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Short Communication

Isolation of *Daucus carota* ethylene insensitive3-like (DcEIL) involved in stress-inducible *DcMYB1* expression in suspension-cultured carrot cells

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Abstract In carrot suspension-cultured cells, the expression of phenylalanine ammonia-lyase 1 (*DcPAL1*) gene was crucially regulated by the transcription regulatory factor *Daucus carota* MYB1 (DcMYB1). To elucidate the regulatory mechanism of *DcMYB1* expression, we isolated and identified the transcription regulatory factor *D. carota* ethylene-insensitive3 (EIN3)-like protein (DcEIL) from a cDNA library prepared from suspension-cultured carrot cells treated with the "dilution effect" using a yeast one-hybrid system. DcEIL bound to a region of the *DcMYB1* promoter containing a putative *cis*-element, which suppressed *DcMYB1* promoter expression. The amino acid sequence of DcEIL contained the predicted signal sequence for nuclear localization, and transportation to the nucleus was confirmed using a green fluorescent protein-DcEIL fusion protein expressed in suspension-cultured *Arabidopsis thaliana* cells. The *DcEIL* gene was constitutively expressed irrespective of the elicitor treatment or the dilution effect. These results suggest that the binding of DcEIL to a *cis*-element of the *DcMYB1* promoter might be regulated at the posttranslational level as a consequence of the regulation of *DcPAL1* gene expression via DcMYB1 expression.

Key words: EIN3 family, myb, phenylalanine ammonia-lyase, phenylpropanoid metabolism, transcription factor.

The phenylpropanoid metabolic pathway produces phenolic compounds such as hydroxycinnamate and benzoic acid derivatives, coumarins, and lignins. This pathway supplies the starting materials for the flavonoid and anthocyanin metabolic pathway. Substances derived from the phenylpropanoid pathway absorb ultraviolet (UV) light, playing an effective role in protecting plant DNA from damage (Solovchenko and Schmitz-Eiberger 2003). Lignins are accumulated and polymerized in plant cell walls to give mechanical strength to resist wounding or pathogens (Vanholme et al. 2008). Thus, phenylpropanoid metabolism plays an important role in protecting plants from environmental stresses. Phenylalanine ammonia-lyase (PAL) is a key enzyme involved in regulating the metabolic flow from the primary metabolism to phenylpropanoid metabolism in higher plants. It has been reported that box-L sequences, which are located in the proximal region of PAL promoters, are key *cis*-elements that regulate PAL promoter activity in many plant species (Lois et al. 1989, Osakabe et al. 2009). Box-L-like sequences are important regulatory *cis*-elements of genes for enzymes, such as cinnamate 4-hydroxylase and 4-coumarate: CoA ligase, which are involved in phenylpropanoid metabolism (Logemann et al. 1995). Consequently, it is important to determine the *cis*-element-mediated regulatory mechanism of *PAL* gene expression by transcription regulatory factors.

There are at least two different *PAL* genes, *Daucus carota* phenylalanine ammonia-lyase 1 (*DcPAL1)* and *DcPAL3*, in the carrot genome. *DcPAL1* expression is induced by environmental stresses, such as treatment with a fungal elicitor, irradiation with UV light, and by transferring and diluting cells with fresh medium (the dilution effect). *DcPAL3* expression is regulated by developmental cues, such as plant growth regulators, during differentiation to anthocyanin or lignin synthesis (Ozeki et al. 1990; Takeda et al. 1997; Takeda et al. 2002). The *DcPAL1* and *DcPAL3* genes might play different roles in accordance with environmental and developmental cues to provide different final metabolites. It is important to elucidate the different mechanisms that regulate the expression of each *PAL* gene. Here, we focused on the regulatory mechanisms for *DcPAL1* gene expression.

