# Isoquinoline alkaloid biosynthesis is regulated by a unique bHLH-type transcription factor in *Coptis japonica*

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**Abbreviations**: BBE, berberine bridge enzyme; bHLH, basic helix-loop-helix; CaMV, cauliflower mosaic virus; CM, chorismate mutase; CNMT, coclaurine-*N*-methyltransferase; CYP719A1, canadine synthase;CYP80B2, (*S*)-*N*-methylcoclaurine 3'-hydroxylase;DAH7PS, 3-deoxy-*d*-arabino heputulosonate 7-phosphate synthase; DQSDH, dehydroquinate shikimate dehydrogenase;GAPDH,

glyceraldehydes-3-phosphate dehydrogenase; NCS, norcoclaurine synthase; 6OMT, norcoclaurine 6-*O*-methyltransferase; 4'OMT, 3'-hydroxy-*N*-methylcoclaurine-4'-*O*-methyltransferase; PR10, pathogenesis-related protein 10; RAR1, required for Mla12 resistance; SMT, (*S*)-scoulerine 9-*O*-methyltransferase; SOD, superoxide dismutase; THBO, tetrahydroprotoberberine oxidase; TYDC, tyrosine decarboxyrase

**Footnotes**: Nucleotide sequences of full length cDNAs of CjbHLH1 (AB564544), CjbHLH2 (AB564545), CjMYB1 (AB564546), CjMYB3 (AB564547), CjMYB4 (AB564548) as well as other EST clones (AB565440-AB565452, see Supplemental Table) have been deposited in the DDBJ/Genbank/EMBL database.

**[Abstract]**

Specific plant species produce unique isoquinoline alkaloids (IQAs); however, the mechanism of their evolution and the regulation of their biosynthesis are largely unknown. We report here the isolation of a novel basic helix-loop-helix protein, CjbHLH1, from IQA-producing *Coptis japonica*. A BLAST search indicated that CjbHLH1 homologues were only found in plant species that produce IQAs. Transient RNAi and overexpression of CjbHLH1 in *C. japonica* protoplasts revealed the activity of CjbHLH1 in transcription of IQA biosynthetic genes, and little activity in the transcription of genes involves in primary metabolism or the stress response. A chromatin immuno-precipitation experiment using CjbHLH1-specific antibodies revealed the direct interaction of CjbHLH1 with promoter sequences of IQA biosynthetic genes in vivo. We discuss the unique role of CjbHLH1 in IQA biosynthesis.

**Keywords**: Isoquinoline alkaloid biosynthesis, basic helix-loop-helix, *Coptis japonica*, transcriptional regulation

**[Introduction]**

Higher plants produce quite divergent chemicals, such as terpenoids, phenylpropanoids and alkaloids. Whereas terpenoids and phenylpropanoids are commonly found in higher plants, only a limited number of plant species produce certain types of alkaloids. For example, the distribution of isoquinoline alkaloids (IQAs), such as the analgesic morphine, the anti-tussive codeine, the muscle relaxant papaverine, and the antimicrobial berberine, is limited to specific plant families such as Magnoliaceae, Ranunculaceae, Berberidaceae, and Papaveraceae. How these plants evolved to produce alkaloids and how the molecular mechanisms of biosynthesis are regulated are largely unknown (Liscombe et al. 2008).

The biosynthesis of IQAs is widely distributed among alkaloid-producing plant species and the basic pathway of biosynthesis from norcoclaurine to reticuline is common to all IQA-producing plants. Thus, IQA biosynthesis has been intensively investigated at the enzymological level, particularly using cultured plant cells that produce certain types of IQAs (Sato et al. 1984,Galneder et al. 1988, Facchini et al. 1994, Sato et al. 1994, Kutchan 1995, Takeshita et al. 1995, Pauli et al. 1998, Morishige et al. 2000, Choi et al. 2002, Samanani and Facchini 2002, Sato and Yamada 2008). Indeed, almost all enzymatic genes in the berberine biosynthetic pathway have been isolated and characterized from high berberine-producing suspension-cultured *Coptis*

*japonica* (*Ranunculaceae*) cells (Sato et al. 1994, Takeshita et al. 1995, Morishige et al. 2000, Choi et al. 2002, Ikezawa et al. 2003, Minami et al. 2007, Sato et al. 2007). Furthermore, an expression sequence tag (EST) library has been prepared from high berberine-producing *C. japonica* cells (Morishige et al. 2002), and the transcriptional regulation network in *C. japonica* cells has been characterized to aid investigation of the regulatory network in berberine biosynthesis; For example, from this study *CjWRKY1* was isolated as a general transcription factor gene in berberine biosynthesis in *C. japonica* (Kato et al. 2007).

CjWRKY1 was the first WRKY transcription factor to be identified in alkaloid biosynthesis. When the expression of *CjWRKY1* was reduced by transient RNAi in *C. japonica* protoplasts, the expression of almost all of the berberine biosynthetic genes was reduced simultaneously. Moreover, overexpression of *CjWRKY1* in *C. japonica* protoplasts enhanced the expression of almost all of the berberine biosynthetic genes. After *CjWRKY1* was identified as the first general regulator in IQA biosynthesis, Apuya et al. (2008) reported that the ectopic expression of Arabidopsis WRKY1 induced IQA biosynthesis in California poppy cells. Whereas their results suggest that the alkaloid biosynthetic pathway is regulated by some common transcription factor(s) that is also found in a non-alkaloid-producing plant, i.e. Arabidopsis, we report here a unique

bHLH gene active in IQA biosynthesis in *C. japonica* and the regulation of IQA biosynthesis by CjbHLH1.

**[Results]**

**Screening of novel transcription factors for berberine biosynthesis from cultured *Coptis japonica* cells**

After the successful identification of CjWRKY1 as a general transcription factor in IQA biosynthesis from 1014 EST clones of high berberine-producing *C. japonica* cells, we further screened 4032 EST clones. A sequence analysis and homology search of all 5046 EST clones using BLASTx (http://www.ncbi.nlm.nih.gov/) revealed 49 putative transcription factor genes and/or signal transduction-related genes among the ESTs. Because 12 of the clones had been analyzed previously (Kato et al. 2007) and 10 clones were duplicates or undetected due to insufficient amplification by PCR, 27 clones were subjected to further screening (Supplementary Table S1).

Transient RNAi was used to evaluate the activity of the 27 candidate genes in *C. japonica* protoplasts using the expression level of norcoclaurine 6-*O*-methyltransferase (6OMT) gene as a marker of berberine biosynthetic enzymes (Fig. 1). Whereas the RNA-silencing effect of RNAi on each target gene was

consistently high (Supplementary Fig. S1, data of 13 genes are shown), the expression level of *6OMT* varied considerably. While RNAi of some ESTs such as clone 16 and clone 24 enhanced *6OMT* expression, RNAi of clone 48 significantly reduced *6OMT* expression. The silencing effect of clone 48 was most evident and reproducible, and so we focused on the characterization of this clone, which encoded a partial sequence of a basic helix-loop-helix protein, and we assigned it the name *CjbHLH1*. Another EST clone encoded a basic helix-loop-helix protein, but was omitted from the first transient RNAi assay in the *C. japonica* EST library. Because this bHLH sequence was considerably different from *CjbHLH1*, we assigned it the name *CjbHLH2* and tested the effect of RNAi of this gene (Supplementary Fig. S2). Interestingly, RNAi of *CjbHLH2* did not have an RNA-silencing effect on *6OMT* expression.

**CjbHLH1 sequence and its unique phylogenetic relationship with other bHLH family proteins**

Sequence determination of full-length cDNAs of *CjbHLH1* and *CjbHLH2* clarified that they were different (Fig. 2, Supplementary Fig. S3), although both CjbHLH1 and CjbHLH2 had a basic helix-loop-helix domain at the N terminal region and could be classified into Group B, which has a 5-8-13 configuration BxR with a

basic amino acid (K or H) at site 5 and an R at site 13. As indicated in Fig. 2A, CjbHLH1 formed a different clade from other bHLHs found in Arabidopsis and CjbHLH2.

In all of the angiosperm genomes that have been sequenced so far, more than 600 bHLHs have been reported (Arabidopsis, rice, poplar) (Li et al. 2006, Carretero-Paulet et al. 2010). Some bHLH proteins have also been reported to be involved in transcriptional regulation in secondary metabolism, such as phenylpropanoids.

Because CjbHLH1 was suggested to be a transcription factor in IQA biosynthesis, we searched a database with the CjbHLH1 sequence using BLAST. Highly homologous genes were found among the ESTs of *Aquilegia formosa* × *Aquilegia pubescens* (*Ranunculaceae*), *Liriodendron tulipifera* (*Magnoliaceae*), *Saruma henryi* (*Aristolochiaceae*), and *Aristolochia fimbriata* (*Aristolochiaceae*). Amino acid sequence alignment indicated that CjbHLH1 showed approximately 60-80% identity with these homologues. In particular, a bHLH domain and a carboxyl-terminal region of CjbHLH1 and its homologues showed strong similarity (Fig. 2B). On the other hand, the identity between CjbHLH1 and AtRGE1, which is the closest Arabidopsis homologue of CjbHLH1 in the phylogenetic tree and reported to play an important role

in controlling embryo development (Kondou et al. 2008), was low (approximately 20-30%).

These plant species with highly homologous genes were previously reported to produce IQAs (Constantine et al. 1966, Hufford et al. 1975, Wu et al. 2002) and our HPLC analysis confirmed the production of IQAs in these plants, e.g., magnoflorine (Supplementary Fig. S4). This result, and the absence of homologues in *Arabidopsis thaliana* and *Oryza sativa,* suggest the specificity of CjbHLH1 and the homologues to IQA-producing plants.

