Modulation of benzylisoquinoline alkaloid biosynthesis by overexpression berberine bridge enzyme in *Macleaya cordata*

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*Macleaya cordata* produces a variety of benzylisoquinoline alkaloids (BIAs), such as sanguinarine, protopine, and berberine, which are potential anticancer drugs and natural growth promoters. The genes encoding the berberine bridge enzyme (BBE) were isolated from *M. cordata* and *Papaver somniferum*, and then the two genes were overexpressed in *M. cordata*. Through liquid chromatography with triple-quadrupole mass spectrometry analysis, it was determined that McBBE-OX caused higher levels of (S)-norcoclaurine, (S)-coclaurine, (S)-N-cis-methylcoclaurine, (S)-reticuline, (S)-tetrahydrocolumbamine, (S)-tetrahydroberberine, (S)-cheilanthifoline, and (S)-scoulerine than PsBBE-OX, empty vector or control treatments. qRT-PCR analysis demonstrated that the introduced genes in the transgenic lines were all highly expressed. However, the levels of sanguinarine (SAN) and chelerythrine (CHE) in all the transgenic lines were slightly lower than those in the wild-type lines, possibly because the overexpression of McBBE causes feedback-inhibition. This is the first report on the overexpression of potential key genes in *M. cordata*, and the findings are important for the design of metabolic engineering strategies that target BIAs biosynthesis.

Benzylisoquinoline alkaloids (BIAs) are a large and structurally diverse group of natural products, most of which occur in the families Papaveraceae, Ranunculaceae, Lauraceae, Rutaceae and Menispermaceae\(^1\). Some BIAs have important clinical medicinal benefits; for example, morphine is a narcotic drug, berberine (BBR) has been used to treat bacterial diarrhoea\(^2\), and protopine (PRO) and sanguinarine (SAN) were potential anticancer drugs and have real potential as effective antischistosomal drugs\(^3,4\). More importantly, SAN has been widely used in livestock as an alternative to antibiotic growth promoters\(^6,7\). Recently, the market demand for BIAs has increased steadily every year. However, the use of BIAs is significantly restricted because of its low levels in plants. Fortunately, the synthetic pathways and enzymes of BIAs in the Papaveraceae family of plants have been elucidated in previous studies\(^8,9\). BIA biosynthesis begins with tyrosine; then, two tyrosine derivatives, dopamine and 4-hydroxyphenylacetaldehyde, are condensed to form (S)-norcoclaurine. Then, (S)-norcoclaurine is converted into (S)-reticuline through several steps. If we consider the BIA synthetic pathway to be a network, (S)-reticuline is the key branch-point intermediate of BIAs. This compound is located at a crucial crossroads site, and the N-methyl group of (S)-reticuline is converted into the methylene bridge moiety of (S)-scoulerine by the berberine bridge enzyme (BBE) (Fig. 1). In fact, (S)-scoulerine is the key intermediate in many BIA-producing plants, and BBE is the most critical rate-limiting enzyme in the entire BIA synthesis pathway\(^10\).

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Establishment of Transgenic Plants with the McBBE/PsBBE Gene. We subjected leaf and stem explants to vacuum infiltration with Agrobacterium tumefaciens harbouring McBBE- or PsBBE- and GUS (β-glucuronidase) overexpression vectors, respectively, for 10 min, and then the explants were placed into co-cultivation medium (100 μM acetosyringone) for 3 days. Then, the explants were transferred to selection medium (75 mg/l kanamycin and 400 mg/l Timentin) to determine whether the transgenic lines were established successfully. GUS (β-glucuronidase) histochemical analysis was performed. The GUS histochemical results showed the presence of blue colour in the McBBE-OX and PsBBE-OX plants. In contrast, the wild-type lines did not show GUS activity (Fig. 2A–D). Polymerase chain reaction was performed using GUS, nptII (kanamycin resistance gene) and PsBBE primers for the transgenic lines, one wild type plant (negative control) and pCMVIA2301, pCMVIA2301 + McBBE and pCMVIA2301 + PsBBE plasmid vectors (positive control). The results showed that the reporter gene GUS and the nptII gene were detected in all three vectors and in all the transgenic lines (Fig. 2E–G). Only the 2301 + PsBBE vector and the PsBBE-OX line displayed PsBBE-specific fragments. No PCR amplification product was detected for the wild-type plants.

