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Gene Note

A promoter analysis of the *DcMYB1* gene that encodes the transcriptional activator of the stress-inducible *DcPAL1* gene

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Abstract DcMYB1, a carrot MYB protein, acts as a transcriptional activator of the stress-inducible carrot *PAL* gene *DcPAL1*, and is induced by several environmental cues such as UV-B irradiation and elicitor treatment. Here, the *DcMYB1* promoter region was isolated, and its putative *cis*-elements were characterized using several deletion mutants of the *DcMYB1* gene promoter. This was undertaken with transient expression experiments using carrot protoplasts. The results indicated that GCC-box and one MYC binding sequence might participate in the up-regulation of *DcMYB1* promoter activity induced by UV-B irradiation, and that the AG-motif-like and/or Box-P might undertake this by elicitor treatment. MYB and/or another MYC binding site neighboring the TATA-box might take part in the negative regulation of promoter activity, and the W-box near the TATA-box might be necessary for the up-regulation of promoter activity by both UV-B irradiation and elicitor treatment.

Key words: *Cis*-element, MYB, phenylalanine ammonia-lyase, promoter.

Many *MYB*-related genes exist in the plant genome. For example, it is predicted that about 131 *R2R3-MYB* genes are coded in genome sequences of *Arabidopsis thaliana* (Riechmann et al. 2000). Myb-related proteins in plants can be classified into three subgroups based on the number of MYB repeats. In particular, the R2R3-type MYB subgroup, which has R2 and R3 repeats, forms the largest family among the three subgroups (Kranz et al. 1998; Romero et al. 1998; Stracke et al. 2001). R2R3-type MYB proteins have been known to play important roles in various developmental processes, and in the differentiation and regulation of secondary metabolisms (Grotewold et al. 1994; Borevitz et al. 2000; Jin et al. 2000; Sugimoto et al. 2000; Abe et al. 2003). Some plant MYBs regulate the expression of phenylpropanoid biosynthetic genes to resist abiotic stress and pathogen attack (Jin et al. 2000; Sugimoto et al. 2000).

Phenylalanine ammonia-lyase (PAL) acts as the key enzyme regulating the metabolic flow from primary metabolisms to phenylpropanoid metabolism. Genes encoding PAL are regulated by both environmental and developmental cues (Lois et al. 1989; Logemann et al. 1995). Some phenylpropanoid compounds produced by this pathway act as antimicrobial defense products and UV-protective screens in plants (Dixon and Paiva 1995). It has been revealed that expression of the *DcPAL1* gene in suspension-cultured carrot cells is induced by UV-B irradiation, elicitation, and dilution effects. Here, box-L-like sequences in the promoter, (ACC(A/T)(A/T)CC), which are AC-rich elements, play an important role in the regulation of *DcPAL1* expression (Takeda et al. 1997; Takeda et al. 2002).

We identified and characterized DcMYB1, an R2R3-type MYB protein that acts as a positive regulator of the *DcPAL1* gene in response to UV-B irradiation, elicitor treatment, and dilution effects in suspension-cultured carrot cells (Maeda et al. 2005). It was noted that the *DcPAL1* promoter was activated by DcMYB1 binding to plural box-L-like sequences in the *DcPAL1* promoter following UV-B irradiation, elicitation, and dilution effects. *DcMYB1* gene expression was up-regulated by these stimuli at the transcriptional level 30 min to 1 h before the induction of *DcPAL1* gene expression. We showed that repression of *DcMYB1* expression using the double-stranded RNA interference method halted the up-regulation of *DcPAL1* expression from UV-B irradiation or the elicitor treatment. These results indicated that DcMYB1 might act as the most important and crucial transcriptional activator concerning the induction of *DcPAL1* expression in response to stress (Maeda et al. 2005). As such, the regulatory mechanism of *DcMYB1* gene induction should be determined to understand the signaling pathway of the defense mechanisms induced by environmental stresses.