**Characterization of transcriptional regulation activity of CjbHLH1 on berberine biosynthetic genes**

The regulatory activity of CjbHLH1 in the expression levels of other genes involved in berberine biosynthesis, those involved in primary metabolism including tyrosine biosynthesis, and those involved in stress responses were determined with transient RNAi in *C. japonica* 156-S protoplasts (Fig. 3). RNAi of *CjbHLH1* clearly decreased the expression levels of all berberine biosynthetic enzyme genes examined, i.e., tyrosine decarboxylase (*TYDC*), norcoclaurine synthase (*NCS*), *6OMT*, coclaurine-*N*-methyltransferase (*CNMT*), (*S*)-*N*-methylcoclaurine 3'-hydroxylase

(*CYP80B2*), 3'-hydroxy-*N*-methylcoclaurine-4'-*O*-methyltransferase (*4'OMT*), berberine bridge enzyme (*BBE*), (*S*)-scoulerine 9-*O*-methyltransferase (*SMT*) and canadine synthase (*CYP719A1*), but not that of the tetrahydroprotoberberine oxidase (*THBO*) gene (data not shown). The expression of *PR10-like* gene, which showed sequence similarity with *Thalictrum flavum NCS* gene and coded a protein having NCS enzyme activity, was also suppressed.

On the other hand, the expression of genes involved in primary metabolism, i.e., genes of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 3-deoxy-*D*-arabino heptulosonate 7-phosphate synthase (*DAH7PS*), dehydroquinate shikimate dehydrogenase (*DQSDH*) and chorismate mutase (*CM*), and those involved in the stress response, i.e., pathogenesis-related protein 10 (*PR10*, different from *PR10-like*), required for Mla12 resistance (*RAR1*), superoxide dismutase (*SOD*), cystatin and defensin were not affected by the RNAi of *CjbHLH1*. These results indicate that CjbHLH1 is a general transcription factor that is specific for IQA biosynthesis, and thus shows the similar activity as CjWRKY1.

Transient RNAi analysis suggested that CjbHLH1 acts as a transcription activator in berberine biosynthesis; therefore, we investigated the effect of overexpression of *CjbHLH1* on berberine biosynthetic enzyme gene expression using a

CaMV 35S::CjbHLH1 overexpression vector (Fig. 4). Quantitative RT-PCR analysis showed that overexpression of CjbHLH1 increased the expression levels of all endogenous berberine biosynthetic enzyme genes, i.e., *TYDC*, *NCS*, *6OMT*, *CNMT*, *CYP80B2*, *4'OMT*, *BBE*, *SMT* and *CYP719A1*; however, the levels of increase were moderate in comparison with the large increase in *CjbHLH1* transcripts. On the other hand, transcription of the *THBO* gene was not affected (data not shown). The expression of primary metabolism genes, i.e., *GAPDH* and *CM,* and those of the stress response, i.e., *RAR1* and cystatin, were also not affected by overexpression of *CjbHLH1*. The modest effects of overexpression of *CjbHLH1* might be due to the fine-tuning of this transcription regulator. That is, a moderate increase by *CjbHLH1* overexpression was detected at 6 h after transient expression, but was not detected after longer incubation (Supplementary Fig. S5).

**Additional characterization of bHLH-associated factors, which may affect the transcriptional activity of CjbHLH1 and subcellular localization**

To clarify the role of bHLH, we investigated the involvement of MYB and WD40, which are known to be associated with the transcriptional activation of bHLH in phenylpropanoid biosynthesis. Four MYB and three WD40 homologues were found

among ESTs and examined with RNAi (Supplementary Fig. S6), but no MYB or WD40 genes affected *6OMT* expression.

When the subcellular localization of CjbHLH1 was characterized to examine the possibility of regulation through nuclear localization, the reporter construct sGFP-CjbHLH1 clearly indicated the stable nuclear localization of CjbHLH1 in *C. japonica* 156-S protoplasts and onion cells (Fig. 5).

**Detection of transcriptional activation activity of CjbHLH1 using biosynthetic enzyme gene promoter::luciferase reporter gene**

Because the effect of overexpression of CjbHLH1 on the endogeneous biosynthetic enzyme genes was modest, the transcriptional activation activity of CjbHLH1 was further examined using the co-transformation of an overexpression vector of *CjbHLH1* with a biosynthetic enzyme gene promoter::luciferase (*LUC*) reporter construct (Fig. 6A). Co-transformation experiments showed significant activation of the expression of *4'OMT* promoter::*LUC* or *CYP719A1* promoter::*LUC* reporter by the overexpression of *CjbHLH1*, whereas there was little activation of the expression of *CYP80B2* promoter::*LUC*.

**Detection of the specific binding of CjbHLH1 to biosynthetic enzyme gene promoter by chromatin immuno-precipitation**

Specific binding of CjbHLH1 to the promoter region of the *4'OMT* and *CYP719A1* genes was confirmed by chromatin immuno-precipitation (ChIP) using CjbHLH1-specific antibodies. PCR analysis using primer pairs specifically designed to amplify the promoter regions of the *4'OMT* and *CYP719A1* genes indicated that CjbHLH1 specifically and directly bound to promoters of the *4'OMT* and *CYP719A1* genes close to the translation start site *in vivo*. However, a significant difference between with and without CjbHLH1-specific antibodies was not detected with the promoter region of *CYP80B2* gene used in this experiment (Fig. 6B).

**[Discussion]**

**Unique bHLH1 in isoquinoline alkaloid biosynthesis**

CjbHLH1, identified here, has a unique sequence that is specific to its homologues found in IQA-producing plant species. In contrast, more than 600 bHLH proteins have been found in *Arabidopsis thaliana* and rice genomes (Li et al. 2006) and several bHLH proteins have been identified in secondary metabolism, particularly in phenylpropanoid biosynthesis (such as AtTT8, AtGL3, AtEGL3, PhJAF13, PhAn1, and ZmB/R) (Koes et

al. 2005). CrMYC1, which is involved in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (Chatel et al. 2003), and NbbHLH1 and NbbHLH2, which are involved in nicotine biosynthesis in *Nicotiana benthamiana* (Todd et al. 2010), are also very different from CjbHLH1 and its bHLH homologues (Fig. 2A). Multiple alignments indicate that the presence of Glu at position 9 enables CjbHLH1 to contact with the E-box, whereas several residues of the bHLH domain or C-terminal regions in CjbHLH1 and its homologues are distinct from those in AtRGE1 (Fig. 2B). It would be interesting to investigate whether these residues have a particular role.

Transient assay suggests that CjbHLH1 is specific to berberine biosynthesis and does not affect the expression of genes involved in primary metabolism and the stress response, as is also the case for CjWRKY1. These specific functionalities of the transcriptional regulation of CjbHLH1 and CjWRKY1 in berberine biosynthesis in *C. japonica* cells are clearly different from that of ORCA3, a jasmonate-responsive AP2-domain transcription factor, isolated from indole alkaloid biosynthesis in *C. roseus*, which trans-activated both secondary and primary metabolism (van der Fits et al. 2000).

It is also notable that transgenic approaches using an Arabidopsis transcription factor library with California poppy and a poppy biosynthetic gene promoter::*LUC* assay system did not identify bHLH. As Apuya et al. (2008) mentioned in their report,

Arabidopsis may lack some essential cofactors for transcription, which exist in alkaloid-producing tobacco or poppy cells, or Arabidopsis bHLH may lack affinity for IQA-specific promoters. The unique presence of CYP719-family genes in IQA-producing plants is also known (Sato et al. 2009). While the unique presence of the CYP719 family would depend on the structure of the substrates, the evolutionary force that underlies the unique bHLH transcription factor is an open question.

**Transcriptional regulation network in isoquinoline alkaloid biosynthesis**

In gene regulation of phenylpropanoid biosynthesis, phytochrome signaling and trichome development, bHLH is known to associate with MYB and WD40 repeat proteins (Ramsay et al. 2005, Jang et al.2007, Gonzalez et al. 2008). However, our preliminary studies, with a limited number of EST clones of MYB or WD40 isolated from *C. japonica* cells, did not show an effect of these genes on the expression of *6OMT* in transient RNAi experiments (Supplementary Fig. S6). Because these factors may function redundantly, more detailed investigation is required.

On the other hand, moderate, but statistically significant activation of endogenous berberine biosynthetic enzyme genes by the overexpression of *CjbHLH1* after 6 h incubation suggests the possibility that CjbHLH1 functions as a transcription

activator without other factors (Fig. 4). However, increase in the expression levels of endogenous biosynthetic enzyme genes was not detected after 24 h of transient overexpression of *CjbHLH1*(Supplementary Fig. S5). Further analysis of the expression levels of endogenous biosynthetic enzyme genes after 72 h of transient overexpression of *CjbHLH1* indicated that the overexpression of *CjbHLH1* halted the increase in gene expression found in control 156-S protoplasts without *CjbHLH1* overexpression (Supplementary Fig. S5). A further factor to consider is the effect of protoplast formation and PEG-mediated transformation on gene expression. There would be a steady increase in biosynthetic enzyme genes expression in control 156-S protoplasts, induced by the elicitation stimuli due to protoplast formation and PEG-mediated transformation, whereas *CjbHLH1* and *CjWRKY1* expression was slightly enhanced or not affected (data not shown). These results suggest that a large excess of CjbHLH1 may hinder the endogenous increase in biosynthetic enzyme genes expression, which would be regulated by other transcription factor(s).

