An Anthocyanin Regulator from Grapes, VlmybA1-2, Produces Reddish-Purple Plants

Yoshiko Koshita¹, Shozo Kobayashi^{1*}, Megumi Ishimaru², Yoshio Funamoto³,
Mikio Shiraishi⁴, Akifumi Azuma¹, Hiroshi Yakushiji¹ and Masayoshi Nakayama⁵

¹Grape and Persimmon Research Station, National Institute of Fruit Tree Science, Higashi-hiroshima 739–2494, Japan

²Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Gakuen-cho, Sakai 599–8531, Japan

³Faculty of Agriculture, Yamaguchi University, Yamaguchi 753–8515, Japan

⁴Fukuoka Agricultural Research Center, Chikushino 818–8549, Japan

⁵National Institute of Floricultural Science, Tsukuba 305–8519, Japan

A cDNA, VlmybA1-2, for an anthocyanin regulatory gene of grape was constructed under the control of the cauliflower mosaic virus (CaMV) 35SRNA promoter and introduced into leaf disks and petioles of kiwifruit (*Actinidia deliciosa*) plants by *Agrobacterium*-mediated gene transfer. When the tissues were cultured on selection medium, red cells visible to the naked eye formed at the cut ends of the segments after approximately 2 weeks. The cells developed into calluses, adventitious buds, and reddish-purple plantlets after 2, 3, and 6 months of culture, respectively. The pigment produced in the leaves of the transformed plants was determined to be cyanidin 3-*O*-(2-*O*- β -xylosyl)- β -glucoside (cyanidin 3-*O*-sambubioside). As VlmybA1-2 can also produce red cells in tomato and eggplant, it may be useful as a visible marker to confirm efficient transformation in dicots.

Key Words: anthocyanin, grape, kiwifruit, *myb*, transformant.

Introduction

In many plants, two types of transcription factors (MYB and bHLH) control the expression of anthocyanin biosynthesis genes (Holton and Cornish, 1995; Mol et al., 1998). In addition, a regulatory factor, WD40 repeat protein, is important for the expression of anthocyanin biosynthesis genes, which is controlled by a transcription complex containing MYB, bHLH, and WD40 proteins (Koes et al., 2005). Previously, we showed that *VvmybA1*, the gene for a MYB transcription factor in grape, plays an important role in the regulation of anthocyanin biosynthesis in grapes (Kobayashi et al., 2004, 2005). Genetic analyses of berry color also revealed that *VvmybA1* is the gene underlying the mapped locus controlling skin color (Lijavetzky et al., 2006).

The genes for β -D-glucuronidase (GUS) and green

fluorescent protein (GFP) are widely used as reporter genes in plants, but visualizing their products requires histochemical staining or a fluorescence microscope. On the other hand, a bHLH (*Lc*) gene of maize was proposed as a visible marker (reporter gene), which allows monitoring and quantification of gene expression in living cells of maize without any staining procedure (Ludwig et al., 1990). Such a visible marker would be beneficial in dicot species. Therefore, as a similar reporter gene for dicot species, the possibility of bHLH and MYB (*C1*) genes of maize were examined (Damiani et al., 1998; Lloyd et al., 1992); however, pigment induction by bHLH and *C1* genes varied strongly among species.

When the cDNA of *VvmybA1* was introduced into somatic embryos of grape by particle bombardment, the cDNA induced red cells (Kobayashi et al., 2005). The cDNAs, VlmybA1-1, VlmybA1-2, and VlmybA2, which are homologs of *VvmybA1*, also induced red cells in the embryos (Kobayashi et al., 2002). As VlmybA1-2 seems to have higher potential as a visible marker than bHLH and *C1* genes in dicot species, we examined whether VlmybA1-2 is suitable as a reporter gene and can function to produce purple plants of other species.

Received; June 4, 2007. Accepted; August 13, 2007.

