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Corresponding Author: Fumihiko Sato

Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kyoto, 606-8502, Japan

E-mail, fsato@lif.kyoto-u.ac.jp

Phone, +81-75-753-6381

Fax, +81-75-753-6398

Subject Areas: natural products, regulation of gene expression

Black and white figures: 2

Color figures: 4

Supplementary materials: 19

CjbHLH1 homologues regulate sanguinarine biosynthesis in *Eschscholzia californica* cells.

Running title: **EcbHLH1s regulate sanguinarine biosynthesis**

Yasuyuki Yamada, Yukiya Motomura and Fumihiko Sato*

Department of Plant Gene and Totipotency, Graduate School of Biostudies, Kyoto University, Kitashirakawa, Sakyo, Kyoto 606-8502, Japan

* Corresponding author: Fumihiko Sato; fsato@lif.kyoto-u.ac.jp

Abbreviations: BBE, berberine bridge enzyme; bHLH, basic Helix-Loop-Helix; CNMT, (*S*)-coclaurine-*N*-methyltransferase; CYP719A2/CYP719A3, (*S*)-stylopine synthase; CYP719A5, (*S*)-cheilanthifoline synthase; CYP80B1, (*S*)-*N*-methylcoclaurine 3'-hydroxylase; CYP82N2v2, protopine-6-hydroxylase; DBOX, dihydrobenzophenanthridine oxidase; GFP, green fluorescent protein; IQA, isoquinoline alkaloid; MeJA, methyl jasmonate; MSH, (*S*)-*N*-methylstylopine 14-hydroxylase; NCS, (*S*)-norcoclaurine synthase; SR, sanguinarine reductase; TNMT,

(*S*)-tetrahydroprotoberberine cis-*N*-methyltransferase; 4’OMT, (*S*)-3’-hydroxy-*N*-methylcoclaurine-4’-*O*-methyltransferase; 6OMT, (*S*)-norcoclaurine-6-*O*-methyltransferase

Footnotes: Nucleotide sequence of full length cDNAs of *EcbHLH1-1* (AB910896), *EcbHLH1-2* (AB910897) were deposited in the DDBJ/Genbank/EMBL database.

[Abstract]

Isoquinoline alkaloids (IQAs) like terpenoid indole alkaloid and nicotine are some of the most studied alkaloids. Recently, several groups have reported that the biosynthesis of these alkaloids is regulated by basic helix-loop-helix transcription factors (bHLHs). Whereas the biosyntheses of nicotine and terpenoid indole alkaloid in Nicotiana plants and *Catharanthus roseus* are directly or indirectly regulated by *Arabidopsis thaliana* MYC2 homologues, a non-MYC2-type bHLH transcription factor, CjbHLH1, comprehensively regulates berberine biosynthesis in *Coptis japonica*. Interestingly, *CjbHLH1* homologous genes were found in many IQA-producing plant species, which suggests that non-MYC2-type CjbHLH homologues are specifically associated with IQA biosynthesis. To test whether CjbHLH1 homologues are involved in the biosynthesis of IQA in a plant other than *C. japonica*, we isolated two genes homologous to *CjbHLH1*, i.e., *EcbHLH1-1* and *EcbHLH1-2*, from *Eschscholzia californica* (California poppy). Stable transformants in which the expression levels of *EcbHLH1* genes were constitutively suppressed by RNA interference (RNAi) showed a reduced expression of some IQA biosynthetic enzyme genes. A metabolite analysis confirmed that the suppression of *EcbHLH1*, particularly *EcbHLH1-2*, caused a decrease in sanguinarine accumulation in transgenic cultured cells. These results

indicate that non-MYC2-type EcbHLH1s regulate IQA biosynthesis in California poppy, like CjbHLH1 in *C. japonica*.

Keywords: basic Helix-Loop-Helix, *Eschscholzia californica* (California poppy), isoquinoline alkaloids, transcriptional regulation

[Introduction]

Secondary metabolites that are commonly produced by higher plants can be classified into three groups: phenylpropanoids and aromatic polyketides, terpenoids, and alkaloids. These low-molecular-weight chemicals help defend the plant against pathogens and herbivores, and attract pollinators, and are also used by humans in flavorings, dyes, and pharmaceuticals (Pichersky and Gershenzon 2002). In particular, alkaloids, which are nitrogen-containing compounds, have potent biological activity, and are used as important pharmaceuticals, stimulants, and narcotics. Among them, isoquinoline alkaloids (IQAs) are the most structurally diverse and pharmaceutically valuable, and include the analgesics morphine and codeine, the antitumor agent noscapine, and the antimicrobial agents sanguinarine and berberine (Facchini 2001).

IQA biosynthesis starts with the conversion of L-tyrosine to dopamine and 4-hydroxyphenylacetaldehyde, which are then condensed to (*S*)-norcoclaurine by (*S*)-norcoclaurine synthase (NCS) (Samanani and Facchini 2002, Samanani et al. 2004, Minami et al. 2007). Three methyltransferases [(*S*)-norcoclaurine 6-*O*-methyltransferase (6OMT), (*S*)-coclaurine-*N*-methyltransferase (CNMT), and (*S*)-3’-hydroxy-*N*-methylcoclaurine-4’-*O*-methyltransferase (4’OMT)] and a cytochrome P450 [(*S*)-*N*-methylcoclaurine 3’-hydroxylase (CYP80B1)] catalyze the

conversion of (*S*)-norcoclaurine to (*S*)-reticuline, which is a common intermediate of morphine, sanguinarine, and berberine (Sato et al. 1994, Pauli and Kutchan 1998, Morishige et al. 2000, Choi et al. 2002). The IQA biosynthetic pathway has been intensively investigated at the molecular level, such as for berberine in *Coptis japonica* (Ranunculaceae), sanguinarine in *Eschscholzia californica* (Papaveraceae), and morphine in *Papaver somniferum* (Papaveraceae) (Hagel and Facchini 2013, Sato 2013). Almost all of the enzymes involved in the biosynthesis of these alkaloids have been characterized and this information has enabled us to reconstitute the IQA biosynthetic pathway in microorganisms and to manipulate the IQA biosynthetic pathway by metabolic engineering (Sato et al. 2001, Hawkins and Smolke 2008, Minami et al. 2008, Nakagawa et al. 2011, Fossati et al. 2014). However, little is known about transcriptional regulation in the biosynthesis of IQA.

Berberine, which has antimicrobial activity and has recently been reported to lower cholesterol levels, is synthesized from (*S*)-reticuline by berberine bridge enzyme (BBE), (*S*)-scoulerine-9-*O*-methyltransferase (SMT), (*S*)-canadine synthase (CYP719A1), and (*S*)-tetrahydroprotoberberine oxidase (THBO) in *C. japonica* (Dittrich and Kutchan 1991, Takeshita et al. 1995, Ikezawa et al. 2003, Gesell et al. 2011, Matsushima et al. 2012, Fig. 1). The enzyme genes involved in the biosynthesis

from (*S*)-norcoclaurine to berberine have been completely identified and two general transcription factors, CjWRKY1 and CjbHLH1, that are involved in the regulation of berberine biosynthesis, have also been isolated from *C. japonica* using high berberine-producing cultured cells (Kato et al. 2007, Yamada et al. 2011a). Transient RNA-silencing of *CjWRKY1* or *CjbHLH1* significantly decreased the expression of berberine biosynthetic enzyme genes, which indicates that both CjWRKY1 and CjbHLH1 act as transcriptional activators in berberine biosynthesis in *C. japonica* cells.

Recently, some Arabidopsis MYC2-type bHLH transcription factors, such as NbbHLH1/NbbHLH2/NtMYC2 and CrMYC2, have been reported to be involved in the regulation of nicotine biosynthesis in Nicotiana plants and terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (Todd et al. 2010, Shoji and Hashimoto 2011, Zhang et al. 2011, 2012). These bHLHs have been confirmed to function through a jasmonate (JA) signaling cascade, like AtMYC2.

Intriguingly, the predicted amino acid sequence of CjbHLH1 showed that CjbHLH1 belongs to group B of the bHLH-family proteins. CjbHLH1 is a non-MYC2-type bHLH protein, which lacks the Jasmonate-ZIM domain protein (JAZ)-interaction-domain (JID) sequence of MYC2 in the interaction with JAZ, although *CjbHLH1* has been shown to be responsive to methyl jasmonate (MeJA), like

MYC2 genes (Yamada et al. 2011b, Yamada and Sato 2013). Genes that are highly homologous to *CjbHLH1* have been found in many IQA-producing plant species, such as *Aquilegia formosa*, *Liriodendron tulipifera*, *Saruma henryi*, and *Aristholocia fimbriata,* and not in *Arabidopsis thaliana* or *Oryza sativa* (Yamada et al. 2011a). A phylogenetic tree based on the bHLH domain of several plants indicated that CjbHLH1 and its homologues were distinct from these MYC2-type transcription factors. These results suggest that CjbHLH1 and its homologues might be unique bHLH transcription factors in IQA biosynthesis.

To evaluate this hypothesis that CjbHLH1 homologues play a role in IQA biosynthesis, we tried to isolate CjbHLH1 homologues from another IQA-producing plant, *E. californica* (California poppy), and investigated its physiological role in the regulation of IQA biosynthesis. *E. californica* mainly produces sanguinarine, which, while a different type of IQA than berberine, shares a common pathway from L-tyrosine to (*S*)-scoulerine. Sanguinarine is synthesized from (*S*)-scoulerine by (*S*)-cheilanthifoline synthase (CYP719A5), (*S*)-stylopine synthase (CYP719A2/3), (*S*)-tetrahydroprotoberberine cis-*N*-methyltransferase (TNMT), (*S*)-*N*-methylstylopine 14-hydroxylase (MSH), protopine-6-hydroxylase (CYP82N2v2), and dihydrobenzophenanthridine oxidase (DBOX) (Ikezawa et al. 2007, 2009, Hagel et al.

2012, Beaudoin and Facchini 2013, Takemura et al. 2013, Fig. 1). Furthermore, California poppy offers the advantage of easy transformation with *Agrobacterium tumefaciens*. Thus, we analyzed the effects of the modification of *bHLH1* expression on biosynthetic enzyme gene expression and metabolic profiles using transgenic cells.

In this study, we report two genes (*EcbHLH1-1* and *1-2*) from California poppy that are homologous to *CjbHLH1*, and their different expression profiles in California poppy tissues. We also report that transgenic cells, in which the expression of *EcbHLH1* genes was decreased by RNAi, showed correlated decreases in the expression of some sanguinarine biosynthetic enzyme genes and sanguinarine content. Our results suggest that these EcbHLH1s function in the regulation of IQA biosynthesis in California poppy, like CjbHLH1 in *C. japonica* cells.

[Result]

Isolation of *CjbHLH1* homologues in *E. californica*

To characterize CjbHLH1 homologues in *E. californica*, we isolated genes that were homologous to *CjbHLH1* from *E. californica* using degenerate primers based on the highly conserved amino acid sequences of CjbHLH1 and its homologues in IQA-producing plants. Next, we tried to establish transformants, in which a *CjbHLH1* homologous gene was suppressed by RNAi in *E. californica,* to clarify its physiological role in IQA biosynthesis. Nucleotide sequencing of PCR products revealed two different fragments of genes that were highly similar to *CjbHLH1*. We then separately isolated each full-length cDNA by 3'RACE and 5'RACE with specific primer pairs and designated them *EcbHLH1-1* (accession number AB910896) and *EcbHLH1-2* (accession number AB910897), respectively (Supplementary Fig. S1).

The amino acid sequences of EcbHLH1-1 and EcbHLH1-2 were highly homologous to that of CjbHLH1 (74% and 66% identity, respectively), and the identity between EcbHLH1-1 and EcbHLH1-2 was 79%. Analysis of domain organization and amino acid sequence similarity of bHLH domain indicated that EcbHLH1-1 and EcbHLH-2 were non-MYC2-type bHLH transcription factors like CjbHLH1 (Supplementary Fig. S2). Both EcbHLH1-1 and EcbHLH1-2 also showed clear nuclear

localization like CjbHLH1, when expression vectors of *EcbHLH1-1* and *EcbHLH1-2* fused to the *synthetic green fluorcescent protein* (*sGFP*) gene were transiently introduced into *C. japonica* protoplasts (Niwa et al. 1999, Supplementary Fig. S3). These results suggest that both EcbHLH1-1 and EcbHLH1-2 are homologues of CjbHLH1. The latest transcriptome data for *E. californica* on the PhytoMetaSyn website (http://www.phytometasyn.ca/index.) also confirmed the expression of *EcbHLH1-1* and *EcbHLH1-2* in cultured cells.

The expression of *EcbHLH1-1* and *EcbHLH1-2* in MeJA-treated *E. californica* seedlings and different tissues of *E. californica* plant.

MeJA is known to be an inducer in the biosynthesis of alkaloids and has been shown to affect the expression of *CjbHLH1* (Yamada et al. 2011b). MeJA has also been reported to affect the expression of sanguinarine biosynthetic enzyme genes in California poppy (Ikezawa et al. 2007, 2009). Thus, we analyzed the MeJA-responsiveness of *EcbHLH1* genes in seedlings of *E. californica*. Both the *EcbHLH1-1* and *EcbHLH1-2* genes showed a rapid increase in expression at 1 h after treatment with MeJA, and a subsequent gradual decrease over 24 h (Fig. 2). As

previously shown (Ikezawa et al. 2007), the expressions of sanguinarine biosynthetic enzyme genes; *Ec6OMT*, *Ec4'OMT*, *EcBBE*, *EcCYP719A3*, and *EcTNMT*, were also transiently increased after treatment with MeJA, although the transcript levels were highest at 6 h. These results were similar to the MeJA-responsiveness of *CjbHLH1* and biosynthetic enzyme genes in *C. japonica* cells (Yamada et al. 2011b).

Since genes for sanguinarine biosynthesis are highly expressed in root where sanguinarine accumulates (Ikezawa et al. 2007, Supplementary Fig. S4), we analyzed the expression of *EcbHLH1-1* and *EcbHLH1-2* in leaf blades, petioles, roots, and cultured cells of *E. californica*. Real-time PCR analysis revealed that the expression level of *EcbHLH1-1* in leaf blades was higher than that in petioles, roots, and cultured cells, whereas that of *EcbHLH1-2* in roots and cultured cells was higher than that in leaf blades and petioles (Supplementary Fig. S4). Furthermore, when we estimated the transcript levels of *EcbHLH1-1* and *EcbHLH1-2* using standard plasmids containing each gene, *EcbHLH1-1* showed higher expression than *EcbHLH1-2* in leaf blades, whereas *EcbHLH1-2* showed higher expression than *EcbHLH1-1* in roots and cultured cells (Supplementary Fig. S5). These results suggest that *EcbHLH1-1* and *EcbHLH1-2* might have different physiological functions.