Longer enhancement of gene expression of *LUC,* driven by either the *4'OMT* promoter or *CYP719A1* promoter, with overexpression of *CjbHLH1* may reflect the dose-dependent effect of CjbHLH1 in berberine biosynthesis. That is, a large excess of gene expression of *CjbHLH1* may inhibit the interaction of other endogenous regulatory

factors to promoter regions of berberine biosynthetic enzyme genes, which Gill and Ptashne (1988) and Natesan et al. (1997) reported as “squelching” of an accessory protein. An increase in promoter sequences by the introduction of a berberine biosynthetic enzyme gene promoter::reporter construct would reduce this constraint of transcription factors (Supplementary Fig. S7). Indeed, when the amount of berberine biosynthetic enzyme gene promoter::*LUC* construct was decreased, activation of *LUC* gene expression was reduced (data not shown). These results suggest that CjbHLH1 works with other interacting factor(s) or other transcription factors. Recently, CrMYC2, a homolog of NbbHLH1 and NbbHLH2, was reported to work as a transcriptional regulator for transcription factor ORCA in indole alkaloid biosynthesis of *Catharanthus roseus* (Zhang et al. 2011). On the other hand, NbbHLH1 was reported to work cooperatively with ORC1, an ORCA homolog, for the maximum transactivation of nicotine alkaloid biosynthesis in jasmonate-inducible signaling cascades (De Boer et al. 2011). These findings, including the role of CjbHLH1 in isoquinoline alkaloid biosynthesis, support the possibility of the existence of a complicated transcriptional network of bHLH in alkaloid biosynthesis. Successful immuno-precipitation of chromatin would provide useful material to study these interacting proteins or modifications of transcription factors.

The above model suggests that CjWRKY1 is one of the other regulatory factors working together with CjbHLH1 in gene regulation of berberine biosynthesis in *C. japonica* cells. The two transcription factors, however, would work as independent transcription activators. When we examined the interaction of these genes, dsRNA of *CjbHLH1* did not affect the level of *CjWRKY1* mRNA and vice versa (data not shown). Furthermore, co-expression of *CjbHLH1* and *CjWRKY1* did not overcome the overexpression effect of *CjbHLH1*; the expression of biosynthetic enzyme genes in *CjbHLH1* overexpression was not increased by additional expression of *CjWRKY1* (data not shown). How these signal pathways are incorporated to regulate the biosynthetic activity is an interesting subject for future study.

## [Materials and Methods]

### Plant material and construction and sequencing of EST library of *C. japonica*

*C. japonica* 156-1, a cell line with high berberine productivity, and 156-S, cell line 156-1 transformed with overexpression vector of *SMT*, were maintained as described previously (Sato et al. 1984, Sato et al. 2001).

An EST library of *C. japonica* 156-1 cultured cells was constructed and sequencing of the EST library was performed as described previously (Morishige et al.

2002, Kato et al. 2007). ESTs obtained from the library were annotated using a tBLASTX search.

**In vitro double-stranded (ds) RNA synthesis and transient RNAi in *C. japonica* protoplasts**

In vitro dsRNA preparation was performed using a T7 RiboMAX Express RNA Production System (Promega) with PCR. The templates for dsRNA preparation were produced with primers harboring the T7 RNA polymerase promoter at both ends (see Supplementary Table S1). *C. japonica* protoplasts were prepared from 2- to 3-week-old 156-S cultured cells as reported previously (Dubouzet et al. 2005). Transient RNAi analysis was also performed as described previously (Kato et al. 2007).

**Quantitative RT-PCR**

Total RNA extraction, reverse transcription and quantitative RT-PCR with specific primers (see Supplementary Table S2) were performed with the obtained cDNAs and iQTM SYBR® Green Supermix (Bio-Rad) using a DNA Engine Opticon Continuous Fluorescence Detection system (MJ Research) or CFX96 Real-time PCR Detection System (Bio-Rad). The data were analyzed as described previously (Kato et

al. 2007).

**Sequence alignment analysis**

Other bHLH amino acid sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/), and bHLH domain sequences were searched for in SMART (http://smart.embl-heidelberg.de/). Sequences were aligned by ClustalW. The phylogenetic tree was created with MEGA 5.0 software (Tamura et al. 2007) using ClustalW and the neighbor-joining method. Bootstrap tests were performed using 1000 replicates.

**Subcellular localization analysis**

To determine the subcellular localization of CjbHLH1 of *C. japonica*, the sGFP-CjbHLH1 fusion vector was constructed. In brief, *CjbHLH1* cDNA was amplified with primers that have *BsrGI* sites at both ends. The PCR products were cloned into pGEM-T Easy vector (Promega) and the sequence integrity was confirmed. The resulting plasmid was digested with *BsrG1* and the *CjbHLH1* fragment was ligated to the cloning site of the Cauliflower Mosaic Virus (CaMV) *35S*::*sGFP* (S65T) vector (Niwa et al. 1999) to produce *35S*::*sGFP-CjbHLH1* construct. The *sGFP* fusion

construct was transformed into 156-S protoplasts of *C. japonica* by PEG transformation or into onion epidermal cells by particle bombardment using PDS-1000/He (Bio-Rad). Bombardment was performed under the following conditions: vacuum of 71 mm Hg, helium pressure of 7585 kPa, and target distance of 6-8 cm using 1.0 μm gold microcarriers. GFP fluorescence was observed by fluorescence microscopy (KEYENCE, BZ9000) in *C. japonica* protoplasts or onion epidermal cells after incubation at 28°C or 23°C for 24 h, respectively.

**Preparation of CjbHLH1-specific antibodies**

An expression vector to produce recombinant CjbHLH1 protein in *Escherichia coli* was constructed. *CjbHLH1* cDNA was amplified by PCR with the designed primers to introduce *BamHI* and *EcoRI* sites at the 5' and 3' ends, respectively. The PCR product was cloned into pT7Blue vector (Novagen) and the sequence integrity was confirmed. The resulting plasmid was digested with *BamHI* and *EcoRI*, and the CjbHLH1 fragment was ligated into pET-22b vector. Recombinant *CjbHLH1* was produced in *E. coli* Rosetta-gami B (DE3) with this construct at 37°C for 3 h after 1 mM isopropylthiogalactoside induction. The recombinant protein formed an inclusion body, and therefore the pellet was solubilized with 8 M urea buffer [8 M urea, 100 mM

$NaH_2PO_4$, 10 mM Tris-HCl (pH 8.0)], and purified on a Ni column (Qiagen, Ni-NTA Agarose) and a cation exchange column (GE Healthcare, Hi Trap SP). The purified recombinant CjbHLH1 protein was injected into rabbits to prepare polyclonal antibodies by Keari (Osaka, Japan).

**Transient luciferase reporter assay**

The transcriptional activation activity of CjbHLH1 was determined with a transient luciferase reporter assay using a Dual-Luciferase Reporter Assay System (Promega). *CjbHLH1* overexpression vector was constructed as a *CjWRKY1* construct (Kato et al. 2007). Three berberine biosynthetic gene promoters were amplified by thermal asymmetric interlaced (TAIL)-PCR and luciferase reporter constructs were prepared. The assay conditions were as described previously except that plasmid concentrations of 5 μg effector construct, 4 μg reporter construct and 0.05 μg reference construct were used (Kato et al. 2007).

**Chromatin immunoprecipitation**

To detect the direct interaction of CjbHLH1 with the promoters of genes coding for berberine biosynthesis enzymes, chromatin fragments were precipitated with

CjbHLH1-specific antibodies, and genomic DNAs of biosynthetic genes were detected.

For preparation of crude nuclei, 156-S protoplasts ($2.0\times10^7$) were suspended in 25 ml of W5 solution [154 mM NaCl, 125 mM $CaCl_2\cdot2H_2O$, 5 mM KCl, and 5 mM glucose] containing 1% formaldehyde, for 10 min, for cross-linking. Nuclei were isolated according to the method described previously (Lee et al. 2007, Koyama et al. 2010). Protoplasts were suspended in 15 ml of extraction buffer I [0.4 M Sucrose, 10 mM Tris-HCl (pH8.0), 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, and protease inhibitor cocktail without EDTA (Roche)], fractured and centrifuged at 4000 rpm for 20 min at 4°C. The pellet was suspended in 1 ml of extraction buffer II [0.25 M Sucrose, 10 mM Tris-HCl (pH8.0), 10 mM $MgCl_2$, 1% Triton X-100, 5 mM β-mercaptoethanol, and protease inhibitor cocktail without EDTA (Roche)] and centrifuged at 13000 rpm for 10 min at 4°C. Then, the resultant pellet was suspended in 300 μl of extraction buffer II and layered on top of an equal volume of extraction buffer III [1.7 M Sucrose, 10 mM Tris-HCl (pH8.0), 2 mM $MgCl_2$, 0.15% Triton X-100, 5 mM β-mercaptoethanol, and protease inhibitor cocktail without EDTA (Roche)] and centrifuged at 13000 rpm for 1 h at 4°C. The resultant nuclei pellet was suspended in 500 μl of lysis buffer [50 mM Tris-HCl (pH8.0), 10 mM EDTA, and 1% SDS] and sonicated to yield small DNA fragments with an average length of 0.2 to 2 kb (centered around 0.5 kb). For

immuno-precipitation of chromatin, the 200 μl of sonicated chromatin solution was diluted 10-fold with dilution buffer [1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH8.0), and 167 mM NaCl] and incubated with 40 μl of Dynabeads Protein A (Invitrogen) that was pre-blocked with salmon sperm DNA. The pre-treated chromatin fragments were incubated with 5 μl CjbHLH1-specific antibodies serum overnight at 4°C. Immuno-complexes were recovered by incubation with Dynabeads Protein A (Invitrogen) for 1 h at 4°C. After several washings, immuno-complexes were eluted with 500 μl of elution buffer [1% SDS, 100 mM $NaHCO_3$] and subjected to reverse cross-linking by incubation at 65°C overnight. DNA was isolated from chromatin by the incubation with protease K (Invitrogen), phenol-chloroform extraction, and ethanol precipitation. The isolated DNA was subjected to PCR for 39 cycles with sets of primers specific to the corresponding promoter regions (see Supplementary Table S3).

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**[Figure legends]**

Fig. 1 Effect of RNAi of 27 candidate genes on *6OMT* in berberine biosynthesis.

*Coptis japonica* 156-S protoplasts were treated with dsRNA of each candidate gene, and the *6OMT* transcript level was determined by quantitative RT-PCR after 72 h incubation. The relative transcript level was calculated using the V-type ATPase c subunit gene as an internal control, and dsRNA of GFP was used as a control (C) of RNAi. Error bars indicate the standard deviation (n=3).

