Plant Biotechnology 27, 131–139 (2010)

Original Paper

Characterization of MYB proteins acting as transcriptional regulatory factors for carrot *phenylalanine ammonia-lyase* gene (*DcPAL3*)

Tatsuya Wako, Soichi Kimura, Yukie Chikagawa, Yoshihiro Ozeki*

Department of Biotechnology and Life Science, Faculty of Engineering, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan
* E-mail : ozeky@cc.tuat.ac.jp Tel & Fax : +81-42-388-7239

Received December 4, 2009; accepted January 18, 2010 (Edited by M. Yamazaki)

Abstract In suspension-cultured carrot cells, a *phenylalanine ammonia-lyase* gene, *DcPAL3*, plays an important role in regulation of anthocyanin synthesis. In the *DcPAL3* promoter region, a putative *cis*-element, box-L, which is committed to the upregulation of promoter activity, has been identified. Here, we isolated *DcMYB5* cDNA using yeast one-hybrid screening with box-L as a bait from a cDNA library prepared from cells of a variant cultured cell line constitutively synthesizing anthocyanin. Although expression of *DcMYB2*, *3*, and *4* was observed, all of which were previously isolated by plaque hybridization from a subtracted cDNA library of anthocyanin-synthesizing cells of a normal cultured cell line cultured in medium lacking 2,4-dichlorophenoxyacetic acid, *DcMYB5* expression was not observed in cells of the normal cultured cell line; high expression of *DcMYB5* was observed in cells of the variant cultured cell line. Although not only DcMYB5 but also DcMYB2, 3, and 4 could bind to the box-L sequence in yeast, DcMYB3 and 5 showed strong transcriptional activation activity for *DcPAL3* promoter in carrot protoplasts. These results suggest that DcMYB3 and 5 might play an important role in the upregulation of *DcPAL3* promoter activity in the different regulatory paths between the normal and variant cultured cell lines.

Key words: Anthocyanin, carrot (*Daucus carota*), 2,4-dichlorophenoxyacetic acid, MYB, phenylalanine ammonia-lyase.

The phenylpropanoid pathway is one of the fundamental secondary plant metabolic pathways producing a wide variety of metabolites having a C6–C3 skeleton, such as lignin, anthocyanins, flavonoids, and phytoalexins, some of which play important roles in resistance to environmental stresses such as pathogen infection, ultraviolet (UV) irradiation, and injury (Dixon and Paiva 1995; Dixon 2001; Hahlbrock and Scheel 1989). In this pathway, phenylalanine ammonia-lyase (PAL) catalyzes the conversion of phenylalanine to *t*-cinnamate, which is the first step in and acts as the key enzyme in regulating metabolic flow from primary metabolism to phenylpropanoid metabolism. It has been revealed that *PAL* genes comprise a small gene family in many plant species (Lois et al. 1989; Ohl et al. 1990; Ozeki et al. 1990) and *PAL* gene expression is regulated by environmental and developmental cues (Bevan et al. 1989; Liang et al. 1989; Shufflebottom et al. 1993). In previous research on *PAL* genes that were analyzed in many plant species, the common *cis*-elements in the promoter regions were reported as box-P ($CCA^{A}/_{C}C^{A}/_{T}AAC^{C}/_{T}CC$), box-A ($^{C}/_{A}$ $CGTC^{T}/_{C}$) and box-L ($^{T}/_{A}CT^{C}/_{A}ACCTA^{C}/_{A}{}^{C}/_{A}$). It is known that these elements are essential to activate the gene expression in responses to environmental and developmental cues (Lois et al. 1989; Logemann et al. 1995).

We established a system in which anthocyanin synthesis can be induced under defined conditions using suspension-cultured carrot cells (Ozeki and Komamine 1981; designated as "the normal cultured cell line"). The suspension-cultured carrot cells grew and were subcultured under dedifferentiating conditions in medium containing 2,4-dichlorophenoxyacetic acid (2,4-D). When the cells were transferred to and cultured in a medium lacking 2,4-D, anthocyanin synthesis was induced (Ozeki and Komamine 1981). In these cells, two *PAL* genes, *DcPAL1* and *DcPAL3*, are regulated by different cues. The gene expression of *DcPAL1* is rapidly

Abbreviations: 3AT, 3-amino-1,2,4-triazole; bHLH, basic helix-loop-helix; CaMV, cauliflower mosaic virus; CHS, chalcone synthase; 2,4-D, 2,4-dichlorophenoxyacetic acid; DBD, DNA binding domain; ERF, ethylene-responsive element binding factor; GA_3, gibberellic acid A3; Luc, firefly luciferase; PAL, phenylalanine ammonia-lyase; UV, ultraviolet; VP16, viral protein 16.

The nucleotide sequence reported in this paper have been submitted to DDBJ under accession numbers, AB298505 (*DcMYB2*), AB298506 (*DcMYB3*), AB298508 (*DcMYB4*) and AB298509 (*DcMYB5*).
This article can be found at http://www.jspcmb.jp/

and transiently induced by stresses, such as osmotic shock, UV irradiation, and elicitor treatment (Ozeki et al. 1990; Takeda et al. 2002) and that of *DcPAL3* is slowly induced concomitant with that of the chalcone synthase gene (*DcCHS*) during anthocyanin synthesis, which was regulated by 2,4-D (Ozeki et al. 1990; Ozeki et al. 2000). Analysis of the nucleotide sequence of the proximal promoter region of *DcPAL3* revealed the existence of several putative *cis*-elements, G-box (CCACGTC), GCC-box (ATGAACGGCGG), box-P (TTCCAACAAACCCC), box-A (CCGTCC), box-P′ (AACCCAACCCACCC), and box-L (CCCCACCTACC) (Ozeki et al. 2000). Transient expression experiments using deletion mutants of the *DcPAL3* promoter connected with the firefly *luciferase* gene (*Luc*) as a reporter gene suggested that GCC-box and box-L were essential to confer full promoter activity (Ozeki et al. 2003).

The sequences of box-L are similar to the binding site, ACCTACCA, of *MYB* homologous genes, *C1* (Sainz et al. 1997), *P* (Grotewold et al. 1994), *AtMYB6* and *7* (Li and Parish 1995), suggesting that MYB homologs may play an important role in the expression of *DcPAL3* promoter activity. In our previous study, four cDNAs for *MYB* homologous genes, *DcMYB8*, *10*, *12* and *14* cDNAs, were obtained from a subtracted carrot-cDNA library by plaque hybridization using cDNA fragments corresponding to *Arabidopsis thaliana* MYB DNA binding domain as probes (Ozeki et al. 2000). However, the characterization of the expression profiles of these transcripts, the binding activity of these MYB proteins to the box-L sequence, and their *trans*-activation activity on the *DcPAL3* promoter remain to be characterized.

