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The sweet potato *IbMYB1* gene as a potential visible marker for sweet potato intragenic vector system

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MYB transcription factors play important roles in transcriptional regulation of many secondary metabolites including anthocyanins. We cloned the R2R3-MYB type *IbMYB1* complementary DNAs from the purple-fleshed sweet potato (*Ipomoea batatas* L. cv Sinzami) and investigated the expression patterns of *IbMYB1* gene with *IbMYB1a* and *IbMYB1b* splice variants in leaf and root tissues of various sweet potato cultivars by reverse transcription-polymerase chain reaction. The transcripts of *IbMYB1* were predominantly expressed in the purple-fleshed storage roots and they were also detectable in the leaf tissues accumulating anthocyanin pigments. In addition, transcript levels of *IbMYB1* gene were up-regulated by treatment with methyl jasmonate or salicylic acid in leaf and root tissues of cv. White Star. To set up the intragenic vector system in sweet potato, we first evaluated the utilization of the *IbMYB1* gene as a visible selectable marker. The *IbMYB1a* was transiently expressed in tobacco leaves under the control of a constitutive cauliflower mosaic virus *35S* promoter, a root-specific and sucrose-inducible sporamin promoter, and an oxidative stress-inducible sweet potato anionic peroxidase2 promoter. We also showed that overexpression of *IbMYB1a* induced massive anthocyanin pigmentation in tobacco leaves and up-regulated the transcript levels of the structural genes in anthocyanin biosynthetic pathway. Furthermore, high-performance liquid chromatography analysis revealed that the expression of *IbMYB1a* led to production of cyanidin as a major core molecule of anthocyanidins in tobacco leaves. These results suggest that the *IbMYB1* gene can be applicable to a visible marker for sweet potato transformation with intragenic vectors, as well as the production of anthocyanin as important nutritive value in other plant species.

Abbreviations – AN2, anthocyanin2; ANS, anthocyanidin synthase; bHLH, basic helix-loop-helix; CaMV, cauliflower mosaic virus; cDNA, complementary DNA; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3H, flavanone-3-hydroxylase; GM, genetically modified; HPLC, high-performance liquid chromatography; MJ, methyl jasmonate; Or, orange; PAP1, PRODUCTION OF ANTHOCYANIN PIGMENT1; RT-PCR, reverse transcription-polymerase chain reaction; SA, salicylic acid; SPO, sporamin; SWPA2, sweet potato anionic peroxidase2; T-DNA, transfer DNA; TFs, transcription factors.

Introduction

Sweet potato (*Ipomoea batatas* (L.) Lam) is the seventh most important food crop in the world, after wheat, rice, maize, potato, barley and cassava (FAO 1993). It is used not only as a staple food, but it also an important industrial raw material for animal feed and alcohol production. Sweet potato is rich in secondary metabolites, especially antioxidant compounds including anthocyanins, carotenoids and vitamin C (Teow et al. 2007, Yoshinaga et al. 1999). Antioxidants have been attracted special attention because they can protect human body from oxidative stress which may promote aging and many diseases including cancer (Halliwell 2007, Luceri et al. 2008). Furthermore, anthocyanins are secondary metabolites known as flavonoids responsible for coloration of flowers, fruits and vegetables. Despite the importance of sweet potato as a food source, relatively little research has been carried out on sweet potato at the molecular level.

Flavonoid biosynthetic pathways appear to be mostly regulated at the transcriptional level (Quattrocchio et al. 2007, Taylor and Grotewold 2005). MYB transcription factors (TFs) play important roles in transcriptional regulation of diverse secondary metabolites (Jin and Martin 1999, Martin and Paz-Ares 1997, Quattrocchio et al. 2007, Stracke et al. 2001, Taylor and Grotewold 2005). Plant MYB TFs are classified into three major groups on the base of the sequence of the DNA-binding domain: R2R3-MYB, with two adjacent repeats; R1R2R3-MYB, with three adjacent repeats; a heterogenous group collectively referred to as the MYB-related proteins with a single MYB repeat (Jin and Martin 1999, Rosinski and Atchley 1998, Stracke et al. 2001). A specific subgroup of R2R3-MYB genes, including maize *C*1 (Paz-Ares et al. 1987) and *P1* (Grotewold et al. 1991), petunia anthocyanin2 (*AN2*) (Quattrocchio et al. 1999), Arabidopsis *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*) (*AtMYB75*, At1g56650) and *PAP2* (*AtMYB90*, At1g66390) (Borevitz et al. 2000), grapevine *VvMYB5a* (Deluc et al. 2006) and sweet potato *IbMYB1* (Mano et al. 2007), was shown to be responsible for the regulation of anthocyanin biosynthesis genes and the enhanced anthocyanin pigmentation in plant species.

Crop genetic engineering is based on the integration of foreign DNA into plant genomes. The lingering presence of foreign DNA, especially antibiotic marker or herbicide resistance and insect tolerance genes in genetically modified (GM) crops negatively affects public acceptance of GM foods and is often perceived as undesirable. Recent progress in designing vectors for *Agrobacterium*-mediated plant transformation has contributed to the development of the intragenic vector concept (Conner et al. 2007, Rommens 2004, Rommens et al. 2004, 2005, 2007). This involves identifying functional equivalents of vector components in the targeted plant genomes and using these plant-origin DNA sequences to assemble vectors for plant transformation without foreign DNA. One of the important requirements for intragenic vector system is a plant-derived selectable marker gene instead of bacterial selectable marker genes that provide resistance to antibiotics, herbicides or drugs. The *MYB* and basic helix-loop-helix (*bHLH*) transcription factor genes involved in anthocyanin biosynthetic pathways, and the orange (*Or*) gene responsible for carotenoid accumulation provide potential color markers that can be adapted to a visual identification system in plants (Chawla et al. 1999, Quattrocchio et al. 2007, Zhou et al. 2008a, b). A second important component of intragenic vector systems involves the regulatory elements of appropriate promoters and terminators from the target genome for transcriptionally regulating the gene of interest in the appropriate and desired manner. Finally, because *Agrobacterium*-derived transfer DNA (T-DNA) border sequences are widely used for plant transformation with foreign DNA, it is absolutely essential to find plant-derived replacements for the *Agrobacterium* T-DNAs (Conner et al. 2007, Rommens 2004, Rommens et al. 2005). In this context, we are currently exploring functional equivalents of important vector components to develop a sweet potato intragenic vector system.