* Corresponding author (E-mail: skobaya@affrc.go.jp).

This work was supported in part by a Grant-in-Aid for Young Scientists (B) (No. 18789006) from the Ministry of Education, Culture, Sports, Science and Technology.

Materials and Methods

1. Vector construction and transformation

A cDNA, VlmybA1-2 (DDBJ accession number AB073011), for a *Myb*-related gene isolated from a mature-berry cDNA library of 'Kyoho' grapes (Kobayashi et al., 2002) was used as template DNA. From the sequences of the coding region, we designed a forward primer (5'-CGCTCTAGA(*Xba*I)CTCGATG GAGAGCTTAGGAGTTAG-3') and a reverse primer (5'-CGCGAGCTC(*Sac*I)TAAATCAGATCAAATGAT TACTT-3') to amplify the coding region. The amplified fragment was then digested with *Xba*I and *Sac*I and integrated into a binary vector, pBI121 (Clontech, USA), from which the β -glucuronidase (*gus*) gene sequence had been removed by the same digestion. The chimeric gene was introduced into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating. Leaf disks and petioles of kiwifruit (*Actinidia deliciosa* 'Hayward') were infected with this *Agrobacterium*, the tissues were cultured on solidified MS medium containing kanamycin (50 mg·L⁻¹), and plantlets were regenerated as described by Matsuta et al. (1993). To confirm whether this pigmentation could be extended to other species, we infected 7-day-old cotyledons of tomato and eggplant with the *Agrobacterium* according to the method of Deikman and Fischer (1988).

2. Southern and northern blot analyses

Total DNA was extracted from the leaves of five transformants and a control plant according to the method of Kobayashi et al. (1996). For each analysis, 3 μ g of DNA was digested with *Sac*I followed by *Xba*I, and another 3 μ g of DNA was single-digested with *Xba*I at 37°C for more than 5 h in both digestions. Southern blot analysis was performed according to the method of Kobayashi et al. (2005) using DIG-labeled probes prepared from a coding region of VlmybA1-2. Northern blot analysis was also performed as described by Kobayashi et al. (2005) using RNA extracted from the leaves of the plants and the same DIG-labeled probes.

3. Anthocyanin analysis

Endogenous anthocyanins in the transformants were quantified and analyzed by high-performance liquid chromatography (HPLC). The major anthocyanin detected by HPLC analysis was further purified, and its structure was determined by ¹H-nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (ESIMS). Analytical procedures of endogenous anthocyanins were as follows.

3.1 Quantification of anthocyanins

One g of young leaves (5–7 cm wide) from each of five selected transformants and one control plant were collected, and the anthocyanins extracted according to the method of Jeong et al. (2004), with the following modifications. Each sample was ground with a mortar

and pestle and extracted three times with 20 mL of 50% (v/v) acetic acid. The mixture was then centrifuged at 2000 × g at 4°C for 5 min, and the supernatants from the three extractions were combined. After dilution of the extract to 1/10 with 50% acetic acid, absorbance was measured at 530 nm with a spectrophotometer (UV-260, Shimadzu, Japan). Total anthocyanin concentrations were expressed as milligrams of cyanidin-3-glucoside (Extrasynthese, France) equivalents per gram of fresh weight.

3.2 HPLC analysis

Anthocyanins in the kiwifruit plants were extracted with 5% aqueous acetic acid. We analyzed 10 μ L of the extract using an HP1100 system with a photodiode array detector (Agilent Technologies/Yokogawa Analytical Systems, Japan) and an Intersil ODS-2 column (4.6 mm × 250 mm, GL Science, Japan) at 40°C with a flow rate of 0.8 mL·min⁻¹. Absorption spectra were monitored at 240 to 580 nm. A linear gradient of 10% to 50% of solvent B (1.5% H₃PO₄, 40% acetonitrile, 50% acetic acid) in solvent A (1.5% H₃PO₄) was run over 40 min.