Fig. 2 Phylogenetic tree of plant bHLH transcription factors and sequence alignment of CjbHLH1 and its homologs.

(A) A phylogenetic tree was constructed with amino acid sequences of typical bHLH domains obtained for Arabidopsis and some secondary metabolism-related genes which were obtained from NCBI and SMART. CjbHLH1 and its homologues found in isoquinoline alkaloid-producing plant species are shown in red and bHLH in other secondary metabolism such as phenylpropanoid biosynthesis (AtTT8, PhAN1, AtGL3, AtEGL3, AmDEL, ZmLc), indole alkaloid biosynthesis (CrMYC1) or nicotine biosynthesis (NbbHLH1, NbbHLH2) are shown in blue. Accession numbers are: Q9FN69, Arabidopsis AtGL3; Q9CAD0, AtEGL3; Q39204, AtMYC2; O80536, AtPIF3 (PHYTOCHROME-INTERACTING FACTOR 3); Q8W2F3, AtPIF4; Q8W2F1,

AtMYC1; Q8GZM7, AtPIL5 (PHYTOCHROME-INTERACTING FACTOR 3-LIKE 5); Q9ZPY8, AtAIB (ABA-INDUCIBLE bHLH-TYPE); O81900, AtDYT1 (DYSFUNCTIONAL TAPETUM 1); Q9FUA4, AtSPATULA; Q9FE22, AtHRF1 (LONG HYPOCOTYL IN FAR-RED 1); Q9SND4, AtHEC2 (HECATE 2); O81313, AtORG3 (OBP3-responsive gene 3); Q9M1K0,AtIND (INDEHISCENT); Q9FT81, AtTT8; Q9LXD8, AtHEC3; Q9M8K6, AtMUTE; Q9LEZ3, AtBIM1 (BES1-interacting Myc-like protein 1); O22768, AtUNE12 (UNFERTILIZED EMBRYO SAC 12); Q9ZVX2, AtAMS (ABORTED MICROSPORES); NP_195179, AtCIB1 (CRYPTOCHROME-INTERACTING BASIC-HELIX-LOOP-HELIX 1); Q9FHA2, AtALC (ALCATRAZ); NP_173950, AtCIB5; Q9FXA3, AtRGE1 (RETARDED GROWTH OF EMBRYO 1); Q56YJ8, AtFMA; Q700C7, AtSPCH (SPEECHLESS); Q9ZVB5, AtbHLH100; Q9CAA4, AtBIM2; Q9CAA4, AtICE1 (INDUCER OF CBF EXPRESSION 1); Q8L5W8, AtPIL1; Q8L5W7, AtPIL2; Q9FMB6, AtBIM3; AAQ14331, Catharanthus CrMYC1; P13526, Maize ZmLc; AAG25928, Petunia PhAN1 (ANTHOCYANIN 1); AAC32828, soybean GmSAT (symbiotic ammonium transporter); AAA32663, snapdragon AmDEL; FAA00382, rice OsIRO2; AAO73566, OsPTF1 (Pi starvation-induced transcription factor 1); GQ859152, OsbHLH144; BAF14724, OsbHLH146; EEC73367, poplar PtbHLH130; EEE73911, *Nicotiana*

*benthamiana* NbbHLH1; GQ859153, NbbHLH2.

(B) Sequence alignment of bHLH domains of CjbHLH1 and its homologues in comparison with AtbHLH100, AtRGE1 and CjbHLH2. Identical residues are shown as white letters shaded in black, and similar residues are shown as black letters shaded in gray. AfbHLH1; CjbHLH1 homolog in EST of *Aquilegia formosa* x *Aquilegia pubescens* (accession number DT752478), LtbHLH1; CjbHLH1 homolog in EST of *Liriodendron tulipifera* (accession number FD498024), ShbHLH1; CjbHLH1 homolog in EST of *Saruma henryi* (accession number DT584473), ArbHLH1; CjbHLH1 homolog in EST of *Aristolochia fimbriata* (accession number FD755492). Asterisks indicate conserved His (position 5), Glu (position 9) and Arg (position 13) residues of bHLH domain which recognizes the E-box.

Fig. 3 Effect of dsRNA of *CjbHLH1* on gene expression in berberine biosynthesis (A), primary metabolism (B), stress response (C), and that of *CjbHLH1* (D).

Expression levels of the genes were analyzed by quantitative RT-PCR. The relative transcript level was calculated with V-type ATPase c subunit gene as an internal control, and dsRNA of *GFP* was used as a control. Error bars indicate the standard deviation (n=3).

Fig. 4 Effect of overexpression of *CjbHLH1* on gene expression in berberine biosynthesis (A), primary metabolism (B), stress response (C), and that of *CjbHLH1* (D).

The expression levels of genes were analyzed by quantitative RT-PCR after 6 hours incubation. The relative transcript level was calculated with V-type ATPase c subunit gene as an internal control, and *GUS* overexpression was used as a control. Error bars indicate the standard deviation (n=3). Asterisks indicate significant differences from the values of the control ($p<0.01$, Student's *t*-test).

Fig. 5 Nuclear localization of CjbHLH1 protein *in vivo*.

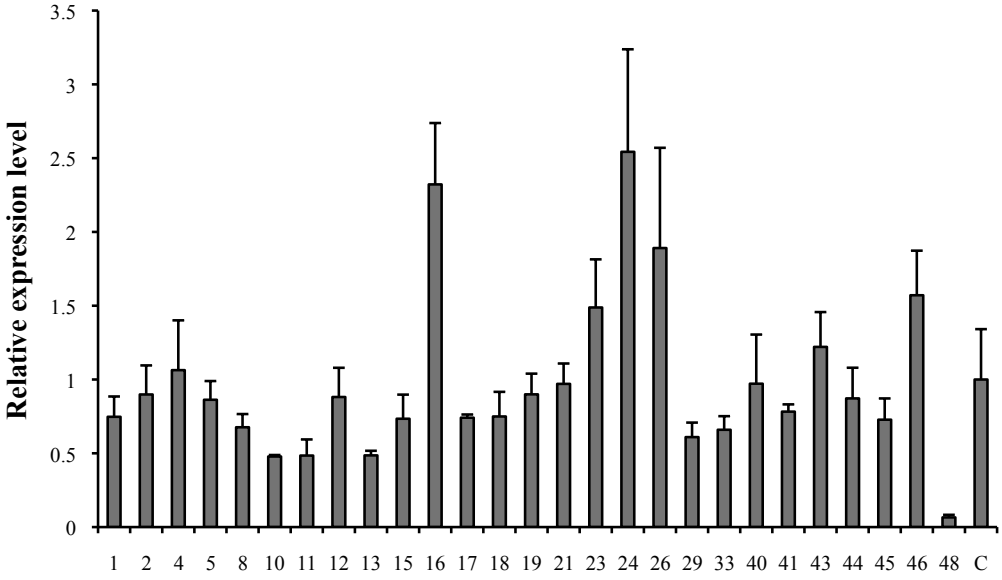
CaMV *35S*::*sGFP-CjbHLH1* fusion or CaMV *35S*::*sGFP* constructs were introduced into *C. japonica* 156-S protoplasts or onion epidermal cells, and the fluorescence was detected after 24 hours incubation.

Fig. 6 Trans-activation analysis and chromatin immuno-precipitation assay of CjbHLH1.

(A)Trans-activation of berberine biosynthetic gene promoter::*LUC* reporter gene by

ectopic expression of *CjbHLH1*. *C. japonica* 156-S protoplasts were co-transfected with the reporter construct (*CYP80B2* promoter::*PpLUC*, *4'OMT* promoter::*PpLUC* and *CYP719A1* promoter::*PpLUC*), effector construct (CaMV *35S*::*GUS* as a control and CaMV *35S*::*CjbHLH1*), or reference construct (CaMV *35S*::*RrLUC*). *Photinus pyralis* (Pp) and *Renilla reniformis* (Rr) LUC activities were determined by a dual-luciferase reporter assay. Error bars indicate the standard deviation (n=3). Asterisks indicate significant differences from the control ($p<0.01$, Student's *t*-test).