Here, we describe the identification of *IbMYB1a* and *IbMYB1b* complementary DNA (cDNA) clones from the purple-fleshed storage roots of sweet potato (cv. Sinzami) and the potential of *IbMYB1a* as a useful visible marker gene for transformation approach in various plant species. Overexpression of *IbMYB1a* splice variant conferred an increased expression of a number of structural genes in anthocyanin biosynthetic pathway and resulted in anthocyanin pigmentation in tobacco leaves.

Materials and methods

Plant material

Sweet potatoes including cv. Yulmi, cv. Sinzami and cv. Sinhwangmi were obtained from plants grown at the Bioenergy Crop Research Center, National Institute of Crop Science, Rural Development Administration (RDA), Korea in the 2007 growing season. Storage roots were harvested approximately after 4 months of planting. Sweet potato (cv. White Star) was grown in a small pot in the growth chamber at 25 ± 3°C. For expression analysis of the *IbMYB1* gene in leaf and root tissues, fully expanded mature leaves were obtained from sweet potato vines at approximately 2 months after planting

the upper part of the vine with three to four young leaves into a small pot. Root tissues were also harvested from these sweet potato plants.

Plant treatments and RNA extraction

For hormone treatments, 2-month-old sweet potato plants were sprayed with solutions containing 0.01% (approximately 424 μ*M*) methyl jasmonate (MJ) (95% MJ solution, Sigma-Aldrich, St Louis, MO) or 100 μ*M* salicylic acid (SA) (Sigma-Aldrich) and water was used as control. Tissue samples were kept in the solutions for various periods, harvested and immediately immersed in liquid nitrogen. Total RNA was extracted from the frozen samples using the easy-spin Total RNA Extraction Kit (iNtRON, Daejeon, Korea). The concentration of RNA was quantified at 260 nm by spectrophotometric measurement.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

First-strand cDNA was synthesized from 2 μg of total RNA using the First-Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada) according to the manufacturer's instructions. Reverse transcription-polymerase chain reaction (RT-PCR) was performed in 20 μl reactions containing 2 μl of 1:10 diluted cDNA samples, 0.2 μ*M* of a pair of primers using the *Accel Taq* 2× PCR Pre-mix Kit (GenDocs, Daejeon, Korea) under the following conditions: an initial denaturation step at 95°C for 2 min followed by 30–35 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, polymerization at 72°C for 1 min and a final extension at 72°C for 10 min. Control RT-PCR was performed using a primer pair specific to the *α-tubulin* gene (accession no. DV037573) under the same condition. Ten μl of each RT-PCR product was analyzed on a 1.0% (w/v) agarose gel to visualize the amplified cDNAs. The primers used in this study are shown in Table 1: For *IbMYB1*, IbMYB1-specific-For and IbMYB1-specific-Rev primers; for *IbMYB1a*, IbMYB1a-specific-For and IbMYB1-specific-Rev primers; for *IbMYB1b*, IbMYB1-specific-For and IbMYB1b-specific-Rev primers; for *α-tubulin*, IbTubA-For and IbTubA-Rev.

Cloning and constructs of *IbMYB1* cDNA

The *IbMYB1* cDNA (accession no. AB258984) and genomic (accession no. AB258985) sequences were available in the GenBank database. The coding region of *IbMYB1* were amplified from the storage root of purple-fleshed sweet potato (cv. Sinzami) by RT-PCR using Advantage 2 polymerase mix (Clontech, Tokyo, Japan) with a *IbMYB1* gene-specific oligonucleotide primer set: IbMYB1-For and IbMYB1-Rev primers as shown in Table 1. The resulting *IbMYB1* cDNA products were cloned into the pGEM-T Easy vector according to the manufacturer's instructions (Promega, Madison, WI). The full-length cDNAs of *IbMYB1* gene were cloned and confirmed by sequencing. The genomic clone corresponding to the *IbMYB1* cDNA was amplified by Advantage 2 polymerase mix (Clontech) using genomic DNA prepared from cv. Sinzami with the *IbMYB1* gene-specific primer set. The PCR product was cloned into the pGEM-T Easy vector according to the manufacturer's instructions (Promega). The complete sequence of the *IbMYB1* genomic clone was determined by sequencing.

Table 1. Primer sequences used in the paper. Restriction sites in the primers are underlined

Primer name	Primer sequence
IbMYB1-specific-For	5′-CCCCTTAGAGCTGGATTGAATAG-3′
IbMYB1-specific-Rev	5′-TTAAAATAGTAAGATGAAAGTGAATTTAAC-3′
IbMYB1a-specific-For	5′-CTTAGGCAACAGGTGGTCGCTT-3′
IbMYB1b-specific-Rev	5′-CTGCTACGGTGGATCTCAGCAGT-3′
IbTubA-For	5′-CGGATTCAAGTGTGGTATCAATTA-3′
IbTubA-Rev	5′-AGTACACATAGCACAAGGAAAACG-3′
IbMYB1-For	5′-AGCTAAGAATTTCCGACACCCTTCAATA-3′
IbMYB1-Rev	5′-GTGAATTTAACGCTTAGCTTAACAGTTCT-3′
SPO-Pro-For (*Hin*dIII)	5′-AAGCTTTGCCAAACAGAGCCTAAATCCA-3′
SPO-Pro-Rev (*Pst*I)	5′-CTGCAGGGTGGCAGATGAGATGACAACTTGG-3′
SPO-Ter-For (*Kpn*I)	5′-GGTACCCAGTGAAAAGTGCCGGTTATGAGGT-3′
SPO-Ter-Rev (*Sac*I)	5′-GAGCTCGATTTGATTCTCTGAGTTGTTACCCA-3′
NtCHS-For	5′-ATAGGTTCTGATCCAATTCCAGAG-3′
NtCHS-Rev	5′-CTGTGGAGAACAACAGTCTCAACT-3′
NtCHI-For	5′-ACGGGTAAGCAATACTCAGAGAAG-3′
NtCHI-Rev	5′-TAGACTCCAATTTCTGGAATGGT-3′
NtF3H-For	5′-TATCCAATTCGGGCTAGAGACTAC-3′
NtF3H-Rev	5′-GGGTACACTATAGCTTCTGGTGCT-3′
NtDFR-For	5′-CCTAGCTTAATCACTGCACTTTCA-3′
NtDFR-Rev	5′-ATTGGTTGACTTTCCTGTACCATT-3′
NtANS-For	5′-CTGGCCTAAAATCCCTACTGACTA-3′
NtANS-Rev	5′-TCTCTTTATTCACAACCCCTCTGT-3′
NtACTIN-For	5′-TGGACTCTGGTGATGGTGTC-3′
NtACTIN-Rev	5′-CCTCCAATCCAAACACTGTA-3′