Here, we isolated the *DcMYB1* promoter region and prepared internal deletion mutants of the *DcMYB1*

Abbreviations: *Dc*, *Daucus carota*; PAL, phenylalanine ammonia-lyase
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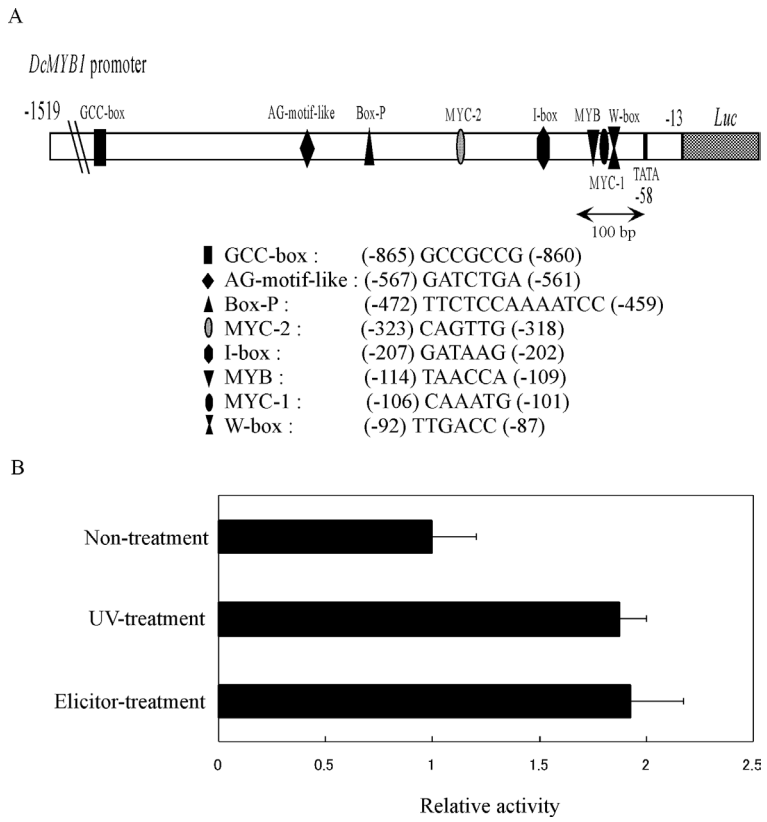

Figure 1. A, candidates of stress-related *cis*-elements and the location of the *DcMYB1* promoter. The putative translation-starting site is numbered +1, and the *luc* reporter gene was fused at −13. Nucleotide sequences and positions of *cis*-element candidates previously reported to play important roles in the expression of genes in response to stress (GCC-box, AG-motif-like, Box-P, I-box, and W-box homologue sequences, as well as the recognition sites of MYB and MYC proteins) are shown with symbols. B, up-regulation of LUC activity in protoplasts transfected with the *DcMYB1* promoter:*luc* construct from UV-B irradiation and the elicitor treatment. To normalize transfection efficiency, LUC activity was divided by Rluc activity. Means (boxes) and standard errors (bars) were derived from three independent measurements of each construct.

promoter conjugated to the firefly luciferase (*luc*) gene. Transient expression experiments were undertaken by transferring these constructs into carrot protoplasts with UV-B irradiation or the elicitor treatment. They indicated that putative *cis*-elements in the promoter region of *DcMYB1* involved with up-regulation by UV-B irradiation might partially differ from those of the elicitor treatment.