Establishment of transgenic cells of California poppy with RNA silencing of *EcbHLH1*

To evaluate the physiological functions of each *EcbHLH1* gene, we constructed three distinct 37-bp RNAi vectors to suppress the *EcbHLH1-1* and *EcbHLH1-2* genes independently or simultaneously (Supplementary Fig. S6). Segments of *E. californica* leaves were infected with *Agrobacterium tumefaciens* LBA4404 harboring RNAi vectors and a control vector without an RNAi insert. After three months of culture, 20-30 calli in each vector transformation were obtained, and *EcbHLH1-1*-specific RNAi, *EcbHLH1-2*-specific RNAi, and both *EcbHLH1-1*- and *EcbHLH1-2*-RNAi were designated 1-RNAi, 2-RNAi, and 12-RNAi, respectively. After repeated culture on selection medium, and following subculture in liquid medium for more than 6 months, we obtained several well-growing cell lines. Each cell line showed similar cell growth, whereas some cells and media of RNAi lines were slightly colorless (Supplementary Fig. S7A). Genomic PCR analysis confirmed the integration of transgenes in all cell lines (Supplementary Fig. S7B).

The effects of RNAi on the expression of *EcbHLH1-1* and *EcbHLH1-2* genes in transgenic cells were measured by real-time PCR. Vector control (VC) cells showed considerable variation in transcript levels of *EcbHLH1-1* and *EcbHLH1-2*, as reported

for biosynthetic enzyme genes (Inui et al. 2007), and VC lines that showed relatively moderate levels of transcripts were selected as control lines (Supplementary Fig. S8A). Similarly, representative 1-RNAi cell lines (RNAi lines for *EcbHLH1-1*; 1-L7, L14, and L16), 2-RNAi cell lines (RNAi lines for *EcbHLH1-2*; 2-L13, L14, and L18), and 12-RNAi cell lines (RNAi lines for both *EcbHLH1-1* and *EcbHLH1-2*; 12-L1 and L2), which showed a clear decrease in the expression of target *EcbHLH1* genes, were selected (Supplementary Figs. S8B, S9). Unexpectedly, two of the 2-RNAi cells (2-L13 and L14) also showed a low transcript level of *EcbHLH1-1* in addition to the silencing of *EcbHLH1-2*. The exact mechanism of this concomitant decrease in the *EcbHLH1-1* gene in 2-RNAi cells is not clear, but a high expression level of *EcbHLH1-2* might interfere with the low-level expression of *EcbHLH1-1*. Preliminary analyses of the expression levels of several sanguinarine biosynthetic enzyme genes and of the contents of benzophenanthridine alkaloids showed that these values were decreased in RNAi transgenic cells (Supplementary Figs. S10, S11).

Down-regulation of gene expression for biosynthetic enzymes in the sanguinarine pathway in transgenic cells with *EcbHLH1* RNA silencing

After preliminary experiments were performed using a single flask culture, we

repeated the measurements using an increased number of cultures; i.e., two for the VC, 1-RNAi, and 2-RNAi cell lines and three for the 12-RNAi cell line (Figs. 3, 4). Due to the variation in the cultured cell conditions during successive cell cultures, the expression levels of *EcbHLH1-1* and *EcbHLH1-2* in VC cells were dispersed and the decrease of *EcbHLH1-2* expression in 12-RNAi cells became low. The effects of RNA silencing of EcbHLH1 in transgenic cells were, however, still observed. Whereas a box plot analysis revealed that the expression level of sanguinarine biosynthetic enzyme genes was dispersed in all of the cell lines and the differences in the expression level between VC and RNAi cells became small, 2-RNAi cells showed moderate decreases in the expression of *6OMT*, *CNMT*, *CYP719A5*, *CYP719A2*, *CYP719A3*, and *sanguinarine reductase* (*SR*) compared to VC cells. On the other hand, 1-RNAi cells showed modest decreases in the expression of *CNMT*, *CYP719A2, CYP719A3*, and *MSH*. The effects on the expression levels of *NCS* (putative homolog of the *Thalictrum flavum NCS* gene), *CYP80B1*, *4'OMT*, *BBE*, *TNMT*, *CYP82N2v2*, and *DBOX* were not distinguishable among RNAi transformants and control cells.

Since the expression levels of *EcbHLH1-1*, *EcbHLH1-2*, and sanguinarine biosynthetic enzyme genes were considerably dispersed among the control and RNAi cell lines, we analyzed the correlations between the expression of *EcbHLH1-1* or

EcbHLH1-2 and that of each sanguinarine biosynthetic enzyme gene (Fig. 5, Supplementary Fig. S12). Correlation plot showed relatively high correlations between the expression of *EcbHLH1-1* and those of *6OMT*, *CYP719A3*, and *TNMT* (R=0.573, 0.732, and 0.497, respectively), and between the expression of *EcbHLH1-2* and those of *6OMT*, *4'OMT*, and *CYP719A3* (R=0.716, 0.506, and 0.624, respectively). These results suggest that the expression of these biosynthetic enzyme genes is regulated by EcbHLH1-1 and EcbHLH1-2. On the other hand, *EcbHLH1*-RNAi did not affect the expression of genes involved in primary metabolism, such as *3-dehydroquinate dehydratase/shikimate dehydrogenase* (*DHQ/SDH*) and *chorismate mutase* (*CM*). Also, the decrease in *EcbHLH1* gene expression did not affect the expression of other potential transcription factor genes, such as a putative *CjWRKY1* homologous gene (*EcWRKY1*) and an *AtMYC2* homologous gene (*EcMYC2*) (Supplementary Figs. S13, S14).

***EcbHLH1* RNAi cells showed reduced sanguinarine production**

The effects of the suppression of *EcbHLH1-1* and *EcbHLH1-2* on the accumulation of sanguinarine, a major IQA in California poppy cells, were confirmed by liquid chromatography-mass spectrometry (LC-MS) analysis (Supplementary Figs.

S15, S16), since the metabolite composition was nearly identical in VC and RNAi cells. The accumulation of sanguinarine in VC cells varied greatly, similar to gene expression, but 2-RNAi and 12-RNAi cells showed a relatively clear reduction of sanguinarine content compared to VC cells (Fig. 6A). 1-RNAi cells did not show a reduction of alkaloid compared to the control. The correlation between the expression of *EcbHLH1-2* and sanguinarine accumulation was relatively strong (R=0.706), whereas that between the expression of *EcbHLH1-1* and sanguinarine accumulation was weak (R=0.156) (Fig. 6B). Further analysis of the correlation between biosynthetic enzyme gene expression and alkaloid accumulation showed that the expressions of *6OMT* and *4OMT*, which were significantly correlated with that of *EcbHLH1-2*, were highly correlated with sanguinarine accumulation (R=0.377 and 0.453, respectively), whereas there was little correlation between the expression of *CYP719A3* and sanguinarine accumulation (R=0.161) (Fig. 6C). These results suggest that the suppression of *EcbHLH1*, particularly *EcbHLH1-2*, decreased sanguinarine accumulation in cultured cells.

[Discussion]

Non-MYC2 type bHLH in isoquinoline alkaloid biosynthesis

Recent studies indicate the physiological importance of bHLH transcription factors in several alkaloid pathways. Several MYC2-type bHLH transcription factors, such as NbbHLH1/NbbHLH2/NtMYC2 and CrMYC2, have been reported to be involved in the regulation of nicotine biosynthesis in Nicotiana plants and terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* (Todd et al. 2010, Shoji and Hashimoto 2011, Zhang et al. 2011, 2012). On the other hand, CjbHLH1, transcriptional regulator in berberine biosynthesis in *C. japonica,* is a non-MYC2-type bHLH protein distinct from MYC2 (Yamada et al. 2011a). In this study, we isolated two *EcbHLH1* genes (*EcbHLH1-1* and *EcbHLH1-2*), which were highly homologous to *CjbHLH1*, from California poppy (Supplementary Fig. S1). Functional analyses of stable transgenic California poppy cells, in which the expressions of *EcbHLH1-1* and *EcbHLH1-2* were decreased by RNAi, showed that EcbHLH1 regulates the biosynthesis of sanguinarine (Fig. 4-6). Our findings indicate the physiological importance of non-MYC2-type bHLHs in the regulation of IQA biosynthesis.

Functional characterization of EcbHLH1 in transgenic California poppy cells

Whereas the functions of CjbHLH1 in berberine biosynthesis were characterized using transient expression system in *C. japonica* protoplasts due to the difficulty of stable transformation, the functions of CjbHLH1 homologues, EcbHLH1-1 and EcbHLH1-2 in IQA biosynthesis were investigated in stable transgenic cultured California poppy cells produced by RNA silencing of *EcbHLH1* (Fig. 3, Supplementary Fig. S9). Importantly, *EcbHLH1-2*-knockdown cell lines (2-RNAi cell lines) with a concomitant reduction of *EcbHLH1-1* as 12-RNAi cell lines (RNAi for both *EcbHLH1-1* and *EcbHLH1-2*) showed significant reduction in sanguinarine content (Fig. 6A), whereas the reduction in the expression of sanguinarine biosynthetic enzyme genes was relatively weak in 2-RNAi and 12-RNAi cells (Fig. 4). Whereas these weak differences in the expression of biosynthetic enzyme genes among VC and RNAi cells were due to a decrease in *EcbHLH1* expression in some of the VC cell lines during successive subculture and hindered our analysis, repeated experimental data clearly supported the positive effects of *EcbHLH1* expression in sanguinarine production (Supplementary Figs. S9-S12).

Furthermore, correlation analysis showed a significant correlation between the expression of *EcbHLH1-2* and both the expression of *6OMT*, *4'OMT*, and *CYP719A3* genes and sanguinarine content (Figs. 5B, 6B). On the other hand, a reduction in the

expression of *EcbHLH1-1* with a concomitant reduction in the expression of *6OMT*, *CYP719A3*, and *TNMT* genes did not affect the sanguinarine content (Figs. 5A, 6B). These results suggest that *EcbHLH1-2* has a greater effect than *EcbHLH1-1* in transgenic cultured cells. Interestingly, the expression of *6OMT* and *4'OMT* was more strongly correlated with the sanguinarine content, which suggests that these enzymes would be rate-limiting in sanguinarine biosynthesis (Fig. 6C). The notion that *EcbHLH1-1* expression has little effect on the expression of *4'OMT* might explain why there was no reduction in sanguinarine content in 1-RNAi cells, since Ec4'OMT would be crucial in cultured cells for 6-*O*-methylation (Inui et al. 2007).

Functional diversification of bHLH transcription factors in IQA biosynthesis

Functional analyses with stable transformants indicated that the function of EcbHLH1-1 and EcbHLH1-2 in IQA biosynthesis was not redundant. Intriguingly, a tissue-specific expression analyses indicated that *EcbHLH1-1* was more highly expressed in leaves, and *EcbHLH1-2* was more highly expressed in roots (Supplementary Figs S4, S5). It is known that California poppy shows the exclusive production of pavine-type alkaloids and benzophenanthridine-type alkaloids in aerial parts and underground parts, respectively. Therefore, EcbHLH1-2 may show the greater

effect than EcbHLH1-1 in roots as well as in culture cells, while EcbHLH1-1 may regulate the biosynthesis of pavine-type alkaloids through regulation of the expression of several biosynthetic enzyme genes investigated in this study and *EcCYP719A9* and *EcCYP719A11*, which were highly expressed in aerial tissues of California poppy (Ikezawa et al. 2009). Shoot and root culture systems are currently being prepared to confirm this hypothesis.

The different roles of EcbHLH1s in California poppy also indicate the functional diversification of CjbHLH1 homologues in IQA biosynthesis. For example, whereas CjbHLH1 was a comprehensive regulator in berberine biosynthesis in *C. japonica* cells, EcbHLH1-1 and EcbHLH1-2 would be more specific regulators in this pathway. In fact, the expression of *NCS*, *CNMT*, *CYP80B1*, *CYP719A5*, *CYP719A2*, *MSH*, *CYP82N2v2*, *DBOX* and *SR* genes was not significantly correlated with that of *EcbHLH1-1* or *EcbHLH1-2* (Supplementary Fig. S12). This difference might be caused by different regulatory mechanism(s) between *C. japonica* and California poppy cells with bHLH1 transcription factors, such as post-translational, epigenetic regulation, or a difference in co-activators in the transcriptional machinery. In fact, the overexpression of *CjbHLH1* did not affect IQA biosynthesis in California poppy cells (Yamada et al. unpublished data). Further molecular characterization of these transcription factors in

different IQA-producing plant species is needed to understand their physiological functions.

MYC2-type bHLH transcription factors, which are clearly different from CjbHLH1 and EcbHLH1, are involved in the regulation of nicotine or terpenoid indole alkaloid biosynthesis, whereas *MYC2* homologous genes have also been found in *C. japonica* and *E. californica* (Yamada et al. 2011b, Yamada et al. unpublished data, Supplementary Fig. S2). Whereas RNAi of *EcbHLH1* only weakly affected the expression level of *EcMYC2* (Supplementary Fig. S13), a correlation analysis suggested that EcbHLH1-1 is involved in the regulation of *EcMYC2* gene expression (Supplementary Fig. S14A). Since EcbHLH1-1 is speculated to be involved in the regulation of IQA biosynthesis in aerial parts, a functional characterization of MYC2-type bHLHs in IQA-producing plants should provide clues for understanding the diversification of the bHLH family to regulate alkaloid biosynthesis and the signal transduction network of the jasmonate (JA) pathway in alkaloid biosynthesis. It is well known that MYC2 is one of the key components in the JA signal pathway (De Geyter et al. 2012), while the relation of CjbHLH1 and EbHLH1 to them is not characterized.

The identification of cis-regulatory elements in IQA biosynthesis is another unanswered question. In IQA biosynthesis, a WRKY-type transcription factor has been

reported in *C. japonica* and *P. somniferum* in addition to bHLHs (Kato et al. 2007, Mishra et al. 2013). While genome data are not yet available for IQA-producing plants, we determined a draft genome sequence of *E. californica* and found many putative target sequences of WRKY and bHLH transcription factors in the promoter region of sanguinarine biosynthetic enzyme genes (Yamada et al. unpublished results). This preliminary result suggests the direct regulation of the expression of sanguinarine biosynthetic enzyme genes through a direct interaction with promoter regions. In addition, the different expression of *EcbHLH1-1* and *EcbHLH1-2* in different tissues suggests that the different regulatory elements may present in their promoter regions. Further progress in the analysis of genome sequencing technologies and the analysis of transgenic cells with altered transcription factor gene expression should enable us to elucidate the transcriptional networks that regulate IQA biosynthesis spatially and temporally, and the genome-wide interaction between transcription factors and gene promoters in IQA biosynthesis.