Construction of *IbMYB1*-expression cassettes

For construction of *SPO-IbMYB1a* expression cassette, the pGEM-T::IbMYB1a clone was first digested with

*Sph*I restriction enzyme and blunted by Klenow enzyme (TaKaRa, Shiga, Japan). After fill-in reaction, the *IbMYB1* cDNA was cut out by *Sal*I restriction enzyme and then introduced into the pUC19 vector (*Sal*I–*Sma*I restriction enzyme sites) to generate the pUC19::IbMYB1 clone. The sporamin (*SPO*) promoter (1124 bp) and *SPO* terminator (257 bp) of the SPO *gSPO-A1* gene (accession no. X13509) were amplified from genomic DNA of White star cultivar by Advantage 2 Polymerase Mix (Clontech) using a pair of SPO-Pro-For primer (introduction of a *Hin*dIII restriction site) and SPO-Pro-Rev primer (introduction of a *Pst*I restriction site), and a set of SPO-Ter-For primer (introduction of a *Kpn*I restriction site) and SPO-Ter-Rev primer (introduction of a *Sac*I restriction site). The resulting PCR products were introduced into the pGEM-T easy vector after gel purification. The *SPO* promoter region obtained after digestion with *Hin*dIII–*Pst*I restriction enzymes and the *SPO* terminator region produced from digestion with *Kpn*I–*Sac*I restriction enzymes were introduced into the pUC19::IbMYB1 clone. To get the *SPO-IbMYB1a* expression cassette, the *SPOpro-IbMYB1a-SPOter* construct after cutting with *Hin*dIII–*Sac*I restriction enzymes was integrated into the corresponding sites of the pCambia2300 binary vector, and thus generating the pCam-SPO-IbMYB1a.

For construction of *SPO-IbMYB1a* expression cassette, the pCam-SPO-IbMYB1a clone was digested using *Hin*dIII–*Spe*I restriction enzymes to replace *SPO* promoter with a stress-inducible sweet potato anionic peroxidase2 (*SWPA2*) promoter described by Kim et al. (2003). The pGEM-T::SWPA2 clone harboring the *SWPA2* promoter (−1314 bp) was first cut with *Spe*I restriction enzyme and then partial digested with *Hin*dIII restriction enzyme. The *SWPA2* promoter fragment (*Spe*I–*Hin*dIII) was integrated into the *Hin*dIII–*Spe*I restriction enzyme sites of the pCambia-*IbMYB1a-SPOter*, and thus generating the pCam-SWPA2-IbMYB1a.

For construction of *35S-IbMYB1a* expression cassette, the *IbMYB1a* fragment (*Sal*I–*Sph*I blunt) was ligated into the pRTL2 binary vector (*Xho*I–*Sma*I restriction enzyme sites) and the resulting 2*x*35*S* promoter-*IbMYB1a*-35*S* terminator construct was again excised with *Hin*dIII restriction enzyme and was introduced into the corresponding sites of the pCambia2300 binary vector, and thus generating the pCam-35S-IbMYB1a. The amplified PCR products were confirmed by sequencing and all the constructs were verified by restriction enzyme mapping. The constructed binary vectors and the empty control vector (pCambia2300) were transformed into the *A. tumefaciens* strain GV3101 via a freeze-thaw method (Höfgen and Willmitzer 1988).

Agrobacterium-mediated transient expression

For *Agrobacterium*-mediated transient expression assay, *Agrobacterium* (strain GV3101) harboring different constructs was grown overnight in Luria Bertani medium containing 25 μg ml^{-1} of rifampicin, 50 μg ml^{-1} of kanamycin and 150 μ*M* acetosyringone. Cells were collected by centrifugation (10 000 *g*), resuspended to an OD_{600} of 1.0 in infiltration medium (10 m*M* Mes, pH 5.6 plus 10 m*M* $MgCl_2$) with 150 μ*M* acetosyringone, and infiltrated into the leaves of 4-week-old tobacco (*Nicotiana benthamiana*) plants. Leaf discs were harvested at 5-days post-inoculation of *Agrobacterium* cells, immediately frozen in liquid nitrogen, and stored at −80°C until use.

For RT-PCR analysis, total RNA were isolated from the agro-infiltrated leaf tissues and cDNA was synthesized as described above. In this study, 25 cycles of PCR amplification were performed as described above. Primers for anthocyanin biosynthetic genes in tobacco were synthesized based on the GenBank nucleotide sequence database. Specific primers used in this study are shown in Table 1: For chalcone synthase (CHS) (accession no. AF311783), NtCHS-For and NtCHS-Rev primers; for chalcone isomerase (CHI) (accession no. AB213651), NtCHI-For and NtCHI-Rev primers; for flavanone-3-hydroxylase (F3H) (accession no. AB289450), NtF3H-For and NtF3H-Rev primers; for dihydroflavonol 4-reductase (DFR) (accession no. AB289448), NtDFR-For and NtDFR-Rev primers; for anthocyanidin synthase (ANS) (accession no. AB289447), NtANS-For and NtANS-Rev primers; for ACTIN, NtACTIN-For and NtACTIN-Rev primers.

