glcA H-5), 3.42 (1H, t, J=9 Hz, glcA H-4), 3.30-3.40 (2H, m, glcA H-2, 3), 3.90 (3H, s, -OMe). ^{13}C-NMR (126 MHz, DMSO-d_6)：δ 163.6 (C-2), 105.3 (C-3), 182.4 (C-4), 149.2 (C-5), 98.7 (C-6), 156.0 (C-7), 129.3 (C-8), 156.0 (C-9), 105.3 (C-10), 130.7 (C-1'), 126.4 (2C, C-2', 6'), 129.3 (2C, C-3', 5'), 132.2 (C-4'), 99.6 (glcA C-1), 72.9 (glcA C-2), 75.3 (glcA C-3), 71.2 (glcA C-4), 75.8 (glcA C-5), 170.0 (glcA C-6), 61.4 (-OMe). ESI-MS: m/z 495 $[M-H]^-$. Chrysin 6-*C*-arabinoside 8-*C*-glucoside[4]：^{1}H-NMR (500 MHz, DMSO-d_6)：δ 8.19 (2H, m, H-2', 6'), 7.60 (3H, m, H-3'-5'), 7.01 (1H, s, H-3), 4.80 (1H, d, J=9 Hz, glc H-1), 4.75 (1H, d, J=9 Hz, ara H-1), 3.92 (1H, dd, J=9, 10 Hz, glc H-2), 3.25-3.80 (10H, m, glc H-3-6, ara H-2-5). ^{13}C-NMR (126 MHz, DMSO-d_6)：δ 163.6 (C-2), 104.9 (C-3), 182.5 (C-4), 155.3 (C-5), 108.4 (C-6), 161.3 (C-7), 105.3 (C-8), 158.3 (C-9), 104.1 (C-10), 131.0 (C-1'), 127.0 (2C, C-2', 6'), 129.1 (2C, C-3', 5'), 132.1 (C-4'), 74.2 (ara C-1), 68.5, 70.6 (ara C-2, 3), 73.9 (ara C-4), 69.7 (ara C-5), 73.3 (glc C-1), 71.0 (glc C-2), 78.9 (glc C-3), 70.2 (glc C-4), 82.0 (glc C-5), 61.3 (glc C-6). ESI-MS m/z 547 $[M-H]^-$. 標準品5化合物の構造をFig. 1に示す．その他の試薬は全て特級又は高速液体クロマトグラフィー用を用いた．NMRは，Bruker AVANCE500（Bruker BioSpin社製），MSはBruker micrOTOF-Q（Bruker Daltonics社製）

Table 1 List of Market Samples "Scutellariae Radix" Used in the Present Investigation

No.	Code No.	Place of production or habitat	Collection date (year)	Notes
1	NIB−001	Hebei, China	2010	Cultivated
2	NIB−002	Hebei, China	2009	Wild
3	NIB−035	Hebei, China	2010	unknown
4	NIB−036	Hebei, China	2009	unknown
5	NIB−057	Shangdong, China	2009	unknown
6	NIB−059	Hebei, China	2010	Cultivated
7	NIB−073	Hebei, China	2008	Wild
8	NIB−089	Hebei, China	2009	unknown
9	NIB−105	Hebei, China	2009	Cultivated
10	NIB−106	Inner Mongolia, China	2008	Wild
11	NIB−142	Hebei, China	2001	unknown
12	NIB−145	Hebei, China	2010	unknown
13	NIB−167	Shaanxi Province, China	2009	Cultivated
14	NIB−174	Hebei, China	2010	Wild
15	NIB−175	Shaanxi Province, China	2009	Cultivated

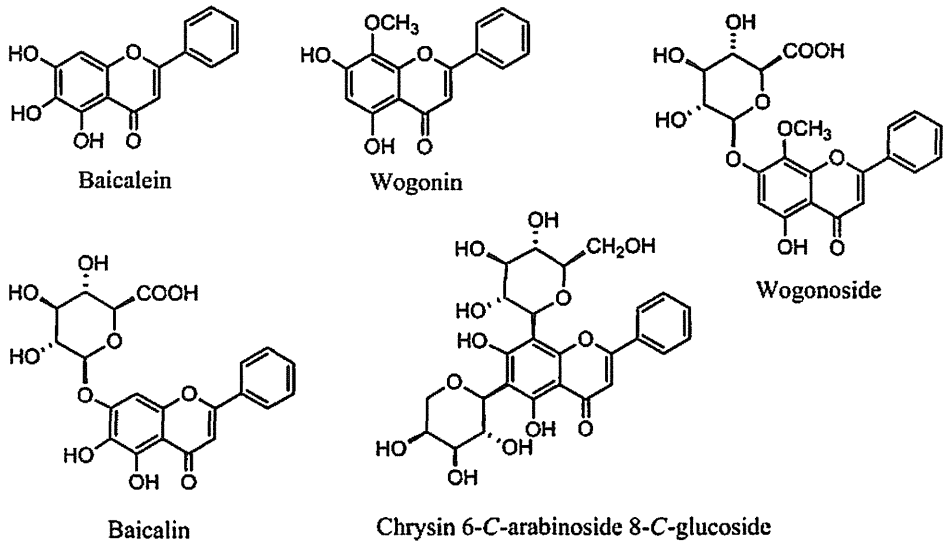

Fig. 1 Structures of 5 Flavones from the Crude Drug "Scutellariae Radix"

