

# **Basic Helix-Loop-Helix transcription factors and regulation of alkaloid biosynthesis**

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## **Addendum to:**

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## **Abstract**

Transcription factors of the basic Helix-Loop-Helix (bHLH) family play a central role in cell proliferation, determination, and differentiation. In plants, the regulatory functions of bHLHs in phenylpropanoid biosynthesis have been well established with regard to other interacting-proteins; i.e., MYB and WD40 repeat proteins. On the other hand, those in alkaloid biosynthesis are greatly limited due to the limited distribution of alkaloids in plant species. Recently, several groups have reported the regulatory functions of bHLH in alkaloid biosynthesis: novel CjbHLH1 in isoquinoline alkaloid biosynthesis in *Coptis japonica*, and Jasmonate-inducible MYC2-type bHLHs in nicotine-alkaloid biosynthesis in *Nicotiana* plants and indole alkaloid biosynthesis in *Catharanthus roseus*. We report here the JA-inducibility of CjbHLH1 and discuss the similarity and differences of non-MYC2-resemblant CjbHLH1 and MYC2-type bHLHs in nicotine and indole alkaloid biosynthesis.

## **TEXT**

The bHLH family of proteins is a wide group of functionally diverse transcription factors that are distributed in both animals and plants. In plants, bHLHs are key components of transcriptional networks that regulate several biological processes, such as light signaling,

hormone signaling, development, and secondary metabolism.<sup>1,2</sup> In secondary metabolism, bHLHs involved in phenylpropanoid biosynthesis, which is found in all plant species, have been well characterized in association with other interacting-proteins; i.e., MYB and WD40 repeat proteins.<sup>3</sup> On the other hand, those in alkaloid biosynthesis are greatly limited due to the limited distribution of alkaloids in specific plant species and the scarce characterization of the molecular mechanism of biosynthesis.<sup>4</sup> The first bHLH to be identified in alkaloid biosynthesis was CrMYC1, in terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*, although its function remained unclear.<sup>5</sup> Recently, some MYC2-type bHLHs, such as NbbHLH1/NbbHLH2/NtMYC2 and CrMYC2, have been reported to be directly involved in nicotine biosynthesis in *Nicotiana* plants and terpenoid indole alkaloid biosynthesis in *C. roseus*, respectively.<sup>6-8</sup> These bHLHs function commonly through a signaling cascade of jasmonate (JA), a well-known phytoalexin inducer.

On the other hand, we recently identified a unique bHLH-type transcription factor, CjbHLH1, which regulates isoquinoline alkaloid biosynthesis in *C. japonica*.<sup>9</sup> Since CjbHLH1 showed low sequence similarity to MYC2-type bHLHs and formed a distinct isoquinoline alkaloid-specific clade, we have characterized the responsiveness of

CjbHLH1 to JA and discuss the similarity and differences in the signaling cascade among alkaloid biosynthesis.

### **MeJA rapidly induces the expression of the *CjbHLH1* gene**

JA, an efficient inducer of secondary metabolism in the defense response, has also been reported to influence isoquinoline alkaloid biosynthesis.<sup>10, 11</sup> The involvement of *CjbHLH1* in MeJA induced-isoquinoline alkaloid biosynthesis was examined with low berberine-producing suspension-cultured *C. japonica* cells (CjY line), since high-secondary metabolite-producing lines are often insensitive to treatment with JA. Treatment of CjY cells with MeJA clearly induced the expression of (*S*)-scoulerine-9-*O*-methyltransferase (*SMT*), which converts (*S*)-scoulerine to (*S*)-tetrahydrocolumbamine, while the expression of 3'-hydroxy-*N*-methylcoclaurine-4'-*O*-methyltransferase (*4'OMT*), which converts (*S*)-4'-hydroxy-*N*-methylcoclaurine to (*S*)-reticuline in berberine biosynthesis, was scarcely induced (Figure 1). The transcript rapidly increased at 1 h after treatment with MeJA and remained at a high level for 24 h compared to treatment with dimethyl sulfoxide (DMSO mock). Treatment with MeJA also significantly induced the expression of *CjbHLH1*. The expression level was about 5 times higher than that with mock treatment at 0.5-1 h after treatment with MeJA, but decreased to the same level as with mock treatment after

24 h. This result and previous results<sup>9</sup> on the transcriptional activity of CjbHLH1 clearly indicated that *CjbHLH1* was involved in the response to MeJA in berberine biosynthesis.