3.3 Purification of endogenous anthocyanin and spectroscopic analyses by ¹H-NMR and ESIMS

Anthocyanins in transformed kiwifruit shoots (3 g fresh weight) were extracted with 5% acetic acid (200 mL × 2). The extract was partitioned with diethyl ether (50 mL × 2), and the acetic acid fraction was evaporated to dryness *in vacuo*. The residue was dissolved in 1% acetic acid, passed through an ODS column (5 g, Nacalai Tesque Inc., Japan), and eluted with water (100 mL) containing acetic acid increasing in 1% steps. The major anthocyanin was eluted in the 6% aqueous acetic acid fraction. This compound was purified using an HP1100 with a Senshu Pak ODS-4253-D (10 mm × 250 mm; Senshu Scientific Co., Ltd, Japan) at 40°C, with a flow rate of 3.0 mL·min⁻¹, under isocratic solvent (5% acetic acid, 23% methanol, 72% water), and about 3 mg of compound was obtained. ESIMS spectra were analyzed on a TSQ system (Thermo Quest Finnigan, USA). ¹H-NMR spectra of the extracted compounds were analyzed by ARX400 (Bruker Biospin K. K., Germany) in *d*₆-dimethyl sulfoxide containing 10% (V/V) trifluoroacetic acid-*d*. Cyanidin: 8.83 (1H, s, H-4), 6.70 (1H, d, *J* = 1.9 Hz, H-6), 6.87 (d, *J* = 1.9 Hz, H-8), 8.01 (d, *J* = 2.3 Hz, H-2'), 7.01 (d, *J* = 8.7 Hz, H-5'), 8.25 (dd, *J* = 2.3, 8.7 Hz, H-6'); glucose: 5.56 (d, *J* = 7.6 Hz, H-1), 3.86 (t, *J* = 8.3 Hz, H-2), 3.62 (t, *J* = 8.9 Hz, H-3), 3.32 (t, *J* = 9.2 Hz, H-4), 3.55 (m, H-5), 3.70 (brd, *J* = 10.4 Hz, H-6a), 3.51 (dd, *J* = 5.7, 12.2 Hz, H-6b); xylose: 4.64 (d, *J* = 7.7 Hz, H-1), 2.97 (t, *J* = 8.3 Hz, H-2), 3.10 (t, *J* = 8.8 Hz, H-3), 3.22 (m, H-4), 3.42 (dd, *J* = 6.3, 10.3 Hz, H-5a), 2.87 (t, *J* = 10.9 Hz, H-5b).

Results

1. Transformation

Leaf disks and petioles inoculated with *Agrobacterium* were cultured on kanamycin selection medium. After

approximately 2 weeks of culture, red cells, which were visible to the naked eye, formed at the cut ends of the segments (Fig. 1A–C). The cells developed into calluses after 2 months of culture (Fig. 1D), and adventitious buds differentiated from the calluses after 3 months of culture (Fig. 1E). When the adventitious buds had developed into shoots about 5 mm long (after 6 months of culture), they were removed from the calluses and transferred to a medium for plant regeneration. One plant was selected from each segment and proliferated by subculturing on the same medium (Fig. 1F). In all, 19 plants were regenerated from 309 treated segments. Figure 1G shows a 10-month-old transformant and a control plant. All organs (leaves, stems, and roots) of the transformants were pigmented. No differences in their growth and morphological characteristics were observed between transformed and control plants. When cotyledons of tomato and eggplant were inoculated, purple calluses also formed at the cut ends (Fig. 1H, I).