HPLC analysis of anthocyanidins

Leaf sections of tobacco plants infiltrated with *A. tumefaciens* cells harboring the *SPO-IbMYB1a* expression cassette were taken at 5-days post-infiltration and leaf tissues (approximately 200 mg) were incubated with 500 μl of acidic methanol containing 1% (v/v) hydrochloric acid (HCl). To analyze anthocyanidins, anthocyanins were hydrolyzed into anthocyanidins and glycones by acidic methanol. An equal volume of water (200 μl) and 2.5 times of chloroform (500 μl) were added to separate anthocyanidins and lipophilic pigments. The supernatant was taken prior to analysis after centrifigation at 20 000 *g* for 10 min. Anthocyanin analysis was carried out according to Zhou et al. (2008a, b) with Shimadzu 10AD system (Shimadzu, Kyoto, Japan) on a C18 reverse-phase column (250 × 4.6 mm, Waters). The mobile phase solvents were composed of 0.1% (v/v) phosphoric acid (solvent A) and acetonitrile (solvent B). A gradient solvent system was composed of

ratios of solvent A to B of 95:5 (0–5 min), 95:5 to 90:10 (5–10 min), 90:10 to 83:17 (10–25 min), 83:17 to 77:23 (25–30 min), 77:23 to 50:50 (30–65 min) and 50:50 to 0:100 (65–69 min) at a flow rate of 1 ml min^{-1}. Anthocyanidins were monitored at 530 nm. Products were identified by comparison with authentic cyanidin and delphinidin (Sigma-Aldrich). To quantify total anthocyanin contents, absorbance was read at 530 and 657 nm by spectrophotometer. Total anthocyanins were quantified using the following equation described by Mehrtens et al. (2005): $Q_{\text{Anthocyanins}} = (A_{530} - 0.25 \times A_{657}) \times M^{-1}$, where $Q_{\text{Anthocyanins}}$ is the amount of anthocyanins, A_{530} and A_{657} are the absorptions at the indicated wavelengths and M is the fresh weight (in grams) of the plant tissues used for extraction.

Results

Alternative splicing of the *IbMYB1* gene produces two splice variants

The *IbMYB1* cDNA was previously reported to have an open reading frame of 750 bp with 249 amino acids, including 312 bp of the R2R3-type MYB domain at the N-terminus (Mano et al. 2007). In an attempt to amplify the *IbMYB1* cDNA from the purple-fleshed sweet potato (cv. Sinzami) using sequence-based primer sets at 5′- and 3′-untranslated region (UTR) sites, we found that two different sizes of transcripts are expressed in the storage roots of purple-fleshed sweet potato by RT-PCR. By cloning and sequencing the two expressed PCR products, we confirmed the identities of two types of spice variants for *IbMYB1* gene in the storage roots of purple-fleshed sweet potato. The two splice variants were named *IbMYB1a* (750 bp) and *IbMYB1b* (1063 bp). A sequence comparison of the cDNAs to the *IbMYB1* genomic clone that was amplified from the genomic DNA in the purple-fleshed sweet potato revealed the presence of introns in the *IbMYB1* gene. Schematic structure of the two splice variants detected for *IbMYB1* gene based on both database analysis and our experimental results is shown in Fig. 1. The coding region of *IbMYB1* gene consists of three exons (139, 130 and 481 bp) interrupted by two introns (131 and 313 bp). These data suggest that the *IbMYB1* gene undergoes alternative splicing, resulting in the two different splice variants. The *IbMYB1a* splice variant is produced by removal of the two introns, whereas the *IbMYB1b* splice variant is generated by retention of the second intron, causing premature termination of mRNA translation with 104 amino acids.

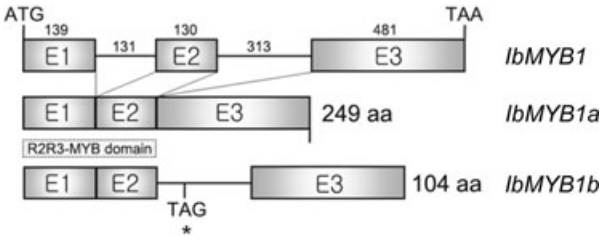

Fig. 1. Schematic representation of alternative splicing variants derived from *IbMYB1* gene. Alternative splicing of *IbMYB1* gene produces two splice variants, *IbMYB1a* and *IbMYB1b*. Position of R2R3 DNA-binding domain (R2R3-MYB domain) is indicated at the N-terminus of IbMYB1. Exons and introns are shown in boxes and lines, respectively, and the coding regions are indicated by gray-filled boxes. A star indicates the premature stop codon of *IbMYB1b* transcript. Translational start codon (ATG) and termination codon (TAA) of *IbMYB1* gene product were indicated.

IbMYB1 gene is expressed in leaf and storage roots of sweet potato

We investigated expression level of *IbMYB1* in leaf tissues of various cultivars of sweet potato by RT-PCR. The transcripts of *IbMYB1* gene were detectable in the leaf tissues of the three cultivars, White Star, Sinhwangmi and Sinzami (Fig. 2A). Under our RT-PCR conditions, the amount of *IbMYB1a* spice variant was more abundant, whereas the *IbMYB1b* transcript was expressed at very low level in these cultivars. No *IbMYB1* transcripts were detectable in the leaves of cultivar Yulmi (Fig. 2A). To further examine expression patterns of *IbMYB1* in relation to flesh colors in different cultivars, we performed RT-PCR on RNA samples from the storage roots of white-, purple- and orange-fleshed sweet potato (Yulmi, Sinzami and Sinhwangmi, respectively). Expression of *IbMYB1* transcripts with *IbMYB1a* and *IbMYB1b* variants was detected only in the storage roots of purple-fleshed sweet potato (Sinzami), but not in other cultivars (Fig. 2B). We also monitored whether accumulation of *IbMYB1* transcripts is enhanced by phytohormones in the white-fleshed cultivar White Star using *IbMYB1* gene-specific or its spice variant-specific primers (Fig. 3). The *IbMYB1* transcripts including *IbMYB1a* and *IbMYB1b* were detectable in untreated tissues of cv. White Star. Expression levels of both variant transcripts were induced by treatment of MJ in leaf and root tissues. We observed that the enhanced accumulation of *IbMYB1* transcripts by MJ came from the greater induction of the *IbMYB1a* splice variant, which exhibited marked up-regulation 12 h after the application of MJ. Similarly, SA induced high accumulation of the *IbMYB1a* transcript in leaf tissues of cv. White Star 12 h after SA treatment. These results suggest that *IbMYB1* gene, comprising *IbMYB1a*