Figure 1. The metabolic pathway of sanguinarine and chelerythrine. Berberine bridge enzyme (BBE) was studied in this work. 6OMT, norcoclaurine 6-O-methyltransferase; CNMT, cocaicine-N-methyltransferase; NMCH, N-methylcoclaurine hydroxylase; 4OMT, 4′-O-methyltransferase; BBE, berberine bridge enzyme; GFS, chelanthifoline synthase; SPS, stylopine synthase; TNMT, tetrahydroprotoberberine cis-N-methyltransferase; MSH, (S)-cis-N-methylstylopine 14-hydroxylase; P6H, protopine 6-hydroxylase; DBOX, dihydrobenzophenanthridine oxidase; TDC, (S)-canadine synthase; SMT, (S)-scoulerine 9-O-methyltransferase.
Modulation of BIA biosynthesis by overexpression of McBBE and PsBBE in *Macleaya cordata* and transcriptional activation of several genes encoding BIA biosynthetic enzymes. We analysed the BIA content of 3-month-old plants using liquid chromatography with triple-quadrupole mass
and McTDC, McP6H for McBBE-OX lines and were more similar to those for the EV and WT lines, except regarding the expression of gene. However, the results for PsBBE-OX plants were quite different from those,

Furthermore, the reduction in the expression of downstream genes (McBBE positive feedback derived from higher expression of the McBBE transgenic lines. A possible reason for the reduction in SAN content and the increase in reticuline is that (the carbon flow and caused the upstream intermediate substances to accumulate slowly. A similar result was metabolite profiles (Figs 3 and 4). Another unexpected observation in this study was that transformation with gene expression, we found that 9 genes were significantly influenced in transgenic plants, which affected the reason is that the accumulation of a large number of synthetic precursors in the McBBE-OX line resulted in (S-transgenic plants and wild-type plants in McCFS and McTNMT mRNA expression, but (S)tetrahydrocolumbamine, and (S)-reticuline) and the expression levels of upstream genes (Mc6OMT, McCNMT, McNMCH and Mc4OMT) in the different transgenic lines (Figs 3 and 4). Compared with those in the PsBBE-OX, EV and WT lines, the expression levels of upstream genes (Mc6OMT, McCNMT, McNMCH and Mc4OMT) were significantly increased (P > 0.05) in the McCBBE-OX lines. Correspondingly, the metabolite levels ((S)-norcoclaurine, (S)-coclaurine, (S)-N-cis-methylcoclaurine, and (S)-reticuline) in McBBBE-OX lines were also higher than those in the other transgenic lines. A possible reason for the reduction in SAN content and the increase in reticuline content is that positive feedback derived from higher expression of the McBBE gene activates the expression of upstream genes. Furthermore, the reduction in the expression of downstream genes (McTDC, McP6H, and McDBOX) blocked the carbon flow and caused the upstream intermediate substances to accumulate slowly. A similar result was observed in 1-deoxy-D-xylulose 5-phosphate synthase (DXS)-overexpressing tissues in Catharanthus roseus. These unintended consequences often appear when engineering single enzymes to increase flux. For example, overexpression of pythoene synthase in tomato increases lycopene content but downregulates the gibberellin pathway, ultimately resulting in dwarf plants. However, overexpression of BBE in E. californica hairy root culture increases total benzophenanthridines and slightly increases SAN content, indicating that there are difference between species even when the same metabolic pathways are disturbed. On the other hand, the gene expression level of McTDC was significantly decreased in McBBBE-OX lines, while the content of (S)-tetrahydroberberine in the McBBE group was the highest among all the groups (Fig. 3). Similarly, there were no differences between transgenic plants and wild-type plants in McCF5 and McCNMT mRNA expression, but (S)-chelanthifoline and (S)-tetrahydroberberine levels were significantly higher in McBBBE-OX plants than in WT plants. The possible reason is that the accumulation of a large number of synthetic precursors in the McBBBE-OX line resulted in higher levels of downstream products and caused a corresponding impact on metabolites. Through analysis of gene expression, we found that 9 genes were significantly influenced in transgenic plants, which affected the metabolite profiles (Figs 3 and 4). Another unexpected observation in this study was that transformation with PsBBBE did not increase any BIAS. However, the overexpression of PsBBBE in Eschscholzia californica significantly increases the levels of the end products. Because the focus of this study was to use overexpression of McBBE to enhance BIA content, we will study this phenomenon in the PsBBBE plant in a future study.

Previous researchers have succeeded in achieving high production of BIAS by overexpressing the key enzyme in Papaveraceae plants. However, it is difficult to cultivate many Papaveraceae plants; for example, the cultivation of poppy is regulated by the government. In contrast, M. cordata is free of addictive compounds and has a good prospect of cultivation. The alkaloid profile in transgenic M. cordata was clearly made more diverse through overexpression of the key step gene and introduction of the exogenous gene. This article is the first to report the successful overexpression of key genes in M. cordata.

Materials and Methods

CDNA Synthesis and PsBBBE Synthetic. RNA from M. cordata root was isolated using the RNA prep pure Plant Kit (TIANGEN, CHINA) and reverse transcribed into cDNA using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TAKARA, CHINA) according to the manufacturer’s instructions. The cDNA was used as a template for subsequent vector construction. The PsBBBE (GenBankAF025430) synthetic from (Genscript, China).