で測定した．HPTLCの試料溶液注入にはTLCサンプルアプリケーター リノマートV，画像の撮影にはTLC撮影システム TLCビジュアライザー（いずれもCAMAG社製）を使用した．HPLCはShimadzu Prominenceシステム（島津製作所製）を使用した．HPLC条件を以下に記す．カラム：L-column ODS（2.1 i.d. × 150 mm）（化学物質評価研究機構製），カラム温度：40°C，流速：0.3 mL/min，試料注入量：2 μL，測定波長：200-400 nm，移動相：(A) 5%酢酸水溶液及び (B) アセトニトリル〔濃度勾配条件（B in A）：0 → 30 min（0 → 50%），30 → 35 min（50 → 85 %），35 → 40 min（85 %），40 → 50 min（85 → 90%），50 → 55 min（90 → 100%），55 → 60 min（100%）〕．

2.2 試料溶液の調製

HPTLC用試料溶液は，局方の確認試験法[5]を一部改変して調製した．すなわち，各オウゴン試料の粉末1 gにメタノール10 mLを加え，10分間超音波処理後，ろ過して得られたろ液を各試料溶液とした．標準溶液は，各標準品1 mgをメタノール1 mLに溶解し調製した．HPLC用試料溶液は，試料粉末0.5 gを使用し，還流抽出を行う局方の調製法[5]に準じて調製した．

2.3 HPTLC分析条件

試料溶液及び標準溶液各2 μL（wogonoside及びchrysin 6-*C*-arabinoside 8-*C*-glucosideは各5 μL）を，TLCサンプルアプリケーター（リノマート）を使用してHPTLCガラスプレートにスポット（バンド幅8.0 mm，バンド間隔2.0 mm）し，約7 cm展開した．展開溶媒として，*n*-ブタノール/水/酢酸混液（4:2:1）を用いた．UV照射（254，366 nm）による検出後，塩化鉄（III）・メタノール試液を噴霧して検出した．得られた画像は，TLC撮影システム TLCビジュアライザーにより撮影した．

3. 結果

3.1 HPTLC分析

Table 1に示したオウゴン15検体について試料溶液を調製し，実験の部に記した方法でHPTLC分析を行った．試料溶液のスポットには，ばらつきをなくす目的でTLCサンプルアプリケーター（リノマートV）を用い，TLC画像の撮影には専用機であるTLCビジュアライザーを用いた．その結果，すべての検体において，Fig. 2に示すように大きく3つの領域に明瞭なスポットが観察された．それら成分を明らかにするため，標準品のスポットと直接比較したところ，R_f 0.50付近にある暗緑色のスポットがbaicalinと一致し，本化合物であると同定した．その他，溶媒フロントの近くに観察された褐色のスポットはwogoninと一致した．また，baicalinのすぐ上にあるスポット（R_f 0.58付近の褐色スポット）については，別途分取TLCを行い，2成分（wogonoside及びchrysin 6-*C*-arabinoside 8-*C*-glucoside）を単離，同定することができた．一方，標準品であるbaicalinのアグリコンbaicaleinについては，本条件では分離せず，明確なスポットとして観察されなかった．

