Isolation of anthocyanin-related MYB gene, GbMYB2, from Gynura bicolor leaves

Yasuhiro Shimizu1,2, Kazuhiro Maeda2,*, Mika Kato1,2, Koichiro Shimomura1,3

1 Graduate School of Life Sciences, Toyo University, Ora, Gunma 374-0193, Japan; 2 San-Ei Gen F.F.I., Inc., Toyonaka, Osaka 561-8588, Japan; 3 Plant Regulation Research Center, Toyo University, Ora, Gunma 374-0193, Japan

* E-mail: kazuhiro-maeda@saneigenffi.co.jp Tel: +81-6-6333-0521 Fax: +81-6-6333-2076

Abstract The leaves of field-cultivated Gynura bicolor DC. are reddish purple on the abaxial side and green on the adaxial side. The leaves of cultured G. bicolor plantlets, however, appear almost completely green on both sides. Cultured plantlet leaves treated with sucrose accumulated anthocyanins on the abaxial side. In this study, to investigate the anthocyanin-accumulation mechanism in G. bicolor leaves, we isolated the MYB transcription regulatory gene from the leaves using a degenerate PCR method. The isolated gene, GbMYB2, was approximately 10-fold up-regulated in sucrose-treated leaves. In addition, co-expression of GbMYB2 and bHLH-type transcription factor, GbMYC1, activated GbDFR and GbANS promoters in tobacco leaf protoplasts. These results suggest that GbMYB2 might regulate anthocyanin biosynthesis genes in G. bicolor leaves.

Key words: Anthocyanin, bHLH, Gynura bicolor, R2R3 MYB, transcription factor.
The results of isolation of the GbMYB2 gene.

To identify the regulatory mechanism of sucrose-induced anthocyanin accumulation in *G. bicolor* leaves, we isolated the MYB gene and then analyzed the expression in *G. bicolor* sucrose-treated leaves. The cDNA-encoding MYB protein was isolated from *G. bicolor* sucrose-treated leaves using a degenerate PCR method with primer pairs, 5′/H11032-TGY ATHRA Y AARTA Y-GGIGARGGIAARTGG-3′ as the sense primer and 5′/H11032-GTRTTCCARTARTYTTCRTTNGC-3′ as the antisense primer, designed in the R2R3 MYB domain.

Total RNA was isolated with RNeasy Plant Mini kit (Qiagen, Hilden, Germany) and reverse transcribed into cDNA using TaKaRa RNA PCR kit (TaKaRa, Tokyo, Japan). Amplified cDNA fragments by PCR were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA). The full-length sequence of the cDNA was determined by 5′/H11032 and 3′/H11032 rapid amplification of the cDNA ends (5′-RACE and 3′-RACE) using a GeneRacer kit (Invitrogen). Isolated MYB gene was termed GbMYB2 (accession number, AB550245). GbMYB2 encoded a predicted protein of 276 amino acids. This protein shared high homology with MYBs, PAP1 in *Arabidopsis*, GMYB10 in *Gerbera*, and AN2 in *Petunia*, all of which regulate anthocyanin biosynthesis gene expression.

Phylogenetic analysis showed that GbMYB2 is part of a group of MYB proteins that regulate anthocyanin biosynthesis (Figure 1A). We have found that GbMYB1, which has high homology with anthocyanin-related MYB proteins in the R2R3 domain, was up-regulated in MJ-treated roots and coordinate action of GbMYB1 with GbMYC1, bHLH transcription factor, activated GbDFR promoter, and GbANS promoter activity in tobacco protoplasts (Shimizu et al. personal communication). GbMYB2 shared 93.3% similarity with GbMYB1 in the R2R3 MYB domain, and it showed 60.8% similarity to GbMYB1 over the entire protein. In addition, GbMYB2 shared 91.3% similarity with GMYB10, 82.7% similarity with AN2, and 77.8% similarity with AtPAP1 in the R2R3 MYB domain, and has the amino acid residues [DE]Lx2[RK]x3Lx6Lx3R that interact with bHLH (Grotewold et al. 2000; Zimmermann et al. 2004) (Figure 1B). This suggests that GbMYB2 might be related to the regulation of anthocyanin accumulation in *G. bicolor* leaves.
For sucrose-treatment experiments, plantlets were cultured on MS solid medium (pH 5.7) containing 3% sucrose and 0.22 (w/v) Gelrite at 25°C under 14 h of light (70 \mu mol m^{-2} s^{-1})/day for 6 weeks, and leaves excised from the stem were incubated in a 9% sucrose solution for 5 days. Anthocyanins were extracted with 0.5% H$_2$SO$_4$ from treated leaves, and absorbance of the extract was measured. Anthocyanin content was calculated as cyanidin 3-O-glucoside equivalent from a standard curve using cyanidin 3-O-glucoside. Anthocyanins in leaves of cultured G. bicolor plantlets were barely accumulated; however, excised sucrose-induced leaves accumulated anthocyanins in the abaxial side (Figure 2).