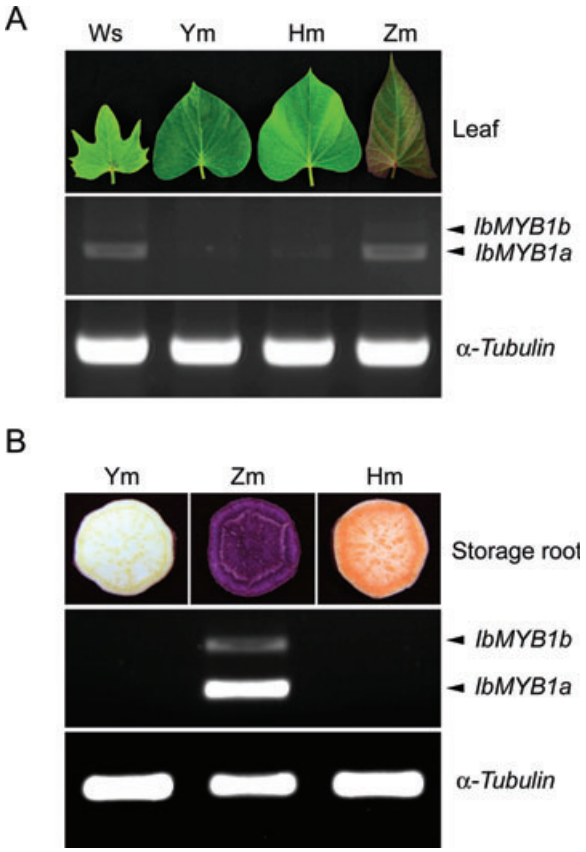

Fig. 2. Expression analysis of *IbMYB1* gene by RT-PCR. Expression patterns of *IbMYB1* gene in leaf tissues of various cultivars (A) and in the storage roots of white-, purple- and orange-fleshed cultivars (B). RT-PCR was carried out using the *IbMYB1*-specific primer sets. *α-Tubulin* gene primers were used to confirm that equal amounts of RNA were used for cDNA synthesis. Ws, White Star; Ym, Yulmi; Hm, Sinhwangmi; Zm, Sinzami. 30 cycles (A) and 25 cycles (B) of PCR amplification were performed as described in Materials and methods.

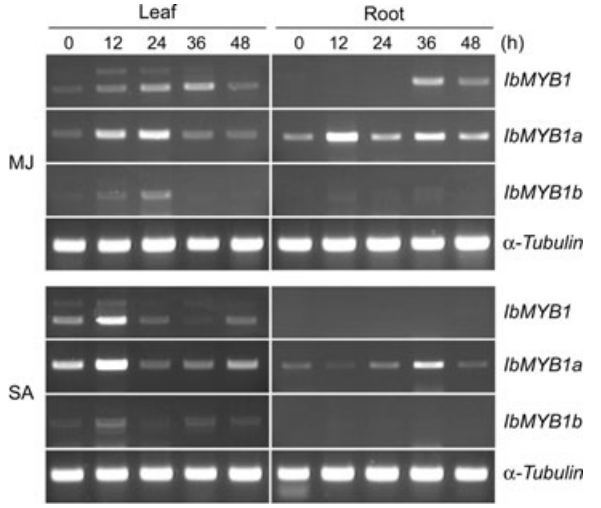

Fig. 3. Expression analysis of *IbMYB1* gene by RT-PCR. Expression patterns of *IbMYB1* gene in leaves and roots of white-fleshed cultivar (White Star) under the stress conditions. RT-PCR was carried out using the *IbMYB1* gene-specific and its splice variant-specific primer sets. *α-Tubulin* gene primers were used to confirm that equal amounts of RNA were used for cDNA synthesis. MJ, SA. 30–35 cycles of PCR amplification were performed as described in Materials and methods.

and *IbMYB1b* splice variants, is responsive to stress hormones of MJ and SA in leaf and root tissues.

Overexpression of *IbMYB1a* results in anthocyanin accumulation

In an effort to develop all-native DNA transformation vectors for sweet potato intragenic vector system, we have identified vector components including promoter, terminator and selectable marker gene from the sweet potato genome. To control the expression of the *IbMYB1a* splice variant, we examined the two promoters derived from sweet potato, a root-specific and sucrose-inducible SPO *SPO-A1* gene promoter (Hattori et al. 1990, Ohta et al. 1991) and an oxidative stress-inducible peroxidase *SWPA2* gene promoter (Kim et al. 2003). In addition, *SPO*-terminator (3′-untranslated region) was used to terminate the transcription of *IbMYB1* gene. As a positive control, the constitutive cauliflower mosaic virus (*CaMV*)35*S* promoter and terminator were used to drive the expression of *IbMYB1* gene. Therefore, the three promoter-*IbMYB1a* expression cassettes were constructed in the pCambia2300 binary vector (Fig. 4). We then evaluated the utilization of the *IbMYB1* gene as a visible selectable marker. To examine whether the promoters effectively induce the expression of *IbMYB1* gene, we conducted transient expression assays by infiltrating *A. tumefaciens* harboring each promoter-*IbMYB1a* expression cassette into *N. benthamiana* leaves. The expression of *IbMYB1a* under the control of 35*S*, *SPO* and *SWPA2* promoters conferred a visual pigmentation of anthocyanin accumulation in the leaf area infiltrated with *A. tumefaciens* cells. Pigmentation was evident 2 days after infiltration and became progressively stronger. Furthermore, the *35S-IbMYB1a* and *SWPA2-IbMYB1a* expression cassettes resulted in cell death-like phenotype after prolonged time in the agro-infiltrated leaf area. To address whether the visible symptom is related to an increased expression of *IbMYB1* gene, we performed RT-PCR analysis on RNA from leaf discs collected at the infiltration sites of *N. benthamiana* leaves. Both the 35*S* and *SPO* promoters induced high expression of *IbMYB1a* in leaf samples harvested at 5 days after agro-infiltration (Fig. 5B). Furthermore, we investigated whether an increased induction of *IbMYB1*