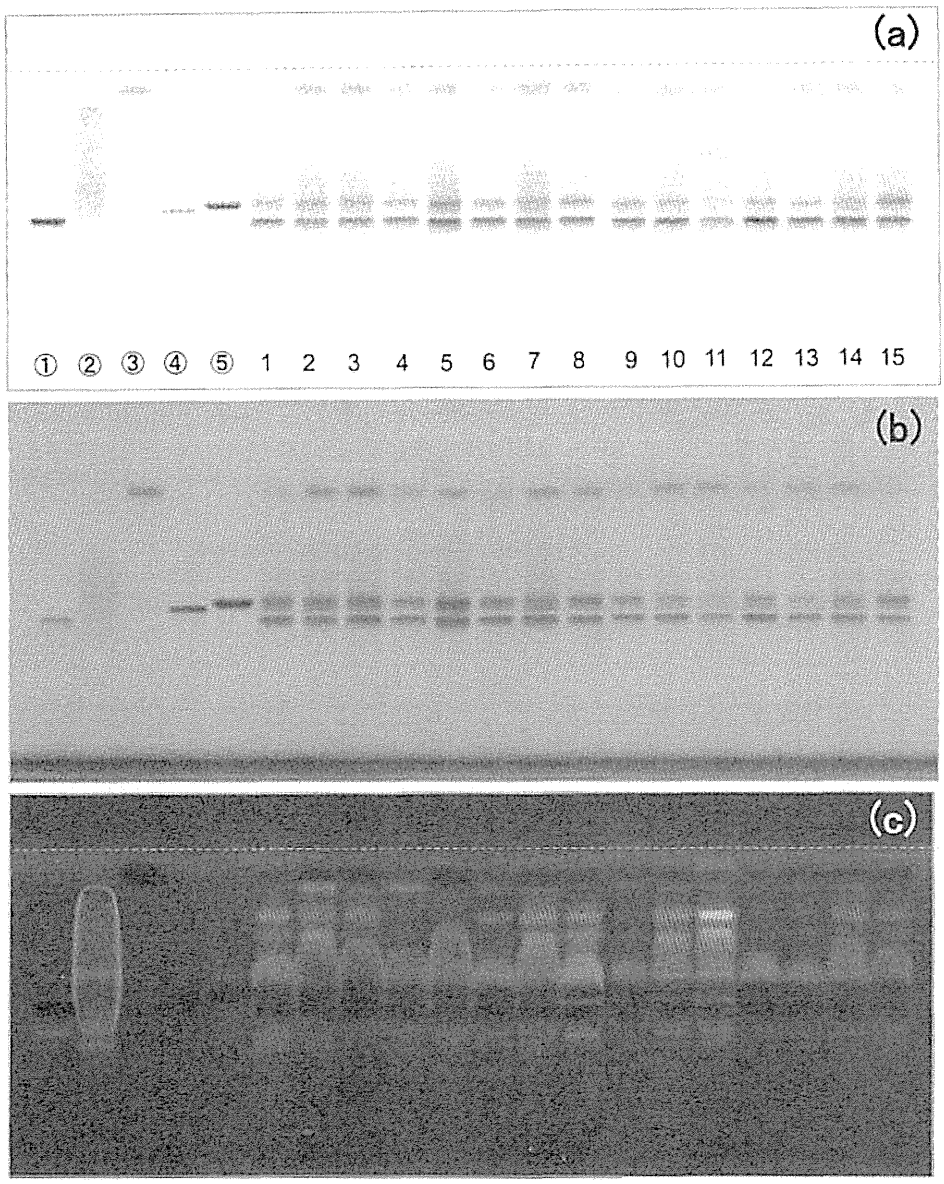

Fig. 2 HPTLC Profiles of 15 Market Samples of "Scutellariae Radix"
1-15: Sample number. ① baicalin, ② baicalein, ③ wogonin, ④ wogonoside, ⑤ chrysin 6-*C*-arabinoside 8-*C*-glucoside.
(a) Iron (III) chloride-MeOH test solution, (b) UV 254 nm, (c) UV 366 nm.

3.2 HPLC 分析

HPTLC 分析したオウゴン 15 検体の試料溶液について，併せて逆相 HPLC 分析を行った．その結果を Fig. 3 に示す．HPTLC の結果と同様，全ての試料において，標準品 baicalin が主ピークとして検出された．Baicalin の他，baicalein, wogonin, wogonoside, chrysin 6-*C*-arabinoside 8-*C*-glucoside の計 5 フラボン類のピークが観察された．

4. 考察

オウゴンの国内流通品 15 検体について，HPTLC による成分比較を実施し，全ての試料において R_f 0.5 付近に標準品 baicalin のスポットが明瞭に検出された．本試験は再現性もよく，塩化鉄 (III)・メタノール試液噴霧による検出で，標準品との比較により判別可能であった．また，baicalin 以外のスポットについても同定することができ，製品中の含有成分の傾向を観察する際の参考データになると考えられる．得られた TLC の結果から産地による成分差異を比較検討したが，明確な区別はできなかった．

HPLC 分析においても baicalin が主要成分として検出され，その他 4 種のフラボン類のピークも観察された．Baicalin と wogoniside の間に数個のマイナーピークが

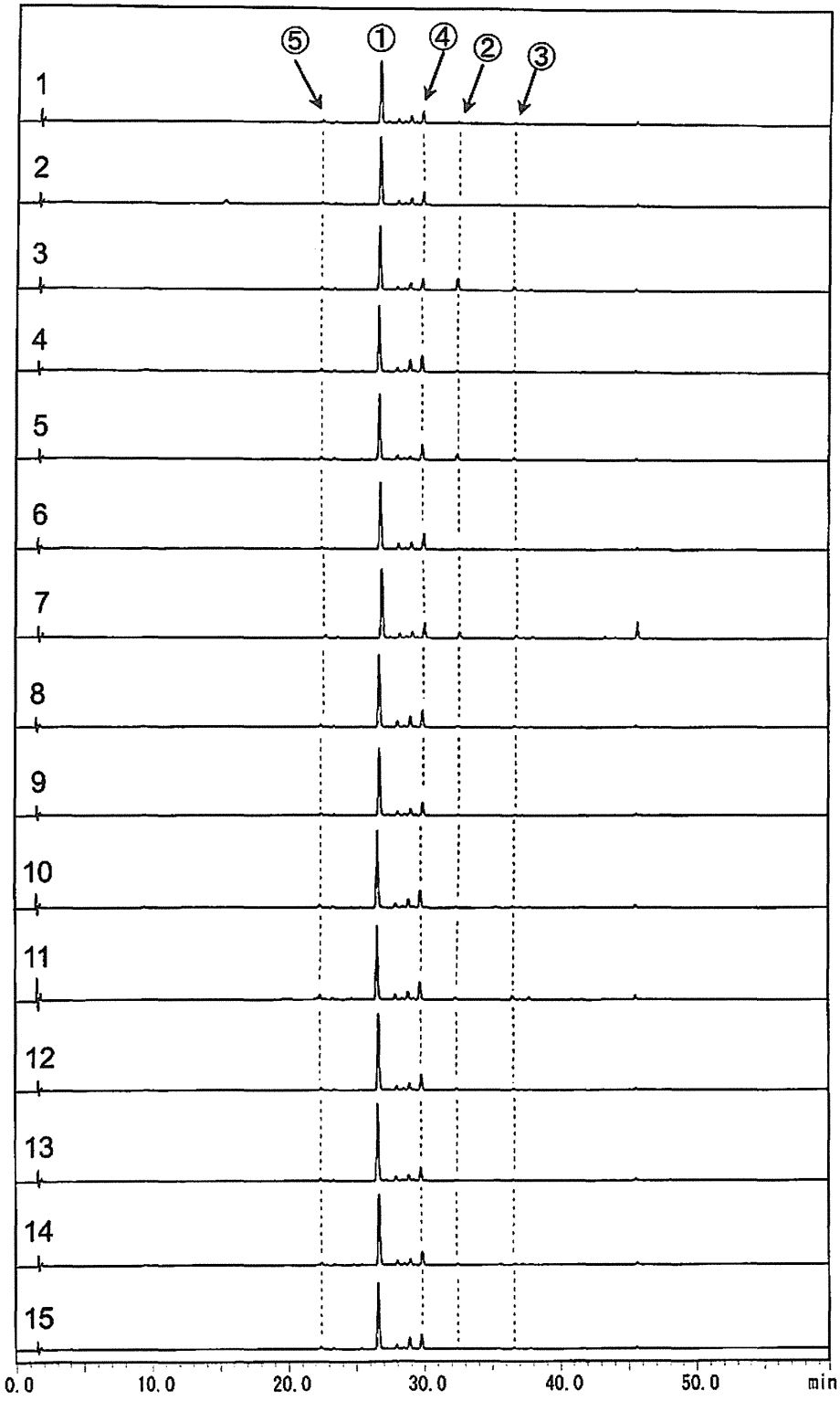

Fig. 3 HPLC Profiles of 15 Market Samples of "Scutellariae Radix"

1-15: Sample number. ① baicalin, ② baicalein, ③ wogonin, ④ wogonoside, ⑤ chrysin 6-*C*-arabinoside 8-*C*-glucoside. Signals were detected at 280 nm.