We established another anthocyanin-synthesizing cultured cell line with undifferentiated growth in the medium containing 2,4-D using a cell aggregate cloning method (Itoh and Ozeki 2002; here, designated as "the variant cultured cell line"). Our previous study of the GCC-box of the *DcPAL3* promoter, which is, besides box-L, the other important *cis*-element in the activation of the *DcPAL3* promoter, indicated that two proteins belonging to the ethylene-responsive element binding factor (ERF) family, DcERF1 and DcERF2, were isolated from two independent cDNA libraries prepared from anthocyanin-synthesizing cells of the normal and variant cultured cell lines, respectively, by yeast one-hybrid screening using the GCC-box sequence as a bait (Kimura et al. 2008). No *DcERF1* cDNA was isolated from the cDNA library for the variant cultured cell line and no *DcERF2* was from that for the normal cultured cell line, indicating that each of them was differentially expressed in each cell lines. Different properties in *trans*-activation activity of DcERF1 and DcERF2 were observed in transient expression experiments using carrot protoplasts. These findings suggested that DcERF1 and DcERF2 might function in different ways to the upregulation of *DcPAL3* promoter activity in the normal and variant cultured cell lines. This suggests the hypothesis that MYB homolog(s) in cells of the variant cultured cell line, distinct from those in cells of the normal cultured cell line identified as described above, might play an important role in the activation of *DcPAL3* expression by acting on box-L.

We showed here that *DcMYB5* cDNA could be isolated from a cDNA library prepared from the cells of the variant cultured cell line using yeast one-hybrid screening. The expression profiles of the transcripts and the properties of their binding activity to the box-L sequence and *trans*-activation activity for the *DcPAL3* promoter in a transient expression system for formerly isolated *DcMYB*s together with *DcMYB5* were studied. In our previous paper, we isolated *DcMYB8*, *10*, *12* and *14* cDNAs (Ozeki et al. 2000). *DcMYB8* and *14* were renamed here as *DcMYB2* and *4*, respectively. The nucleotide sequence of *DcMYB10* cDNA had 98% identity to *DcMYB12*, including the 5′ and 3′ noncoding regions. Three amino acid sequences differed between them. Here, *DcMYB10* cDNA was designated as *DcMYB3*, but *DcMYB12* was not assessed.

Materials and methods

Plant materials

Suspension-cultured carrot cells (*Daucus carota* cv. Kurodagosun) of the normal cultured cell line were subcultured in a modified Lin and Staba medium containing 0.5×10^{-7} M 2,4-D as previously described (Ozeki and Komamine 1981; Kimura et al. 2008). When the subcultured cells from the normal cultured cell line were transferred to and cultured in the modified Lin and Staba medium lacking 2,4-D, induction of anthocyanin synthesis began 4–5 days after the transfer and the maximum synthesis and accumulation of anthocyanin occurred 6–7 days after the transfer. The variant cultured cell line, in which the cells continuously synthesized anthocyanin in a dedifferentiated condition grown in the medium containing 2,4-D, was established as reported previously (Itoh and Ozeki 2002) and subcultured at 14-day intervals. The cells were harvested on the days after culturing noted in the legend to Figure 3, immediately frozen in liquid nitrogen, and stored at −80°C until RNA preparation.

Yeast one-hybrid screening for cDNA clones encoding the protein binding to the box-L sequence and characterization of binding nucleotide sequences of the protein in yeast

Two oligonucleotides, the upper strand sequence, 5′–AATTGAAATCATAATCCCCACCTACCAAAGAAATCATAATCCCCACCTACCAAAGAAATCATAATCCCCACCTACCAAA–3′ (the box-L core sequence is underlined), and its complement sequence, 5′–TTTGGTAGGTGGGGATTATGATTTCTTTGGTAGGTGGGGATTATGATTTCTTTGGTAGGTGGGGATTATGATTTC–3′, which consisted of three repeats of

the −120 to −96 sequence of the *DcPAL3* promoter, were annealed and introduced between the *Eco*RI and *Sma*I sites of pLacZi and pHISi1 (Matchmaker One-Hybrid System; Clontech, Tokyo, Japan). They were then recombined into the genome of yeast strain YM4271 to obtain a yeast reporter strain having dual reporter genes, *LacZ* and *His3*. The phagemid plasmids containing cDNAs prepared from an activation domain-tagged cDNA library (ca. 3.0×10^6 independent clones) of the cells of the variant cultured cell line (Kimura et al. 2008) were transformed to the reporter yeast, which was plated on medium containing 3-amino-1,2,4-triazole (3AT) as a selection marker. The 3AT-resistant yeasts were assessed in a filter assay to detect β-galactosidase activity to confirm that the yeast clones isolated in this manner showed both 3AT resistance and β-galactosidase activity.

For the binding sequence analysis of DcMYBs in yeast, the yeast strain harboring wild and mutated box-L sequences in front of the *LacZ* reporter gene were prepared in a similar manner to that described above, using oligonucleotides containing the sequence of ACggACC (designated to "Lm" as follows) for mutated box-L sequence instead of that of ACCTACC for wild box-L. The *DcMYB* cDNAs were introduced into pGAD425, which was constructed from pGAD424 by replacing the *ADH1* promoter with a *GAL1* promoter derived from pYES2, and were then used as effector plasmids in the reporter yeasts (see Figure 3). The β-galactosidase activity in the extract of the reporter yeasts harboring the effector plasmid was measured using *o*-nitrophenyl-β-D-galactopyranoside as a substrate, according to the manufacturer's protocol.

Northern blot analysis

Preparation of poly(A)$^+$ RNAs from the carrot cells and agarose gel electrophoresis under denaturing conditions, followed by northern blotting and hybridization using ^{32}P-labeled DNA probes were conducted as previously described (Ozeki et al. 1990). An exception was hybridization at 60°C and washing once with 2×SSC and 0.5% SDS followed by 0.1×SSC and 0.1% SDS once at 68°C. To confirm that an equal amount of RNA was loaded into each lane on the agarose gel, the cDNA fragment for B4 (Itoh and Ozeki 2002), which is constitutively expressed in all cells independent of cultured cell line and irrespective of presence or absence of plant growth regulators and culturing period, was used as a probe for hybridization.