[Materials and Methods]

Plant material

Seeds of *E. californica* were obtained from Takii Seed Co., Ltd (cultivar, Hitoezaki). Suspension cultured cells were grown in Linsmaier-Skoog (LS) medium (pH5.7) containing 3% sucrose, 10 μM 1-naphthylacetic acid (NAA), and 1 μM benzyladenine (BA) on a gyratory shaker (90 rpm) at 23°C in the dark. Plants used for expression analysis were grown on soil at 20°C under white light (100-200 μ mol·photons·m^{-2}·s^{-1}) and a 16 h light/8 h dark cycle for about 4 months. Plants used for transformation were grown in a 1/2 concentration of LS medium (pH 5.7) with 1% agar at 23°C under continuous white light for about 2 months. Seedlings used for the analysis of MeJA-responsiveness were grown in LS medium (pH5.7) supplemented with 1% sucrose and 0.8% agar at 23°C under continuous white light for 2 weeks, and then transferred to LS medium with 100 μM MeJA or the same volume of ethanol as a control.

Isolation of *EcbHLH1* genes from *E. californica*

Degenerate PCR was performed with primers based on amino acid sequences of CjbHLH1 and its homologues as described previously (Yamada et al. 2011a) as well

as template cDNAs synthesized from total RNA of cultured California poppy cells treated with 100 μM MeJA, and two different sequences were obtained. 3’ and 5’ RACE were independently performed with specific primer pairs based on different sequences in two *EcbHLH1* genes by a Gene Racer^{TM} Kit (Invitrogen). The sequences of the primers are given in Supplementary Table 1.

Construction of RNAi vectors

RNAi vectors were constructed as described previously (Ifuku et al. 2005). Oligonucleotide fragments were amplified by PCR using primers containing 37 bp sequences of *EcbHLH1-1*, *EcbHLH1-2*, or both and a 20 bp sequence of β-*glucuronidase* with *Bam*HI and *Sac*I target sites, and subcloned into pT7blue vector (Novagen). After nucleotide sequences were confirmed, the obtained plasmids were digested with *Bam*HI and *Sac*I. The resulting fragments were introduced into pBIE binary vector cut by *Bam*HI and *Sac*I. The pBIE vector contains the enhancer-equipped cauliflower mosaic virus (CaMV) 35S promoter (El2 promoter). The sequences of the primers are given in Supplementary Table 2.

Transformation of California poppy cells

Petioles of California poppy plants were cut into 5-10 mm segments and used for transformation. These segments were co-cultured with *Agrobacterium tumefaciens* LBA4404 harboring each 37 bp RNAi vector and pBIE vector as a control, and inoculated on LS medium (pH 5.2) containing 3% sucrose, 1% agar, 100 μg/ml acetosyringone, 10 μM NAA, and 1 μM BA at 23°C in the dark. After 2d of co-culture, transformants were selected on LS medium (pH 5.7) supplemented with 3% sucrose, 1% agar, 10 μM NAA 1 μM BA, 150 μg/ml kanamycin, and 200 μg/ml cefotaxime at 23°C in the dark. Every 2-3 weeks, inoculated segments were transferred into fresh selection medium. After about 9 months, obtained cells were transferred into liquid LS medium and cultured every 2-3 weeks.

Quantitative RT-PCR

Total RNA extraction from 7-day-old *E. californica* suspension cultured cells was performed using an RNeasy Plant Mini Kit (Qiagen). Single-stranded cDNAs were synthesized from 1 μg total RNA by Super Script III reverse transcriptase (Invitrogen). Real-time PCR was performed with specific primer pairs (see Supplementary Table 3) using iQ^{TM} SYBR Green Supermix and a CFX96 Real-Time PCR Detection System (Bio-Rad). Conditions for the PCR reaction were 95°C for 3 min, followed by 40 cycles

of 95°C for 10 s, 60°C for 20 s and 72°C for 20 s. The data were calculated by a standard curve method and the relative expression levels between the samples were standardized by the amplification of *β-actin* as an internal control.

Metabolite analysis

Transgenic California poppy cells were subcultured for more than 6 months at 2-3 week intervals. Stably growing cells were harvested after 7d of culture and about 200 mg-fresh weight cells were extracted in 800 μl of methanol containing 10 μl of 1N HCl at room temperature overnight. Alkaloids in the culture medium were recovered with Sep-Pak plus C18 cartridges (Waters) and eluted with 3 ml of methanol. The alkaloid contents were determined by an LC-10A system and LC-MS 2010 (Shimadzu) under the following conditions; acetonitrile/H_2O solvent gradient containing 0.05% (v/v) trifluoroacetic acid; 30 to 45% (v/v) for 45 min or LC-MS 2020 (Shimadzu) under the following conditions; acetonitrile/H_2O solvent gradient containing 1% acetic acid; 40 to 80% (v/v) for 45 min. Flow speed; 0.5 ml/min, column temperature, 40°C; analytical mode; SIM-SCAN(+), Q-array voltage; 100-150 V. Sanguinarine (Sigma-Aldrich) was purchased and used as a standard. Each peak was identified based on its retention time and mass-to-charge ratio (m/z) by LC-MS as described previously

(Takemura et al. 2010).

[Funding]

This research was supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) [Grant-in-Aid nos. 21248013, 25108713, 26252012, and 26221201 to F. S.].

[Acknowledgements]

We thank Dr. Yasuo Niwa for the kind gift of sGFP vector.

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[Figure legends]

Fig. 1. Berberine and sanguinarine biosynthetic pathways in *C. japonica* and *E. californica*, respectively. NCS, (*S*)-norcoclaurine synthase; 6OMT, (*S*)-norcoclaurine 6-*O*-methyltransferase; CNMT, (*S*)-coclaurine-*N*-methyltransferase; CYP80B1, (*S*)-*N*-methylcoclaurine 3'-hydroxylase; 4'OMT, (*S*)-3'-hydroxy-*N*-methylcoclaurine-4'-*O*-methyltransferase; BBE, berberine bridge enzyme; SMT, (*S*)-scoulerine-9-*O*-methyltransferase; CYP719A1, (*S*)-canadine synthase; THBO, (*S*)-tetrahydroprotoberberine oxidase; CYP719A5, (*S*)-cheilanthifoline synthase; CYP719A2/3, (*S*)-stylopine synthase; TNMT, (*S*)-tetrahydroprotoberberine cis-*N*-methyltransferase, MSH, (*S*)-*N*-methylstylopine 14-hydroxylase; CYP82N2v2, protopine-6-hydroxylase; DBOX, dihydrobenzophenanthridine oxidase; SR, sanguinarine reductase. Broken lines indicate uncharacterized enzyme reactions.

Fig. 2. MeJA-induced transcript levels of *EcbHLH1-1* and *EcbHLH1-2* in California poppy seedlings treated with 100 μM MeJA. The expression levels of transcription factors; *EcbHLH1-1* and *EcbHLH1-2* (A) and biosynthetic enzyme genes; *Ec6OMT*, *Ec4'OMT*, *EcBBE*, *EcCYP719A3*, and *EcTNMT* (B) were determined by quantitative

RT-PCR. The relative expression level shows the values standardized by that of the 0 h sample as 1. Error bars indicate the standard deviation calculated from three technical replicates.

Fig. 3. Expression levels of *EcbHLH1-1* and *EcbHLH1-2* in transgenic RNAi cell lines. The transcript levels of *EcbHLH1-1* and *EcbHLH1-2* were determined by quantitative RT-PCR. Gray boxes indicate VC cell lines, blue boxes indicate 1-RNAi cell lines, green boxes indicate 2-RNAi cell lines, and red boxes indicate 12-RNAi cell lines. The relative expression level shows the values standardized by that of the VC4-1 sample as 1. Each cell line shows six biological replicates (n=6).

Fig. 4. The expression levels of sanguinarine biosynthetic enzyme genes in transgenic RNAi cell lines. The transcript levels of *EcNCS*, *Ec6OMT*, *EcCNMT*, *EcCYP80B1*, *Ec4'OMT*, *EcBBE*, *EcCYP719A5*, *EcCYP719A2*, *EcCYP719A3*, *EcTNMT*, *EcMSH*, *EcCYP82N2v2*, *EcDBOX*, and *EcSR* were determined by quantitative RT-PCR. The relative expression level shows the values standardized by that of the VC4-1 sample as 1. Each cell line shows six biological replicates (n=6).

Fig. 5. Correlation analysis between the expression of *EcbHLH1-1* (A) or *EcbHLH1-2* (B) and that of *Ec6OMT*, *Ec4'OMT*, *EcCYP719A2*, *EcCYP719A3*, or *EcTNMT*. The relative expression level shows the values standardized by that of the VC4-1 sample as 1. Asterisks indicate significant correlation between the expression of two genes (df=22, *P<0.05, **P<0.01).

Fig. 6. Total accumulation of sanguinarine in transgenic RNAi cell lines. Sanguinarine content was calculated based on a peak area compared to that of an authentic sample. Each cell line shows six biological replicates (n=6) (A). Correlation between the expression of *EcbHLH1-1* or *EcbHLH1-2* (B) and *Ec6OMT*, *Ec4'OMT*, or *EcCYP719A3* (C) and sanguinarine content. Asterisks indicate significant correlation (df=22, *P <0.05, **P<0.01).

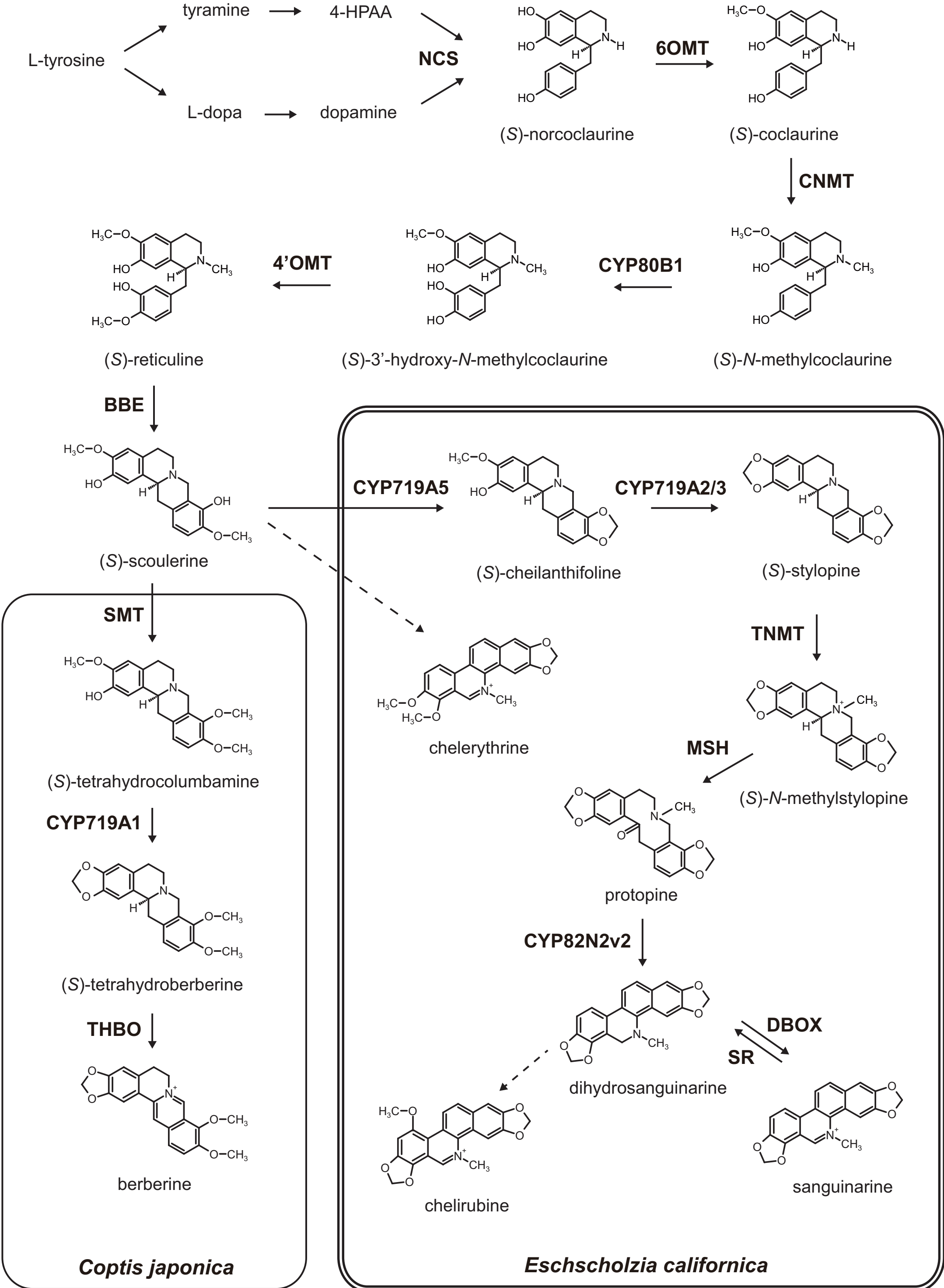
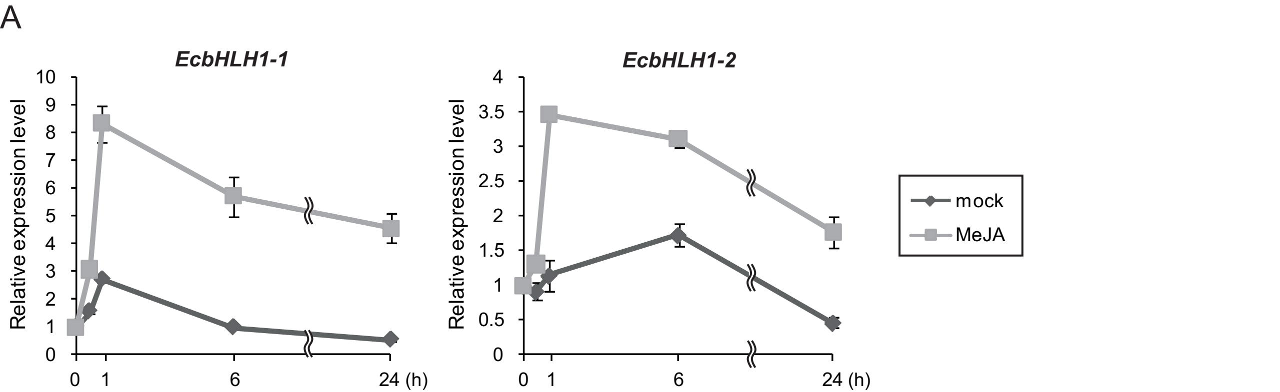