(B) Chromatin immuno-precipitation with anti-CjbHLH1 antibodies confirmed the specific binding of CjbHLH1 to berberine biosynthetic gene promoter *in vivo*. The left panel shows the structure of the *CYP80B2, 4'OMT, CYP719A1* and *ATPase* genes. Gray boxes indicate the coding region of each gene and thick black bars indicate the regions amplified by PCR. The right panel shows the PCR-amplification of DNA fragments from immune-precipitated chromatin (IP) and input control (Input). Chromatins were incubated without (-Ab) or with (+Ab) CjbHLH1-specific antibodies and immuno-precipitated.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3
2.5
2
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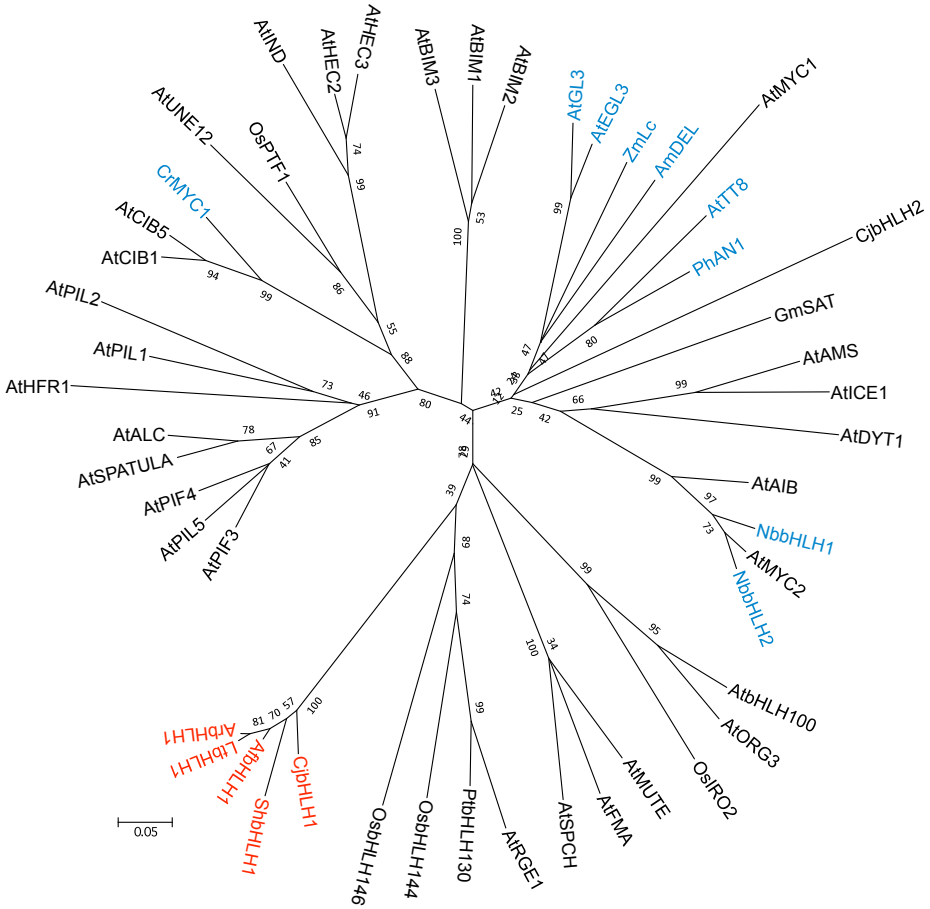
1
0.5
0
1 2 4 5 8 10 11 12 13 15 16 17 18 19 21 23 24 26 29 33 40 41 43 44 45 46 48 C

Fig. 1

A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AtHEC3
AtBIM3
AtBIM1
AtBIM2
AtGL3
AtEGL3
ZmLc
AmDEL
AtMYC1
AtUNE12
OsPTF1
AtTT8
CrMYC1
CjbHLH2
AtCIB5
PhAN1
AtCIB1
GmSAT
AtPIL2
AtAMS
AtPIL1
AtICE1
AtHFR1
AtDYT1
AtALC
AtAIB
AtSPATULA
NbbHLH1
AtPIF4
AtMYC2
AtPIL5
NbbHLH2
AtPIF3
AtbHLH100
AtORG3
ArbHLH1
LtbHLH1
AbHLH1
CjbHLH1
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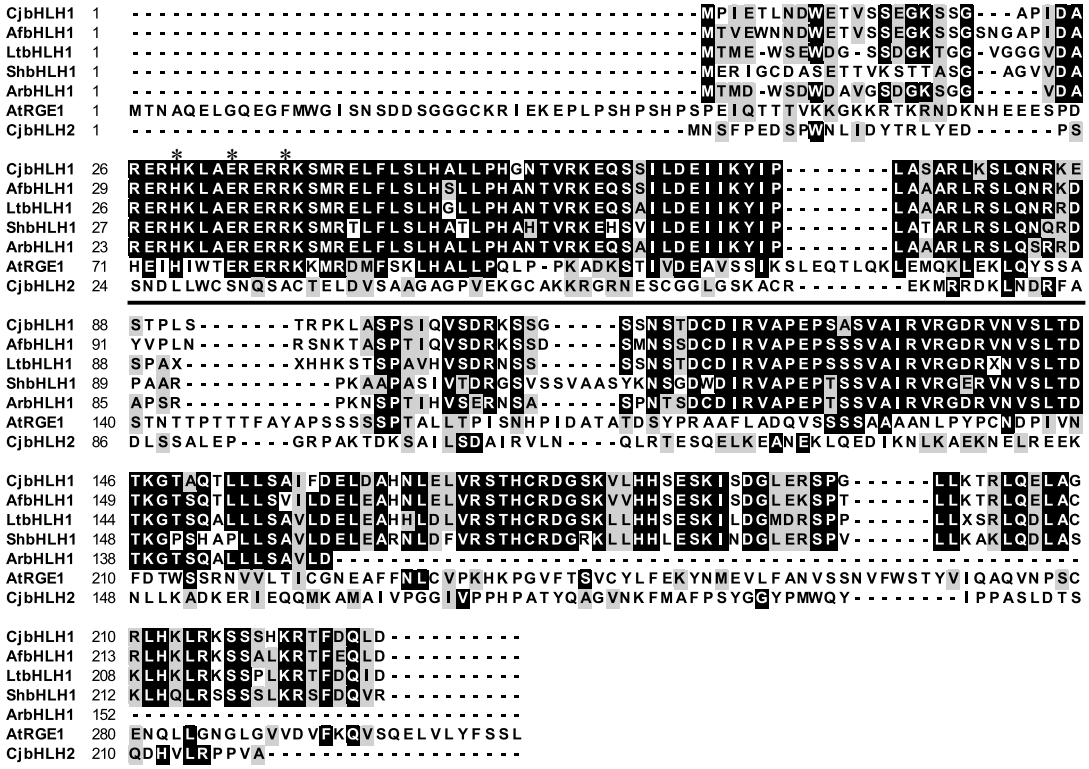
B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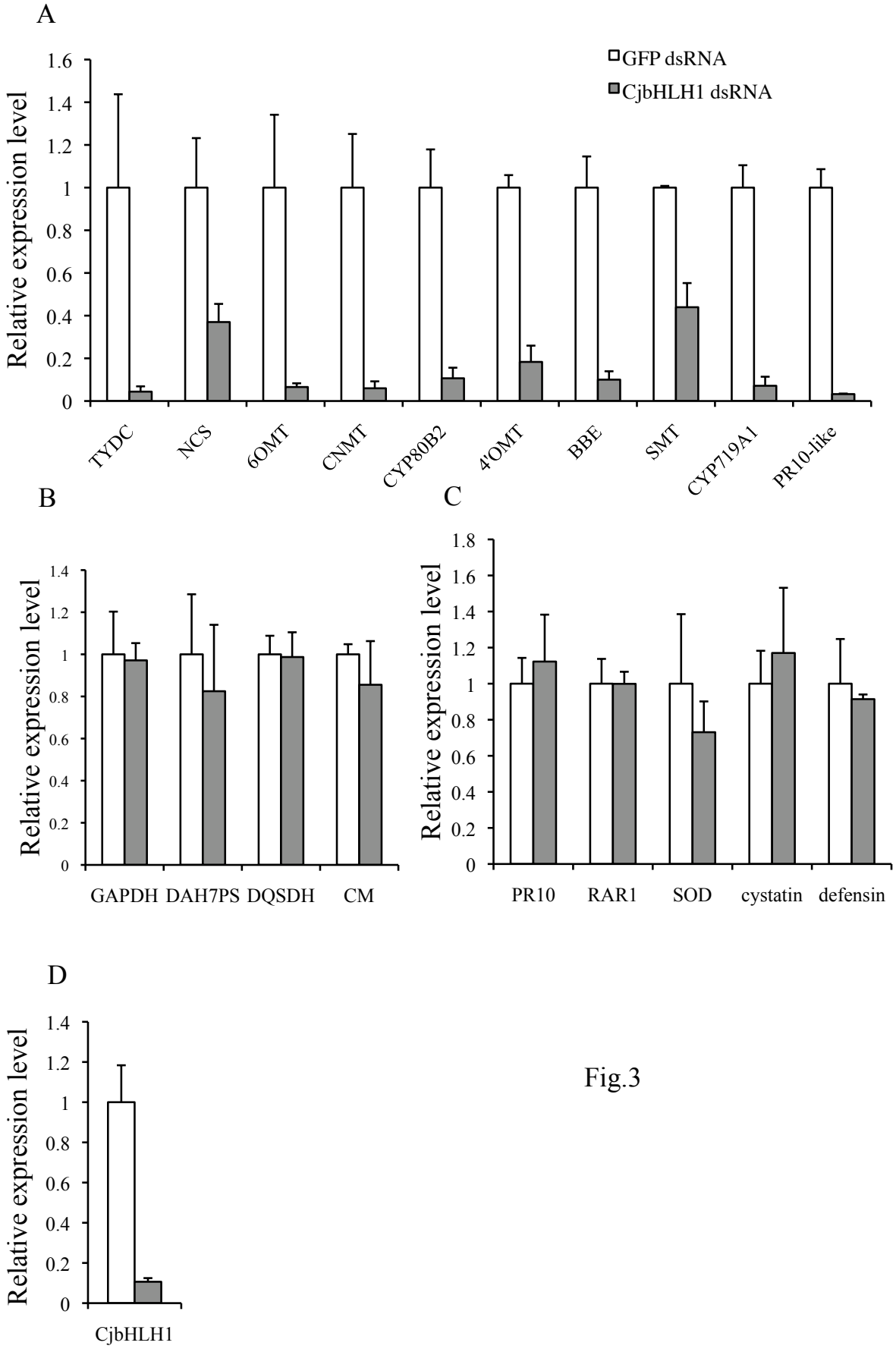
AfbHLH1
LtbHLH1
ShbHLH1
ArbHLH1
AtRGE1
CjbHLH2