In the proximal promoter region of *DcPAL1*, five box-

Abbreviations: Dc, *Daucasu carota*; EIN3, ethylene insensitive3; EIL, EIN3-Lik; PAL, phenylalanine ammonia-lyase
This article can be found at http://www.jspcmb.jp/

L-like sequences (box-L1–box-L5) have been identified, and box-L1 and box-L5 are proposed to play important roles in the activation of *DcPAL1* expression (Takeda et al. 2002). In our previous research, a transcription regulatory factor for the R2R3-type MYB protein, *D. carota* MYB1 (DcMYB1), binding to the box-L1 and box-L5 sequences was isolated by a yeast one-hybrid system using a cDNA library prepared from carrot suspension-cultured cells (Maeda et al. 2005). The binding specificity of DcMYB1 to box-L1 and box-L5 sequences was shown by an *in vitro* gel retardation assay and an *in vivo* yeast one-hybrid system. Transient expression experiments in carrot protoplasts showed that DcMYB1 binds to box-L1 and box-L5 and activates minimal 35S promoter activity, whereas the repression of *DcMYB1* gene expression by RNA interference in protoplasts could be because of inhibition of the upregulation of *DcPAL1* promoter by elicitor treatment or UV-B irradiation. These results suggest that DcMYB1 might play a key role as a transcription regulatory factor in *DcPAL1* expression (Maeda et al. 2005).

Because *DcMYB1* expression might be regulated at the transcriptional level by elicitor treatment or UV-B irradiation, as shown in our previous report (Maeda et al. 2005), the regulatory mechanism of *DcMYB1* promoter activity by these stimuli is an important next step in these experiments. The nucleotide sequence of the proximal promoter region of *DcMYB1* was isolated and transient expression analysis in carrot protoplasts showed that the region from nucleotides −135 to −105 of the *DcMYB1* promoter might act as a negative *cis*-element to suppress promoter activity (Maeda et al. 2006). A mutant promoter with deletion of this region resulted in high levels of *DcMYB1* expression in carrot protoplasts without environmental stimuli, such as elicitor treatment or UV-B irradiation, and expression levels were not further upregulated when these stimuli were added. It was proposed that some factors that bind to this negative *cis*-element in the native full-length promoter might repress *DcMYB1* expression when environmental stimuli were not added. In this study, we have undertaken to isolate and identify proteins that interact with nucleotides −135 to −105 of the *DcMYB1* promoter and analyze the regulatory mechanism of *DcMYB1* gene expression.

Suspension-cultured cells of carrot, *D. carota* L. cv. Kurodagosun, were grown in a modified Lin and Staba (LS) medium containing 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) at 27°C, and subcultured every 7 days (Ozeki and Komamine 1981). cDNA-encoding proteins that bound to the −135 to −105 sequence of the *DcMYB1* promoter were isolated using a yeast one-hybrid system. A yeast reporter strain was constructed that carried the reporter genes, *His3* and *LacZ*, with minimal promoters. Three repeats of the nucleotide sequence corresponding to the promoter region from −135 to −105, TTTGTATTCAGCTTTTTCATATAACCATTCA, of the *DcMYB1* promoter were inserted in front of the two reporter genes. A cDNA library in the pAD-GAL4-2.1 phagemid vector consisting of 3.6×10^6 independent clones was prepared from carrot suspension-cultured cells treated with the dilution effect (Maeda et al. 2005) and transformed to the reporter yeasts. Twenty-nine positive clones showing both 3-amino-1,2,4-triazole resistance and β-galactosidase activity were isolated. The nucleotide sequences of cDNAs of the 29 positive clones were determined and revealed that 11 of the 29 cDNAs contained the same nucleotide sequence that encoded a protein with high similarity to ethylene-insensitive3 (EIN3) and EIN3-like (EIL) proteins, which was designated *D. carota* EIL (DcEIL). *DcEIL* cDNA consisted of 1,857 bp and encoded a protein of 619 amino acids (Figure 1). The DcEIL amino acid sequence had regions characteristic of the EIN3 family proteins, which include an acidic region, a coiled region, five basic domains, and a proline-rich region from the N terminal to the C terminal. It is known that a-helix basic regions and proline-rich regions in the N terminal are important DNA-binding domains for the EIN3-like transcription regulatory factor in tobacco (TEIL) (Kosugi and Ohashi 2000). The EIN3 homologous in *Arabidopsis thaliana* (At), AtEIL1 and AtEIL2, are also transcriptional factors involved in ethylene signal transduction (Chen et al. 2005). Ethylene is a plant growth regulator and is involved in many aspects of plant growth and development, including seed germination, root hair development, root nodulation, flower senescence, abscission, and fruit ripening (Gou and Ecker 2004). The ethylene signal transduction pathway is also induced in response to environmental stimuli from biotic and abiotic stresses such as wounding (Wang et al. 2002). Similarly, phenylpropanoid metabolism is induced for phytoalexin or lignin synthesis to resist environmental stresses (Ververidis et al. 2007). Our results presented here demonstrated that DcEIL, which belongs to the EIN3 family, might play an important role in regulating *DcMYB1* expression by acting as a transcription regulatory factor in phenylpropanoid metabolism.