観察されるが，これらはオウゴン成分分析の文献[6]から，oroxylin A 7-*O*-glucuronide, norwogonin 7-*O*-glucuronide, wogonin 7-*O*-glucoside などのフラボン類のピークと推測される．

今回のような15検体に及ぶ国内流通品生薬の成分評価を同一条件で一斉に実施した例は少なく，更に各スポットを同定したHPTLCによる分離良好な画像データを新たに得ることができた．本結果は今回の目的であるデータベース構築時などの有効な資料になると考えられる．

謝　辞

本研究は，厚生労働科学研究費補助金（創薬基盤推進研究事業）「漢方薬に使用される薬用植物の総合情報データベース構築のための基盤整備に関する研究」により実施された．

文　献

1) 厚生労働省：第十六改正日本薬局方，2011.
2) 厚生労働省：第十六改正日本薬局方，2011，p. 164.
3) Kang, J., Choi, MY., Kang, S., Kwon, HN., Wen, H., Lee, CH., Park, M., Wiklund, S., Kim, HJ., Kwon, SW., Park, S.: *J. Agric. Food Chem.*, 56, 11589-11595 (2008).
4) Takagi, S., Yamaki, M., Inoue, K.: *Phytochemistry*, 20, 2443-2444 (1981).
5) 厚生労働省：第十六改正日本薬局方，2011，p. 1457.
6) Liu, G., Ma, J., Chen, Y., Tian, Q., Shen, Y., Wang, X., Chen, B., Yao, S.: *J. Chromatogr. A*, 1216, 4809-4814 (2009).

J. Trad. Med. 29, 115−123, 2012

Medical and
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The relationship between the color value and pungent compound contents of ginger subjected to heating, soaking in hot water, or steaming

Misato Doui[a,b] and Masayuki Mikage[*a]

[a]*Herbal Medicine and Natural Resources, Division of Pharmaceutical Sciences, Graduate School of Medical Sciences, Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa, 920-1192, Japan.* [b]*Uchida Wakanyaku Co., Ltd., 4-4-10 Higashi-nippori, Arakawa, Tokyo, 116-8571, Japan.* *(Received March 8, 2012. Accepted April 24, 2012.)*

Abstract

Medicinal ginger is produced by various processes in Japan and China, e.g., ginger that has been soaked in hot water or steamed is called *kankyo* in Japan, and roasted ginger is called *hokyo* (*paojiang* in Chinese) in China. However, the heating method differs between the two countries, and the quality of processed ginger might be affected by differences in the heating period and strength. The color of ginger changes markedly during heating. Therefore, in this study, we analyzed the color value and pungent compound contents of our processed ginger samples and elucidated the differences in the quality of these samples. In addition, we investigated the relationship between the color values and pungent compound contents of processed ginger samples.

As a result, we found that the a^* value (indicating redness) of steamed (St) ginger was positively correlated with its 6-shogaol to 6-gingerol ratio ([S/G]), the a^* value of ginger soaked in hot water (Soh) remained constant regardless of the [S/G], and the a^* value of ginger heated at 180℃ (H180) was correlated on a logarithmic curve with [S/G]. In addition, the b^* values (indicating yellowness) of the Soh, St, and H180 ginger samples were negatively correlated with their 6-shogaol concentrations. Therefore, we confirmed that color values are suitable indices for evaluating the quality of processed ginger because they can be used to evaluate its [S/G] ratio and the processing method by this value.

Key words ginger, processing, color, gingerol, shogaol.

Abbreviations H100, heated at 100℃; H180, heated at 180℃; [S/G], 6-shogaol to 6-gingerol ratio; Soh, soaked in hot water; St, steamed; St-AC, steamed by autoclaving; St-P, steamed in a pot.

Introduction

Ginger is a crude drug that is processed for specific medicinal purposes in Japan. In the 16th Japanese Pharmacopoeia,[1] dried ginger is called *shokyo*, and ginger that has been soaked in hot water or steamed before being dried is called *kankyo*. In the Chinese Pharmacopoeia,[2] fresh ginger is called *shokyo* (*shengjiang* in Chinese), dried ginger is called *kankyo* (*ganjiang* in Chinese), and roasted ginger is called *hokyo* (*paojiang* in Chinese). Although heated ginger is used in both countries, the heating methods used differ between the two countries. In addition, the quality of processed ginger can differ due to differences in the heating period and strength.

*To whom correspondence should be addressed.
e-mail : mikage@p.kanazawa-u.ac.jp

Color is an important criterion for evaluating the quality of crude drugs. As for ginger, Japanese *kankyo* is redder than *shokyo*. However, there might be significant interindividual variation in the ability to detect such color changes with the naked eye. Mikage *et al.*[3] previously developed a method for objectively evaluating the quality of crude drugs using a colorimeter and reported that strength of processing given to ginseng could be estimated by measuring its color value. Therefore, in this study, we prepared five samples of ginger that were heated in an oven at 100 or 180℃, soaked in hot water, or steamed in a pot or by autoclaving and analyzed their color values using an objective method.