Construction of plasmids and transient-expression assay in carrot protoplasts

The reporter construct of the DcPAL3-LUC was *Luc* driven by the *DcPAL3* full promoter (−369 to +1) as previously constructed (Kimura et al. 2008). The reporter construct, designated as 3× box-L-LUC, consisted of three repeats of the −120 to −96 sequences of the *DcPAL3* promoter including the box-L sequence followed by the cauliflower mosaic virus (CaMV) 35S minimum promoter from −45 to +5 (35S-mini) into p*luc*-SK, which contained *Luc* cDNA as a reporter gene and the *NOS* terminator (Ozeki et al. 1993). As the effector constructs, the 35S promoter followed by *DcMYB2*, *3*, *4*, and *5* cDNAs (designated to 35S-DcMYBs as follows) were prepared using pBI221 by replacing the *GUS* gene with each *DcMYB* cDNA. The GAL4-responsive reporter plasmid (GAL4-LUC) containing five copies of binding sites for GAL4 was constructed using the 5×GAL DBS-Luc reporter plasmid (Ohta et al. 2000). To prepare the effector constructs of GAL4-DcMYBs, the open reading frame regions of *DcMYB* cDNAs were amplified by PCR using appropriate primers adding *Bam*HI and *Kpn*I sites at each end using *DcMYB* cDNAs as templates; the amplified fragments were inserted, in frame, downstream of the coding sequence of GAL4 DNA binding domain (GAL4DBD) of *Bam*HI and *Kpn*I sites of 35S-GAL4DBD (Ohta et al. 2000). For the effector constructs of DcMYBs-VP16, the activation domain of viral protein 16 (VP16) was amplified by PCR using appropriate primers with 35S-GAL4DBD-VP16 as a template (Fujimoto et al. 2000) and the amplified fragment was inserted, in frame, downstream of the coding sequence of the *DcMYB*s of *Bam*HI and *Eco*RI sites of 35S-DcMYBs. Isolation of carrot protoplasts from cells of the normal cultured cell line, DNA transfection using a polyethylene glycol method, and measurement of the enzyme (LUC) activity derived from the *Luc* gene after protoplast culturing were performed as described previously (Kimura et al. 2008).

Results and discussion

Isolation of DcMYB5 cDNAs and phylogenetic analysis of DcMYB2, 3, 4, and 5

Previously, we isolated cDNAs encoding R2R3 MYB proteins, DcMYB2, 3, and 4, from a subtracted cDNA library prepared from anthocyanin-synthesizing cells of the normal cultured cell line using plaque hybridization screening with the nucleotide sequences of the *Arabidopsis* R2R3 DNA binding region as probes; however, we failed to isolate any cDNAs for MYB homologous sequences from another subtracted cDNA library prepared from anthocyanin-synthesizing cells of the variant cultured cell line (Ozeki et al. 2000; Ozeki et al. unpublished data). Here, we attempted to screen the library of the variant cultured cell line using another method, the yeast one-hybrid system, to isolate cDNAs encoding the proteins that bind to the box-L sequence of the *DcPAL3* promoter. cDNAs of the library in the pAD-GAL4-2.1 phagemid plasmids consisting of 3.4×10^6 independent clones were transformed into the reporter yeast carrying the reporter genes, *His3* and *LacZ*; 33 positive clones that showed both 3AT resistance and β-galactosidase activity were obtained. The nucleotide sequences of 14 cDNAs contained in the positive clones were identical to each other, having high similarity to R2R3 type MYB protein (designated as DcMYB5). The deduced amino acid sequence of DcMYB5 had the greatest similarity to that of DcMYB3 and to that of GhMYB36 from upland cotton (*Gossypium hirsutum*) as deposited in the databases (Figure 1).

It is known that plants contain a large number of *MYB* genes with diverse structures, and the individual genes

play important roles in different regulatory aspects of gene expression in cellular morphogenesis, secondary metabolism, response to environmental stresses and plant growth regulators (Jiang et al. 2004; Stracke et al. 2001). Jiang et al. (2004) categorized MYBs into 42 subgroups and seven singletons based on similarities in the encoded open reading frame and intron–exon structures. They clustered 19 of 42 subgroups as "G" subgroups, in which at least one consensus motif was found in the C-terminal region of each member, but 23 as "N" subgroups without remarkable motifs were identified. DcMYB5 and DcMYB3 belonged to the N8 subgroup without remarkable motifs in their C-terminal region, close to *Zea mays* C1 and Pl playing regulatory factors in anthocyanin synthesis and *A. thaliana* TT2 participating in proanthocyanidin and anthocyanin synthesis (Shirley et al. 1995) (Figure 1). In other MYBs isolated previously from anthocyanin-synthesizing cells of the normal cultured cell lines, DcMYB2 became close to *A. thaliana* MYB16 (Mixta), MYB106 and *Petunia hybrida* MYBPh3, but the conserved motif that was found in the C-terminal domain of MYB16, MYB106 and MYBPh3 was not found in DcMYB2 together with *A. thaliana* MYB17 (Jiang et al. 2004; Zhang et al. 2009) (Figure 1). DcMYB4 was closed to *A. thaliana* MYB37 and MYB68 and tomato (*Lycopersicon esculentum*) LeBlind (Schmitz et al. 2002) of subgroup N4 without any conserved motif in the C-terminal domain (Figure 1). As functional conservation of MYBs clustered in the same subgroup was observed in several cases (Jiang et al. 2004), our phylogenetic analysis implied that DcMYB3 and 5 belonging to N8, some members of which have been revealed to function in the regulation of anthocyanin synthesis, might be expected to play an important role in the regulation of *DcPAL3* expression in carrot cells.

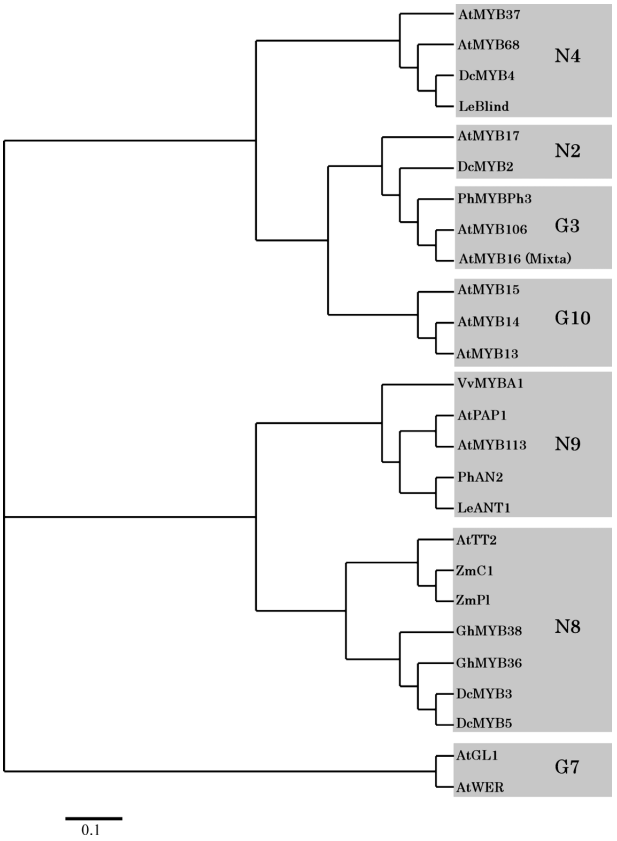

Figure 1. A molecular phylogenetic tree of DcMYBs and other representative R2R3 MYB proteins based on amino acid sequences. Origin of the various proteins is indicated by two-letter prefix: At is *Arabidopsis thaliana*, Zm is maize *(Zea mays*), Ph is petunia (*Petunia hybrida*), Vv is grape (*Vitis vinifera*), Le is tomato (*Lycopersicon esculentum*), Gh is cotton (*Gossypium hirsutum*), Dc is carrot (*Daucus carota*). The gray boxes indicate representatives of subgroup of R2R3 MYB proteins defined previously according to Jiang et al. (2004). The scale bar under the tree represents 0.1 substitutions.