It is reported that A(T/C)G(A/T)A(C/T)CT is a candidate consensus nucleotide sequence to be bound by AtEIN3 and TEIL (Solano et al. 1998). Despite the estimated 88% similarity in amino acid sequence between the DNA binding domain of DcEIL and TEIL, we did not find this consensus nucleotide sequence within the region from −135 to −105 of the *DcMYB1* promoter sequence. Consequently, it is possible that DcEIL has a different binding recognition sequence to that previously reported for members of the EIN3 family. From phylogenetic analysis based on amino acid

Figure 1. DcEIL amino acid sequences. The regions conserved in other EIN3 proteins are shown below; predicted DNA binding domains (basic domain (BD)I–BD V) are shown by italic type. Acidic region and asparagine (Asn)-rich region are indicated by gray. A proline-rich region is underlined. Arrows indicate putative DNA-binding domains. Asterisks (*) indicate putative nuclear localization signals.

sequences of the EIN3 family proteins, DcEIL was found to belong to a different clade to that of AtEIN3 and AtEIL1 (Figure 2). However, recent studies have revealed that AtEIL3, which is also a member of the EIN3 family, might play an important role in sulfur transportation in response to sulfur deficiency in *Arabidopsis* rather than the ethylene signaling pathway (Maruyama-Nakashita et al. 2006). In addition, the *Orysa sativa* sulfur limitation1 (OsSLIM1; 1 and OsSLIM1; 2) proteins, which belong to the EIN3 family, might be involved in sulfur transport responses in rice (Maruyama-Nakashita et al. 2006). These EIN3-like proteins might be functionally distinct from other EIN3 family members that mediate ethylene responses by playing a role in regulating responses to sulfur in plants. DcEIL, which was isolated here and found to belong to a different clade in the phylogenetic tree of EIN3 as well as showing different properties to the binding recognition sequence, might have an as yet unidentified role in the regulation of phenylpropanoid metabolism by environmental stimuli.

Although DcEIL might play different roles in different signal transduction pathway(s) compared with other previously reported EIN3 homologues, it has a similar minimal nuclear localization signal (Chelsky et al. 1989). To confirm this localization signal, a green fluorescent protein (GFP)-DcEIL fusion-construct was prepared using the pEGFP-N1 vector (Clontech, Mountain View, CA, USA). First, the GFP sequence was amplified by the forward primer, 5′-ATACTAGTATGGTGAGCAAGGGC-3′, and the reverse primer, 5′-TAACTAGTCTTGTACAGCTCGTCCAT-3′, which was introduced at the *Spe*I site of a pBluescript II SK vector. Then, *DcEIL* cDNA was ligated between *Sal*I and *Bam*HI sites in the translational fusion followed by GFP. This construct was subcloned into the pBI-OX-GW vector, which was driven by the 35S promoter (Inplanta Innovations, Kanagawa, Japan), and transformed into *A. thaliana* T87 suspension-cultured cells via *Agrobacterium tumefaciens* LBA4404. For the control, an unfused GFP-construct driven by the 35S promoter was introduced into T87 suspension-cultured cells in the same way. Nuclei were stained with 1 mM Hoechst 33258. Transformed *A. thaliana* protoplasts were then observed by fluorescence microscopy, which showed that the GFP-DcEIL fusion protein was localized in the nucleus (Figure 3), suggesting that the translated DcEIL protein was imported into the nucleus.

To investigate the relationship between the expression levels of *DcEIL*, *DcMYB1*, and *DcPAL1* genes using real-time RT–PCR, suspension-cultured carrot cells were treated with elicitor or dilution (transferring into distilled water). The experimental conditions of the elicitor treatment and the dilution effect on the cells were the same as in our previous reports (Takeda et al. 1997, 2002). Five days after subculturing, the cells were given the elicitor treatment or the dilution effect and harvested at 0, 0.5, 1, and 3 h after treatment. Total RNA was