Plant Materials. The line originated from Fujian province (FJ1, available as seeds upon request; geographic coordinates: 27°46′23.408″N and 117°25′12.560″E) and cultivated at Hunan Agricultural University. Then further propagated via tissue culture to transgenic, metabolic analysis, and qRT-PCR, which would ensure the samples
used in the following experiments have the same genetic background. All the tissue culture and the genetic transformation protocol for *M. cordata* was according to the previous paper.

**Plant Expression Vector Construction.** The plant expression vector pCAMBIA2301 (Fig. 5) was purchased from (Miaolingbio, China). McBBE and PsBBE was amplified using the primer listed in Table 1 by
Figure 4. The analysis of gene expression levels in transgenic lines (pcambia2301 + McBBE, pcambia2301 + PsBBE, pcambia2301) and WT lines. Data represent means ± SD (n = 3). Asterisks denote the significant changes (* means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.005$, **** means $P < 0.001$). EV means empty vector, WT means wild type.

Figure 5. The plant expression vector pCAMBIA2301, pCAMBIA2301 + PsBBE, pCAMBIA2301 + McBBE used for M. cordata transformation. The T-DNA region contains the antibiotic selection marker, NPTII, driven by CaMV35 promoter and polyA signal. The uidA (GUS) reporter is under CaMV35S promoter and nopalinesynthase (Nos) terminator.
polymerase (NEB, Q5® High-Fidelity DNA Polymerase, England). Then purified PCR products were subse-
quently cloned into pCAMBIA2301 using the In-Fusion Cloning Kit (Clontech, USA), and the pCAMBIA2301
was used as the empty vector control. These three vectors were introduced into
A. tumefaciens
strain GV3101,
respectively for transformation in
M. cordata
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Genomic DNA Isolation and PCR Amplification. Genomic DNA was isolated from putative trans-
genic lines and wild type lines using TIANGEN DNeasy Plant Mini Kit (China, TIANGEN). The GUS (750 bp),
npt II (364 bp) and PsBBE (750 bp) primers were used in PCR analysis to confirm the integration of T-DNA
in transgenic lines formation (Table 2). Plasmid of pcambia2301 (positive control), pcambia2301-McBBE and
pcambia2301-PsBBE and wild type (negative control). The PCR amplification program (China, TAKARA): dena-
turation at 94 °C for 5 min, followed by 30 cycles, 94 °C for 1 min, 58 °C for 30 second, and 72 °C for 30 second,
final extension 72 °C for 5 min. The products were amplified products were analyzed by 1% (w/v) agarose gels
prepared in 0.5XTBE (Tris/Borate/EDTA) buffer.
GUS Staining Analysis. We performed GUS histochemical staining in wild-type lines (WT) and putative
transgenic lines. All tissues were infiltrated in phosphate buffer (pH 7.0, 0.1 M), EDTA (pH 8.0, 0.5 M), mannitol
(0.3 M), X-Gluc (pH 7.0, 0.1 M) and 0.5% Triton X-100 for overnight at 37 °C. Then, Stained tissues were cleared
in 70% ethanol.
Metabolite Extraction and LC-QQQ MS Analysis. The WT and putative transgenic lines were collected
after 3-month-old days of culture and ground into a fine powder using liquid nitrogen and then freeze-dried.
Then, use ultrasonic extraction for 30 min at room temperature 1 mL of methanol, followed by ultrasonic extrac-
tion for 60 min at room temperature to isolate metabolites from 50 mg tissues. After filtration through a 0.22-mm
membrane filter (Pall, USA), the solution was quantitative analyzed by LC/triple-quadrupole (QQQ) MS. An
ultra-HPLC Agilent 1290 instrument coupled to a QQQ mass spectrometer (6460 A, Agilent) with a BEH C18
column (2.1 3 100 mm, 1.8 mm; Waters, Ireland) was used for the determination of 18 target alkaloids. The
quantitative analyzed for metabolite according to our previous research9. The LC-QQQ MS data were processed
using the Agilent Mass hunter Quantitative Analysis software (B.07.00). For absolute quantification analysis, the
method was validated using the mixed standard solution, which was diluted with methanol to produce a at least
5 points and was used to evaluate absolute quantification of the target compound.
Gene Expression Analysis by qPCR. Total RNA was isolated from putative transgenic line and wild-type
of M. cordata using MiniBEST Plant RNA Extraction Kit (TaKaRa). The quality of RNA was checked by agarose
gel electrophoresis and quantity was confirmed by Qubit 2.0. One micrograms of total RNA from each sample was
reverse transcribed into cDNA using the PrimeScript RT reagent kit with gDNA eraser (TaKaRa, Dalian, China).
The resulting cDNA products were diluted to 100 μL used as templates for subsequent experiments. PCR was
performed on an ABI 7300 using FastStart Universal SYBR Green Master (ROX) according to the manufacturer’s
instructions. The total volume of Quantitative real-time PCR assay was performed in a 20 μL (10 μL of PCR Mix,
0.5 μL of specific primers, 4 μL of cDNA and 5 μL of water). The qPCR cycling conditions were as follows: 95 °C
for 15 min; 95 °C, 15 s; 55 °C 15 s; 72 °C 20 s, with 40 cycles. In this method, three replicates were performed in all
cases. Relative gene expression was performed using the comparative $2^{-ΔΔCT}$method. We have been proved that
only one gene involved in two methylation steps (4OMT and 6OMT) in previous study. Therefore, the primers
used in 4OMT and 6OMT are the same. All the primer sequence (Table 3) was checked via blast analysis and the
18S gene as the internal reference.