Expression profiles of the DcMYB genes

The expression patterns of *DcMYBs*, *DcPAL3*, and *DcCHS* genes in suspension-cultured carrot cells of the normal and variant cultured cell lines were investigated by a northern hybridization analysis. In the cells of the normal cultured cell line cultured in the medium containing 2,4-D in which anthocyanin synthesis was completely repressed, but dedifferentiated growth occurred (Ozeki and Komamine 1981), the expression of *DcPAL* and *DcCHS* genes were repressed and that of all *DcMYB* genes was under the level of detection (Figure 2, lane 1). When the cells of the normal cultured cell line were transferred to and cultured in the medium lacking 2,4-D, anthocyanin synthesis was induced 4–5 days after the transfer, when cell division was ceased but cell elongation was started (Ozeki 1996). The highest rate of the synthesis and accumulation of anthocyanin in the cells was observed 6–7 days after the transfer (Ozeki and Komamine 1981). The high level of the expression of *DcPAL3* and *DcCHS* genes, together with *DcMYB2*, *3* and *4*, but not *5*, was observed 7 days after the transfer (Figure 2, lane 2). When 2,4-D (Figure 2, lane 3) or gibberellic acid A3 (GA_3) (Figure 2, lane 4) was added to the medium in which the anthocyanin-synthesizing cells of the normal cultured cell line were growing at day 6, anthocyanin synthesis was completely suppressed (Ozeki and Komamime 1981, 1986). In the case of the addition of 2,4-D (Figure 2, lane 3), the expression of all *DcPAL3*, *DcCHS, DcMYB2*, *3* and *4* genes was strongly repressed. On the other hand, GA_3 strongly suppressed *DcCHS* gene expression, but had almost no effect on the expression of *DcPAL3* and *DcMYB3*, whereas *DcMYB2* and *4* gene expression was upregulated (Figure 2, lane 4). These expression profiles suggested that DcMYB2, 3 and 4, but not 5, might play important roles in the regulation of *DcPAL3* gene expression regulated by 2,4-D. On the other hand, in the anthocyanin-synthesizing cells of the variant cultured cell line grown in the

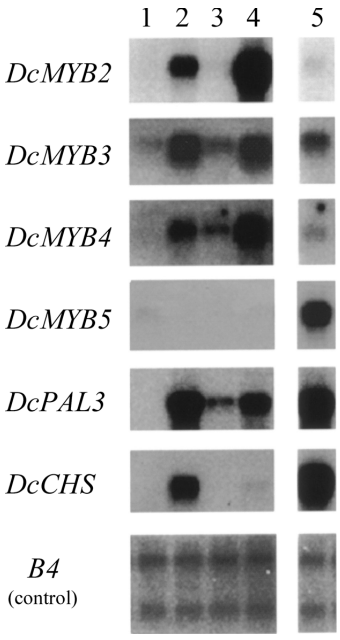

Figure 2. The expression patterns of *DcMYBs*, *DcPAL3*, and *DcCHS* genes in carrot suspension-cultured cells of the normal and variant cultured cell lines. Northern blot analysis was performed with poly(A)$^+$ RNA prepared from the cells of the normal cultured cell line cultured for 5 days in the medium containing 2,4-D under the undifferentiated condition without anthocyanin synthesis (lane 1), that from the anthocyanin-synthesizing cells of the normal cultured cell line cultured for 7 days in the medium lacking 2,4-D under the differentiated condition (lane 2). 2,4-D (final concentration at 5×10^{-7} M) (lane 3) or GA_3 (final concentration at 10^{-6} M) (lane 4) was added to the medium in which the anthocyanin-synthesizing cells of the normal cultured cell line were cultured for 6 days in the medium lacking 2,4-D and the cells were further cultured one more day followed by RNA preparation. RNA was prepared from the cells of the variant cell line (lane 5) in which anthocyanin was constitutively synthesized in the medium containing 2,4-D cultured for 7 days.

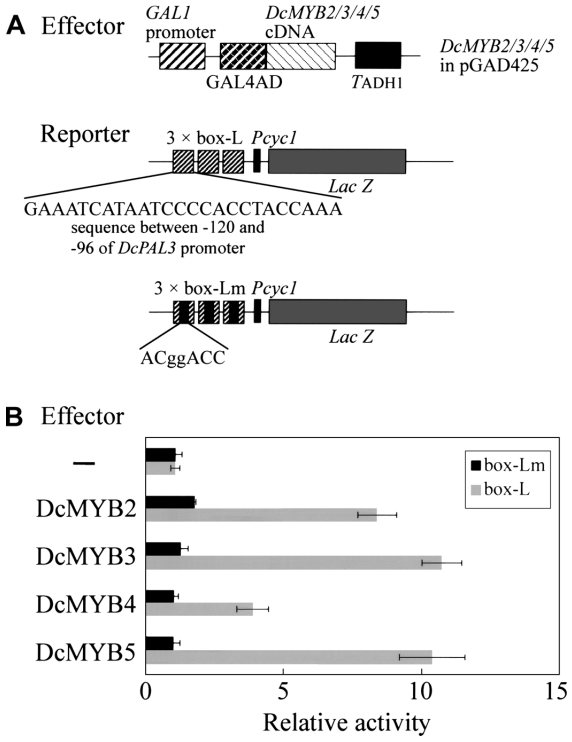

Figure 3. DNA-binding activities of DcMYBs in yeasts. (A) Effector and reporter constructs used in these experiments. The cDNA fragments of each *DcMYB* were inserted into pGAD425 for the effector. The reporter constructs contained three repeats of the box-L sequence (the core sequence was ACCTACC) of *DcPAL3* promoter and mutated box-L (box-Lm; ACggACC) which contained *LacZ* as a reporter gene, and were recombined into the genome of yeast. (B) DNA-binding activities of DcMYBs analyzed by β-galactosidase activity. The values shown are averages of results from three independent experiments. Error bars are standard deviations. All activities are expressed relative to that in the reporter yeasts without the effectors.

medium containing 2,4-D (Figure 2, lane 5), a high level of the expression of the *DcMYB5* gene was observed concomitant with that of *DcMYB3* and *DcPAL3* genes, but not with that of *DcMYB2* and *4*. These results suggest that DcMYB3 and DcMYB5 may possibly play important roles in the expression of *DcPAL3* genes in the cells of the variant cultured cell line, but that in cells of the normal cultured cell line DcMYB5 may not.