### **Diversified transcriptional regulation network in alkaloid biosynthesis**

CjbHLH1 is a bHLH protein that is distinct from the MYC2 clade,<sup>9</sup> but its expression was induced by MeJA, similar to Arabidopsis MYC2 (AtMYC2). AtMYC2 is a key component of the JA signaling pathway, and binds to the G-box in the promoter of JA-induced genes (Figure 2A).<sup>12,13</sup> In addition to JA-responsiveness, the transcriptional activity of AtMYC2 is also regulated by JAZ repressors in the JA signal pathway. Thus, JA promotes the interaction of JAZ repressors with the SCF<sup>CO11</sup> complex, and degrades JAZ by 26S proteasome to activate the expression of JA-responsive genes.<sup>14-16</sup>

NbbHLH1/2 and NtMYC2, which show high sequence similarity to AtMYC2, were also JA-responsive and interacted with tobacco JAZ repressor in yeast (Fig. 2B).<sup>7, 17</sup> Nicotiana MYC2-type bHLHs also directly bind to G-box in the target promoter of alkaloid biosynthetic genes, i.e., putrescine *N*-methyltransferase (*PMT*). Thus, MYC2-clade bHLH proteins directly regulated tobacco nicotine alkaloid biosynthesis in the JA-response, whereas tobacco MYC2-type bHLHs also regulated AP2/ERF-domain transcription factors

ERF189/ORC1, and both transcription factors coordinately regulated the biosynthetic enzyme genes.<sup>7,17,18</sup>

On the other hand, CrMYC2, the closest homologue of AtMYC2 in *C. roseus*, did not directly bind to the promoter of biosynthetic enzyme genes, i.e., strictosidine synthase (*STR*), but was MeJA-responsive and regulated biosynthetic genes through the activation of Catharanthus AP2-domain protein (ORCA) transcription factor (Fig. 2C).<sup>8,19,20</sup> This amplification of signal of JA via bHLH resembles the regulation of ERF expression via tobacco MYC2 in nicotine biosynthesis. Also, the high sequence similarity between CrMYC2 and AtMYC2 suggests that these activities may be regulated by JAZ repressors.<sup>8</sup>

In the case of isoquinoline alkaloid biosynthesis, CjbHLH1 was MeJA-responsive but showed low homology to AtMYC2, especially at the N-terminal half of the JAZ interaction domain (JID) in MYC2,<sup>21</sup> which suggests that transcriptional regulation by CjbHLH1 might be JAZ-independent (Figure 3). It would be interesting to characterize the JAZ-interacting proteins in isoquinoline alkaloid biosynthesis.

As mentioned above, the cascade of signal transduction in the biosynthesis of different

alkaloids would be diverse. Whereas the transcriptional regulation network is not yet sufficiently characterized, there may be differences in a subset of transcription factors among the pathways, as well as in the cascade (Figures 2A-D). In nicotine biosynthesis, NbARF1 (auxin responsive factor) and NbHB1 (homeobox gene) have also been reported to play a role in transcriptional regulation.<sup>6</sup> In indole alkaloid biosynthesis, AT-hook protein has been reported to regulate the expression of ORCA, although the details of this regulation are not yet clear.<sup>22</sup>

In isoquinoline alkaloid biosynthesis, CjWRKY1 is another direct activator of biosynthetic enzyme genes.<sup>23</sup> In addition to our work, Apuya et al. reported that WRKY, AP2/ERF domain and other transcription factors are involved in isoquinoline alkaloid biosynthesis based on their results with an artificial reporter assay system.<sup>24</sup> Whereas we have not yet detected the transcriptional regulation activity of *ERF* genes in isoquinoline alkaloid biosynthesis, several *ERF-like* genes were found in high berberine-producing *C. japonica* expression sequence tag (EST). Characterization of ERF in isoquinoline alkaloid biosynthesis, WRKY in nicotine or indole alkaloid biosynthesis and non-MYC2 type CrMYC1 in indole alkaloid biosynthesis would clarify the similarities and differences in the transcriptional regulation networks in alkaloid biosynthesis.