| Primer Name  | Oligonucleotide Sequences (5’-to 3’) |
|--------------|-------------------------------------|
| nptII-F      | TGCTCCTCGGCGAGAAGATAT               |
| nptII-R      | AATATCAGGTTAGCACAAGG                |
| McBBE-plant-F| GGTGATAGCGCGCGCATGATACAAAAATCGAAAC |
| McBBE-plant-R| GATGTTTTCGCCGGGTATCTTCCATT          |
| PsBBE-plant-F| GGTGATAGCGCGCGCATGATGCGAAGCTTAACA  |
| PsBBE-plant-R| GATGTTTTCGCCGGGTATCTTCCATT          |
| GUS-F        | CTGGGTGGATCGAGCCGATCTCACCG          |
| GUS-R        | GCGAAATATTCTCGGTGACCC               |

Table 1. Nucleotide sequences of primers.

| Primer Name  | Oligonucleotide Sequences (5’-to 3’) |
|--------------|-------------------------------------|
| npt II-F     | TGCTCCTCGGCGAGAAGATAT               |
| npt II-R     | AATATCAGGTTAGCACAAGG                |
| PsBBE-F      | GGAGCCGATTCTTGACGGTA               |
| PsBBE-R      | TCGAGACGAAGCTGCTCAA                |
| GUS-F        | CTGGGTGGAGCCGATCTCAGG              |
| GUS-R        | GCGAAATATTCTCGGTGACCC               |

Table 2. Nucleotide sequences of primers.
Statistical Analysis. All the transgenic experiments were carried out in triplicate and each treatment contained 100 explants. All metabolic content and qPCR experiments were analyzed by one-way analysis of variance (ANOVA). The data were statistically analyzed using the GraphPad prism statistical software (version 7.0, USA). Differences between means were determined by analysis of variance with Tukey’s test on the level of significance declared at \( P < 0.05 \).

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| Primer Name | Oligonucleotide Sequences (5′ to 3′) |
|-------------|-------------------------------------|
| Mc6OMT/4OMT-QP-F | CTCAGCCTAGGTCCTACGGAGTC |
| Mc6OMT/4OMT-QP-R | ACCACAGTGGTGTTGAGAGAGA |
| McCNMT-QP-F | GATAGTGGGTGAGAGAGGCT |
| McCNMT-QP-R | GCCCACTCGAGTTGAGAC |
| McNMCN-QP-F | ATAACAGGCTGTCATAGG |
| McNMCN-QP-R | GAGGGTGTGGTGAGAGAGG |
| McBEE-QP-F | TTCACAGGGGTTGGAGAGAG |
| McBEE-QP-R | TTGCTACGCGCTGTCAGG |
| McTDC-QP-F | TGGCGAGGAGGTTGAGAG |
| McTDC-QP-R | GGGGCGGAGGTTGAGAG |
| McSMT-QP-F | GCCCGGAGAGAGAGAGAG |
| McSMT-QP-R | GGGGGCGGAGGTTGAGAG |
| McCFS-QP-F | AAATTTGGGTCGGTTCATG |
| McCFS-QP-R | GCCGGGAGGTTGAGAG |
| McTNMT-QP-F | GCGAGGATAGCTTCGCAG |
| McTNMT-QP-R | CCACTATTCCAGCTGAGAC |
| McTDC-QP-F | ATGCGTCAAGGCTGACAG |
| McTDC-QP-R | TGGAGGAGCTTGTCAAC |
| McSMT-QP-F | GGGGCGGAGGTTGAGAG |
| McSMT-QP-R | GGGGGCGGAGGTTGAGAG |

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Author Contributions
P.H. and J.Z. conceived and designed research. P.H., W.L., M.X., L.X., R.J., P.W., H.L., Z.T. and Q.Z. conducted experiments. W.L., M.X. analyzed data. P.H. wrote the manuscript. All authors read and approved the manuscript.

Additional Information
Competing Interests: The authors declare no competing interests.

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