During the heating process, it has been reported that dehydration occurs and leads to the transformation of gingerol to shogaol, and a longer heating time results in an increased amount of shogaol.[4,5] These pungent compounds have antipyretic, antioxidant, anti-inflammatory, and anti-allergic[6-8] effects and also suppress gastric contraction and reduce blood pressure.[6] Meanwhile, previous reports showed that gingerol strongly inhibited the growth of *Helicobacter pylori*,[9] while 6-shogaol had strong antioxidant, anti-inflammatory,[7,10] and anti-allergic[8] effects. In addition, Govindarajan *et al.*[11] reported that the pungency (measured in Scoville units) of 6-shogaol was twice as high as that of 6-gingerol. Therefore, the ratio of 6-shogaol to 6-gingerol ([S/G]) is an important factor affecting the quality of processed ginger because medicinal effects of those compounds differs slightly each other. So, in this study, we also analyzed the 6-gingerol and 6-shogaol contents of processed ginger and elucidated the relationships between the color values and pungent compound contents of processed ginger samples.

Materials and Methods

Ginger material: Fresh ginger (breed name: *Sanshu*) was purchased from Sakata Nobuo Store Co., Kochi, Japan (2011).

Reagents: The 6-gingerol (purity: 98%) was purchased from Nakalai Tesque, Ltd., and the 6-shogaol (purity: more than 98%) was from Wako Pure Chemical Industries, Ltd. All chemicals were of analytical grade, and the chromatographic solvents were of HPLC grade.

Preparation method: Fresh ginger was cleaned, peeled, sliced into 5 mm thick sections, and mixed well. Eighty to 90 grams of fresh ginger were used in all examinations. Then, we processed the ginger using one of the following five methods:

(1) Soaking in hot water (Soh)

Fresh ginger pieces were heated by putting them in a beaker of water and then placing the beaker in boiling water.

(2) Steaming in a pot (St-P)

Fresh ginger pieces were wrapped with gauze and steamed in a pot for 15 to 180 minutes.

(3) Steaming by autoclaving (St-AC)

Fresh ginger pieces were placed into a culture bottle, and the top was covered with gauze. Then, the ginger pieces were steamed by autoclaving (SD-30N, Tomy Seiko Co., Ltd., Tokyo, Japan) in the following conditions: 120℃ and 2 atm for 5 to 20 minutes.

(4) Heating at 100℃ (H100)

Fresh ginger pieces were heated in an oven set to 100 ℃ for 15 to 180 minutes.

(5) Heating at 180℃ (H180)

Fresh ginger pieces were heated in an oven set to 180 ℃ for 15 to 180 minutes.

After the above treatment, the processed ginger pieces were dried for two nights in an oven set to 40 ℃. Fresh unprocessed ginger that had been dried for two nights in an oven was used as a control.

Color analysis: Each powdered sample (less than 300 μm) was placed into a minimal Petri dish (1.2cm×1cm, i.d.), and the light reflected when the dish was exposed to a D65 standard illuminant was analyzed using a spectral photometer (CM-3500d, Konica Minolta Holdings, Inc.). We evaluated the reflected light using the parameters L^* (brightness), a^* (redness), and b^* (yellowness).

HPLC method: HPLC was performed according to the methods described by Smith *et al.*[12] and Kano *et al.*[13]

(1) Preparation of samples

Each powdered sample (100 mg) was extracted with 10 ml of methanol under ultrasonication for 20 minutes. After centrifugation of the samples at 3000 rpm for 10 minutes, the supernatants were filtered through a 0.45

μm membrane filter. The resultant solutions were injected into the HPLC system.

(2) HPLC conditions

The apparatus comprised an L-2400 detector, an L-2130 pump, a D-2500 chromatograph, an L-2200 autosampler (Hitachi, Tokyo, Japan), a CTO-6A column oven (Shimadzu, Kyoto, Japan), and a COSMOSIL 5C18-MS-2 column (150 mm×4.6 mm i.d.; Nacalai tesque, Kyoto, Japan)

The HPLC conditions were as follows: mobile phase: CH_3CN-CH_3OH-0.5% CH_3COOH (42:3:55 v/v); flow rate: 1.0 ml/min; detection wavelength: 280 nm; column temperature: 35℃, injected sample volume: 10 μl.

Under the above conditions, the recovery rates of gingerol and shogaol, which were calculated using the standard addition method, were 103.9 and 94.4%, respectively (mean of three experiments).

Results

1. Changes in the samples' color values

We analyzed the color values of powdered ginger samples that had been heated with different methods and for various durations. Under all conditions, the a^* values of the samples were negatively correlated with their L^* values. Except for in the H100 ginger, the b^* values of the samples were positively correlated with their L^* values and negatively correlated with their a^* values. The b^* value of H100 ginger varied from 35 to 41 although its a^* and L^* values showed little variation **(Fig. 1)**.

As the heating period increased, the L^* and b^* values of H180 ginger decreased markedly, and its a^* value increased markedly. Meanwhile, the L^* and a^* values of H100 ginger were hardly changed compared with those of unheated ginger, even in the samples processed for 180 minutes.

The ginger samples processed using the Soh or St method displayed decreased and increased L^* and a^* values, respectively. However, the L^* and a^* values of Soh ginger became stable after 30 minutes. As for the St-P ginger, its L^* and a^* values were slightly decreased and increased, respectively. In the case of St-AC ginger, its L^* and b^* values were decreased and its a^* value was increased compared with those of the St-P ginger samples processed for 180 minutes **(Fig. 2)**.

The b^* values of the samples tended to decrease in all conditions.