2. DNA detection

The kiwifruit plants were transferred to soil to stimulate their growth, and the integration of VlmybA1-2 was confirmed by Southern blot analysis. When DNA from the plants was double-digested with *Xba*I and *Sac*I, a 0.7-kbp band corresponding to VlmybA1-2 was detected in four of five transformed plants but not in the

control plant (Fig. 2A). In the fourth transformant (#4), a larger band was observed. This may have resulted from loss of the *Sac*I site of the integrated VlmybA1-2. To determine whether each band was the result of integration of the gene into the plant genome or of bacterial contamination, the DNA was single-digested with *Xba*I. All transformed plants had one or three bands that represented junctions between T-DNA and adjacent plant DNA (Fig. 2B). These findings show that the plants had integrated one (#1–4) or three copies (#5) of the gene into their genome.

3. Gene expression and anthocyanin content

The expression of VlmybA1-2 was examined by northern blot analysis. All transformants showed the band, which was lacking in the control plant (Fig. 3), confirming that the introduced VlmybA1-2 was transcribed into mRNA in the transformants. The strength of the band, which reflected the level of mRNA expression, differed among the individual transformants. In transformant #4 and #5, it was stronger than the others. The anthocyanin content of the transformants was quantified using calibration curves for cyanidin-3-glucoside. The highest content was 4.5 mg·g⁻¹ fresh weight in young leaves of transformant #5. mRNA expression and anthocyanin content of the plants appeared to be related.

Fig. 1. Transformation of kiwifruit, tomato, and eggplant with VlmybA1-2. When leaf disks of kiwifruit were cultured on kanamycin selection medium, red cells formed at the cut ends of the segments after approximately 2 weeks (A–C). The cells developed into calluses (D), adventitious buds (E), and reddish-purple plantlets (F) after 2, 3, and 6 months of culture, respectively. G: A 10-month-old transformant (left) and a control plant of equal age (right). Purple callus also formed at the cut end of the leaf segment of tomato (H) and eggplant (I) after 2 months. Scale bars indicate 1 mm (A, B, C, E, H, I), 1 cm (D, F), and 5 cm (G).

Fig. 2. Southern blot analysis of kiwifruit transformants. Lanes: C, DNA from a control plant; 1–5, DNAs from transformants. (A) DNAs double-digested using *Xba*I and *Sac*I. (B) DNAs single-digested using *Xba*I.

4. Identification of endogenous anthocyanins in the leaves

The major anthocyanin, which comprised about 80% of total anthocyanins in kiwifruit shoots, was eluted at 12.1 min in HPLC (Fig. 4). It was extracted and purified by HPLC and then analyzed to determine its structure. The ESIMS spectrum showed the molecular mass to charge ratio to be m/z 581, suggesting an anthocyanidin and two sugar moieties. Signals of the ^1H -NMR spectrum were assigned to protons of a cyanidin moiety, a β -glucosyl moiety and a β -xylosyl moiety on the basis of their chemical shifts and coupling constants. Correlations between H-4 of the cyanidin and H-1 of the glucoside in the NOESY spectrum and a lower field shift of the H-2 signal of the glucoside indicated linkages of these moieties. The anthocyanin was identified as cyanidin 3-*O*-(2-*O*- β -xylosyl)- β -glucoside, that is, cyanidin 3-*O*-sambubioside (Fig. 4). The similarity of the spectrum to previously reported spectra of cyanidin 3-*O*-sambubioside (Cabrita and Andersen, 1999) and cyanidin 3-*O*-

Fig. 3. Northern blot analysis of kiwifruit transformants. Lanes: C, total RNA from a control plant; 1–5, RNAs from transformants. mRNA expression appeared to be related with anthocyanin content of the plants. Transformant No. 5, which has 3 copies of the gene (Fig. 2), shows the highest level of gene expression. The ethidium bromide-stained gel prior to RNA transfer is shown in the bottom panel.

sambubioside-5-*O*-glucoside without the signals of 5-*O*-glucoside (Saito et al., 1995) supports the identification.