Fig. 2


A
GFP dsRNA
CjbHLH1 dsRNA
Relative expression level
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TYDC
NCS
6OMT
CNMT
CYP80B2
4'OMT
BBE
SMT
CYP719A1
PR10-like
B
Relative expression level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
GAPDH
DAH7PS
DQSDH
CM
C
Relative expression level
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PR10
RAR1
SOD
cystatin
defensin
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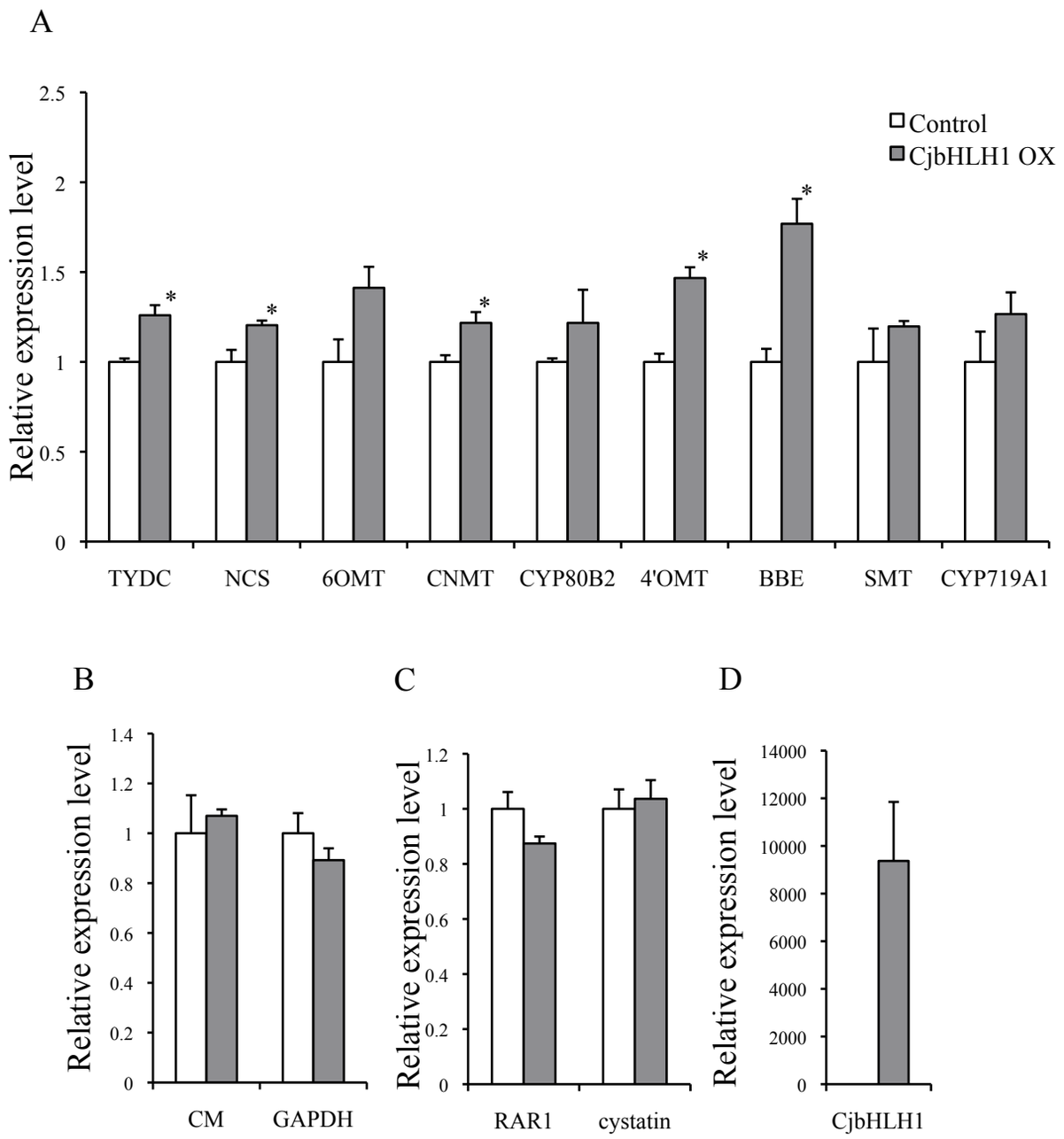
Relative expression level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CjbHLH1

Fig.3


A
Relative expression level
2.5
2
1.5
1
0.5
0
Control
CjbHLH1 OX
*
TYDC
NCS
6OMT
CNMT
CYP80B2
4'OMT
BBE
SMT
CYP719A1
B
Relative expression level
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CM
GAPDH
C
Relative expression level
1.2
1
0.8
0.6
0.4
0.2
0
RAR1
cystatin
D
Relative expression level
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12000
10000
8000
6000
4000
2000
0
CjbHLH1

Fig.4

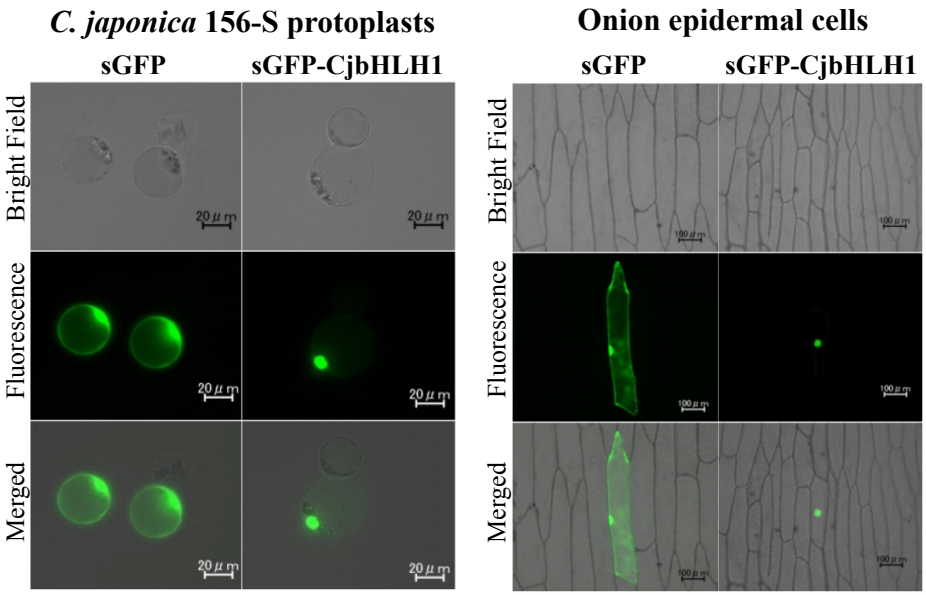
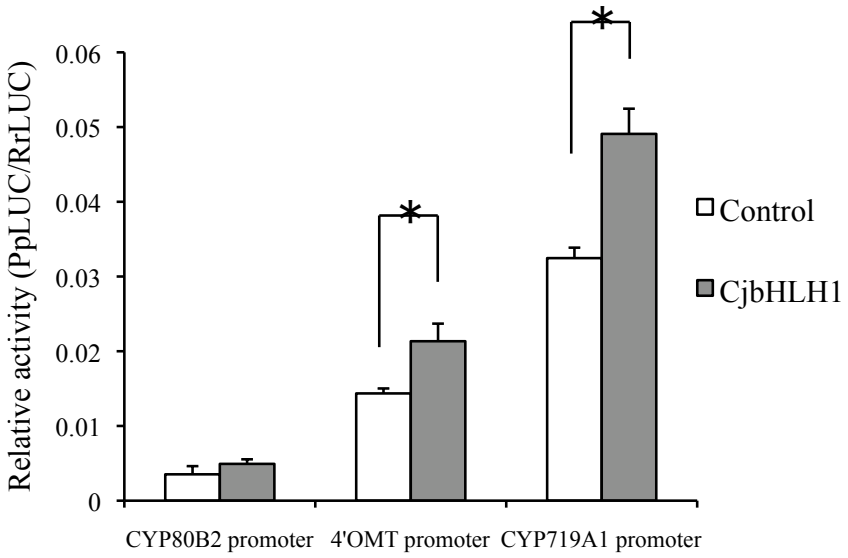

C. japonica 156-S protoplasts
sGFP
sGFP-CjbHLH1
Onion epidermal cells
sGFP
sGFP-CjbHLH1
Bright Field
Fluorescence
Merged
20 μm
100 μm

Fig.5

A


Relative activity (PpLUC/RrLUC)
0.06
0.05
0.04
0.03
0.02
0.01
0
*
*
Control
CjbHLH1
CYP80B2 promoter
4'OMT promoter
CYP719A1 promoter