The possible change in the transcriptional levels of anthocyanin biosynthesis genes and transcription factor genes GbMYB2, GbMYB1, and GbMYC1 in the sucrose-treated leaves of cultured plantlets was tested by standard RT-PCR analysis and quantitative real-time RT-PCR (qRT-PCR) analysis. Total RNA was isolated from sucrose-treated leaves using an RNeasy Plant Mini kit. cDNA was synthesized from the isolated total RNA treated leaves of cultured plantlets was tested by standard RT-PCR analysis and quantitative real-time RT-PCR primers listed in Table 1 were used for standard RT-PCR and qRT-PCR analyses. Standard RT-PCR and qRT-PCR were performed using a SYBR Green Real-time PCR Master Mix-Plus- (Toyobo). The actin gene was used as the control for RT-PCR and qRT-PCR analyses.

Analysis of the expression of anthocyanin biosynthesis and regulatory genes revealed that the structural genes of the anthocyanin biosynthesis pathway were expressed more strongly in the sucrose-treated leaves (+suc) than in the untreated leaves (−suc) (Figures 3, 4). Expression of GbCHS, GbDFR, and GbANS genes was up-regulated strongly in sucrose-treated leaves. In particular, the GbANS expression level increased approximately 3600-fold with the sucrose treatment (Figure 4). In addition, expression of anthocyanin biosynthesis genes, GbCHS and GbDFR, was very low in roots (Figures 3, 4); however, the phenylpropanoid biosynthesis gene, GbPAL, was expressed at almost the same level in both leaves and roots (Figures 3, 4). These results indicate that anthocyanin accumulation in sucrose-treated leaves of cultured G. bicolor was influenced by transcriptional regulation of flavonoid biosynthesis genes. Anthocyanins of red Perilla (P. frutescens) and red cabbage (Brassica oleracea var. capitata) are used as food colorants. In P. frutescens, two formae, an anthocyanin-producing red forma and an anthocyanin nonproducing green forma, are known. Anthocyanin biosynthesis genes are activated in red forma, but not in green forma (Gong et al. 1997). In red forma, MYB-P1, MYB-RP, and PFWD participate in the regulation of the anthocyanin biosynthesis gene (Gong et al. 1999a; Gong et al. 1999b; Sompornpailin et al. 2002). In red cabbage, anthocyanin biosynthesis genes are activated during all stages of vegetative growth, and expression of regulatory genes, bHLH and MYB, are coordinately activated with structural genes (Yuan et al. 2009). In various other plants, it has been
reported that the MYB transcription factors are related to the regulation of anthocyanin biosynthesis genes. In Arabidopsis, it was shown that AtPAP1 regulates anthocyanin accumulation in sucrose-treated leaves (Solfanelli et al. 2006). Expression analysis of regulatory genes revealed that GbMYB2 was expressed strongly in sucrose-treated leaves and was approximately 10-fold up-regulated compared with that in untreated leaves (Figures 4). In roots, expression of GbMYB2 was low, 0.07-fold, compared with that in untreated leaves (Figure 4). Both GbMYB2 and GbMYC1 were up-regulated in sucrose-treated leaves; latter up to 8.1-fold (Figure 4), suggesting that these regulatory factors might be related to anthocyanin regulation in G. bicolor sucrose-treated leaves. This also suggests that expression of these structural genes might affect the expression of GbMYB2 in G. bicolor. On the other hand, expression of GbMYB1 was very low in both leaves and roots, and a drastic expression change of GbMYB1 was not observed, suggesting GbMYB1 might not be involved in the regulation of anthocyanin biosynthesis genes expression in sucrose-treated leaves (Figures 3, 4).