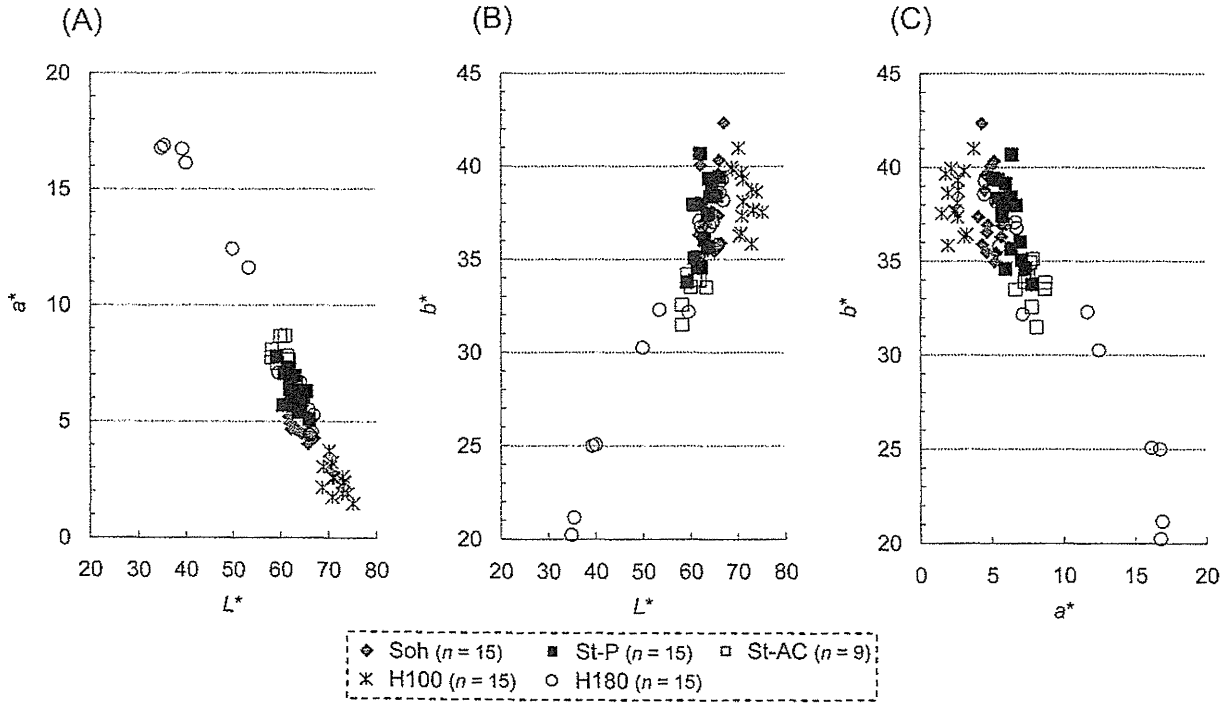

Fig. 1 The color values of powdered ginger samples heated with different methods and for various durations
The relationships between L^* and a^* values (A), L^* and b^* values (B), and a^* and b^* values (C).

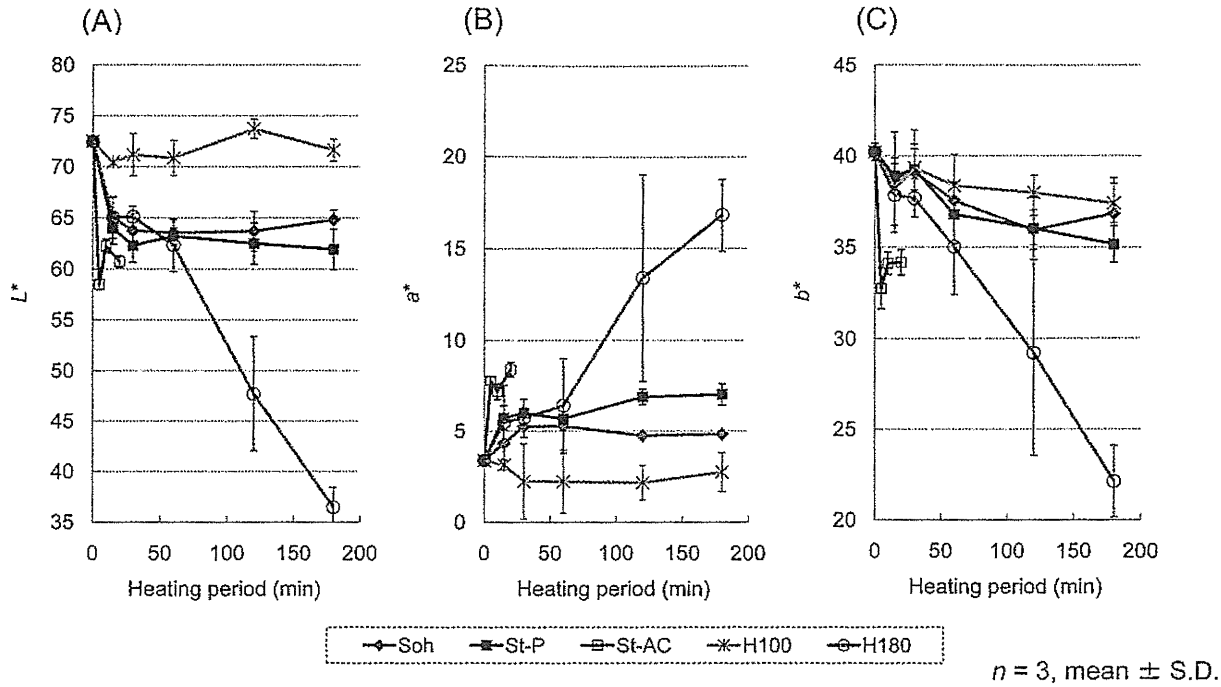

Fig. 2 The changes in the color values of the processed ginger powder samples depended on the heating period
(A) L^*, (B) a^*, (C) b^*