B

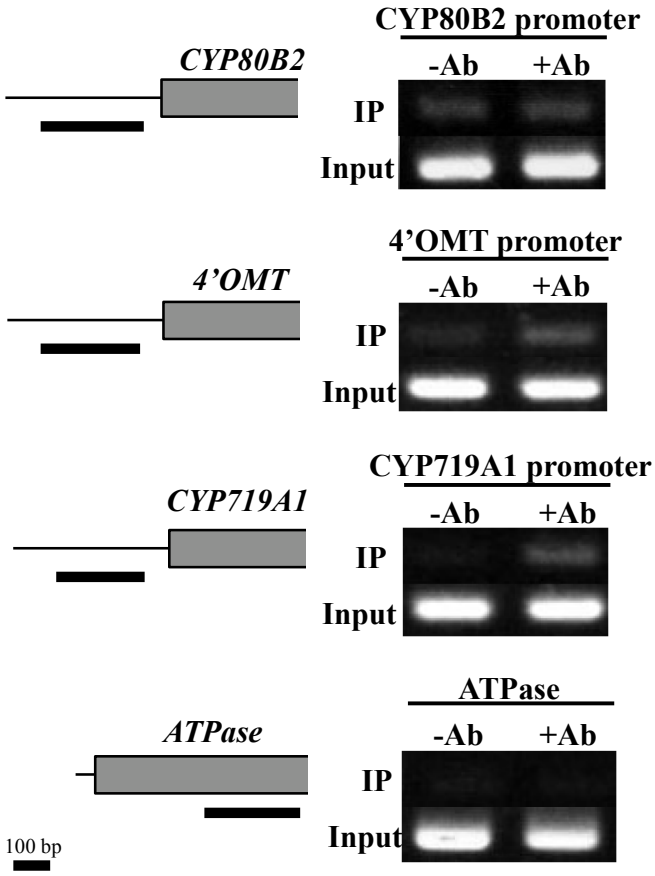
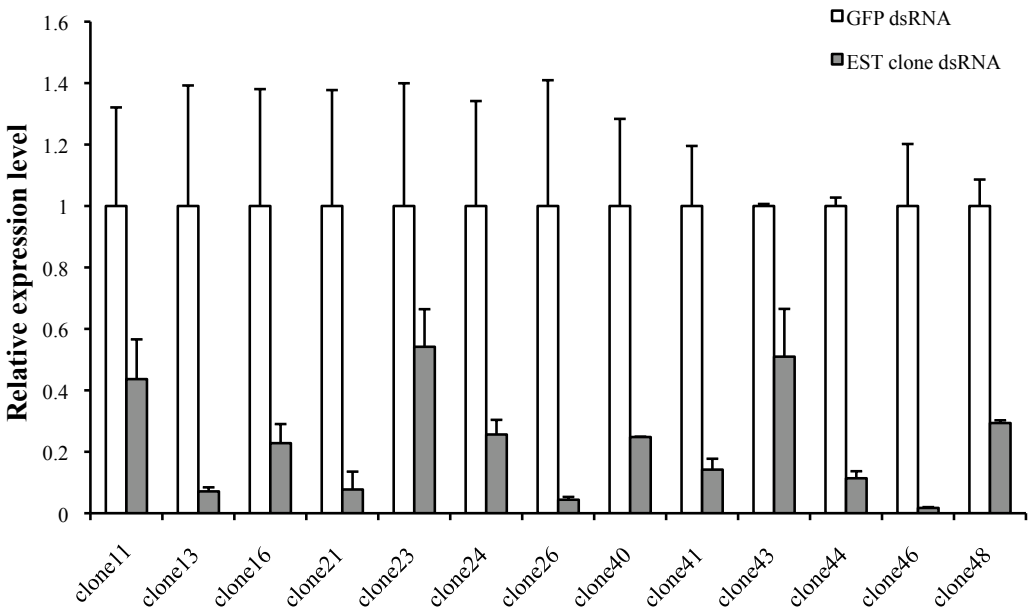
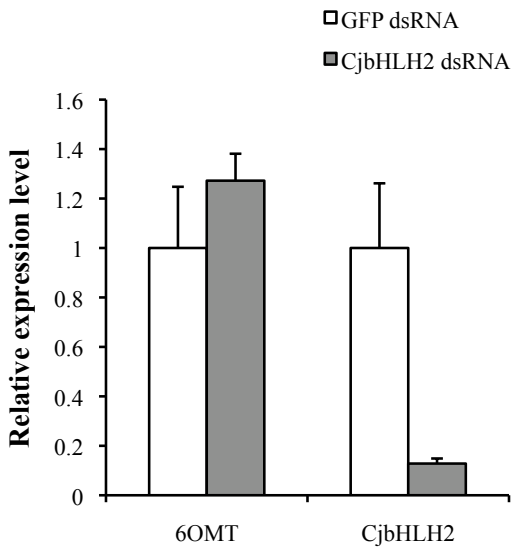

CYP80B2 promoter
CYP80B2
-Ab
+Ab
IP
Input
4'OMT promoter
4'OMT
-Ab
+Ab
IP
Input
CYP719A1 promoter
CYP719A1
-Ab
+Ab
IP
Input
ATPase
ATPase
-Ab
+Ab
IP
Input
100 bp

Fig.6



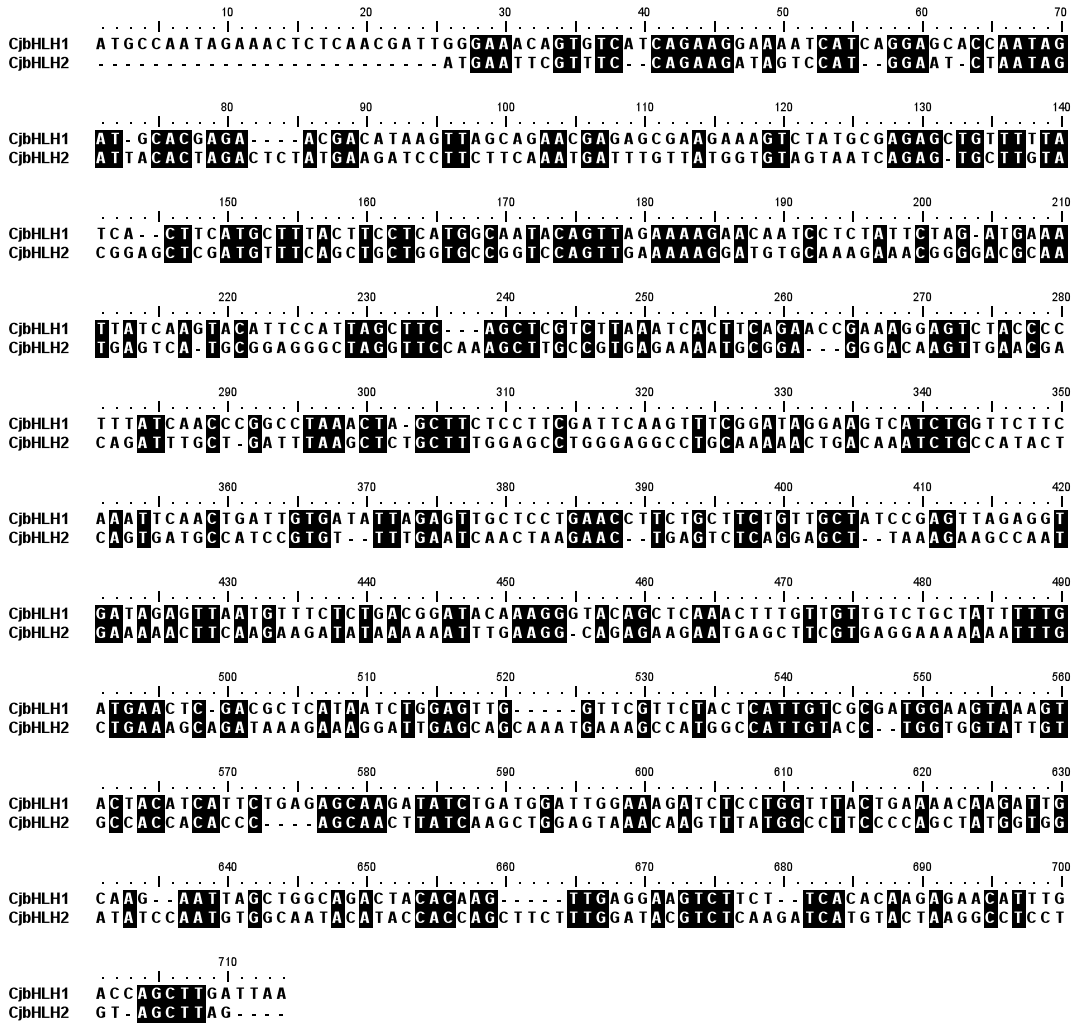

Supplementary Fig. S1. Effect of dsRNA of 13 candidate genes on the expression of each target gene.

*Coptis japonica* 156-S protoplasts were treated with dsRNA of each candidate gene, and its transcript level was determined by quantitative RT-PCR after 72 h incubation. The relative transcript level was calculated using V-type ATPase c subunit gene as an internal control, and dsRNA of *GFP* was used as a control. Error bars indicate the standard deviation (n=3).



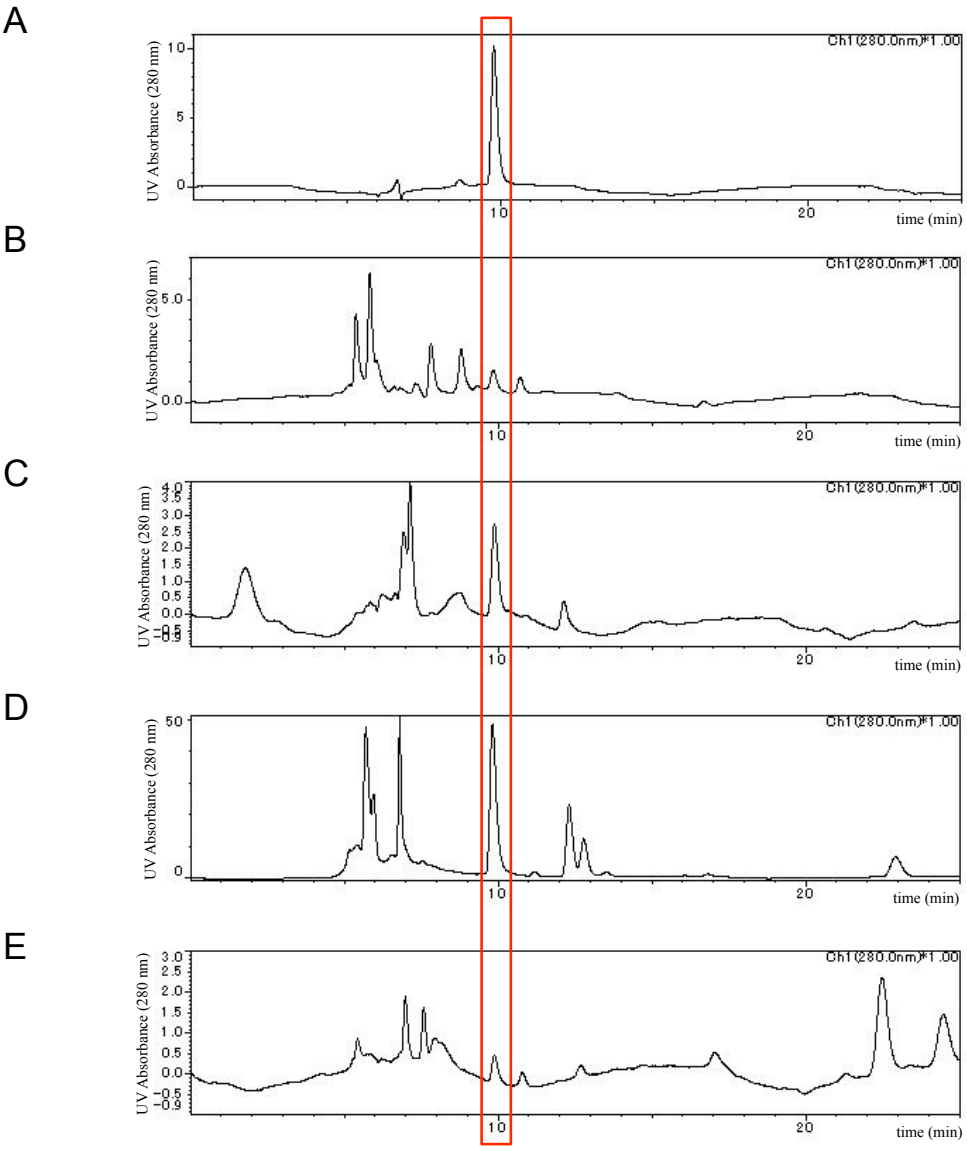

Supplementary Fig. S2. Effect of dsRNA of the *CjbHLH2* gene on *6OMT* gene expression.