DNA fragments of the proximal promoter region of the *DcMYB1* gene were isolated from a carrot genomic library (Ozeki et al. 1993), and subcloned into pBluescriptSK+ to determine the nucleotide sequence (accession number, AB239705). Some putative *cis*-element candidates in other previously reported stress-inducible gene promoter regions were found following surveys of nucleotide sequences using PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE/). These candidates were GCC-box (GCCGCC), Box-P ((C/T)T(T/C)(C/T)(A/C)(A/C)C(C/A)(C/A)C), AG-motif (AGATCCAA), and I- (GATAAG) and W-box (TTGAC) homologues (Figure 1A). The GCC-box is known to play an important role in gene expression in response to ethylene and wounding. Its sequence was recognized by ethylene-responsive element-binding factors (Ohme-Takagi and Shinshi 1995; Fujimoto et al. 2000). Box-P sequences exist in the proximal promoter regions of several defense-related genes such as *PAL* and 4-coumarate: CoAligase (*4CL*) (Lois et al. 1989; Logemann et al. 1995). The AG-motif was found in the *NtMYB2* gene promoter region, and it was recognized by the zinc finger-type transcription factor, AGP1 (Sugimoto et al. 2003). I- and W-boxes are known to play important roles in gene expression in response to light and pathogens or wounding, respectively (Rose et al. 1999; Eulgem et al. 2000; Turck et al. 2004). There were other prominent sequences recognized by MYB and MYC in the *DcMYB1* promoter region that were considered potential cis-elements (Figure 1A).

In order to determine the *cis*-element(s) that might play important roles in the regulation of *DcMYB1* gene expression induced by UV-B irradiation and the elicitor treatment, serial and internal deletion mutants of the *DcMYB1* promoter region were generated by polymerase chain reaction (PCR). This was followed by introduction into p*luc*-SK (Ozeki et al. 1993) to conjugate *luc* cDNA, as a reporter gene, and the *NOS* terminator (as shown on the left sides of Figures 2 and 3). The constructs were