Fig. 1



B

Ec6OMT
Ec4'OMT
EcBBE
EcCYP719A3
EcTNMT
Relative expression level
0 1 6 24 (h)
mock
MeJA

Fig. 2

Fig. 3

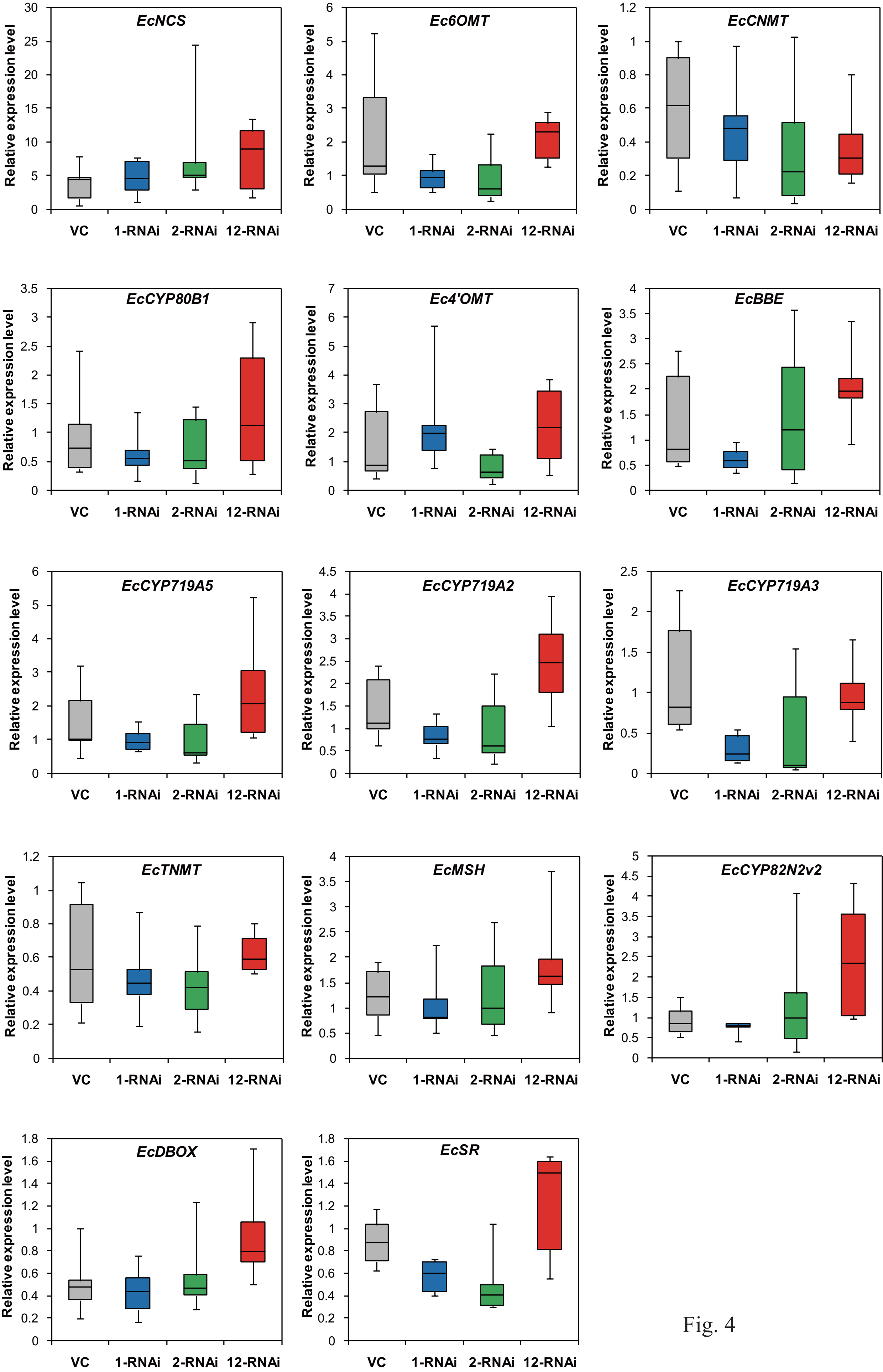


Fig. 4

A

Ec6OMT
$R^2 = 0.328$**
Relative *Ec6OMT* expression
Relative *EcbHLH1-1* expression

Ec4'OMT
$R^2 = 0.005$
Relative *Ec4'OMT* expression
Relative *EcbHLH1-1* expression

EcCYP719A2
$R^2 = 0.117$
Relative *EcCYP719A2* expression
Relative *EcbHLH1-1* expression

EcCYP719A3
$R^2 = 0.535$**
Relative *EcCYP719A3* expression
Relative *EcbHLH1-1* expression

EcTNMT
$R^2 = 0.247$ *
Relative *EcTNMT* expression
Relative *EcbHLH1-1* expression

VC
1-RNAi
2-RNAi
12-RNAi

B

Ec6OMT
$R^2 = 0.512$**
Relative *Ec6OMT* expression
Relative *EcbHLH1-2* expression

Ec4'OMT
$R^2 = 0.255$*
Relative *Ec4'OMT* expression
Relative *EcbHLH1-2* expression

EcCYP719A2
$R^2 = 0.160$
Relative *EcCYP719A2* expression
Relative *EcbHLH1-2* expression

EcCYP719A3
$R^2 = 0.389$**
Relative *EcCYP719A3* expression
Relative *EcbHLH1-2* expression

EcTNMT
$R^2 = 0.080$
Relative *EcTNMT* expression
Relative *EcbHLH1-2* expression

VC
1-RNAi
2-RNAi
12-RNAi

Fig. 5

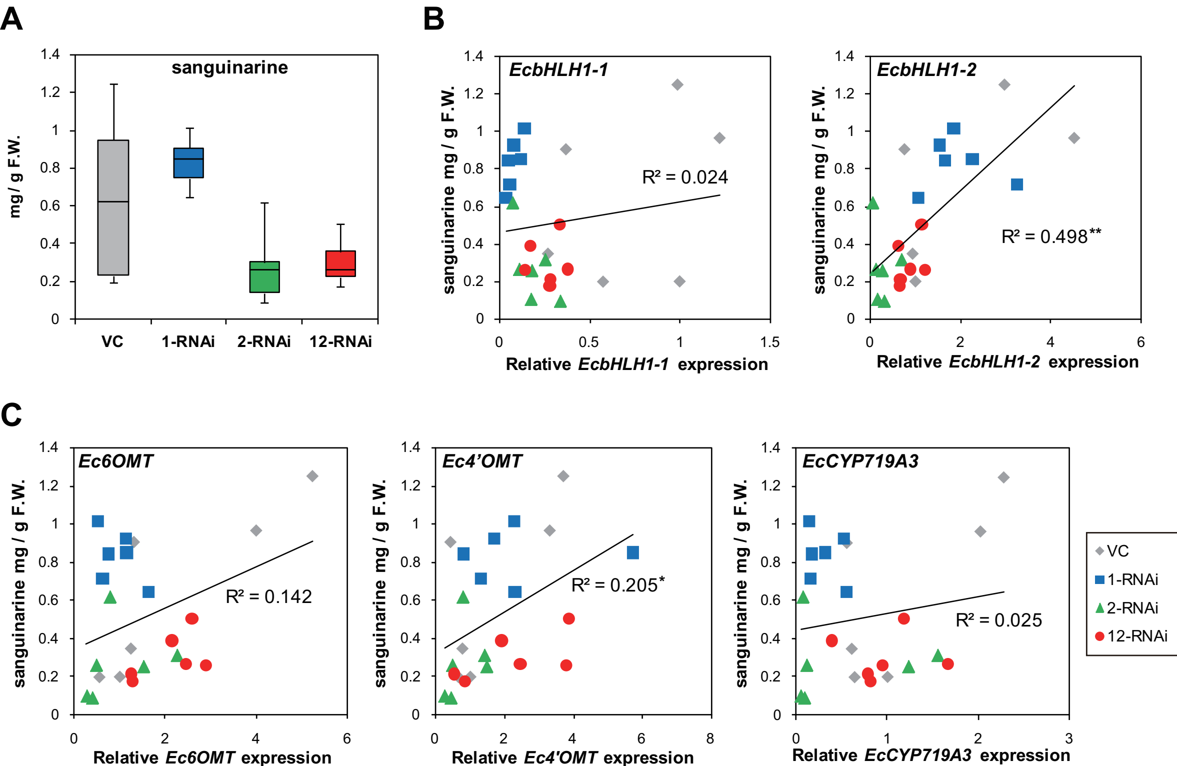


Fig. 6

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EcbHLH1-1   1    MAIDWNSDCEVIGSDVKSTGEGSQSGGSNGGGGGGGIDARERHKLAERERRKSMRELFLS
EcbHLH1-2   1    MAIDWNSDYEQVGSDGKSS-----SGNVNGGG----IDARERHKLAERERRKSMRELFLS
CjbHLH1     1    MPIETLNDWETVSSEGKSS------GAP--------IDARERHKLAERERRKSMRELFLS
                                               bHLH domain ........................

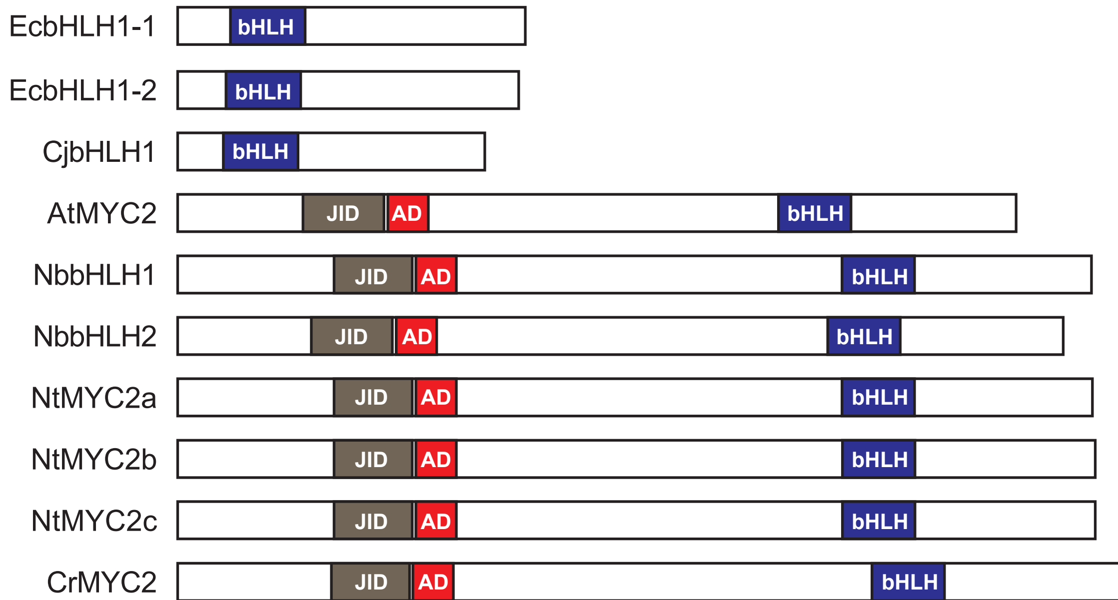
EcbHLH1-1   61   LHSLLPHANTVRKEQSAILDEIIKYIPIAAARLRSLQNRK-NSSSPLMNLNRSKSNSTSK
EcbHLH1-2   52   LHSLLPHANTIRKEQSAILDEIIKYIPIAAARLRSLKNRKESSSSPNWNRSKSSLNSSAS
CjbHLH1     47   LHALLPHGNTVRKEQSSILDEIIKYIPLASARLKSLQNRK-----------ESTPLSTRP
                 ........................................

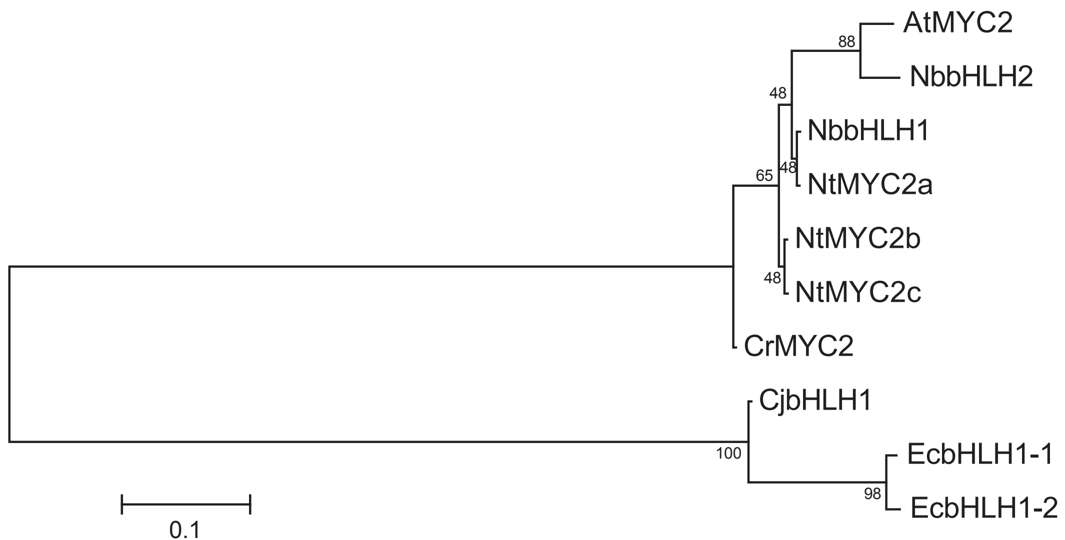
EcbHLH1-1   120  SNSSPSIQVSDRTTT---SNSSNSISCLNTSDCDIRVAPEPSSSVAIRVRGDRVNVSLSD
EcbHLH1-2   112  SSSLPSIQVSDLTCSNVISNSATSSSLINISDCDIRVSPEPSSSVAIRVRGDRVNVSLSD
CjbHLH1     96   KLASPSIQVSDRKSS----GSS------NSTDCDIRVAPEPSASVAIRVRGDRVNVSLTD

EcbHLH1-1   177  TKGTSQTLLLSAILDELEAHQLELVRSTHCRDGSKVLHHSESKICEGLDRSPVLLKARLQ
EcbHLH1-2   172  TKGSSQTLLLSAVLDELEAHQLELVRSTHCRDGSKILHHSESKICDGLDKSPALLKARLQ
CjbHLH1     146  TKGTAQTLLLSAIFDELDAHNLELVRSTHCRDGSKVLHHSESKISDGLERSPGLLKTRLQ