2. Changes in 6-gingerol and 6-shogaol content (Fig. 3)

We compared the changes in the levels of pungent compounds (6-gingerol and 6-shogaol) in ginger heated with various methods. As the heating period increased, the 6-gingerol content decreased and the 6-shogaol content increased, except in the H180 and H100 ginger. As for the H180 ginger, its 6-gingerol and 6-shogaol contents were approximately equal at 60 minutes, and its

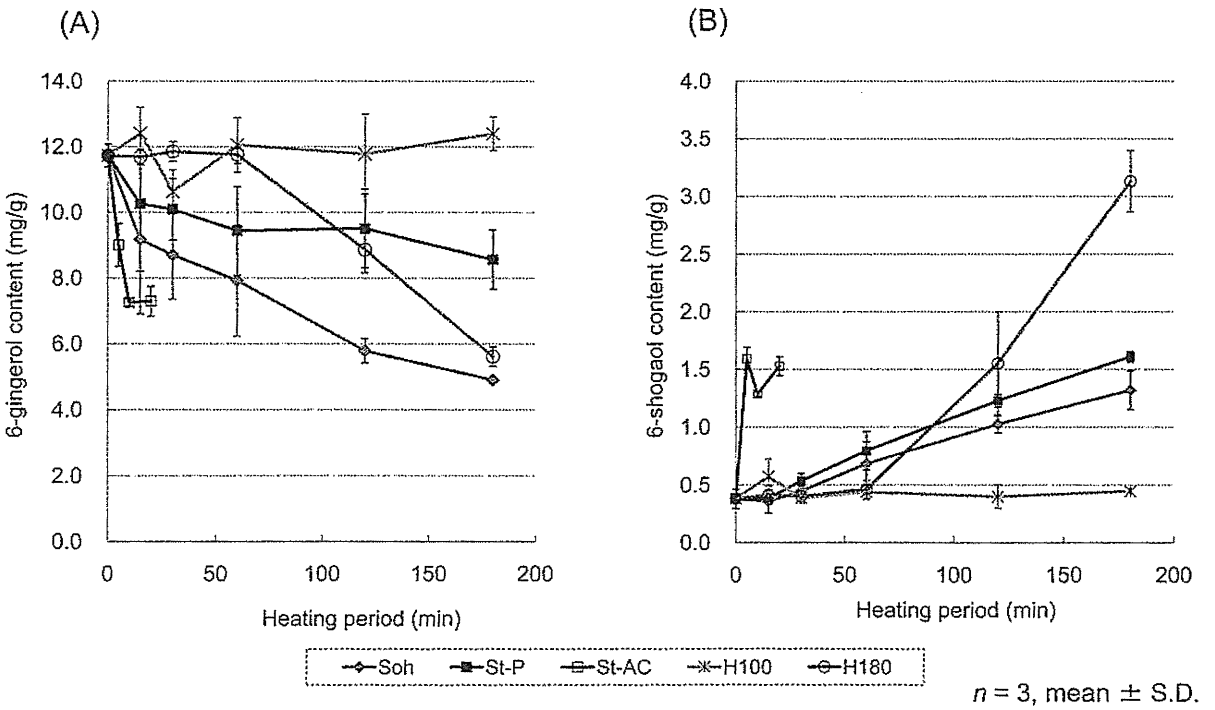

Fig. 3 The changes in the pungent compound levels of the processed ginger depended on the heating period
(A) 6-gingerol, (B) 6-shogaol