Discussion

Although the genes for β -D-glucuronidase (GUS) and green fluorescent protein (GFP) are widely used as reporter genes in plants, a bHLH gene (*Lc*) was proposed as a visible marker (reporter gene), which allows monitoring and quantification of gene expression in living cells without any staining procedure (Ludwig et al., 1990). *Lc* induced pigmented cells in several tissues of maize when introduced by particle bombardment (Ludwig et al., 1990). As a similar reporter gene for dicots, the possibility of another bHLH gene of maize (*Sn:bol3*) was examined in several species (Damiani et al., 1998); however, they stated that the use of *Sn:bol3* as a reporter gene in dicots would be limited, because the induction of tissue pigmentation differed among both

Fig. 4. HPLC chromatogram of anthocyanins from transformant leaves monitored by absorbance at 530 nm. After elution of major anthocyanin at 12.1 min, no other anthocyanin was detected. The anthocyanin identified from the major peak was cyanidin 3-*O*-(2-*O*- β -xylosyl)- β -glucoside.

species and genotypes examined (Damiani et al., 1998). The ability of a MYB gene (*Cl*) to induce pigmentation was also examined in dicots. Lloyd et al. (1992) reported that the *Cl* gene alone could not induce pigmentation in *Arabidopsis thaliana* or *Nicotiana tabacum*. We also showed that the introduction of *Cl* alone or in combination with maize *B-Peru* (bHLH gene) did not induce pigmentation in somatic embryos of grape (Kobayashi et al., 2002). On the other hand, in petunia, when very young leaves were bombarded with *Cl*, *Lc*, and *dfrA-gus* (in which a *dfrA* promoter of petunia is fused to the *gus* reporter gene), red spots were induced, in addition to blue spots that were the result of activation of the *dfrA* promoter (Quattrocchio et al., 1993). Thus, pigment induction by bHLH and *Cl* genes varied strongly among dicot species.

Here, we showed that VmybA1-2, an anthocyanin regulator of grape, can induce pigmented cells, visible to the naked eye, in tissues of kiwifruit, tomato, and eggplant after approximately 2 weeks of culture, and the transgenic kiwifruit produced anthocyanin (cyanidin 3-*O*-(2-*O*- β -xylosyl)- β -glucoside) in all organs (leaves, stems, and roots). In addition to the MYB protein, bHLH and WD40 are essential for the activation of promoters of structural genes for anthocyanin biosynthesis (Koes et al., 2005; Quattrocchio et al., 1993, 1998; Ramsay et al., 2005). The bHLH and WD40 proteins may be constitutively supplied in the kiwifruit, tomato, and eggplant tissues examined, since VmybA1-2 cDNA alone induced pigmentation in the tissues. Similarly, a *Myb-like* transcription factor, *PAP1*, from *A. thaliana* produced purple plants when it was introduced alone into *A. thaliana* and *N. tabacum* by means of *Agrobacterium*-mediated transformation (Borevitz et al., 2000). Since VmybA1-2 displays a high degree of sequence similarity to *PAP1* (data not shown), VmybA1-2 might have a similar function to *PAP1*.

As VmybA1-2 also induces red cells in several dicot plants other than kiwifruit, tomato, and eggplant (M. Wada, personal communication), the gene may thus be useful as a reporter gene for monitoring and quantification of gene expression in living cells and for determining efficient transformation conditions in dicots.

Acknowledgements

We thank Ms. T. Nakasumi, Grape and Persimmon Research Station, National Institute of Fruit Tree Science for technical assistance.