While the biosynthesis of alkaloids, which are powerful components of the plant chemical defense mechanism, is commonly regulated by a key signal regulator, jasmonate, it is also quite plant family-specific<sup>4</sup> since the biosynthetic enzyme genes, such as CYP719A1 in isoquinoline alkaloid biosynthesis, only exist in specific plant families.<sup>25</sup> Different types of alkaloids and secondary metabolites may have different transcriptional networks. Apuya et al. did not identify the transcriptional activity of Arabidopsis bHLH in isoquinoline alkaloid biosynthesis, whereas bHLH is a common transcription factor and AtMYC2 is an ortholog of NbbHLH1 and CrMYC2. It would be interesting to elucidate whether AtMYC2 can substitute for NbbHLH1 or CrMYC2. Our preferred hypothesis is that bHLHs may have diverse functionality in alkaloid biosynthesis. Also, in Arabidopsis, ERF and MYC2 antagonistically regulate the downstream genes, whereas they act synergistically in nicotine and indole alkaloid biosynthesis. Elucidation of the distinct mechanisms by which alkaloid biosynthesis is regulated by transcription factors including bHLHs should help to clarify how specific plant families evolved systems to produce their own alkaloids.

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## Figure legends

### Figure 1

MeJA induced the expression of *CjbHLH1* gene in low alkaloid-producing cultured *C. japonica* cells. Total RNA, prepared from 100 μM methyl-jasmonate (MeJA)- or 0.1 % DMSO (mock)-treated cells, was used for reverse transcription after treatment with DNase I. The expression levels of *SMT* (A), *4'OMT* (B) and *CjbHLH1* (C) were determined using quantitative RT-PCR. The relative expression levels show the values

standardized by that for the 0 h sample as 1. The results shown are mean values  $\pm$ SD of three measurements.

## Figure 2

Simplified model of the MeJA-mediated signaling cascade in *Arabidopsis thaliana* (A), nicotine biosynthesis in *Nicotiana* plants (B), terpenoid indole alkaloid biosynthesis in *C. roseus* (C) and isoquinoline alkaloid biosynthesis in *C. japonica* (D). Solid (blue) lines show transcriptional regulation and dotted (red) lines show post-translational regulation via protein-protein interaction. Arrows indicate activation and T-shaped lines indicate inhibition. The detailed regulation of gene expression is discussed in the text.

## Figure 3

Domain organization in bHLH transcription factors. Gray boxes show the conserved JAZ interaction domain (JID) and black boxes show the bHLH domain.