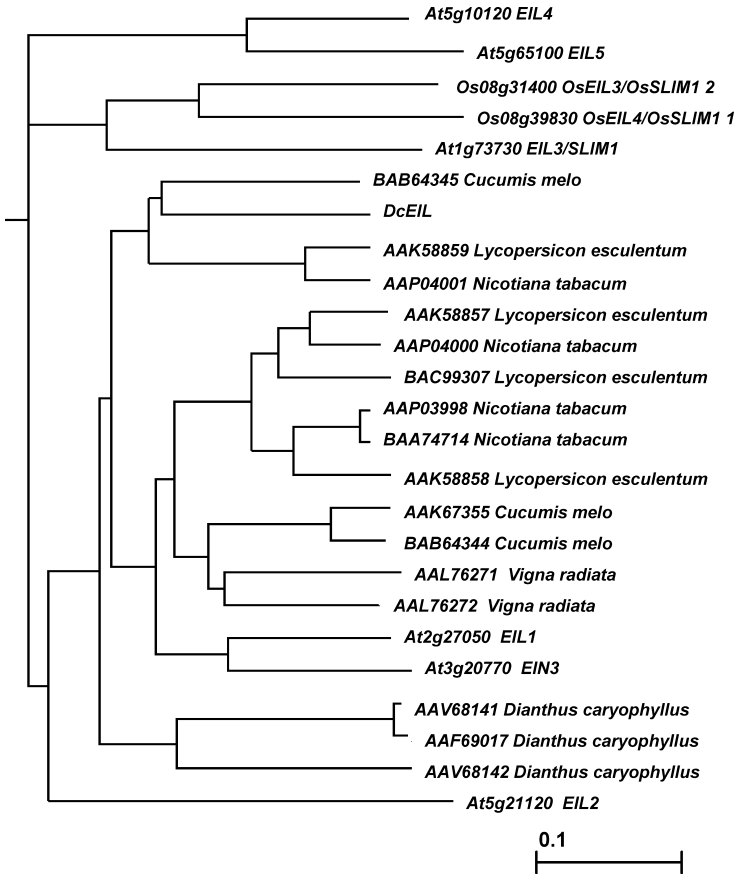

Figure 2. Phylogenetic relationship of amino acid sequences of the EIN3 family. Sequence alignment and the preparation of a phylogenetic tree were performed using the CLUSTALW program at the DNA Data Bank of Japan (DDBJ). Bar=0.1 amino acid substitutions/site.

extracted from suspension-cultured carrot cells using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Total RNA of 1 mg was processed with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and the synthesized cDNA was used as a template for real-time RT–PCR (DNA Engine Opticon 2 system; Bio-Rad Laboratories, Hercules, CA, USA). Reactions were performed using the SYBR Premix Ex Taq II (Takara, Tokyo, Japan). cDNA was amplified using the *DcPAL1* primer for 5′-GAATGTTGTGCTAGGAAACG-3′ and 5′-ACTCAACCTTGACACTCCCT-3′, the *DcMYB1* primer for 5′-CACAGACTCATCAGCCAAGG-3′ and 5′-GCTCCACTGAAAAGATTGCAC-3′, the *DcEIL* primer for 5′-CTGAACCAACCTGTGTACCCA-3′ and 5′-CAGTCCACAGTCAAATGAACCTC-3′, and the carrot *actin* gene as the control normalizing the expression level for 5′-CCTGGTATTGCTGATCGTATGA-3′ and 5′-TCTGTGAACAATTGATGGACCT-3′. *DcPAL1* expression level dramatically increased 3 h after the elicitor treatment and the dilution effect (Figure 4A). Compared with *DcPAL1* expression, *DcMYB1* expression level was slightly higher than that before these treatments, the increase of which was more because of the elicitor treatment than the dilution effect (Figure 4B). Although *DcPAL1* expression level increased about 90 and 70 folds by the elicitor and dilution treatment, respectively, *DcMYB1* expression level did about 1.8 and 1.4 folds. The increment of the latter supposed be insufficient of that of the former. It is thought that, getting the full expression activity of *DcPAL1* gene, expression of some other partner factor(s) was (were) required to interact with DcMYB1 to *DcPAL1* promoter, e.g., maize MYB C1 required R1 as a partner factor (Grotewold et al. 2000). Identification of such factor(s) for DcMYB1 has still remained. However, a change in the level of *DcEIL* expression was not observed before or after these treatments (Figure 4C). AtEIN3 induced the transcription of ethylene-responsive genes in the presence of ethylene and this was also detected in *Arabidopsis* in the absence of ethylene. AtEIN3 is degraded in the ubiquitin–proteasome pathway at the posttranslational level (Guo and Ecker 2004; Potuschak et al. 2003). This fact supports the suggestion that *DcEIL* may be constitutively expressed and controlled at the posttranslational level rather than at the transcription level. Furthermore, the translated DcEIL protein was