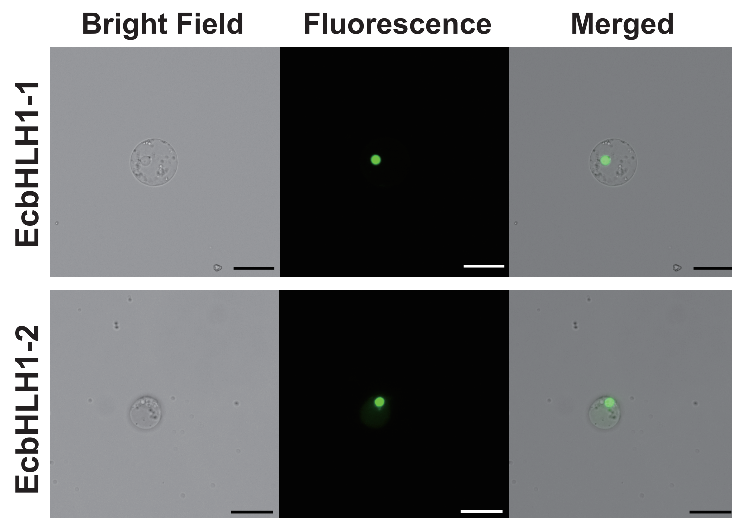
EcbHLH1-1   237  ELARKLHKLRKSSTLKRSFDQI-
EcbHLH1-2   232  ELARKLHNLRKSTSLKRSFDQI-
CjbHLH1     206  ELAGRLHKLRKSSSHKRTFDQLD
```

Supplementary Figure S1. Amino acid sequence alignment of EcbHLH1-1, EcbHLH1-2, and CjbHLH1. The predicted amino acid sequences were aligned using Clustal W. Dotted underlining indicates the highly conserved bHLH domain.

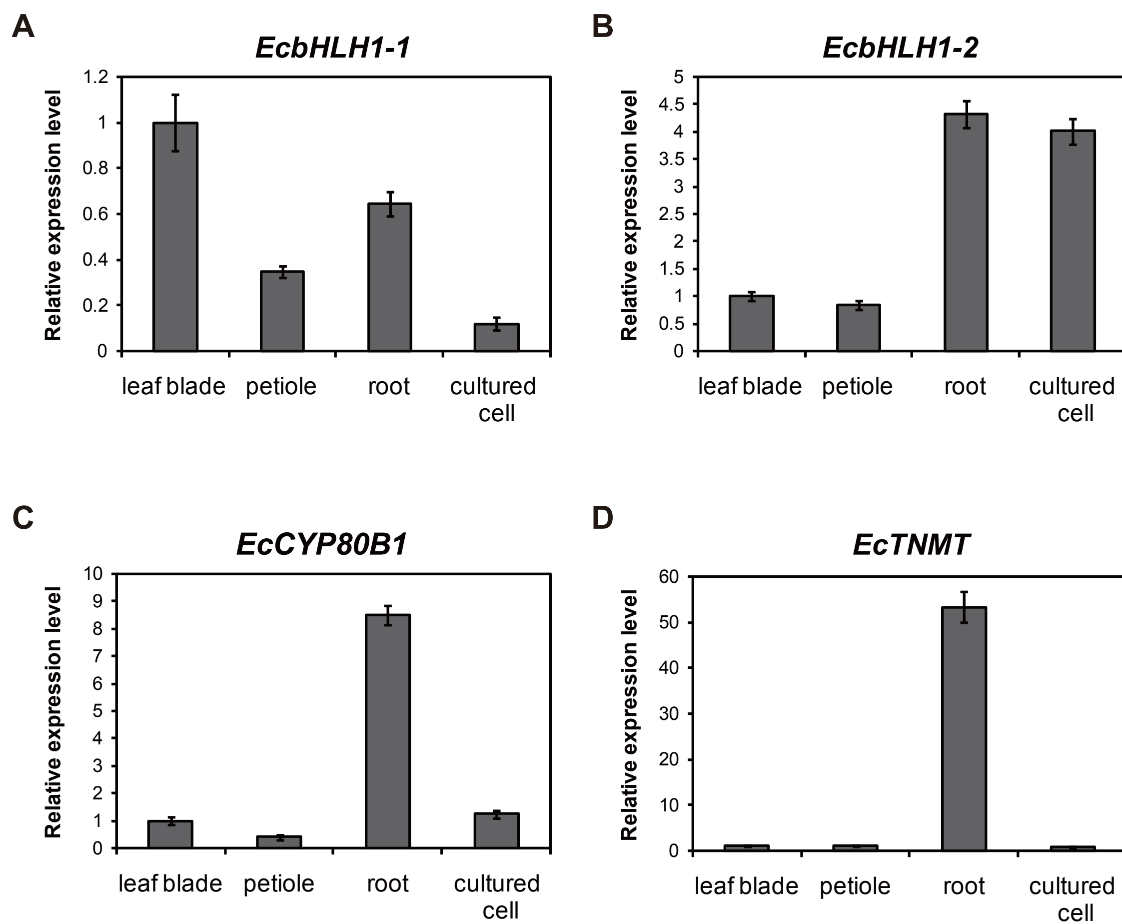




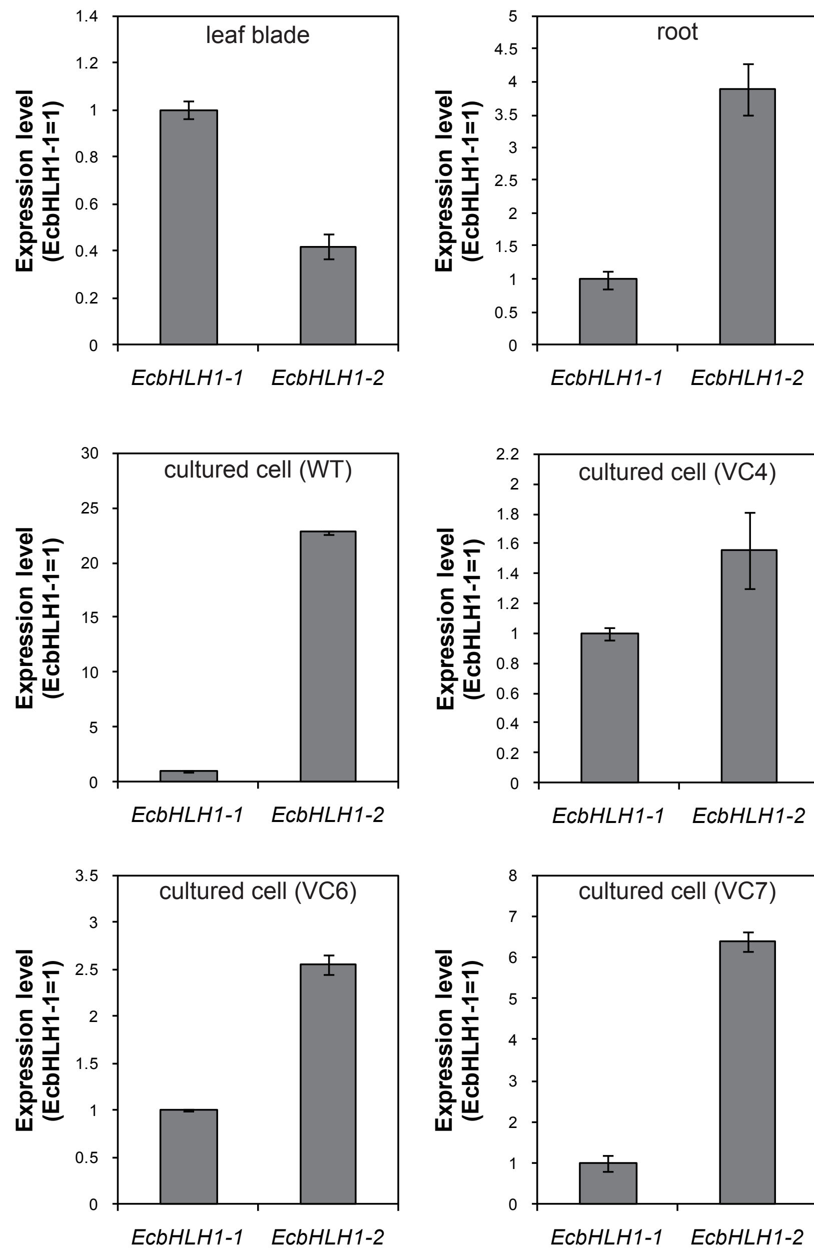
Supplementary Figure 2. Comparison of EcbHLH1 with MYC2-type bHLH transcription factors. (A) Domain organization in bHLH transcription factors. Gray boxes show the JAZ interaction domain (JID), red boxes show the activation domain (AD), and blue boxes show the bHLH domain. (B) A phylogenetic tree of EcbHLH1 and MYC2 homologs was constructed with amino acid sequences of typical bHLH domains using Clustal W and the Neighbor-joining method. Bootstrap tests were performed using 1000 replicates.



Supplementary Figure S3. Nuclear localization of EcbHLH1-1 and EcbHLH1-2. The EcbHLH1-1-sGFP and EcbHLH1-2-sGFP proteins were expressed in 156-S protoplasts and GFP fluorescence was observed by gluorescence microscopy. Scale bar represents 50 µm. GFP fluorescence was observed by a BZ9000 microscope (KEYENCE) in transformed protoplasts after 24 h of culture.



Supplementary Figure S4. Expression profiles of *EcbHLH1-1* and *EcbHLH1-2* in California poppy plant tissues (leaf blade, petiole, and root) and cultured cells. The expression levels of *EcbHLH1-1* (A), *EcbHLH1-2* (B), *EcCYP80B1* (C), and *EcTNMT* (D) were determined by quantitative RT-PCR. The relative expression level shows the values standardized by that of the leaf blade as 1. Error bars indicate the standard deviation calculated from three technical replicates.



Supplementary Figure S5. Relative expression level of *EcbHLH1-1* and *EcbHLH1-2*. The absolute transcript levels were estimated using standard curves drawn with a diluted series of plasmids (pGEM T-Easy vector) containing each gene. The resultant values were standardized by the values of *EcbHLH1-1* as 1. Four different cell lines (WT, VC4, VC6, and VC7) were analyzed due to the large variation of gene expression in cultured cells. Error bars indicate the standard deviation calculated from three technical replicates.

```
EcbHLH1-1   1   ATGGCGATTGATTGGAATAGTGATTGTGAAGTAATAGGATCAGATGTAAAATCAACCGGA
EcbHLH1-2   1   ATGGCTATTGATTGGAATAGTGATTATGAACAAGTAGGATCAGATGGAAAATCATC----

EcbHLH1-1   61  GAAGGATCTCAAAGTGGTGGTAGTAATGGTGGTGGTGGTGGTGGTGGAATTGATGCAAGA
EcbHLH1-2   56  -----ATCT------------------GGTAATGTAAATGGAGGAGGAATTGATGCAAGA

EcbHLH1-1   121 GAAAGACATAAATTAGCAGAGAGAGAAAGAAGAAAATCAATGAGAGAATTATTTTTATCT
EcbHLH1-2   94  GAAAGACATAAATTAGCAGAGAGAGAAAGAAGAAAATCAATGAGAGAATTATTTTTATCA

                                                   EcbHLH1-1/EcbHLH1-2
EcbHLH1-1   181 CTTCATTCTTTACTTCCTCACGCTAATACAGTTAGAAAAGAACAATCAGCAATTCTTGAT
EcbHLH1-2   154 CTTCATTCATTACTTCCTCATGCTAATACAATTAGAAAAGAACAATCAGCAATTCTTGAT

EcbHLH1-1   241 GAAATTATTAAATATATACCCATTGCTGCTGCTAGACTTAGATCATTACAAAATCGTAAA
EcbHLH1-2   214 GAAATTATTAAATATATACCCATAGCTGCTGCTAGATTAAGATCATTAAAAAATCGAAAA

            EcbHLH1-1
EcbHLH1-1   301 AATTCATCATCACCATTAA--TGAATTTGAAT-AGGTCAAAATCAAATTCAACATCAAAA
EcbHLH1-2   274 GAATCATCGTCTTCACCCAATTGGAATCGATCGAAATCTTCATTAAATTCATCAGCTTCT

EcbHLH1-1   358 TCAAATTCATCACCATCAATTCAAGTATCAGATCGTACTACTACTAGTAATAGTAGTAAT
EcbHLH1-2   334 TCTTCTTCTCTACCATCAATTCAAGTATCAGATCTTACTTGTAGTAATGTGATAAGTAAT
                                                      EcbHLH1-2

EcbHLH1-1   418 TCAATTAGTTGTC---------TTAATACTAGTGATTGTGATATTAGAGTTGCTCCTGAA
EcbHLH1-2   394 TCAGCTACTAGTTCTTCTTTGATTAATATAAGTGATTGTGATATTAGGGTTTCTCCTGAA

EcbHLH1-1   469 CCTTCATCTTCAGTGGCGATTCGAGTTAGAGGTGACAGAGTTAATGTCTCACTTAGTGAT
EcbHLH1-2   454 CCTTCATCTTCGGTTGCTATTCGAGTTAGAGGTGATAGAGTTAATGTTTCATTGAGTGAT

EcbHLH1-1   529 ACAAAGGGTACTTCACAAACTCTGTTATTATCTGCAATTTTAGATGAACTTGAAGCTCAT
EcbHLH1-2   514 ACAAAGGGTAGTTCACAAACTCTGTTATTATCAGCTGTTTTAGATGAACTTGAAGCTCAT

EcbHLH1-1   589 CAACTAGAACTTGTTCGTTCTACTCATTGTCGTGATGGAAGTAAAGTTTTACATCATTCT
EcbHLH1-2   574 CAACTCGAACTTGTTCGTTCTACTCATTGTCGCGACGGAAGTAAGATTTTACATCATTCT