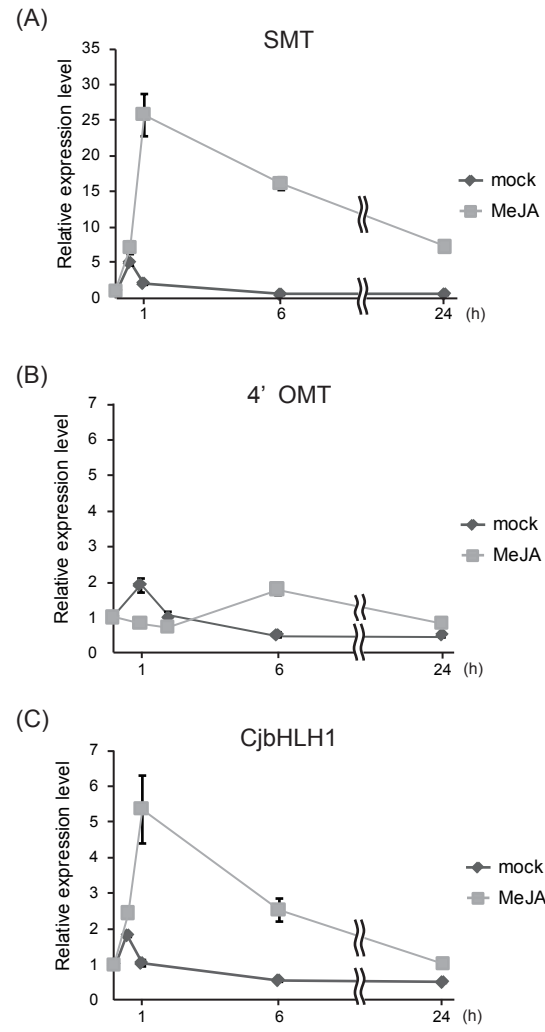


Fig. 1

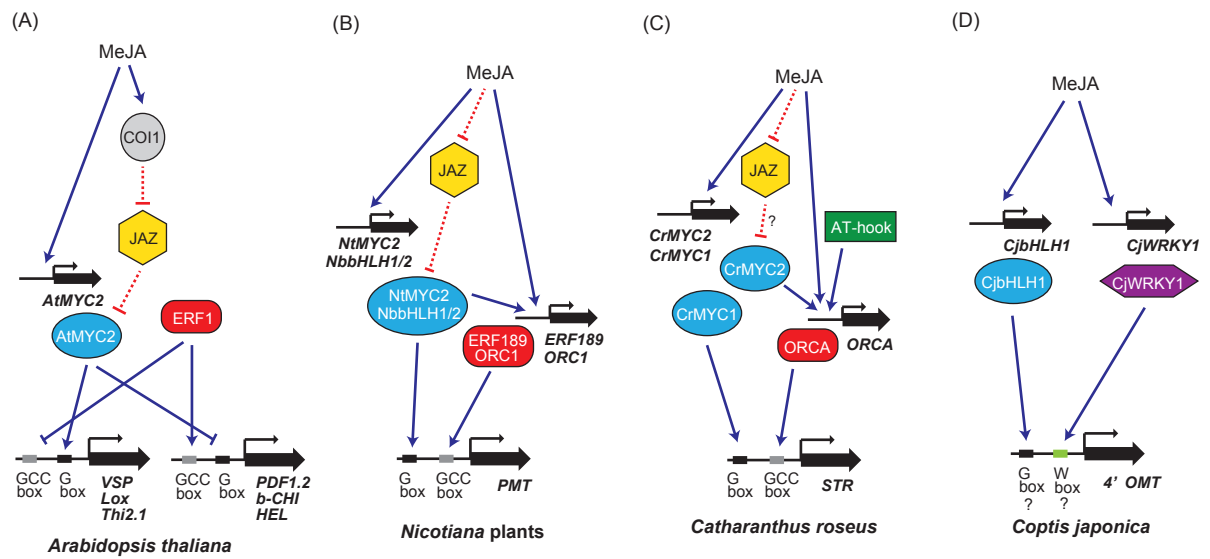


Fig. 2



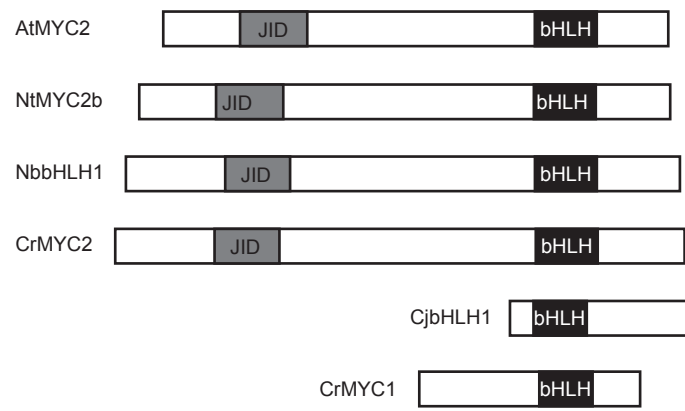


Fig. 3