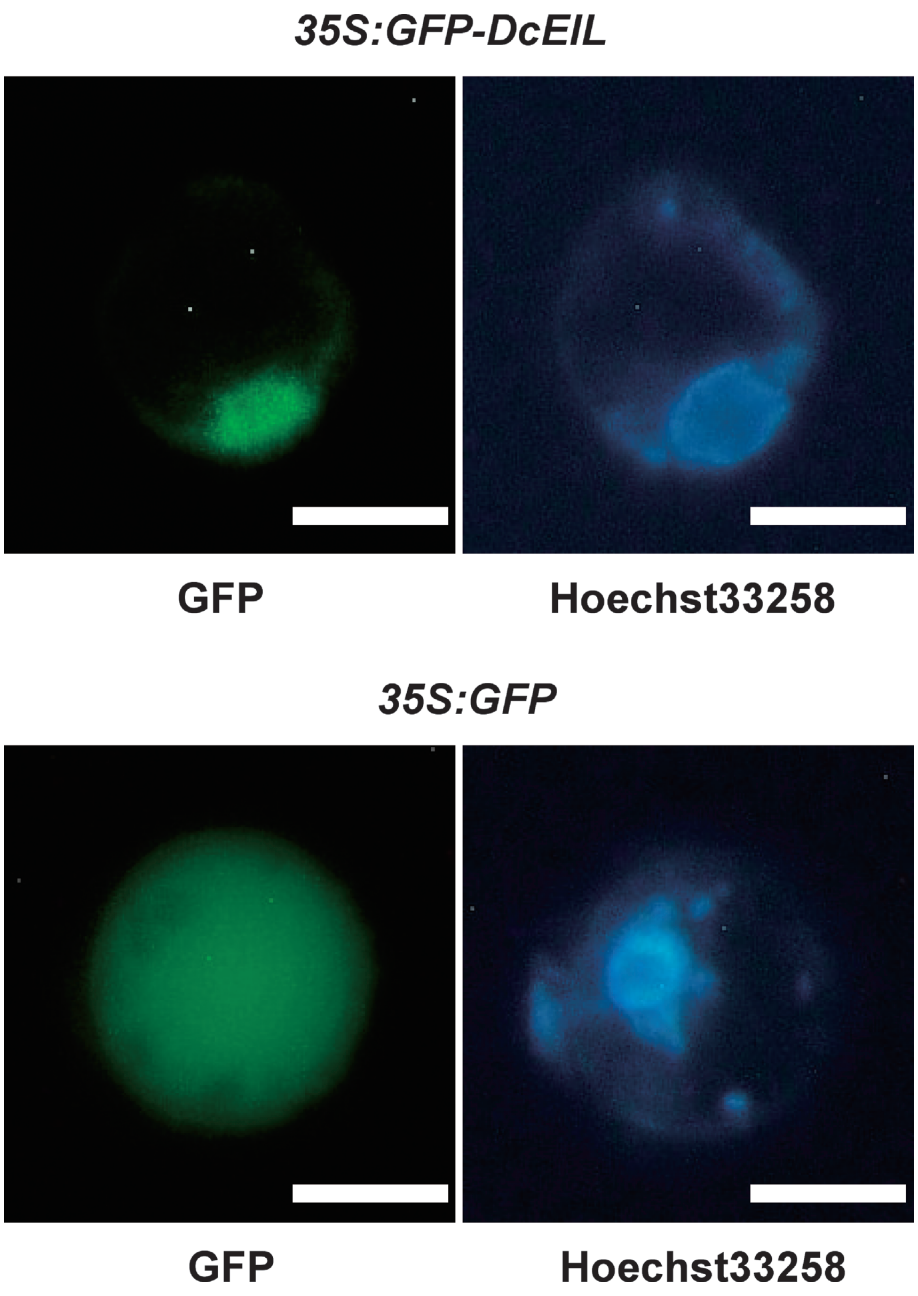

Figure 3. Nuclear localization of the green fluorescent protein (GFP)–DcEIL fusion protein. GFP was fused in frame to the N terminal of the full-length DcEIL (upper panels). The construct was introduced into suspension cultured *Arabidopsis* cells by an *Agrobacterium* method. Expression of the introduced gene was viewed by fluorescence microscopy with 488 nm excitation and 507 nm emission wavelengths. Hoechst 33258 was used to stain nuclei and viewed by fluorescence microscopy with 350 nm excitation and 461 nm emission wavelengths. The lower panels show the expression of GFP without DcEIL as the control. Bar＝20 μm.