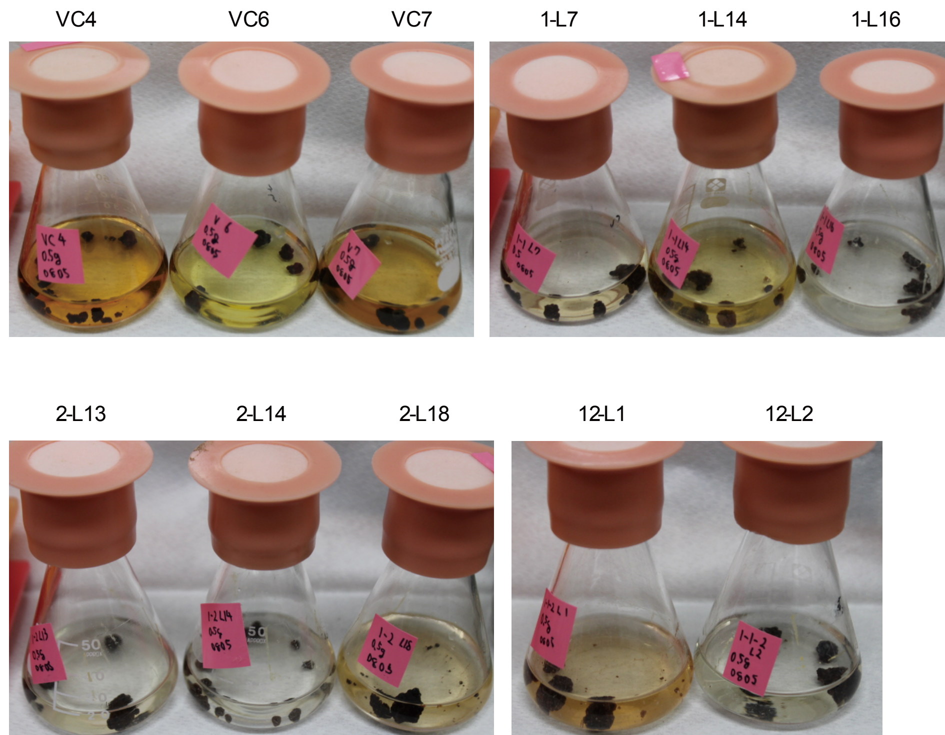
EcbHLH1-1   649 GAAAGCAAGATTTGCGAGGGTCTAGACAGATCGCCTGTTTTGTTGAAAGCAAGATTACAA
EcbHLH1-2   634 GAAAGCAAGATTTGTGATGGGTTGGACAAATCACCAGCTTTACTAAAAGCAAGATTACAA

EcbHLH1-1   709 GAATTGGCTCGAAAACTCCACAAGTTGAGAAAATCATCTACACTTAAGAGATCATTTGAC
EcbHLH1-2   694 GAATTAGCTAGGAAACTTCACAACTTGAGGAAATCAACTTCACTCAAGAGATCATTTGAC