Binding activity of DcMYBs to the box-L sequence in yeast

To determine whether DcMYB2, 3, and 4 isolated by plaque hybridization had binding activity to the box-L sequence in yeast, similar to DcMYB5 isolated by the yeast one-hybrid system, yeast reporter strains were prepared harboring the reporter *LacZ* gene fused to three repeats of the box-L sequence (−120 to −96 sequences of the *DcPAL3* promoter; the core sequence was ACCTACC) and those of the mutated box-L sequence, Lm (ACggACC), at the front of the minimal promoter of the yeast *cytochrome C1* gene (P_{CYC1}) (Figure 3A). DcMYBs were expressed in these yeasts as a translational fusion with the GAL4 activation domain (GAL4DBD) (Figure 3A). The expression of LacZ activity was observed by cotransduction of all DcMYB constructs in the yeast harboring box-L, but not in that harboring the mutated box-Lm (Figure 3B). Although the binding activity of DcMYB4 was weaker than in others, these results suggested that all of DcMYB2, 3, 4, and 5 could specifically bind to the box-L (ACCTACC) sequence in yeast.

Differential activation of the expression of the DcPAL3 promoter by DcMYBs in carrot protoplasts

The transcriptional activation activity of DcMYBs to the expression of the *DcPAL3* gene was demonstrated by the transient expression system using carrot protoplasts. The reporter plasmid was constructed with full promoters of *DcPAL3* (−369 to +1) fused to luciferase (*Luc*) reporter genes and the effector plasmids were constructed with each *DcMYB* cDNA driven by the 35S promoter (Figure

4A). Carrot protoplasts were cotransfected with the DcPAL3-LUC and each 35S-DcMYB. LUC activity increased by cotransfection with an effector plasmid harboring DcMYB3 and DcMYB5 cDNA, but did not by that of either DcMYB2 or DcMYB4 (Figure 4B). Similar upregulation by DcMYB3 and DcMYB5, but not by either DcMYB2 or DcMYB4, was observed in the other reporter construct harboring three repeats of the −120 to −96 sequences of the *DcPAL3* promoter including box-L fused to the 35S minimum promoter (−45 to +5),

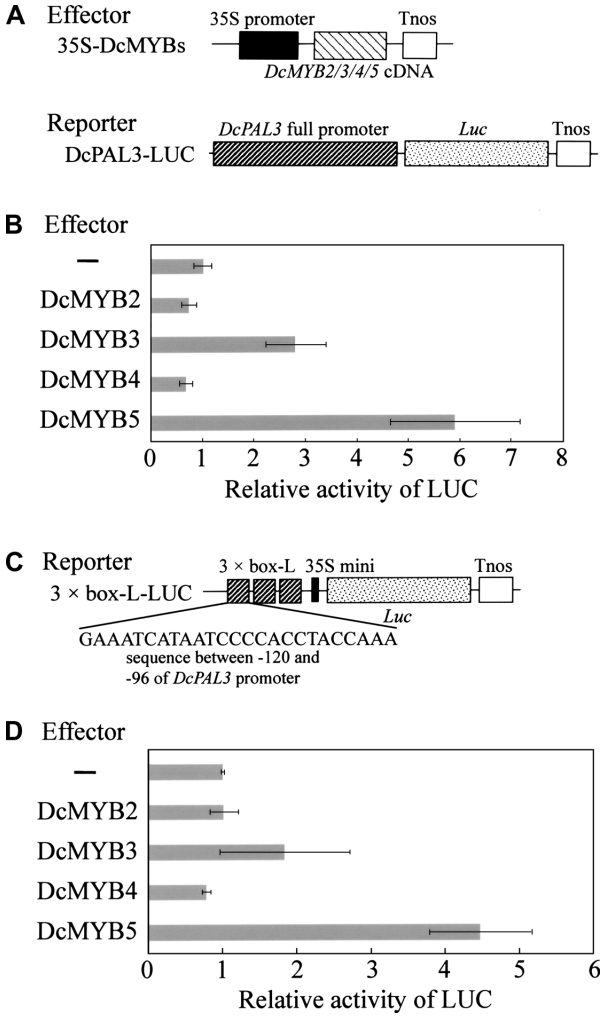

Figure 4. Differential activation of DcMYBs to *DcPAL3* promoter. (A) Constructs of effector and reporter plasmids. The effector constructs 35S-DcMYBs were *DcMYB* cDNAs driven by the 35S promoter, and the reporter construct DcPAL3-LUC was *Luc* gene driven by the *DcPAL3* full promoter (−369 to +1; Kimura et al. 2008). (B) Relative LUC activity in carrot protoplasts after cotransfection with the effector plasmids 35S-DcMYB and the reporter plasmid DcPAL3-LUC. (C) Structure of the reporter plasmid contained three repeats of box-L sequences of *DcPAL3* promoter. (D) Relative LUC activity in carrot protoplasts after cotransfection with effector plasmids 35S-DcMYB and the reporter plasmid containing box-L sequences shown in C. The values shown in B and D are averages of results from three independent experiments. Error bars are standard deviations. All activities in the protoplasts are expressed relative to that the reporter plasmid alone was transfected.

followed by the *Luc* reporter gene (Figure 4C, D). These results suggested that in carrot protoplasts either DcMYB3 or DcMYB5 alone could act as a *trans*-activator of the *DcPAL3* promoter mediated with box-L, but neither DcMYB2 nor DcMYB4 could.

It was thought that the properties of *trans*-activation activity by DcMYBs to the promoter should depend on two parameters; one was the activity of its activation domain acting to the promoter and the other was that of binding to the *cis*-element in the carrot protoplasts. To characterize these properties, first we prepared effector plasmids containing the GAL4 DNA binding domain (GAL4DBD in Figure 5A) including a nuclear localization signal translationally fused to each DcMYB, and prepared the reporter plasmid containing five copies of GAL4 binding sequences fused to *Luc* reporter gene (Ohta et al. 2000) (Figure 5A). This combination of the effector and reporter plasmids could indicate the transcriptional activation activity of each DcMYB protein without influencing the binding activity of the proteins to the *cis*-elements. LUC activity derived from the reporter was upregulated by cotransfection with GAL4-DcMYB3 and GAL4-DcMYB5, and the extent of *trans*-activation by the latter was higher than that of the former (Figure 5B), indicating that the transcriptional activation potential of DcMYB5 for the promoter was higher than that of DcMYB3. By contrast, GAL4-DcMYB2 and GAL4-DcMYB4 did not increase the LUC activity, indicating that DcMYB2 and DcMYB4 might not have transcriptional activation potential in carrot protoplasts. These results were consistent with the results of cotransfection experiments using DcPAL3-LUC or 3×box-LUC with 35S minimal promoter-*Luc* and 35S-DcMYBs (Figure 4B, D).