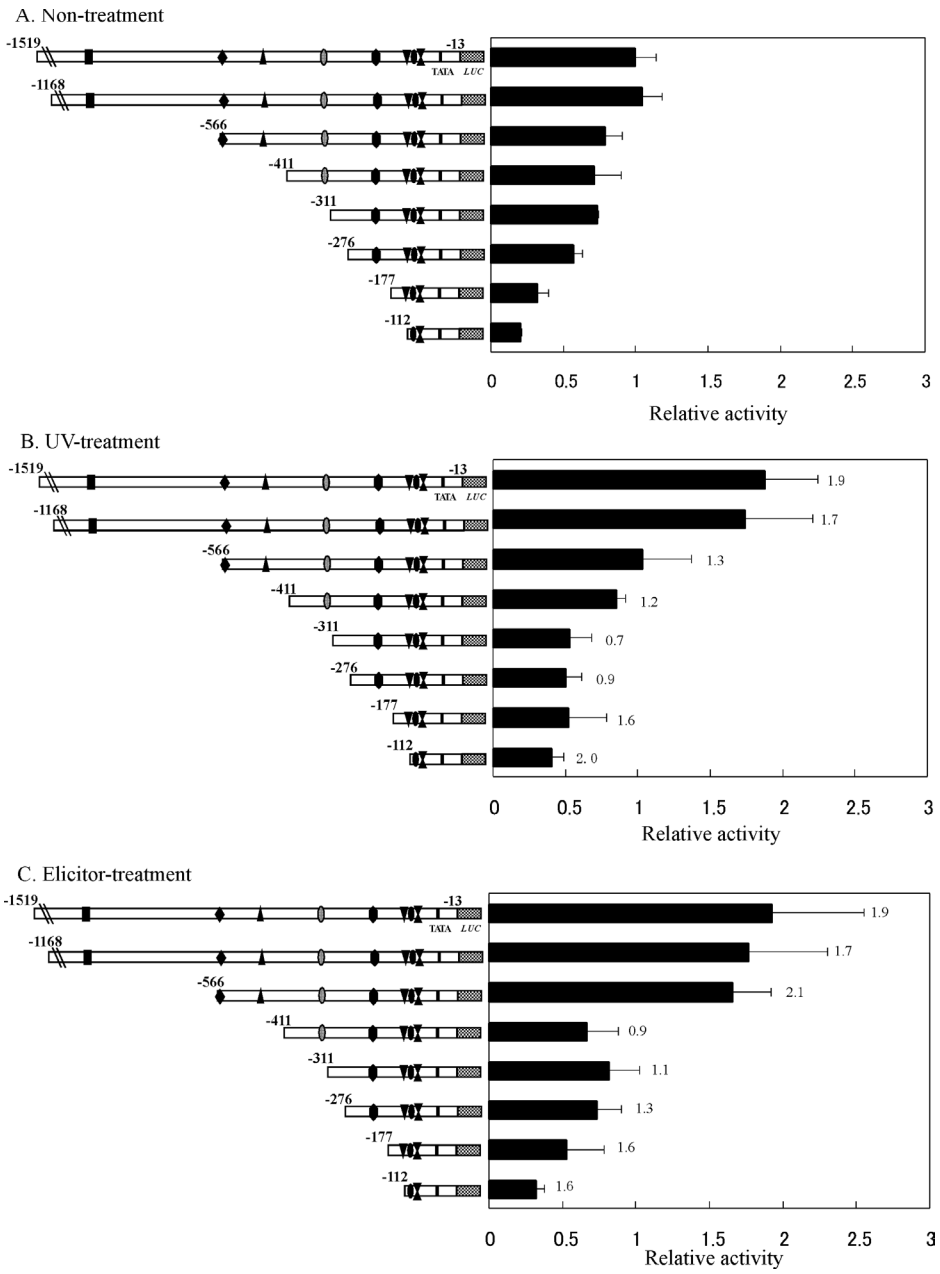

Figure 2. Promoter activity of serially deleted fragments of the *DcMYB1* promoter with UV-B irradiation and the elicitor treatment. 5′ serial deletion mutants of the *DcMYB1* promoter conjugated with the *luc* gene, shown on the schematic diagrams at the left side, and the 35S-*Rluc* construct were co-transfected into cultured carrot cell protoplasts. A, after culturing without any treatment for 26 h, protoplasts were harvested and their LUC and Rluc activities were measured. B, the transfected protoplasts were irradiated with UV-B light. C, the transfected protoplasts were treated with elicitor. Values represent the means and standard errors from three independent experiments for each construct. Numerals on the right of the error bar indicate ratios of LUC activity treated by UV-B (B) or the elicitor (C) versus that of unstressed treatments of each construct.

introduced into protoplasts prepared from suspension-cultured carrot cells using the polyethylene glycol-mediated transfection method (Maeda et al. 2005). As a reference of transfection yield, the *Renilla luciferase* (*Rluc*) gene, which was under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter, was co-transfected with the aforementioned constructs. After culturing transfected protoplasts for 20 h in the dark, treatment with UV-B light or the elicitor was applied to the protoplasts under the same conditions as previously reported (Maeda et al. 2005). The protoplasts were further cultured for 6 h in the dark, and then their LUC activities were measured using the Dual-Luciferase Assay System (Promega K. K. Japan, Tokyo, Japan). For the data in Figures 2 and 3, all transient expression experiments were undertaken using constructs of either the serial deletion series or the internal deletion series from one batch of protoplasts. Treatments with or without either UV-B irradiation or the elicitor treatment were applied to individual batches after culturing. Data