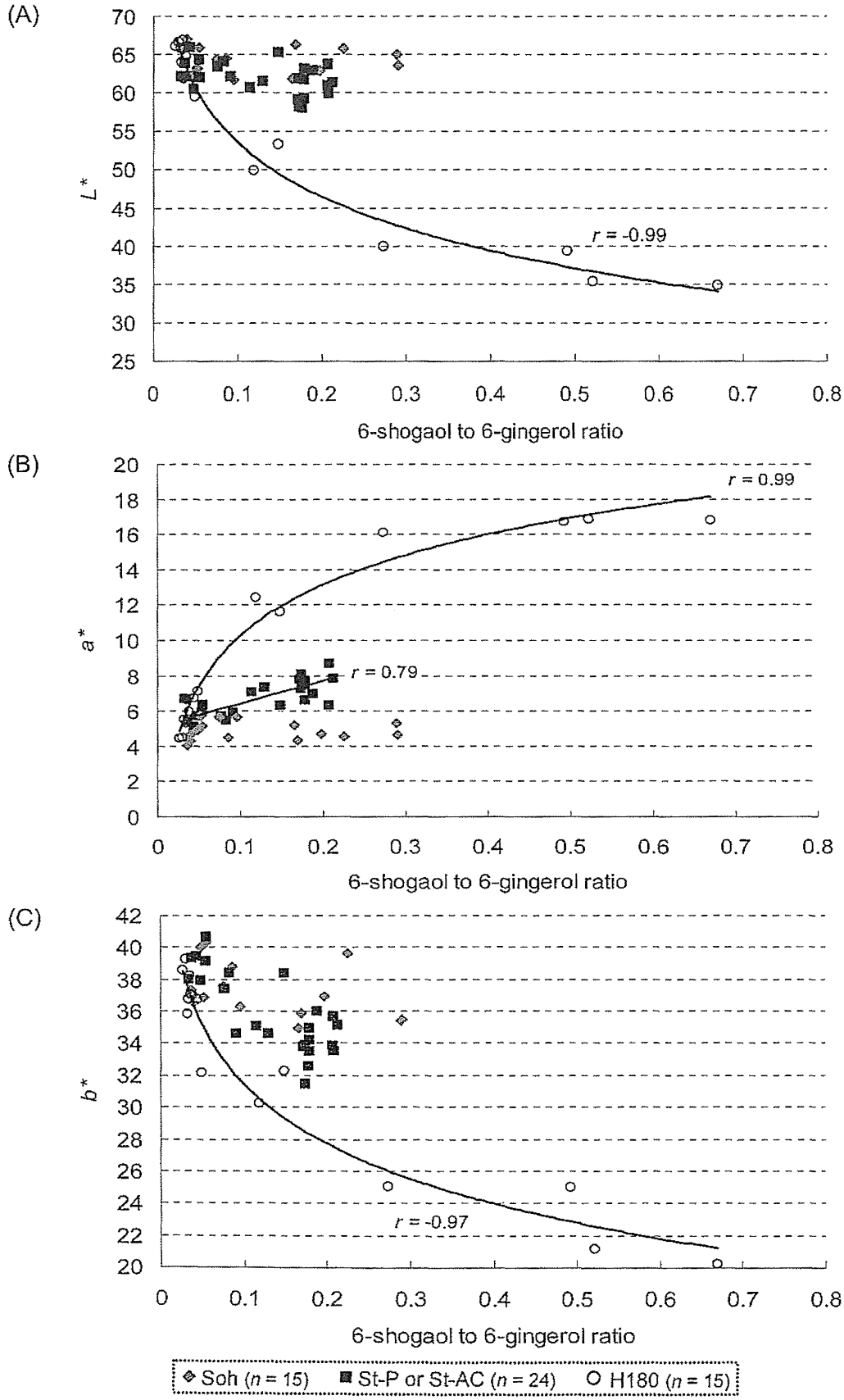

Fig. 4 The relationship between L^* (A), a^* (B), or b^* (C) value and the 6-shogaol to 6-gingerol ratio of processed ginger

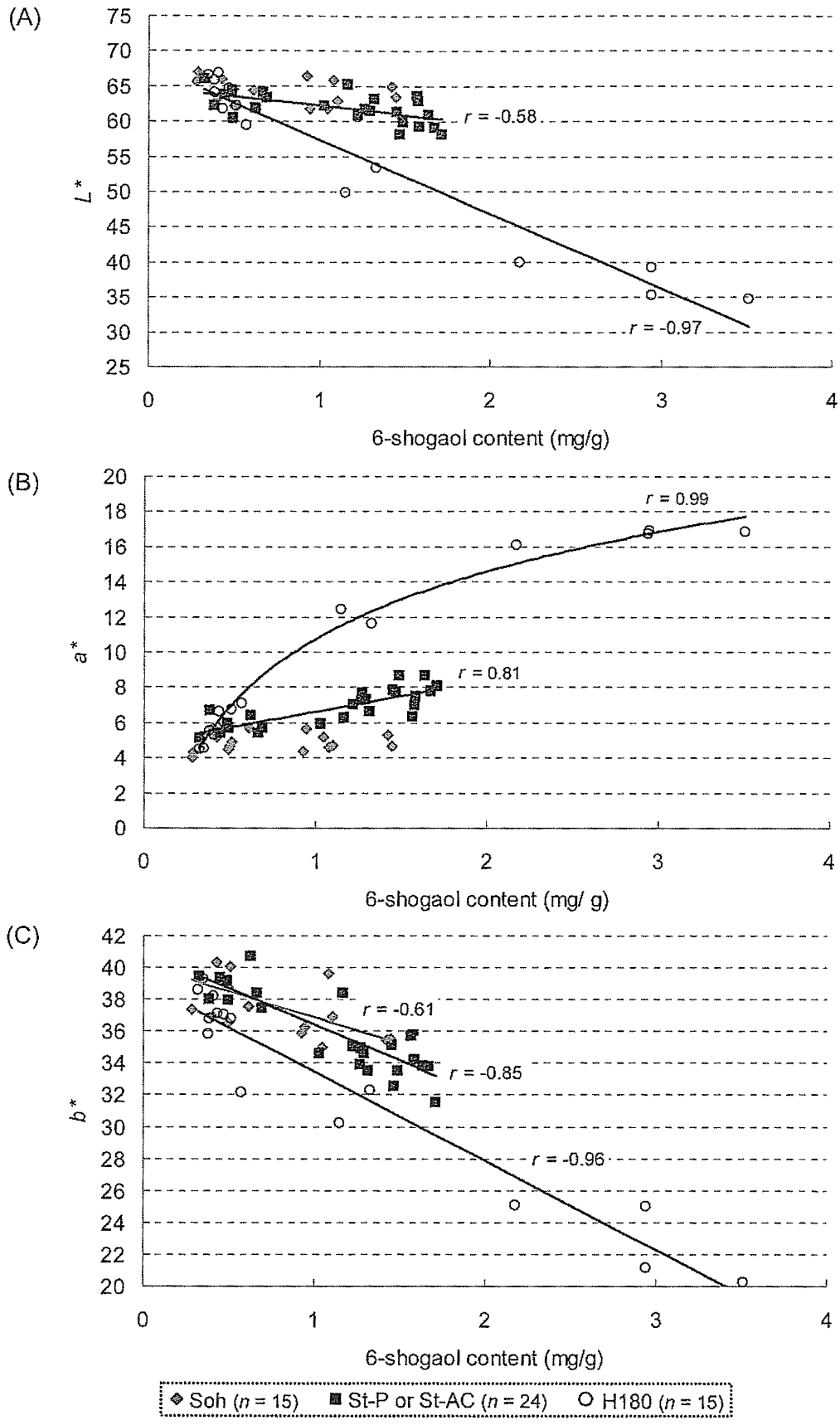

Fig. 5 The relationship between L^* (A), a^* (B), or b^* (C) value and 6-shogaol content of processed ginger