Subsequently, we assessed whether the *cis*-element binding potential of each DcMYB adding the artificial activation domain caused the different migration of the *trans*-activation of the promoter activity in carrot protoplasts. The effector plasmids were constructed to contain the activation domain of viral protein 16 (VP16) from herpes simplex virus (Fujimoto et al. 2000) translationally fused to each *DcMYB* cDNA (Figure 5C), and then cotransfected with DcPAL3-LUC into carrot protoplasts. DcMYB3-VP16 and DcMYB5-VP16 largely enhanced the expression of *DcPAL3* promoter with almost the same extent of *trans*-activation, indicating that the binding activities of DcMYB3 and 5 to the *DcPAL3* promoter in carrot protoplasts might be almost equal. These results coupled with the data shown in Figure 5B suggested that the difference in the extent of *trans*-activation between DcMYB3 and DcMYB5 seen in Figure 4 might be because of the different potential of transcriptional activation to the promoter, but not because of the binding activity to the *cis*-element. Compared with DcMYB3-VP16 and DcMYB5-VP16, a

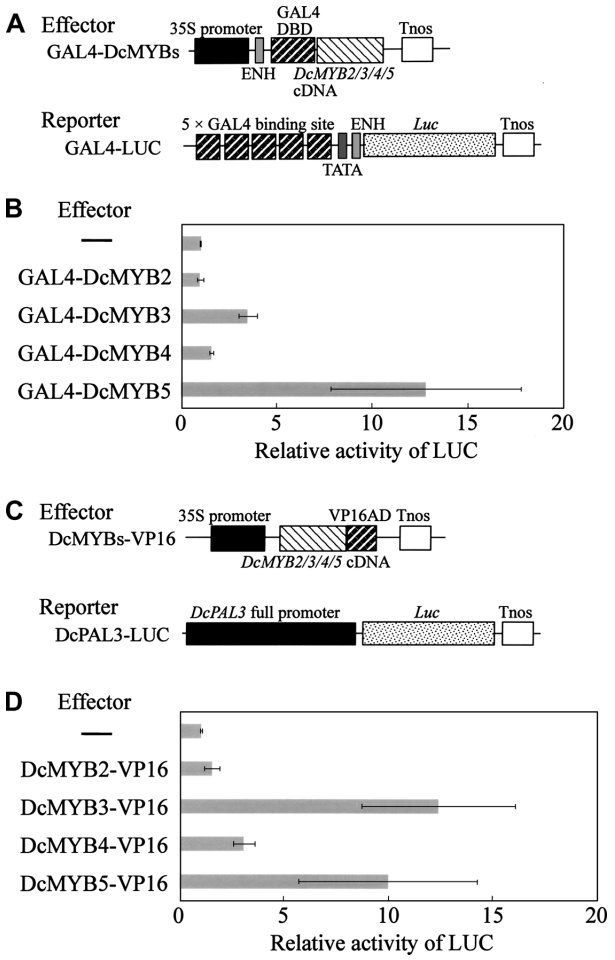

Figure 5. Functional analysis of *trans*-activation potential of DcMYBs in carrot protoplasts. (A) Constructs of effector and reporter plasmids. The effector construct GAL4-DcMYBs contained *DcMYB* cDNAs translationally fused downstream of the GAL4 DNA binding domain (GAL4DBD). The reporter construct GAL4-LUC contained five repeats of the GAL4-binding site upstream of the truncated CaMV 35S promoter (TATA)::*Luc* gene. (B) Relative LUC activity in carrot protoplasts after cotransfection with GAL4-DcMYBs and GAL4-LUC. (C) Effector and reporter plasmid constructs. The reporter construct DcPAL3-LUC was the *Luc* gene driven by the *DcPAL3* full promoter (−369 to +1), and the effector construct DcMYBs-VP16AD contained *DcMYB* cDNAs translationally fused upstream of VP16 activation domain. (D) Relative LUC activity in carrot protoplasts after cotransfection with DcMYBs-VP16 and DcPAL3-LUC. The values shown in B and D are averages of results from three independent experiments. Error bars are standard deviations. All activities are expressed relative to the reporter plasmid alone.

small extent of upregulation of the LUC activity by DcMYB2-VP16 and DcMYB4-VP16 was observed in carrot protoplasts, distinct from the binding activities in yeast (Figure 3).

One remarkable difference between DcMYB3 and 5 was that the potential of transcriptional activation of DcMYB5 was about three times higher than that of DcMYB3 (Figure 5B), although their binding activity to the *DcPAL3* promoter in carrot protoplasts was almost same (Figure 5D). It is presumable that DcMYB5 showing higher activation ability in the transient-expression experiment might efficiently upregulate *in vivo DcPAL3* expression than DcMYB3. However, the possibility remains that *in vivo DcPAL3* expression might be regulated by these DcMYBs cooperated with some other partner factor(s). C1, the first MYB protein reported in the plant kingdom (Paz-Ares et al. 1987), could bind to regulatory elements in the promoters of structural genes for the enzymes involved in the anthocyanin synthetic pathway, which alone was insufficient for transcriptional activation; a partner, R protein having the basic helix-loop-helix (bHLH), was required to show full transcriptional activation activity (Bruce et al. 2000; Grotewold et al. 2000; Hernandez et al. 2004; Sainz et al. 1997). Although *A. thaliana* TT2 and *P. hybrida* AN2 alone could activate the structural genes in transgenic plants, TT2 requires interaction with TT8 having the bHLH and TTG1 having WD40-repeat to upregulate proanthocyanidin synthesis (Baudry et al. 2004; Nesi et al. 2000; Nesi et al. 2001; Zhang et al. 2003), and AN2 is known to regulate the structural genes together with AN1 having the bHLH (Quattrocchio et al. 1999; Spelt et al. 2000). For upregulation of *A. thaliana* and *Lotus japonicus anthocyanidin reductase* promoter activity in the transient expression using particle bombardment to *A. thaliana* leaf, TT2 derived from either *A. thaliana* or *L. japonicus* alone could not or caused less activation of the promoter activity; as the partners for both TT2 of *A. thaliana* and *L. japonicus*, cotransfection of TT8 and TTG1 derived from *A. thaliana* was required for full promoter activity (Yoshida et al. 2008). Although both DcMYB3 and DcMYB5 showed similar binding activity to the *DcPAL3* promoter, the activation ability of *DcPAL3* promoter activity by DcMYB3 was about one-third of that of DcMYB5 (Figure 5A). It is possible that DcMYB3 together with some other partner factor(s) might facilitate much higher activation toward the *DcPAL3* promoter than DcMYB5 with these factor(s). This possibility might be the cause of the absent or reduced activation activity for DcMYB2 and 4 in the carrot protoplasts, but also of their remarkable binding in yeast (Figure 3), that is, because of the absence of reduced expression of their partner factor(s) in the protoplasts. However, this possibility might be lower than that of DcMYB3 or 5 acting as *trans*-activators for the upregulation of *DcPAL3* gene expression via the box-L sequence in carrot cells; the phylogenetic analysis predicted that DcMYB3 and 5 are clustered in the N8 subgroup (Figure 1). In cells of the variant cultured cell line, high expression of *DcMYB3* and *5* were observed, but not of *DcMYB2* and *4*. Although the expression of *DcMYB2* and *4* in cells of the normal cultured cell line was similar to that of *DcMYB3* regulated by 2,4-D, it is possible that DcMYB2 and 4 might play different roles in these cells apart from the