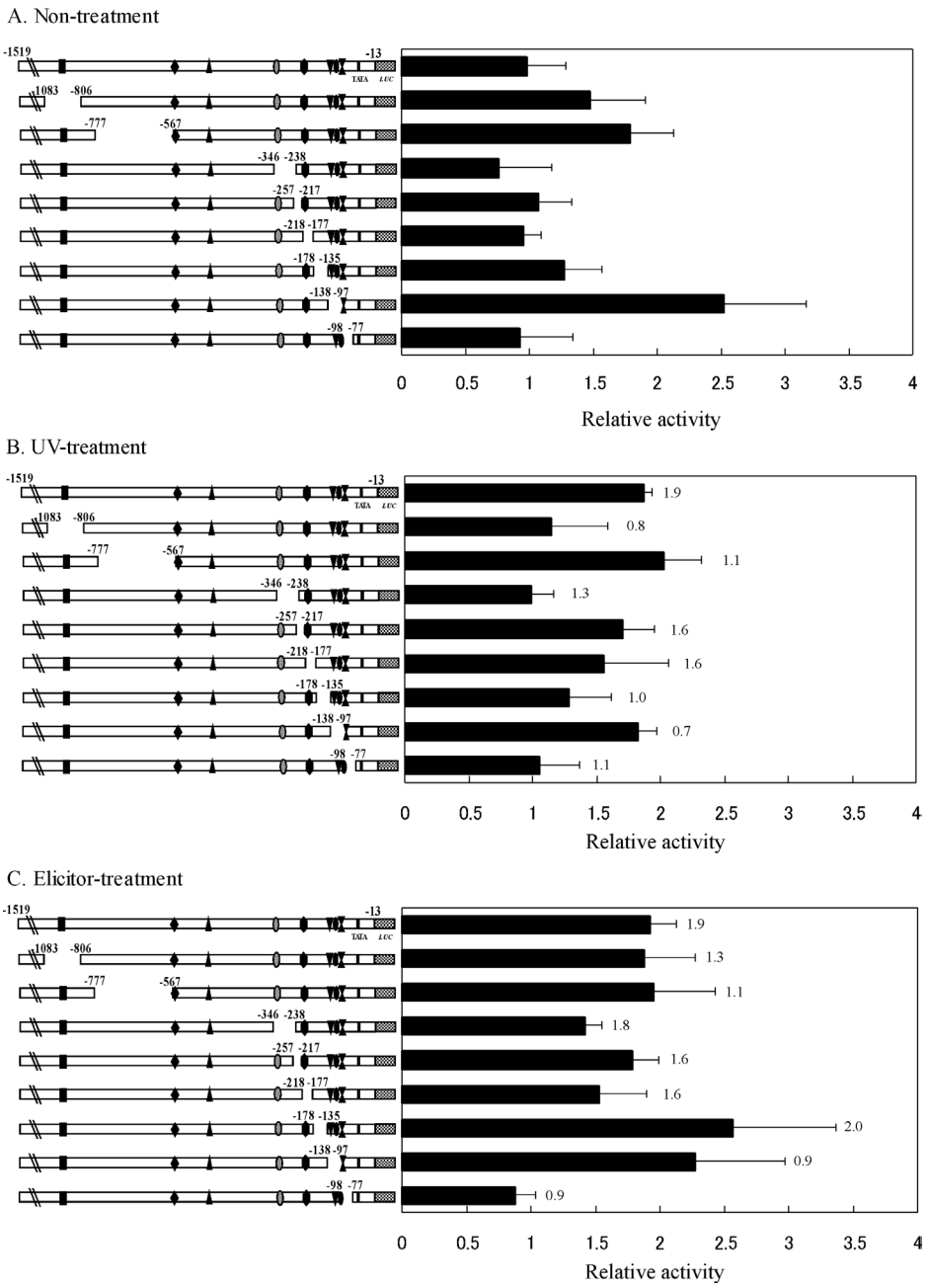

Figure 3. Promoter activity of the internal deletion mutants of the *DcMYB1* promoter with UV-B irradiation and the elicitor treatment. The schematic diagrams of internal deletion constructs are shown on the left side. Internal deletion constructs and 35S-*Rluc* as a reference were co-transfected into cultured carrot cell protoplasts. Experimental treatments and culture conditions are the same as those in Figure 2.

for each individual batch were separately measured, and their relative activities compared with that of the full promoter (−1519).

LUC activity driven by the *DcMYB1* full promoter (−1519 to −13 fragment) was increased by UV-B irradiation and the elicitor treatment to almost double that of the unstressed treatments (Figure 1B). The shorter promoter of the −1168 to −13 fragment displayed almost the same LUC activity as the full promoter, regardless of whether or not stress was applied (Figures 2A, B, and C; second row). This indicated that the region from −1519 to −1169 might not include important regulatory regions. LUC activity decreased gradually along with serial deletions from −1168 to −112 of the *DcMYB1* promoter in unstressed protoplasts (Figure 2A). However, a substantial decrease in LUC activity along with serial deletions was found in the loss of the region from −1168 to −566 with UV-B irradiation compared with the activity of the full promoter. It was also found that the rate of up-regulation for the UV-B treatment versus the control to the −1168 promoter fragment (1.7) decreased to 1.3 in the −566 fragment (Figure 2B; second and third rows). In the case of the elicitor treatment, the decrease along with serial deletions was