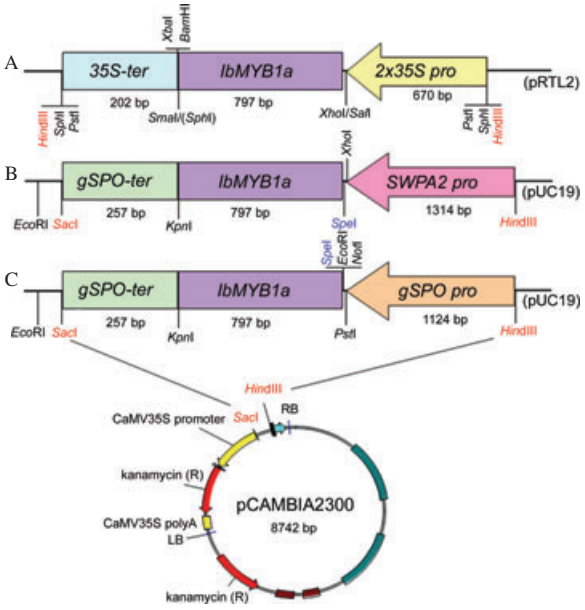

Fig. 4. Diagram of binary vector constructs harboring the promoter-*IbMYB1a* expression cassettes for plant transformation. Expression of *IbMYB1a* was driven by the control of a constitutive *CaMV 35S* promoter (A), a stress-inducible *SWPA2* promoter (B), or a root-specific and sucrose-inducible *SPO* promoter (C) in the pCambia2300 vector. The *CaMV 35S* terminator was replaced by sporamin terminator (*SPO-ter*) in the sweet potato promoter-*IbMYB1a* expression cassettes. The size of promoter, terminator and cDNA was shown in base pair (bp) and the restriction enzyme sites were indicated in name.

gene can induce the expression of a subset of anthocyanin biosynthetic genes in tobacco. Our RT-PCR analysis showed that an increased expression of the *IbMYB1* gene activated a number of structural genes, including *CHS, CHI, F3H*, *DFR* and *ANS* (Fig. 5B). *SPO-IbMYB1a* induced high expression of *CHS* and *DFR* transcripts, but somewhat low expression levels of *CHI, F3H* and *ANS* genes compared with the 35*S-IbMYB1a* control.

HPLC analysis of anthocyanidins in tobacco

To confirm whether the massive pigmentation observed in the leaves of tobacco after expression of *IbMYBa* by agro-infiltration is attributed to anthocyanin accumulation, we first measured total anthocyanin contents in leaf tissues showing pigmentation in tobacco. Almost 10-fold higher anthocyanin levels were detected in the leaf tissues agro-infiltrated with *SPO-IbMYB1a* expression cassette compared with the empty vector control (Fig. 6A). We further analyzed the anthocyanin pigments in the leaf tissues by HPLC (Fig. 6B). A single major peak at 16 min, corresponding to cyanidin based on the HPLC profile, was detected in the extracts from the agro-infiltrated leaf tissues with the *SPO-IbMYB1a* expression cassette (Fig. 6B). These results suggest that *SPO-IbMYB1a* expression in tobacco leaves led to the accumulation of cyanidin as a major core molecule of the anthocyanidins.

Discussion

In an effort to develop intragenic vectors for all-native DNA transformation of sweet potato, we have identified several important components including promoter, terminator and selectable marker gene from the sweet potato genome. We have shown that transient expression of R2R3-MYB *IbMYB1a* cDNA from purple-fleshed sweet potato was necessary and sufficient to induce up-regulation of a number of the structural genes in anthocyanin biosynthetic pathway and production of anthocyanin pigmentation in tobacco leaves. Our data suggest that the *IbMYB1* transcription factor provides a useful selectable marker for sweet potato transformation with intragenic vectors, as well as the production of anthocyanin as an important nutritive value in other plant species.

IbMYB1 gene is differentially expressed in various tissues of sweet potato

Mano et al. (2007) previously reported that the *IbMYB1* gene produces two transcripts of different sizes by alternative splicing in the tuberous roots of cv. Ayamurasaki (purple-fleshed sweet potato cultivar). We also observed that alternative splicing of *IbMYB1* gene was able to generate two different sizes of splice variants, *IbMYB1a* and *IbMYB1b* in the storage roots of purple-fleshed sweet potato, as well as in the leaf tissues of sweet potato. The smaller transcript (*IbMYB1a*) was mainly expressed in the storage roots and the leaves of sweet potato and was shown to be the mature transcript encoding 249 amino acids derived from the removal of two introns. In contrast, the larger transcript (*IbMYB1b*) was expressed at low level and might be produced because of the retention of the second intron from the *IbMYB1* gene. It is possible that the *IbMYB1b* transcript encodes a small MYB-related protein with 104 amino acid residues, which could function as a regulator. The small MYB-domain protein (IbMYB1b) lacking a transactivation domain might compete for DNA-binding activity with the mature MYB protein (IbMYB1a) that possess a transactivation domain and thus it could regulate transcriptional expression levels of any target genes. It is also possible that the small MYB-domain protein (IbMYB1b) interacts with other regulators and, because of the lack of a transcriptional activation domain, forms an inactive complex. However, we do

not exclude the possibility that *IbMYB1b* splice variant exists as only a non-functional pseudogene derived from *IbMYB1* gene.