Literature Cited

- Borevitz, J. O., Y. Xia, J. Blount, R. A. Dixon and C. Lamb. 2000. Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell* 12: 2383–2393.
- Cabrita, L. and O. M. Andersen. 1999. Anthocyanins in blue berries of *Vaccinium padifolium*. *Phytochemistry* 52: 1693–1696.
- Damiani, F., F. Paolucci, G. Consonni, F. Crea, C. Tonelli and S. Arcioni. 1998. A maize anthocyanin transactivator induces pigmentation in hairy roots of dicotyledonous species. *Plant Cell Rep.* 17: 339–344.
- Deikman, J. and R. L. Fischer. 1988. Interaction of a DNA binding factor with the 5'-flanking region of an ethylene-responsive fruit ripening gene from tomato. *EMBO J.* 7: 3315–3320.
- Holton, T. A. and E. D. Cornish. 1995. Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* 7: 1071–1083.
- Jeong, S. T., N. Goto-Yamamoto, S. Kobayashi and M. Esaka. 2004. Effects of plant hormones and shading on accumulation of anthocyanins and other phenolic compounds on expression of anthocyanin biosynthetic genes in the grape berry skins. *Plant Sci.* 167: 247–252.
- Kobayashi, S., N. Goto-Yamamoto and H. Hirochika. 2004. Retrotransposon-induced mutations in grape skin color. *Science* 304: 982.
- Kobayashi, S., N. Goto-Yamamoto and H. Hirochika. 2005. Association of *VmybA1* gene expression with anthocyanin production in grape (*Vitis vinifera*) skin-color mutants. *J. Japan. Soc. Hort. Sci.* 74: 196–203.
- Kobayashi, S., M. Ishimaru, K. Hiraoka and C. Honda. 2002. *Myb*-related genes of the Kyoho (*Vitis labruscana*) regulate anthocyanin biosynthesis. *Planta* 215: 924–933.
- Kobayashi, S., Y. Nakamura, J. Kaneyoshi, H. Higo and K. Higo. 1996. Transformation of kiwifruit (*Actinidia chinensis*) and trifoliate orange (*Poncirus trifoliata*) with a synthetic gene encoding the human epidermal growth factor (hEGF). *J. Japan. Soc. Hort. Sci.* 64: 763–769.
- Koes, R., W. Verweij and F. Quattrocchio. 2005. Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci.* 10: 236–242.
- Lijavetzky, D., L. Ruiz-Garcia, J. A. Cabezas, M. T. De Andres, G. Bravo, A. Ibanez, F. Cabello, J. Ibanez and J. M. Martinez-Zapater. 2006. Molecular genetics of berry colour variation in table grape. *Mol. Gen. Genet.* 276: 427–435.
- Lloyd, A. M., V. Walbot and R. W. Davis. 1992. *Arabidopsis* and *Nicotiana* anthocyanin production activated by maize regulators *R* and *Cl*. *Science* 258: 1773–1775.
- Ludwig, S. R., B. Bowen, L. Beach and R. Wessler. 1990. A regulatory gene as a novel visible marker for maize transformation. *Science* 247: 449–450.
- Matsuta, N., H. Iketani and T. Hayashi. 1993. Transformation in grape and kiwifruit. p. 184–192. In: T. Hayashi, M. Omura and N. S. Scott (eds.). *Techniques on gene diagnosis and breeding in fruit trees*. Fruit Tree Research Station, Tsukuba.
- Mol, J., E. Grotewold and R. Koes. 1998. How genes paint flowers and seeds. *Trends Plant Sci.* 6: 212–217.
- Quattrocchio, F., J. F. Wing, H. T. C. Leppen, J. N. M. Mol and R. E. Koes. 1993. Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. *Plant Cell* 5: 1497–1512.
- Quattrocchio, F., J. F. Wing, K. van der Woude, J. N. M. Mol and R. Koes. 1998. Analysis of bHLH and MYB domain proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. *Plant J.* 13: 475–488.
- Ramsay, N. A. and B. J. Glover. 2005. MYB-bHLH-ED40 protein complex and the evolution of cellular diversity. *Trends Plant Sci.* 10: 63–70.
- Saito, N., F. Tatsuzawa, A. Nishiyama, M. Yokoi, A. Shigihara and T. Honda. 1995. Acylated cyaniding 3-sambubioside-5-glucoside in *Matthiola incana*. *Phytochemistry* 38: 1027–1032.