To investigate the transcriptional activation ability of GbMYB2 from GbDFR and GbANS promoters, PEG-mediated transient expression analyses were carried out with tobacco leaf protoplasts, as described by Takeuchi et al. (2000) in Arabidopsis cultured cells with some modification. GbDFR and GbANS promoters fused to a firefly luciferase (Luc) gene were prepared as reporter constructs (pGbDFRpro-Luc and pGbANSpro-Luc)
GbMYB2 or GbMYC1 cDNA driven by cauliflower mosaic virus 35S (CaMV35S) promoter were prepared as effector constructs (p35S-GbMYB2 or p35S-GbMYC1), which included Arabidopsis ADH1 5′-UTR as a translational enhancer sequence downstream of the CaMV35S promoter (Figure 5A). As a reference, a renilla luciferase (Rluc) gene, which was under the control of the same promoter, was cotransfected (p35S-Rluc). Protoplasts were cotransfected with p35S-GbMYB2, p35S-GbMYC1, pGbDFRpro-Luc or pGbANSpro-Luc, and p35S-Rluc. After culturing in the dark at 25°C for 22 h, Luc and Rluc activities were measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The Luc activity relative to Rluc was calculated to show activation of the GbDFR or GbANS promoter by GbMYB2 and GbMYC1 (Figure 5B, C). Some anthocyanin-related MYB proteins require a partner protein, bHLH transcription factor, for activation of the target gene promoter (Grotewold et al. 2000; Spelt et al. 2000; Zimmermann et al. 2004). It was revealed in our previous study that co-expression of GbMYB1 and GbMYC1 activates GbDFR and GbANS promoters in tobacco leaf protoplasts (Shimizu et al. personal communication). Amino acid sequences

Figure 5. Transient expression analysis in tobacco leaf protoplasts using GbDFR and GbANS promoters and a combination of GbMYB2 and GbMYC1. (A) Schematic drawing of the reporter and effector constructs. In reporter constructs, the putative translation starting site is numbered +1. GbDFRpro and GbANSpro indicate GbDFR and GbANS promoter. 35Spro and Tnos indicate cauliflower mosaic virus 35S promoter and Nos terminator. (B) Activation of GbDFR promoter activity, and (C) GbANS promoter by co-expression of GbMYB2 and GbMYC1. The normalized promoter activity by Rluc was shown as relative activity set to 1 of the activity of the reporter construct only. Error bars indicate standard error of three independent measurements.
between GbMYB2 and GbMYB1 were highly conserved in the R2R3 MYB domain, and in particular, they were conserved completely in the bHLH motif (Figure 1B), and both GbMYB2 and GbMYC1 expressed in cultured sucrose-treated plantlet leaves (Figures 3, 4). This suggests that GbMYC1 might act as a partner protein of GbMYB2 for activation of expression of anthocyanin biosynthesis genes, even in leaves; therefore, GbMYC1 was tested as a partner protein for transient expression assay. As a result, in transient expression analysis, it was shown that co-expression of GbMYB2 and GbMYC1 could activate GbDFR and GbANS promoters (Figure 5B, C). These results suggest that GbMYB2 acts as an anthocyanin regulator in G. bicolor leaves treated with sucrose. The sole expression of GbMYB2 could not completely activate GbDFR and GbANS promoters, GbDFR promoter was not activated by sole expression of GbMYB2 (Figure 5B), whereas GbANS promoter activity increased approximately 2-fold by sole expression of GbMYB2 (Figure 5C). Differences of cis-elements between GbDFR and GbANS promoters might give rise to the difference in these results. Further analyses about the relationship between cis-elements in the promoter regions of these genes and transcription regulatory factors will be required.

In this study, it was shown that GbMYB2 might be related to the regulation of anthocyanin biosynthesis in G. bicolor leaves. In Arabidopsis, it has been shown that PAP1 regulates both MJ- and sucrose-inducible anthocyanin accumulation (Loreti et al. 2008; Solfanelli et al. 2006). In G. bicolor, GbMYB2 might be related to sucrose-inducible anthocyanin accumulation in leaves and GbMYB1 might be related to MJ-inducible anthocyanin accumulation in roots (Shimizu et al. personal communication). The expression of GbMYC1 was up-regulated by MJ treatment (Shimizu et al. personal communication) and sucrose treatment (Figure 4), suggesting that GbMYC1 might be related to both anthocyanin-accumulation phenomena. We are currently creating plants in our laboratory that overexpress these regulatory genes to analyze the regulatory mechanisms of anthocyanin biosynthesis in G. bicolor.

References


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