localized in the nucleus (Figure 3). We hypothesized that DcEIL is transported to the nucleus and binds to a region of the *DcMYB1* promoter that results in suppression of *DcMYB1* expression. This might result in *DcPAL1* downregulation under non-stressed conditions. When stimulus from the environment, such as elicitor treatment or the dilution effect, has an effect on cells, DcEIL might be degraded in the ubiquitin–proteasome pathway, thus resulting in the upregulation of *DcMYB1* expression, and then DcMYB1 might upregulate *DcPAL1* expression. In future studies, we intend to precisely identify the DcEIL-binding nucleotide sequence in the putative *cis*-element of the *DcMYB1* promoter and prepare anti-DcEIL sera to detect degradation of the DcEIL protein.

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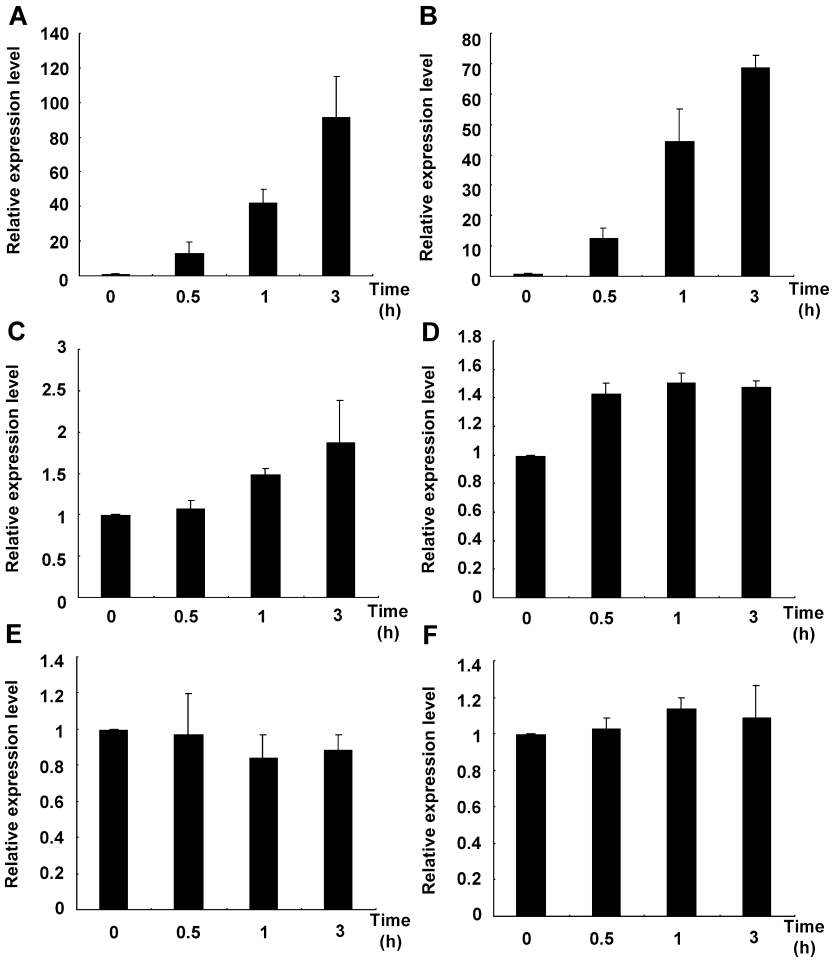

Figure 4. Changes in the level of transcription of *DcPAL1* after the elicitor treatment (A), *DcPAL1* after the dilution effect (B), *DcMYB1* after the elicitor treatment (C), *DcMYB1* after the dilution effect (D), *DcEIL* after the elicitor treatment (E), *DcEIL* after the dilution effect (F). Expression levels were quantified by real-time RT–PCR and normalized using *actin* transcripts as an internal standard. The relative expression level of the transcripts for each gene at 0 h was arbitrarily set at 1 and the values are presented as means±SE (n=3).

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