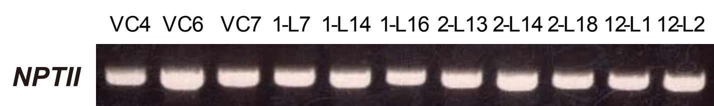
EcbHLH1-1   769 CAGATTTGA
EcbHLH1-2   754 CAGATTTAA
```

Supplementary Figure S6. Selected 37 bp RNAi target sequences specific to *EcbHLH1-1* (blue box), *EcbHLH1-2* (green box), and both *EcbHLH1-1* and *EcbHLH1-2* (red box).

A

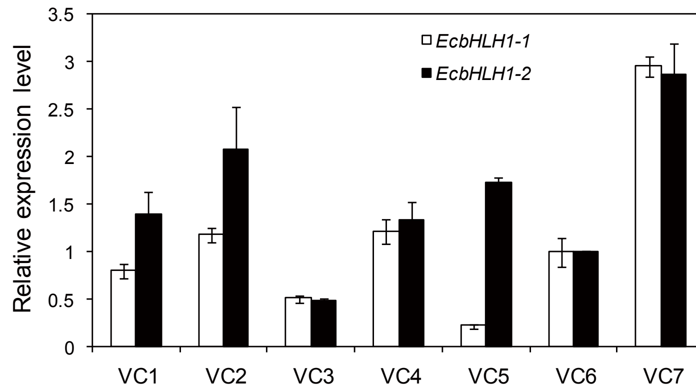


B

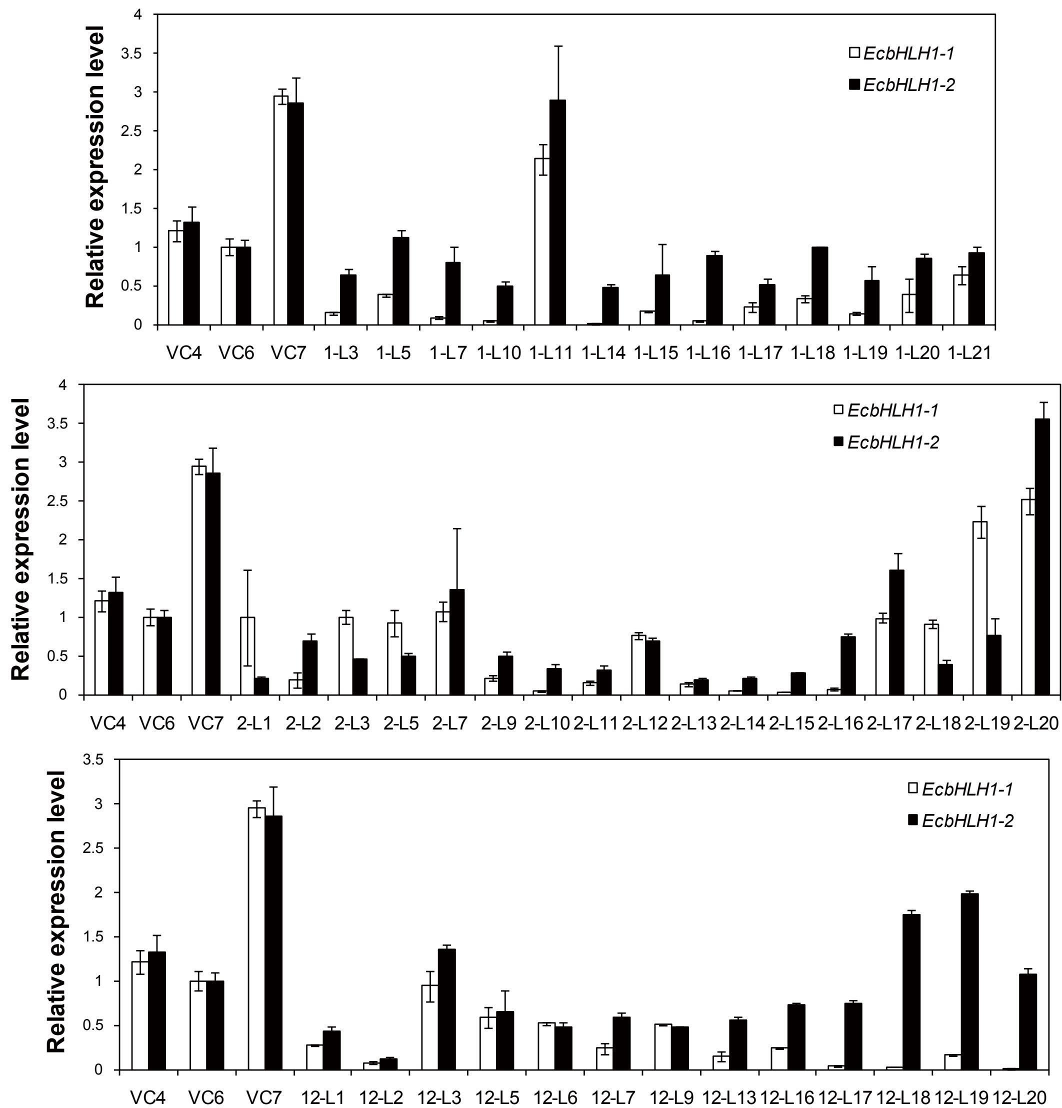


Supplementary Figure S7. Established transgenic cultured cell lines of California poppy. (A) Each VC and RNAi cell line for 16 months of culture. (B) Genomic PCR analysis using primer pairs designed to amplify the *NPT* transgene.

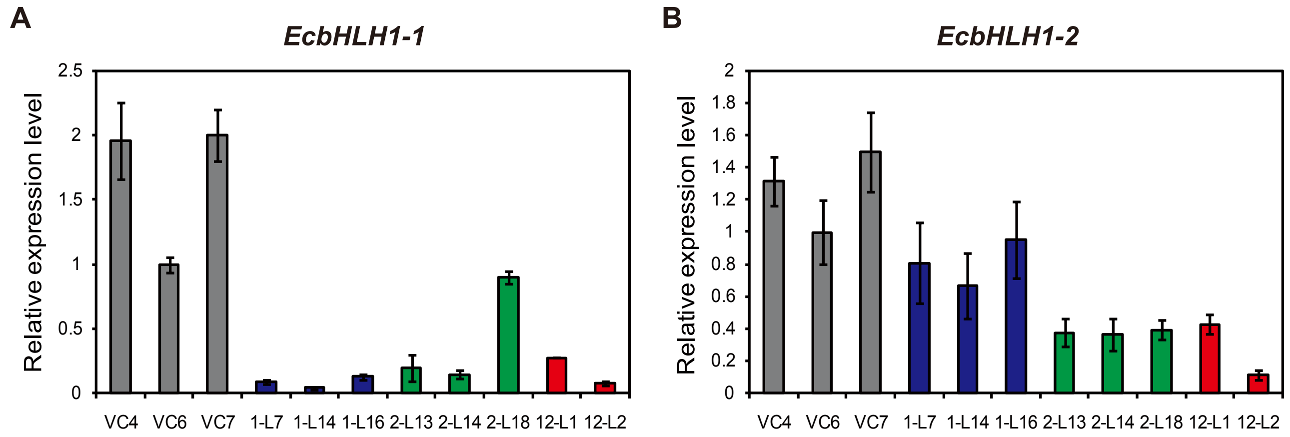
A



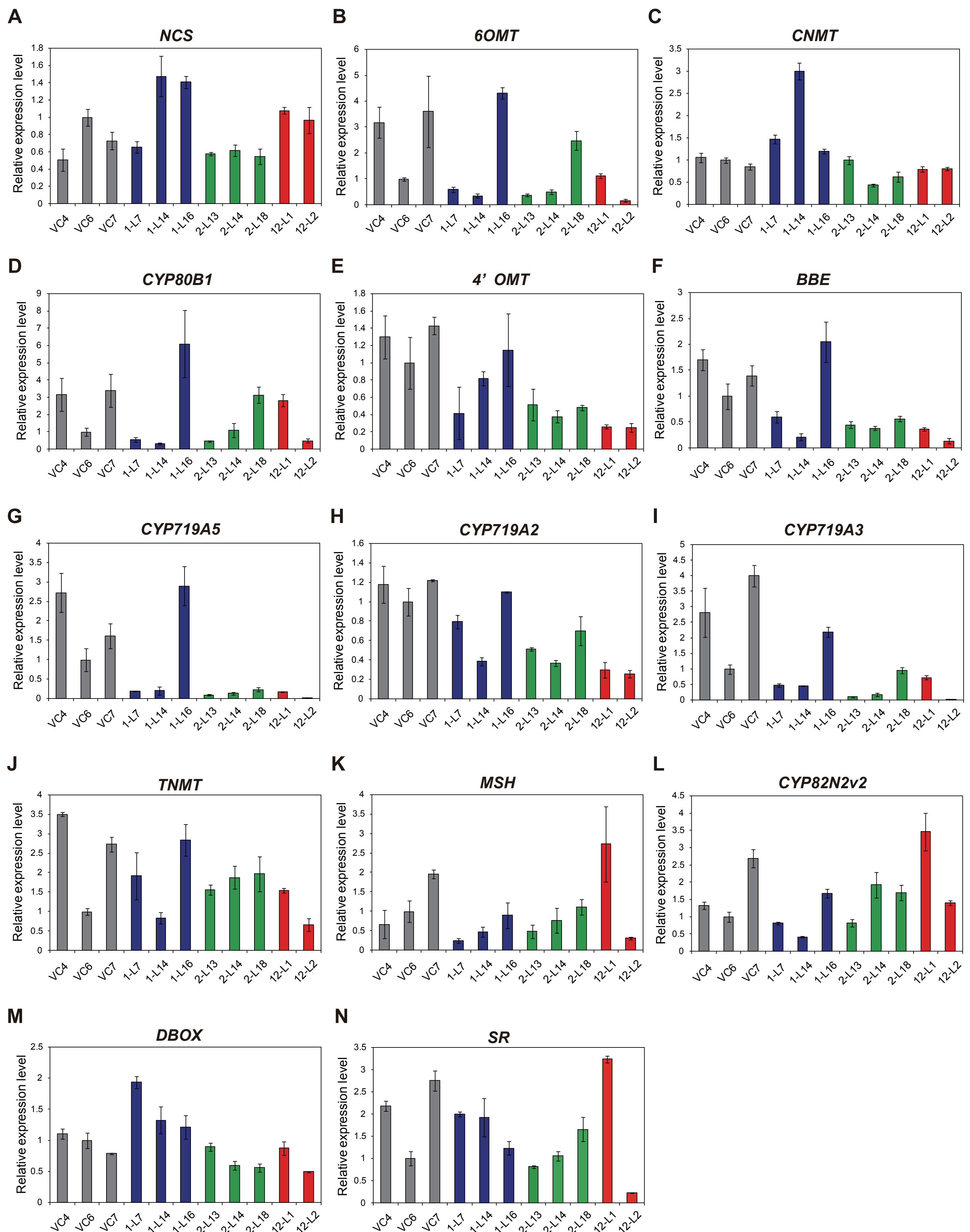
B



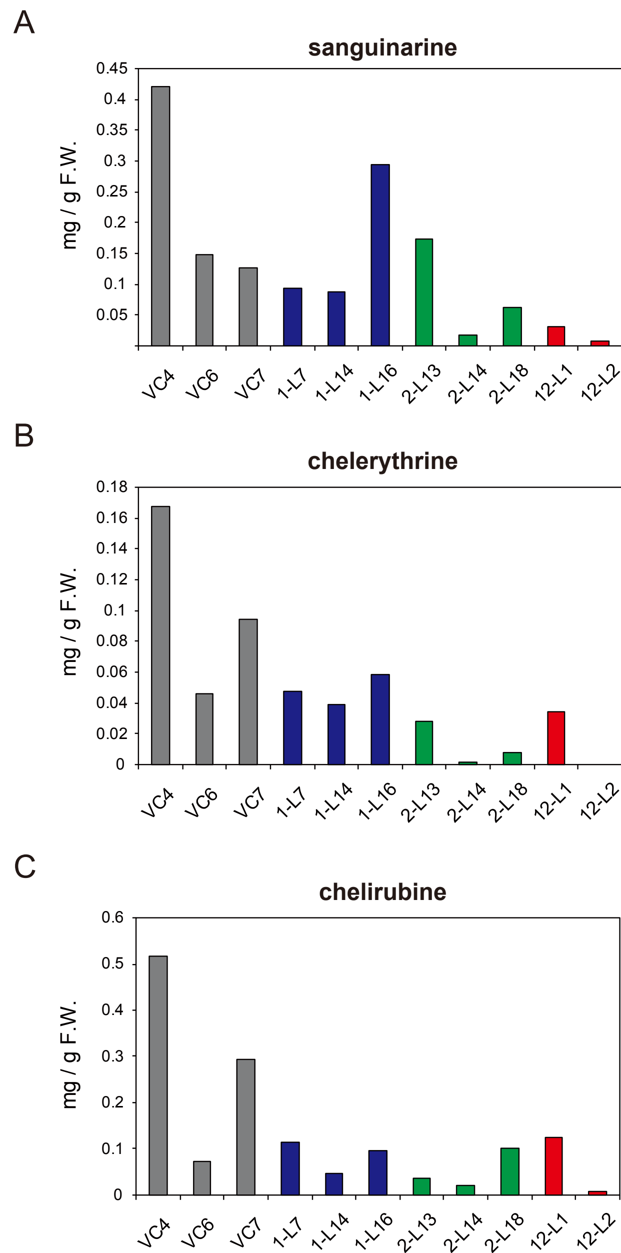
Supplementary Figure S8. The expression levels of *EcbHLH1-1* and *EcbHLH1-2* in each VC cell line (A) and *EcbHLH1* RNAi cell line (B). The transcript levels were determined by quantitative RT-PCR. The relative expression level shows the values standardized by that of the VC6 sample as 1. Error bars indicate the standard deviation calculated from three technical replicates.



Supplementary Figure S9. Expression levels of *EcbHLH1-1* and *EcbHLH1-2* in transgenic RNAi cell lines. The transcript levels of *EcbHLH1-1* (A) and *EcbHLH1-2* (B) were determined by quantitative RT-PCR. Gray boxes indicate VC cell lines, blue boxes indicate 1-RNAi cell lines, green boxes indicate 2-RNAi cell lines, and red boxes indicate 12-RNAi cell lines. The relative expression level shows the values standardized by that of the VC6 sample as 1. Error bars indicate the standard deviation calculated from three technical replicates.

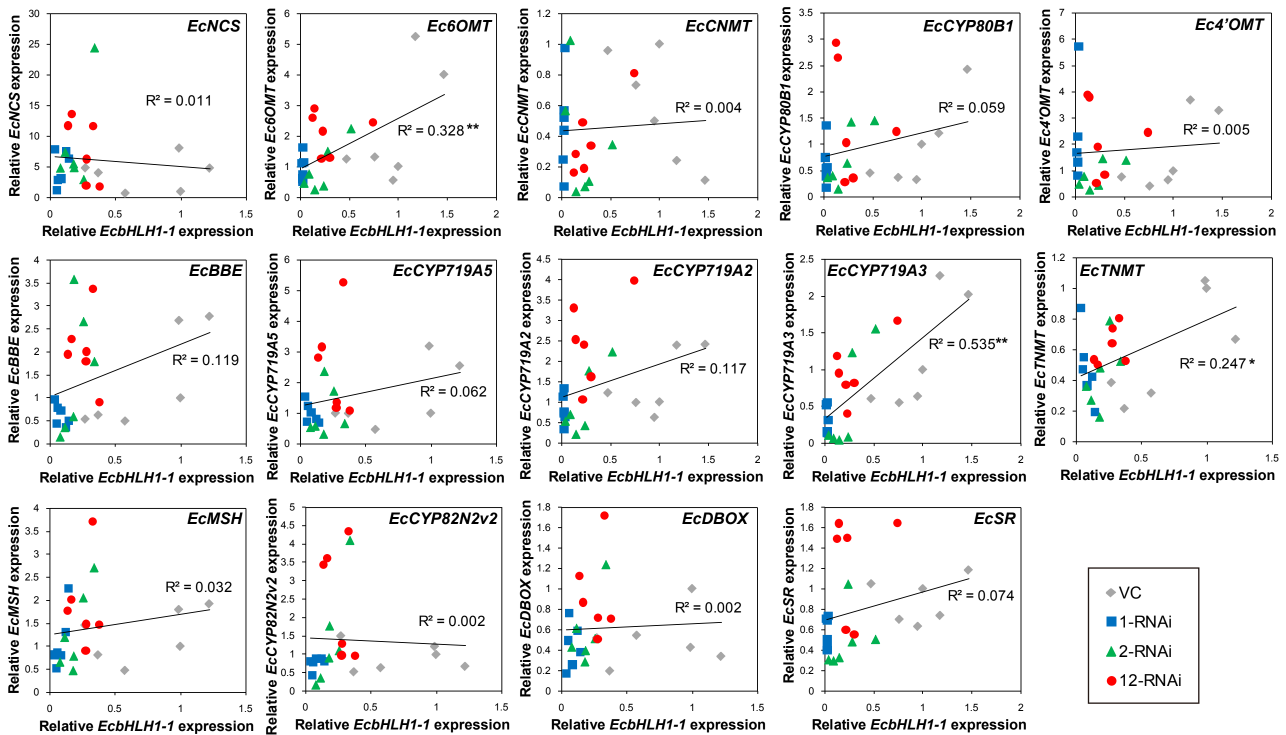


Supplementary Figure S10. The expression levels of sanguinarine biosynthetic enzyme genes in transgenic RNAi cell lines. The transcript levels of *NCS* (A), *6OMT* (B), *CNMT* (C), *CYP80B1* (D), *4' OMT* (E), *BBE* (F), *CYP719A5* (G), *CYP719A2* (H), *CYP719A3* (I), *TNMT* (J), *MSH* (K), *CYP82N2v2* (L), *DBOX* (M), and *SR* (N) were determined by quantitative RT-PCR. Gray boxes indicate VC cell lines, blue boxes indicate 1-RNAi cell lines, green boxes indicate 2-RNAi cell lines, and red boxes indicate 12-RNAi cell lines. The relative expression level shows the values standardized by that of the VC6 sample as 1. Error bars indicate the standard deviation calculated from three technical replicates.

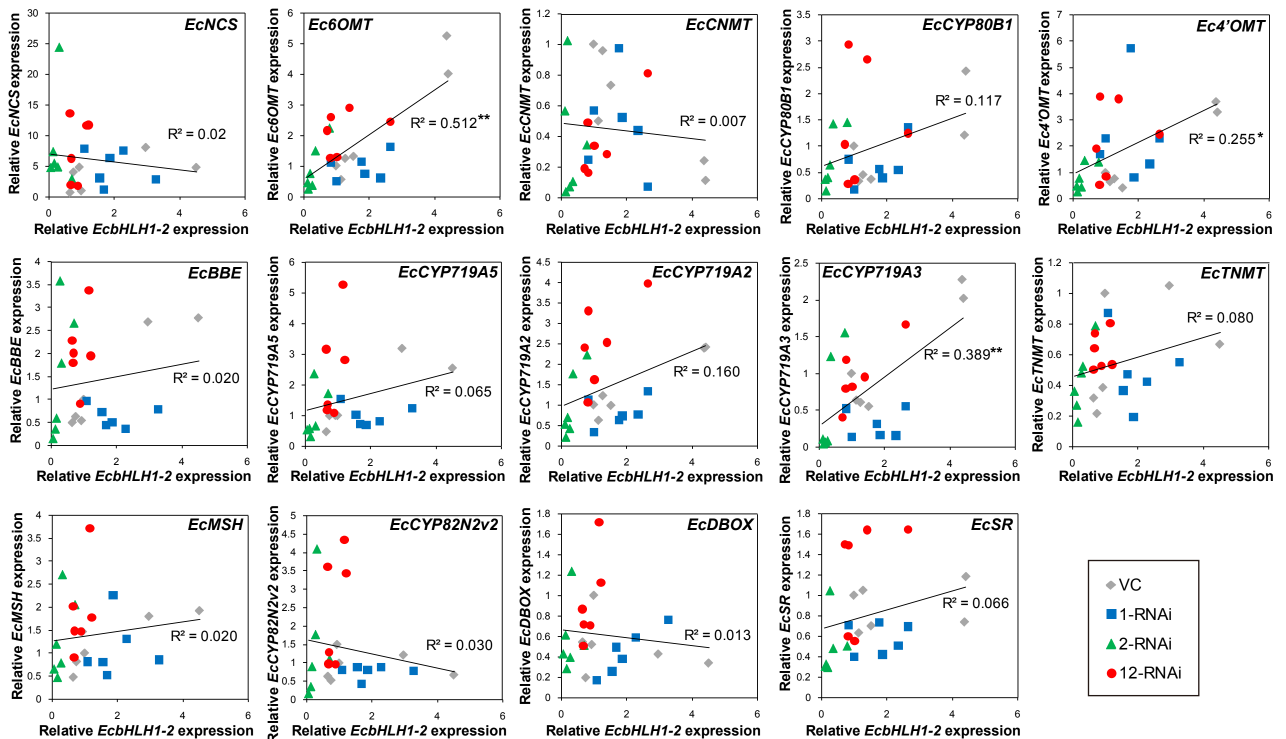


Supplementary Figure S11. The amounts of sanguinarine (A), chelerythrine (B), and chelirubine (C) in cultured cells and medium. Gray boxes indicate VC cell lines, blue boxes indicate 1-RNAi cell lines, green boxes indicate 2-RNAi cell lines, and red boxes indicate 12-RNAi cell lines.

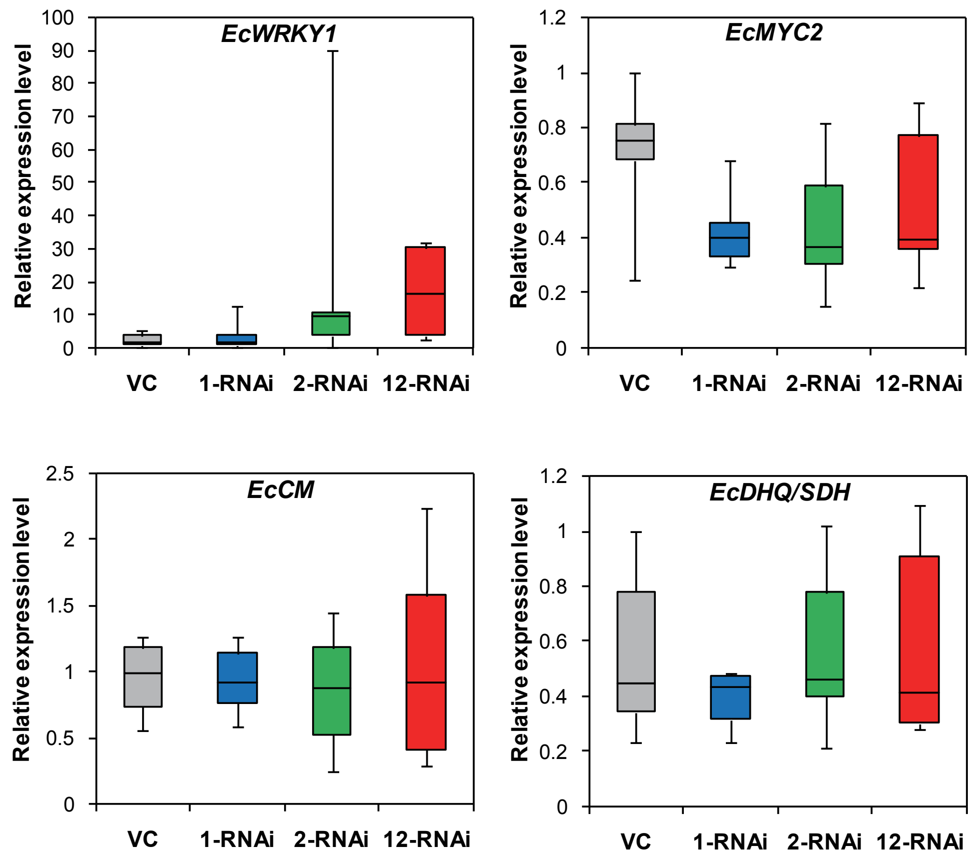
A



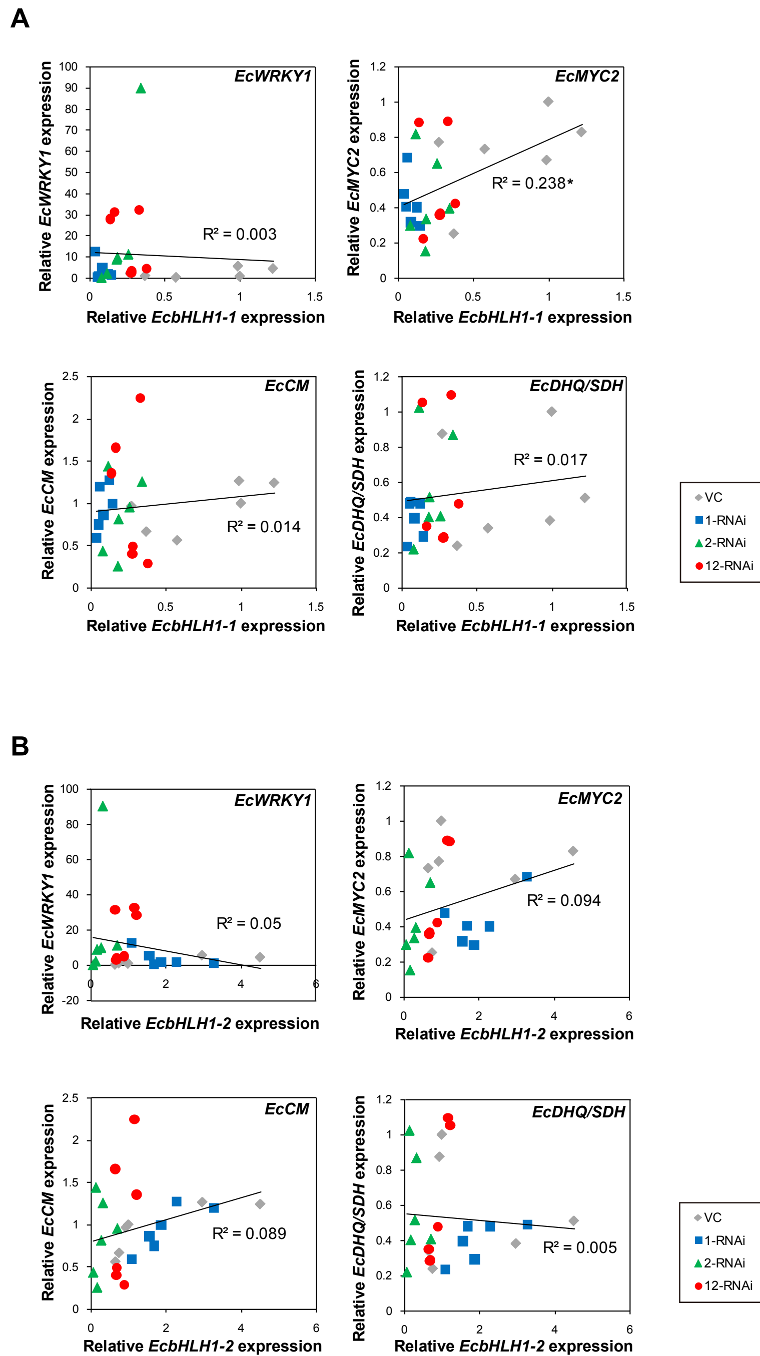
B



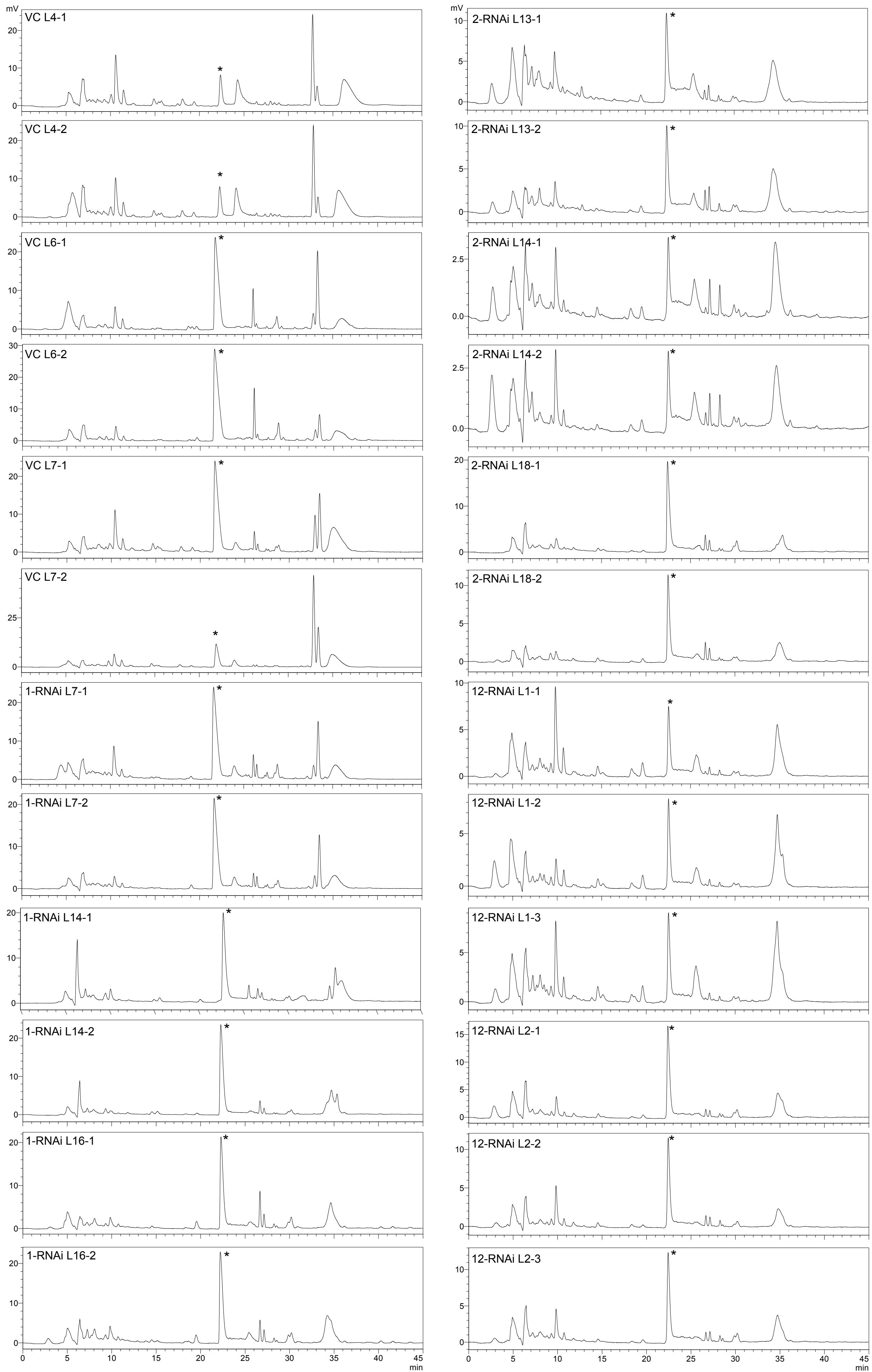
Supplementary Figure S12. Correlation analysis between the expression of *EcbHLH1-1* (A) or *EcbHLH1-2* (B) and that of all sanguinarine biosynthetic enzyme genes. The relative expression level shows the values standardized by that of the VC4-1 sample as 1. Asterisks indicate significant correlation between the expression of two genes (df=22, **P*<0.05, ***P*<0.01)



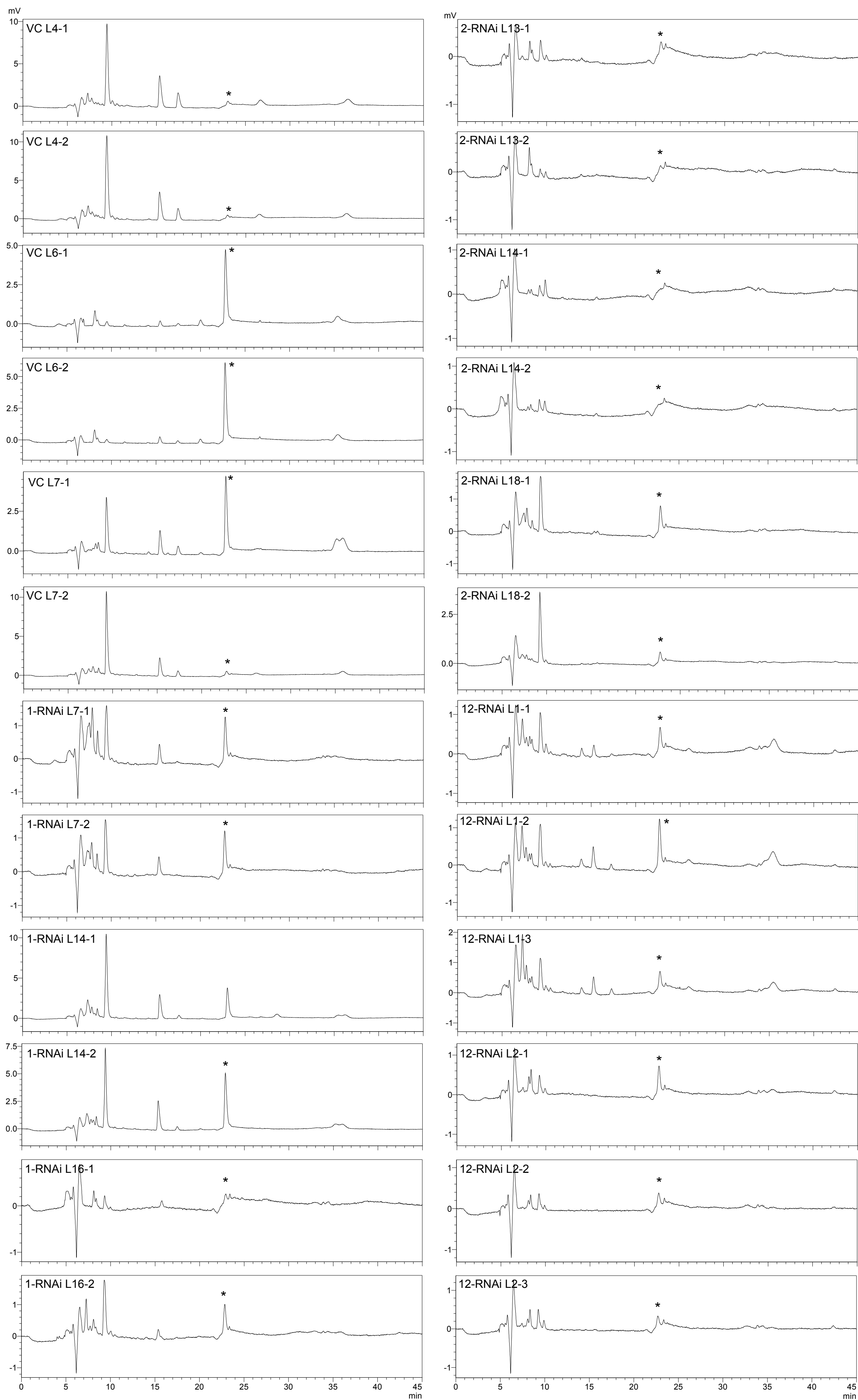