found in the loss of the region from −566 to −411, and the rate of LUC activity of the −411 fragment of the elicitor treatment versus the unstressed control decreased to 0.9 (Figure 2C; fourth row). The decrease in up-regulation by the UV-B treatment resulted in the loss of the GCC-box, and that from the elicitor treatment resulted in the loss of both the AG-motif-like and Box-P sequences. The former result was supported by the internal deletion mutant without the region from −1083 to −806 (Figure 3B; second row). Here, LUC activity in the promoter deleted in this region was about 60% that of the full promoter (compare the second row to the top row in Figure 3B), and the rate of LUC activity of this mutant with the elicitor treatment versus the unstressed control decreased to 0.8. Since we were unable to prepare many fine internal deletion DNA fragments from the region from −567 to −346 by PCR, neither the AG-motif-like nor the Box-P internal deleted mutant promoters were available to provide further evidence that focused independently on AG-motif-like and Box-P elements in the regulation of promoter activity by the elicitor treatment. However, significant decreases in LUC activity from the elicitor treatment were observed with the serial deletion series between the constructs of −566 and −411, and the rate of up-regulation for the −566 fragment (2.1) decreased to 0.9 for the −411 fragment when treated by the elicitor (Figure 2C; third and fourth rows). This contrasted with the constructs of the full promoter and the −1168 and −566 regions, which showed minute decreases in LUC activity and about twice (1.9, 1.7, and 2.1, respectively) the activation rate from the elicitor treatment compared to that of the unstressed treatments (Figure 2C; first, second, and third rows). Previous reports indicated that AG-motif and Box-P sequences were important cis-elements of genes responding to wounding and elicitor treatments (Lois et al. 1989; Logemann et al. 1995; Kawaoka et al. 2000; Sugimoto et al. 2003). As such, the results obtained here imply that the region from −566 to −411 of the *DcMYB1* promoter, which includes the AG-motif-like and Box-P sequences, might play an important role in the up-regulation of the *DcMYB1* promoter by the elicitor. A gain-of-function analysis using the constructs harboring several copies of the core sequence of either the AG-motif-like or Box-P sequences with the 35S minimal promoter conjugating the *luc* gene is required to determine the key *cis*-element for the up-regulation of *DcMYB1* expression in response to elicitor treatment.

In further serial deletion series of the promoter region, a substantial decrease in LUC activity from UV-B irradiation was found between the −441 and −311 constructs (Figure 2B; fourth and fifth rows), where the MYC-2 element was included. The up-regulation rate of 1.2 for the −441 fragment for UV-B irradiation decreased to 0.7 for the −311 fragment. Results for the internal deletion between −346 and −238 showed that LUC activity for this mutant promoter treated with UV-B (Figure 3B, fourth row) decreased to about half of that of the full promoter (Figure 3B; top row). Although the internal deletion between −346 and −238 caused a slight decrease in LUC activity in the unstressed treatments (Figure 3A; fourth row) compared with the activity of the full promoter (Figure 3A; top row), the rate of up-regulation by UV-B irradiation for this internal deletion was reduced to 1.3 (Figure 3B; fourth row). This was lower than that of the full promoter (1.9) (Figure 3B; top row). These results support the belief that deletion of the region including the MYC-2 element was required for the up-regulation of *DcMYB1* promoter activity induced by UV-B irradiation. The recognition site of MYC, and also that of other elements such as the MYB recognition site, the ACGT-core sequence, and the TGA- and W-boxes, was found in the promoter region of plant cytochrome P450 genes induced not only by UV-B irradiation, but also by several abiotic and biotic stresses found in the promoter (Narusaka et al. 2004). However, our results from the serial and internal deletions of the *DcMYB1* promoter indicated that regions including the MYC-2 element played an important role in responses to UV-B irradiation. Despite this, deletion of this region, including the MYC-2 element in the internal deletion mutant, showed a slight decrease in LUC activity with the elicitor treatment (Figure 3C; fourth row). Furthermore, deletion of this region had little effect on the rate of up-regulation by the elicitor treatment (1.8; Figure 3C; fourth row) compared with the control without any deletion (1.9; Figure 3C; top row). This suggested that elements other than MYC-2 might be involved in the regulation of *DcMYB1* expression with the elicitor. In the case of experiments using serial deletion mutants, results from the −411 (Figure 2C; fourth row) to −311 (Figure 2C; fifth row) region might not provide informative results on the role of this region in the regulation of *DcMYB1* promoter activity by elicitor treatment. This is because loss of the region from −566 to −441, which includes the AG-motif-like and Box-P sequences, plays an important role in up-regulation by this elicitor.