IbMYB1 gene was predominantly expressed in the storage roots of purple-fleshed sweet potato and its transcripts were also detected at low level in the leaf tissues which accumulates anthocyanins (Fig. 2). In contrast, we did not detect the *IbMYB1* expression in the leaf tissues of cv. Yumli without anthocyanin accumulation. A slight expression of *IbMYB1* gene was detected in the leaf tissues of cv. Sinhwangmi (Fig. 2A). In cv. White Star, however, we cannot completely explain the reason why *IbMYB1* gene transcript was detectable in the leaf tissues without apparent anthocyanin pigment. One possibility is that environmental stress conditions such as high light and high temperature up-regulated the expression of *IbMYB1* gene in this cultivar. A large number of studies have shown that multiple environmental stress conditions can promote the production of anthocyanins in various plant species (Chalker-Scott 1999, Dixon and Paiva 1995). Such an increased pigmentation of anthocyanins probably results from the up-regulation of the R2R3-MYB TFs. The R2R3-MYB TFs were shown to respond to the phytohormones such as SA, abscisic acid and jasmonic acid (Abe et al. 2003, Lee et al. 2001, Raffaele et al. 2006). We also observed that *IbMYB1* gene was significantly induced by SA and MJ in both leaf and root tissues of White Star cultivar (Fig. 3). These results suggest that *IbMYBl* gene may play a role in SA- and/or MJ-mediated plant stress responses in sweet potato.

Overexpression of *IbMYB1a* leads to up-regulation of anthocyanin biosynthetic genes and anthocyanin accumulation

Ectopic expression of R2R3-MYB genes is well known to induce anthocyanin production in several plant species (Borevitz et al. 2000, Bovy et al. 2002, Deluc et al. 2006, Mano et al. 2007, Mathews et al. 2003, Peel et al. 2009, Rommens et al. 2008, Tohge et al. 2005, Zuluaga et al. 2008). For instance, overexpression of *PAP1/AtMYB75* gene in Arabidopsis, tobacco and tomato plants was reported to induce high accumulation of anthocyanins in all transgenic vegetative tissues (Borevitz et al. 2000, Xie et al. 2006, Zuluaga et al. 2008). In addition, Mano et al. (2007) showed that overexpression of *IbMYB1* gene induced exhibited ectopic pigmentation in seedlings, roots, leaves, stems, flowers and seeds of transgenic Arabidopsis and calli of transgenic sweet potato. They found that most of anthocyanin structural genes were abundantly expressed in the pigmented transgenic calli of sweet potato. Furthermore, by transcriptome analysis of 22 810 *Arabidopsis* genes, Zhou et al. (2008a, b) demonstrated that overexpression of the *PAP1/AtMYB75* gene in tobacco up-regulated transcript expression levels of 38 genes, among which 21 are involved in anthocyanin biosynthetic pathway from phenylalanine to anthocyanins. Recently, Rommens et al. (2008) demonstrated that tuber-specific expression of R2R3-type MYB *StMtf1* activated the phenylpropanoid biosynthetic pathway and accumulated various flavonols and anthocyanins.

Agrobacterium-mediated transient expression assays have been widely used for in vivo analysis of gene expression, gene function and gene silencing in tobacco leaves (Kapila et al. 1997, Schob et al. 1997, Yang et al. 2000). Our agro-infiltration assay has shown that transient expression of *IbMYB1a* under the control of 35*S*, *SPO* and *SWPA2* promoters in tobacco leaves resulted in the up-regulation of anthocyanin biosynthetic genes as well as the production of anthocyanin pigmentation in tobacco leaves, which do not accumulate anthocyanins under normal conditions (Figs 5 and 6). These results suggest that the structural genes of *NtCHS*, *NtCHI*, *NtF3H*, *NtDFR* and *NtANS* in the anthocyanin biosynthetic pathway are the possible targets of sweet potato *IbMYB1a* transcription factor, because their transcript levels were significantly increased compared with untreated tobacco leaves. It is conceivable that sweet potato *IbMYB1a* was able to replace any endogenous TFs in the leaves of tobacco which do not normally produce the anthocyanin pigments. Therefore, our data suggest that *IbMYB1a* is a key factor controlling the up-regulation of the structural genes in anthocyanin biosynthetic pathway.

Many of R2R3-MYB TFs are known to require the presence of a member of the bHLH coactivators and/or a subgroup of WD40 repeat proteins for their function in anthocyanin biosynthesis (de Vetten et al. 1997, Goff et al. 1992, Quattrocchio et al. 1999). The R2R3-MYB/bHLH physical interaction was verified to be mediated by the R3 repeat of the MYB domain and the N-terminal region of the bHLH factors, as reported for the maize *C*1 and *B* regulators (Goff et al. 1992), and the petunia *AN2* and *AN1* TFs (Quattrocchio et al. 1999). The petunia *AN11* WD40 protein was shown to modulate the activity of the R2R3-MYB or bHLH regulators (de Vetten et al. 1997). However, the expression of only the sweet potato *IbMYB1a* transcription factor was sufficient for heterologous activation of anthocyanin biosynthesis in tobacco. Because *IbMYB1a* transcription factor itself could activate the anthocyanin biosynthesis without additional regulatory partners, it provides advantages over other plant MYB TFs such as *C*1 and *AN2* which

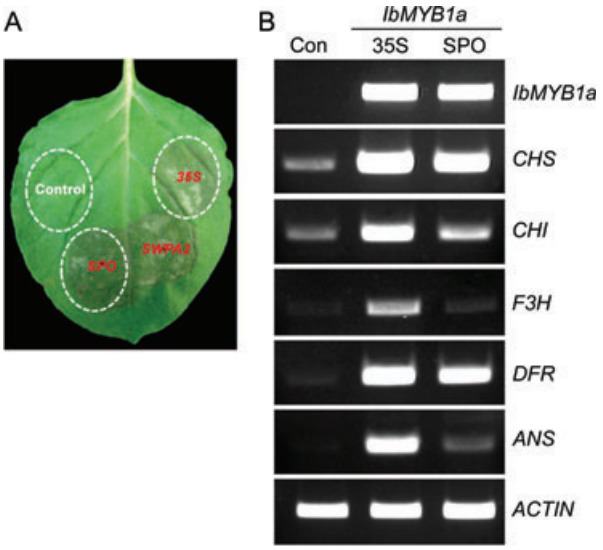

Fig. 5. Anthocyanin accumulation and up-regulation of anthocyanin biosynthetic genes by expression of *IbMYB1a*. (A) Transient agro-infiltration assay of the promoter-*IbMYB1a* expression cassettes in tobacco. *Agrobacterium* cells harboring each binary vector construct were infiltrated into the leaves of 4-week-old tobacco plants. Pictures were taken 5 days after agro-infiltration. Phenotype of leaves began to appear 2 days after agro-infiltration. Control, pCambia2300 empty vector; *SPO*, *SPO-IbMYB1a* expression cassette; 35*S*, *CaMV 35S-IbMYB1a* expression cassette; *SWPA2*, *SWPA2-IbMYB1a* expression cassette. (B) Expression analysis of anthocyanin biosynthetic genes by RT-PCR in tobacco. Leaf tissues corresponding to the agro-infiltrated area were taken 5 days after agro-infiltration for RT-PCR analysis. 25 cycles of PCR amplification were performed as described in the 'Materials and methods' section. *ACTIN* was used as a quantitative control. Tobacco anthocyanin biosynthetic genes used for RT-PCR are as follows: *CHS*, *CHI*, *F3H*, *DFR* and *ANS*.