Supplementary Figure S13. The expression levels of other putative transcription factor genes and the primary metabolism genes in transgenic RNAi cell lines. The relative expression level shows the values standardized by that of the VC4-1 sample as 1. Each cell line shows six biological replicates



Supplementary Figure S14. Correlation analysis between the expression of *EcbHLH1-1* (A) or *EcbHLH1-2* (B) and other transcription factor genes or the primary metabolism genes. The relative expression level shows the values standardized by that of the VC4-1 sample as 1. Asterisks indicate significant correlation between the expression of two genes (df=22, **P*<0.05)



Supplementary Figure S15. UV chromatogram of the cell extract of each VC and RNAi cell line. Asterisks indicate a peak of sanguinarine (m/z 332).



Supplementary Figure S16. UV chromatogram of the culture medium of each VC and RNAi cell line. Asterisks indicate a peak of sanguinarine (m/z 332).

Supplementary Table S1. Primers for the isolation of *EcbHLH1-1* and *EcbHLH1-2* from *E. californica*.

name		Oligonucleotide sequence (5' to 3')
EcbHLH1	Fw	GAYGARATHATHAARTAYATHCC
Degenerate	Rv	CCRTCNCKRCARTGNGTNSWNCK
EcbHLH1-1 3'RACE		GCTGCTGCTAGACTTAGATCATTAC
EcbHLH1-2 3'RACE		CATCGTCTTCACCCAATTGGAATC
EcbHLH1-1 5'RACE first		CAACAAAACAGGCGATCTGTCTAG
EcbHLH1-1 5'RACE nest		CTCTAACTCGAATCGCCACTGA
EcbHLH1-2 5'RACE first		CTGGTGATTTGTCCAACCCATCA
EcbHLH1-2 5'RACE nest		ACCTCTAACTCGAATAGCAACCG

Supplementary Table S2. Primers for 37 bp RNAi

clone		Oligonucleotide sequence (5’ to 3’)
EcbHLH1-1 37 bp RNAi	Fw	CGGGATCCATCATCACCATTAATGAATTTGAATAGGTCAAAATCACGTG GTGATGTGGAGTATTC
	Rv	GCGAGCTCATCATCACCATTAATGAATTTGAATAGGTCAAAATCAGAAT ACTCCACATCACCACG
EcbHLH1-2 37 bp RNAi	Fw	CGGGATCCAATGTGATAAGTAATTCAGCTACTAGTTCTTCTTTGACGTGG TGATGTGGAGTATTC
	Rv	GCGAGCTCAATGTGATAAGTAATTCAGCTACTAGTTCTTCTTTGAGAATA CTCCACATCACCACG
EcbHLH1-1/ EcbHLH1-2 37 bp RNAi	Fw	CGGGATCCCAGCAATTCTTGATGAAATTATTAAATATATACCCATCGTGG TGATGTGGAGTATTC
	Rv	GCGAGCTCCAGCAATTCTTGATGAAATTATTAAATATATACCCATGAATA CTCCACATCACCACG

Supplementary Table S3. Primers for quantitative RT-PCR of target genes.

Gene		Oligonucleotide sequence (5’ to 3’)
EcbHLH1-1	Fw	CTAGACAGATCGCCTGTTTTGTTG
	Rv	GAGATAGATGACCCTTTAGATAATGAGATCC
EcbHLH1-2	Fw	GGTTGGACAAATCACCAGCTTTAC
	Rv	AAGATAGAGGGGTTCATAATGATAGCAAG
NCS	Fw	CTATCACGGAGAGCAACACTTG
	Rv	TCCCCAAGCACATATAACTTCA
6OMT	Fw	CCTGTTCAACCCGTTGACTTAG
	Rv	CCCAACCCTTAATCAGAAATTTG
CNMT	Fw	TGAAGCCAGGCAAAATCTCCT
	Rv	GGTGGCATTTTGGATGAACT
CYP80B1	Fw	TCAAACAGTGGTAGGCGAGAGA
	Rv	CAATGGAGTTGGTGGGTGAA
4’OMT	Fw	CCTAGAAGAGGAATCAGAACATCCA
	Rv	TCACTTCTCTCCCTTCCACCA
BBE	Fw	GAGATTAGTAGGAGTTGGGGTGAGA
	Rv	ATTGGAGGGATACTTTGTGGATG
CYP719A5	Fw	CCTGATCTTAGTGAGGATCATTGC
	Rv	ATGCTAGCACTACATGCCATTTCAC
CYP719A2	Fw	GTCGTAATTAATCACTTAACCGTGCTCG
	Rv	GAAAGAAACAGAGCAAATCTTATCCTTTTACC
CYP719A3	Fw	CCTCGTAACTAATATACCAGTGTGGTG
	Rv	GACAACCAAGCAAACTCTTATTCTTGTAC
TNMT	Fw	TTCAGTAGAGGCATGGAGGA
	Rv	TCTTTACTTCCAAGGCCAGG
MSH	Fw	TTCCATCACTTGGTTGGTTAGA
	Rv	CCCAACTCTCAACTACTGAATCAA
CYP82N2v2	Fw	AACCGTCCTTCCACTAAAGC
	Rv	GGCTCTAACGTCCTTGATGG
DBOX	Fw	AACAAACAGAGCATTCCTCCTC
	Rv	AAAGAAGAATTACGTCGATACGG
SR	Fw	TGAGGAAGTGAAGAACAAAGCA
	Rv	GAACACACCGAGAAACAAAACA
DHQ/SDH	Fw	TGGCATCTTCGGATTTTGTT
	Rv	GTTTTGCCTTACCCATTTCG
CM	Fw	TGCTAGAAAGGGCTCAGCAT
	Rv	CCAAAGAACCATGAAAACCA

EcWRKY1	Fw	GCATTTGACCCAAAATCTGTTC
	Rv	ACAACAAGGATGCAATGTGAAG
EcMYC2	Fw	AGCTGCCATTAATCTAGCAGCTG
	Rv	CTGGGTCAATCTCACAATCTGATC
β-actin	Fw	GGTATTGTGCTGGATTCTGGTG
	Rv	GTAGGATTGCGTGGGGTAGTG