regulation of anthocyanin synthesis. In the medium lacking 2,4-D, cellular events other than synthesis of anthocyanin were induced, namely, cessation of cell division and commencement of cell elongation, which are reversibly regulated by 2,4-D (Ozeki 1996). DcMYB2 belongs to the N2 subgroup together with AtMYB17, which may be involved in the process of the early inflorescence development and seed germination (Zhang et al. 2009), and DcMYB4 belongs to the N4 subgroup together with LeBlind, which controls the formation of lateral meristems (Schmitz et al. 2002), implying that DcMYB2 and 4 may participate in some cellular events regulated by 2,4-D other than regulation of *DcPAL3* gene expression.

Our results suggested that the role and scene for the transcriptional regulation of the *DcPAL3* promoter by DcMYBs might depend on the cells of different cultured cell lines under different differentiation conditions. It is known that *PAL* gene expression is regulated by environmental and developmental cues (Bevan et al. 1989; Liang et al. 1989; Shufflebottom et al. 1993); we have already reported that environmental cues, such as elicitor treatment, UV irradiation, and osmotic stress, induce *DcPAL1* gene expression, and that DcMYB1 plays the most important role in its regulation (Maeda and Ozeki 2006; Maeda et al. 2005, 2006). We showed that *DcPAL3* involved in the synthesis of anthocyanin is regulated by developmental cues, in which absence of 2,4-D induced its synthesis, but its presence strictly repressed it in cells of the normal cultured cell line (Ozeki and Komamine 1981; Ozeki et al. 1990). In these cells, our findings suggested that DcMYB3, but not DcMYB5, might play an important role in the 2,4-D-regulated expression of *DcPAL3*. By contrast, a high level of expression of both *DcMYB3* and *DcMYB5* was observed in cells of the variant cultured cell line, suggesting that both factors could act on the upregulation of *DcPAL3* expression in these cells in the presence of 2,4-D. The expression of *DcMYB3* might be under different regulatory influences from that of *DcMYB5* in anthocyanin-synthesizing cells in the normal and variant cultured cell lines. The expression of *DcMYB5* was supressed in cells of the normal cultured cell line irrespective of presence or absence of 2,4-D, suggesting that the other regulatory way than the way to *DcMYB3* expression regulated by 2,4-D might take part in *DcMYB5* expression. It is supposed that the unknown alteration of expression of some genes during the establishment of the variant cultured cell line caused by somaclonal variation might be due to de-supression of *DcMYB5* expression. It is possible that DcMYB5 might play an important role in dynamic changes in the expression profile of the genes involved in anthocyanin synthesis including *DcMYB3*. Future analysis of the promoter regions on *DcMYB3* and *5* genes will allow us to elucidate further the different regulatory mechanisms for the expression of these genes in the normal and variant cultured cell lines.

Acknowledgements

We thank Dr. Shinshi, Advanced Industrial Science and Technology (AIST), for the kind donation of the 5×GAL DBS-LUC reporter plasmid, and 35S-GAL4DBD and 35S-GAL4DBD-VP16 effector plasmids. Part of this work was supported by the Development of Fundamental Technologies for Controlling the Process of Material Production of Plants (NEDO).

References

Baudry A, Heim MA, Dubreucq B, Caboche M, Weisshaar B, Lepiniec L (2004) TT2, TT8, and TTG1 synergistically specify the expression of *BANYULS* and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. *Plant J* 39: 366–380

Bevan M, Shufflebottom D, Edwards K, Jefferson R, Schuch W (1989) Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. *EMBO J* 8: 1899–1906

Bruce W, Folkerts O, Garnaat C, Crasta O, Roth B, Bowen B (2000) Expression profiling of the maize flavonoid pathway genes controlled by estradiol-inducible transcription factors CRC and P. *Plant Cell* 12: 65–80

Dixon RA (2001) Natural products and plant disease resistance. *Nature* 411: 843–847

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell* 7: 1085–1097

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* 12: 393–404

Grotewold E, Drummond BJ, Bowen B, Peterson T (1994) The *myb*-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. *Cell* 76: 543–553

Grotewold E, Sainz MB, Tagliani L, Hernandez JM, Bowen B, Chandler VL (2000) Identification of the residues in the Myb domain of maize C1 that specify the interaction with the bHLH cofactor R. *Proc Natl Acad Sci USA* 97: 13579–13584

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. *Annu Rev Plant Physiol Plant Mol Biol* 40: 347–369

Hernandez JM, Heine GF, Irani NG, Feller A, Kim MG, Matulnik T, Chandler VL, Grotewold E (2004) Different mechanisms participate in the R-dependent activity of the R2R3 MYB transcription factor C1. *J Biol Chem* 279: 48205–48213

Itoh H, Ozeki Y (2002) Identification of genetic alterations in carrot cell lines using transposable elements belonging to *Tdc1* family. *Plant Biotechnol* 19: 37–44

Jiang C, Gu X, Peterson T (2004) Identification of conserved gene structures and carboxy-terminal motifs in the *Myb* gene family of *Arabidopsis* and *Oryza sativa* L. ssp. indica. *Genome Biol* 5: R46

Kimura S, Chikagawa Y, Kato M, Maeda K, Ozeki Y (2008) Upregulation of the promoter activity of the carrot phenylalanine ammonia-lyase gene (*DcPAL3*) was caused by new members of the transcriptional regulatory proteins, DcERF1 and DcERF2,

which bound to the GCC-box homolog and acted as an activator to the *DcPAL3* promoter. *J Plant Res* 121: 499–508