*C. japonica* 156-S protoplasts were treated with dsRNA of *CjbHLH2* gene, and the *6OMT* and its transcript level was determined by quantitative RT-PCR after 72 h incubation. The relative transcript level was calculated using V-type ATPase c subunit gene as an internal control, and dsRNA of *GFP* was used as a control. Error bars indicate the standard deviation (n=3).



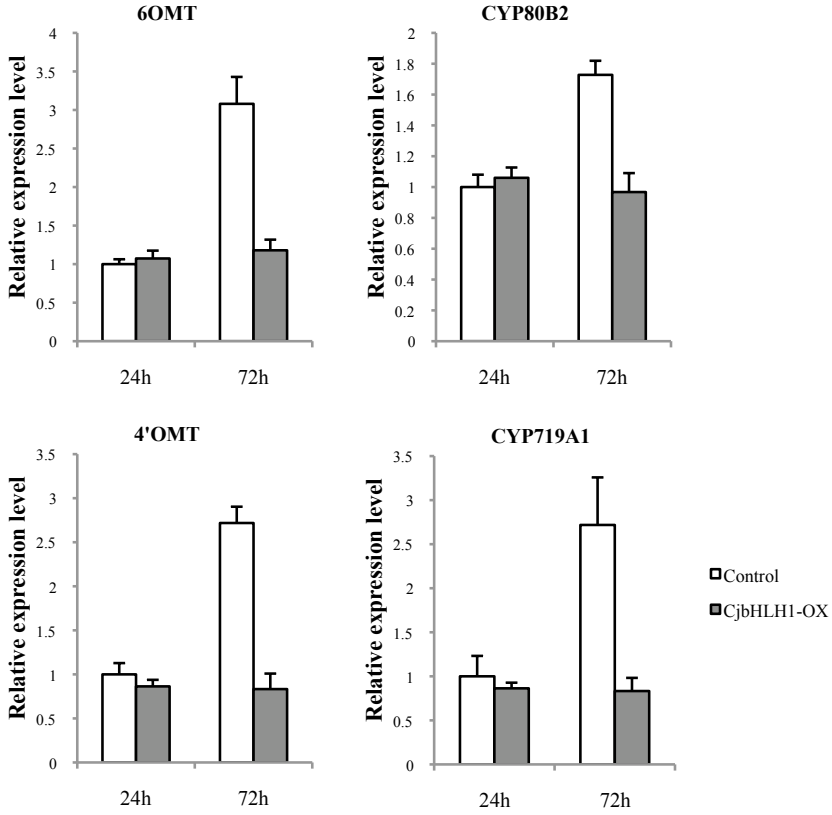

Supplementary Fig. S3. Alignment of cDNA coding sequences of *CjbHLH1* and *CjbHLH2*.

Identical bases are shown as white letters shaded in black.



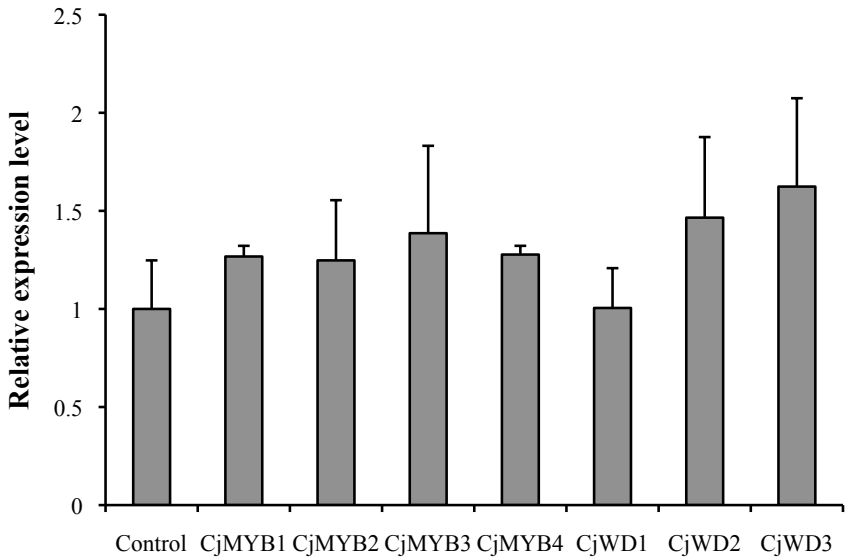

Supplementary Fig. S4. HPLC analysis of cell extracts of isoquinoline alkaloid-producing plants.

Standard magnoflorine (10 μM) (A), extracts of *Aquilegia hybrida* root (B), *Aristolochia debilis* rhizome (C), *Liriodendron tulipifera* root (D), and *Coptis japonica* rhizome (E) were analyzed. Presence of magnoflorine in plant extracts was confirmed by LC-MS in comparison with the standard. The red box indicates the peak of magnoflorine.




Supplementary Fig. S5 Time-dependent change of the expression levels of four biosynthetic enzyme genes by overexpression of *CjbHLH1*.

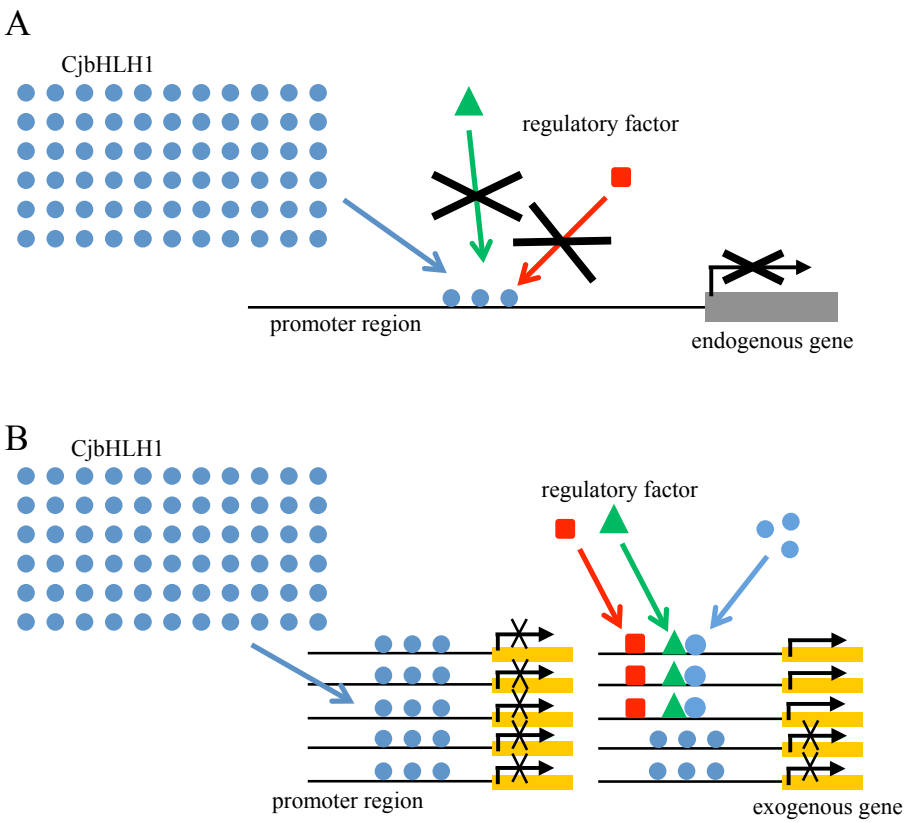
The expression levels of genes were analyzed by quantitative RT-PCR after 24 and 72 h incubation. The relative transcript level was calculated with V-type ATPase c subunit gene as an internal control, and *GUS* overexpression was used as a control. Error bars indicate the standard deviation (n=3).




Supplementary Fig. S6. Effect of dsRNA of *CjMYB* and *CjWD40* EST clones on *6OMT* gene expression.

*C. japonica* 156-S protoplasts were treated with dsRNA of *MYB* and *WD40* genes, and the *6OMT* transcript level was determined by quantitative RT-PCR after 72 h incubation. The relative transcript level was calculated using V-type ATPase c subunit gene as an internal control, and dsRNA of *GFP* was used as a control. Error bars indicate the standard deviation (n=3)

The EST clones analyzed were *CjMYB1* (Supplementary Table 1, clone41), *CjMYB2*, *CjMYB3*, *CjMYB4*, *CjWD1*, *CjWD2* (Supplementary Table 1, clone46) and *CjWD3*.




Supplementary Fig. S7 Working hypothesis to understand the different effect of overexpressed CjbHLH1 on the expression of endogenous and exogenous genes.

(A) Model for endogenous gene. Large excess of CjbHLH1 (blue circle) blocks the binding of other regulatory factors (green triangle, red square) and halt the increase of expression endogenous genes. (B) Model for exogenous reporter genes. Large excess of CjbHLH1 binds to promoter region, but some CjbHLH1 and regulatory factors would bind to the promoter region of exogenous genes introduced at much larger amount than endogenous genes.