MYB, MYC-1, and W-box, which are other elements included in the region between −114 and −87, near the TATA-box, may play complex and important roles in the regulation of *DcMYB1* expression from both the UV-B and elicitor treatments. However, further serial deletion series close to the TATA-box, where other *cis*-elements included in the up-stream region of the promoter sequence were deleted, could not provide distinct information to reveal the roles of MYB, MYC-1, and the W-box. As such, effects of these elements on the regulation of *DcMYB1* promoter activity were considered with results of the internal deletion of the elements

described below. In cases where transfected protoplasts were treated without stress, the internal deletion promoter of the region between −138 and −97, which included MYB and MYC-1, showed LUC activity 2.5 times greater than that of the full promoter (compare the second row from the bottom to the top row in Figure 3A). However, a lack of this region did not result in increases in LUC activity from either UV-B irradiation or the elicitor treatment compared with that of the full promoter (compare the second row from the bottom with the top row in Figures 3B and C). Decreases in the rate of up-regulation of LUC activity by UV-B irradiation or the elicitor treatment to below 1.0 were found (Figures 3B and C; second row from the bottom). These results suggested that the region from −138 to −97 of the *DcMYB1* promoter might act as a negative element in repressing the promoter activity of *DcMYB1*. It was reported that some MYB proteins act as a repressor, e.g., AtMYB4, the derepression of which caused up-regulation of the cinnamate 4-hydroxylase gene, resulting in the production and accumulation of sunscreen compounds for UV protection (Jin et al. 2000). Our preliminary data on co-transformation into carrot protoplasts with both the DcMYB1 over-expressing construct driven by the 35S promoter as an effecter and the *DcMYB1* full promoter conjugating the *luc* gene as a reporter did not show up- or down-regulation of *DcMYB1* full promoter activity (data not shown). This indicated that DcMYB1 might not act on this MYB element to regulate its own promoter activity. Other carrot MYB(s) might act on the negative repressor of the *DcMYB1* promoter. When the neighboring region near the TATA-box between −98 and −77 including the W-box was deleted, up-regulation of LUC activity by UV-B irradiation and the elicitor treatment was reduced to about half that of the full promoter (compare the bottom rows to the top rows in Figures 3B and C). However, both the deleted promoter of this region and the full promoter gave almost the same LUC activity without any stress (compare the bottom row to the top row in Figure 3A). Furthermore, the rate of up-regulation by UV-B irradiation and the elicitor treatment for the deletion between −98 and −77 was 1.1 and 0.9, respectively (Figure 3B and C; bottom rows), indicating that this region might be required for the up-regulation of *DcMYB1* promoter activity by UV-B irradiation and the elicitor treatment. In *Arabidopsis*, it was reported that AtWRKY53 and AtWRKY6, which are transcription factors related to senescence and defense responses bound to the W-box, acted as positive and negative transcription regulators in a complex manner (Robatzek and Somssich 2002; Miao et al. 2004). These results suggest that this region, including MYB, MYC-1, and the W-box, should be examined to reveal the regulatory mechanism of *DcMYB1* expression from UV-B irradiation and elicitor treatments. Fine mutant promoters with altered nucleotide sequences of each element are being prepared to identify core sequences. This will be followed by isolation of the transcription factors that act in the signal transduction pathways of UV-B irradiation and elicitor attack.

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