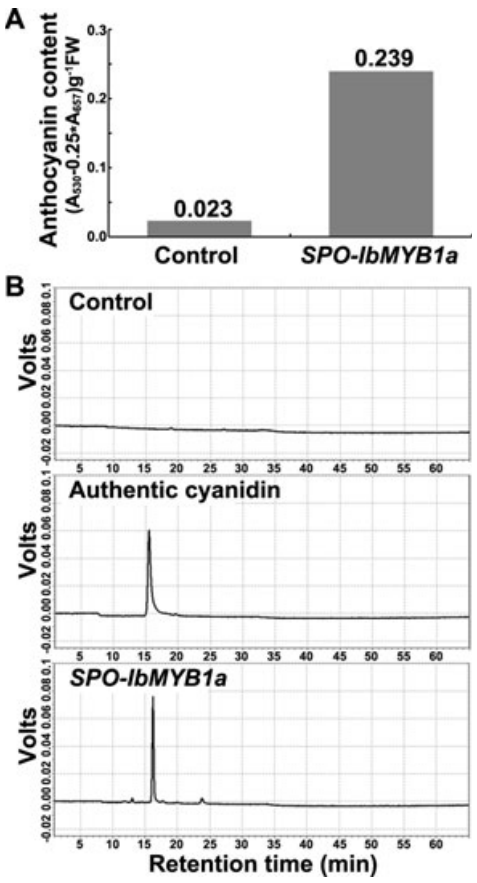

Fig. 6. Anthocyanin quantification and HPLC analysis of anthocyanidins in the leaves of tobacco. *Agrobacterium* cells harboring each binary vector construct were infiltrated into the leaves of 4-week-old tobacco plants. Leaf tissues corresponding to the infiltrated area (empty vector control and *SPO-IbMYB1a* expression cassette) were taken 5 days after agro-infiltration for spectrophotometric (A) and HPLC analysis (B). Anthocyanin contents were determined photometrically by reading absorption of the extracts at wavelengths of 530 and 657 nm as described in Materials and methods (A). Elution profiles of empty vector control and *SPO-IbMYB1a* extracts, and standard cyanidin were monitored at 530 nm.

require additional regulatory partners in anthocyanin applications.

One of the important components of binary vectors for plant transformation is a selectable marker gene. Antibiotic or herbicide resistance genes derived from bacteria are still widely used as selectable marker genes for plant transformation. Although the risks of using antibiotic or herbicide resistance genes in GM plants are considered to be very low, it is still considered important to avoid their presence in the final GM plants (Rosellini et al. 2007). Therefore, the use of an anthocyanin marker gene can offer an alternative to bacterial antibiotic or herbicide resistance genes. In this respect, the *IbMYB1a* gene provides a useful color selectable marker, because the *IbMYB1a* expression was shown to induce anthocyanin pigmentation in various plant species including sweet potato and Arabidopsis (Mano et al. 2007) and tobacco (Fig. 5). It is anticipated that cells expressing the *IbMYB1a* marker gene will be easily visible and detectable from untransformed cells by anthocyanin pigments in transgenic tissues and organs, such as calli and seeds. Furthermore, this approach not only provides visualization for selection of transformed tissues, but also promotes potential nutritive value by accumulation of anthocyanins in transgenic crops. The work reported by Rommens et al. (2008) supports the *IbMYB1* gene as a potential color selectable marker because they also observed that individual plant cells expressing *StMtf1* gene in potato started to accumulate purple pigments at 5 days after *Agrobacterium* infiltration. Therefore, we propose the use of the *IbMYB1a* gene as a visible marker for developing a sweet potato intragenic vector

system, because this transcription factor is a key regulator inducing anthocyanin biosynthesis in sweet potato.

Among the three promoters used to control the *IbMYB1a* expression in tobacco transient assay, we noted that *SWPA2-IbMYB1a* expression cassette led to the strongest pigmentation of anthocyanins in agro-infiltrated leaves of tobacco. The leaf area even exhibited cell death-like phenotype with extended time after agro-infiltration (data not shown). *Agrobacterium* infiltration itself would usually cause abiotic stress responses such as wounding triggered by infiltration in tobacco leaves. *SWPA2* promoter was shown to be highly inducible to oxidative stresses including wounding and H_2O_2 (Kim et al. 2003). Under the abiotic stress including wounding, *SWPA2* promoter was likely activated and its activation would induce strongly the overexpression of *IbMYB1* gene, thus causing the cell death-like phenotype of leaf tissue and it eventually became dry. *SPO* genes exhibit high expression in storage roots, as well as sucrose-inducible expression in vegetative tissues such as stem, leaf and petiole by addition of sucrose (Hattori et al. 1990, Ohta et al. 1991). Our transient assay demonstrated that *SPO* promoter effectively controls the expression of *IbMYB1a*, thereby activating the anthocyanin biosynthetic genes and inducing anthocyanin pigmentation in leaves of tobacco (Figs 5 and 6). Furthermore, because *SPO* promoter responds to exogenous sucrose, we anticipate it will provide a useful marker to select transformed tissues during plant transformation because sucrose is an integral component of plant tissue culture media. The data suggest that *SPO* promoter is an excellent candidate for root-specific and sucrose-inducible expression to regulate the *IbMYB1a* gene as a color marker gene to develop a sweet potato intragenic vector system.

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