Li SF, Parish RW (1995) Isolation of two novel *myb*-like genes from Arabidopsis and studies on the DNA-binding properties of their products. *Plant J* 8: 963–972

Liang XW, Dron M, Schmid J, Dixon RA, Lamb CJ (1989) Developmental and environmental regulation of a phenylalanine ammonia-lyase-β-glucuronidase gene fusion in transgenic tobacco plants. *Proc Natl Acad Sci USA* 86: 9284–9288

Logemann E, Parniske M, Hahlbrock K (1995) Modes of expression and common structural features of the complete phenylalanine ammonia-lyase gene family in parsley. *Proc Natl Acad Sci USA* 92: 5905–5909

Lois R, Dietrich A, Hahlbrock K, Schulz W (1989) A *phenylalanine ammonia-lyase* gene from parsley: structure, regulation and identification of elicitor and light responsive *cis*-acting elements. *EMBO J* 8: 1641–1648

Maeda K, Kimura S, Demura T, Takeda J, Ozeki Y (2005) DcMYB1 acts as a transcriptional activator of the carrot *phenylalanine ammonia-lyase* gene (*DcPAL1*) in response to elicitor treatment, UV-B irradiation and the dilution effect. *Plant Mol Biol* 59: 739–752

Maeda K, Kimura S, Noma W, Ozeki Y (2006) A promoter analysis of the *DcMYB1* gene that encodes the transcriptional activator of the stress-inducible *DcPAL1* gene. *Plant Biotechnol* 23: 203–209

Maeda K, Ozeki Y (2006) Self-interaction of DcMYB1 involved in stress-inducible *DcPAL1* expression. *Plant Biotechnol* 23: 219–225

Nesi N, Debeaujon I, Jond C, Pelletier G, Caboche M, Lepiniec L (2000) The *TT8* gene encodes a basic helix-loop-helix domain protein required for expression of *DFR* and *BAN* genes in Arabidopsis siliques. *Plant Cell* 12: 1863–1878

Nesi N, Jond C, Debeaujon I, Caboche M, Lepiniec L (2001) The Arabidopsis *TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. *Plant Cell* 13: 2099–2114

Ohl S, Hedrick SA, Chory J, Lamb CJ (1990) Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. *Plant Cell* 2**:** 837-848

Ohta M, Ohme-Takagi M. Shinshi H (2000) Three ethylene-responsive transcription factors in tobacco with distinct transactivation functions. *Plant J* 22: 29–38

Ozeki Y (1996) Regulation of anthocyanin synthesis in carrot suspension cultured cells. *J Plant Res* 109: 343–351

Ozeki Y, Chikagawa Y, Kimura S, Soh HC, Maeda K et al. (2003) Putative *cis*-elements in the promoter region of the carrot *phenylalanine ammonia-lyase* gene induced during anthocyanin synthesis. *J Plant Res* 116: 155–159

Ozeki Y, Davies E, Takeda J (1993). Structure and expression of chalcone synthase gene in carrot suspension cultured cells regulated by 2,4-D. *Plant Cell Physiol* 34**:** 1029-1037

Ozeki Y, Itoh Y, Sasaki N, Oyanagi M, Akimoto H, Chikagawa Y, Takeda J (2000) Phenylalanine ammonia-lyase genes involved in anthocyanin synthesis and the regulation of its expression in suspension cultured carrot cells. *J Plant Res* 113: 319–326

Ozeki Y, Komamine A (1981) Induction of anthocyanin synthesis in relation to embryogenesis in carrot suspension culture: Correlation of metabolic differentiation with morphological differentiation. *Phygiol Plantarum* 53: 570–577

Ozeki Y, Komamine A (1986) Effects of growth regulators on the induction of anthocyanin synthesis in a carrot suspension culture. *Plant Cell Physiol* 27: 1361–1368

Ozeki Y, Matsui M, Sakuta M, Matsuoka M, Ohasi Y, Kano-Murakami Y, Yamamoto N, Tanaka Y (1990) Differential regulation of phenylalanine ammonia-lyase genes during anthocyanin synthesis and by transfer effect in carrot cell suspension cultures. *Phygiol Plantarum* 80: 379–387

Paz-Ares J, Ghosal D, Wienand U, Peterson PA, Saedler H (1987) The regulatory *c1* locus of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and with structural similarities to transcriptional activators. *EMBO J* 6**:** 3553-3558

Quattrocchio F, Wing J, van der Woude K, Souer E, de Vetten N, Mol JN, Koes R (1999) Molecular analysis of the *anthocyanin2* gene of petunia and its role in the evolution of flower color. *Plant Cell* 11: 1433–1444

Sainz MB, Grotewold E, Chandler VL (1997) Evidence for direct activation of an anthocyanin promoter by the maize C1 protein and comparison of DNA binding by related Myb domain proteins. *Plant Cell* 9: 611–625

Schmitz G, Tillmann E, Carriero F, Fiore C, Cellini F, Theres K (2002) The tomato *Blind* gene encodes a MYB transcription factor that controls the formation of lateral meristems. *Proc Natl Acad Sci USA* 99: 1064–1069

Shirley BW, Kubasek WL, Storz G, Bruggemann E, Koornneef M, Ausubel FM, Goodman HM (1995) Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis. *Plant J* 8: 659–671

Shufflebottom D, Edwards, K, Schuch W, Bevan M (1993) Transcription of two members of a gene family encoding phenylalanine ammonia-lyase leads to remarkably different cell specificities and induction patterns. *Plant J* 3: 835–845

Spelt C, Quattrocchio F, Mol JN, Koes R (2000) *anthocyanin1* of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. *Plant Cell* 12: 1619–1632

Stracke R, Werber M, Weisshaar B (2001) The R2R3-MYB gene family in *Arabidopsis thaliana*. *Curr Opin Plant Biol* 4: 447–456

Takeda J, Itoh Y, Maeda K, Ozeki Y (2002) Assignment of UVB-responsive *cis*-element and protoplastization-(dilution-) and elicitor-responsive ones in the promoter region of a carrot *phenylalanine ammonia-lyase* gene (*gDcPAL1*). *Photochem Photobiol* 76**:** 232–238

Yoshida K, Iwasaka R, Kaneko T, Sato S, Tabata S, Sakuta M (2008) Functional differentiation of *Lotus japonicus* TT2s, R2R3-MYB transcription factors comprising a multigene family. *Plant Cell Physiol* 49: 157–169

Zhang F, Gonzalez A, Zhao M, Payne CT, Lloyd A (2003) A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. *Development* 130: 4859–4869

Zhang Y, Cao G, Qu LJ, Gu H (2009) Characterization of *Arabidopsis MYB* transcription factor gene *AtMYB17* and its possible regulation by LEAFY and AGL15. *J Genet Genomics* 36: 99–107