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BRIEF COMMUNICATION

A putative *PhODO1* homologous MYB transcription factor gene, *MdMYBB*, is not involved in the regulation of aroma volatile biosynthesis in apple

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Abstract

To get deeper insight on the molecular mechanism underlying production of volatile compounds in apple (*Malus domestica* Borkh.), we performed the isolation and expression analysis of one *R2R3-type MYB* gene named *MdMYBB*. The amino acid sequence and the structural features of MdMYBB highly resembled those of PhODO1, which is a key regulator for floral scent biosynthesis in petunia. The expression of *MdMYBB* was repressed concomitantly with the inhibition of ethylene production, which regulates the volatile synthesis in apple. However, *MdMYBB* expression was not detected in the flesh from nearly ripened apple fruits, although the detection of exogenous volatiles had actually occurred in the same portion. In addition, overexpression of *MdMYBB* did not cause any induction of the volatile compounds in the transgenic tobacco lines. Thus, the features of *MdMYBB* were not in accordance with the aroma volatile emission, unlike the case of *PhODO1*, suggesting that *MdMYBB* may not be involved in the regulation of the biosynthesis for apple aroma volatiles. On the basis of the specific expression patterns, we discussed possible physiological roles of *MdMYBB* in apple.

Additional key words: ethylene, *Malus domestica*, mature fruit skin, *R2R3*-type *MYB* transcription factor.

Apple (*Malus domestica* Borkh.) volatile compounds are one of the important factors that determine apple fruit quality. The majority of aroma compounds are volatile esters and (*E*,*E*)-α-farnesene (Cunningham *et al*. 1986, Olias *et al*. 1992, Brackmann *et al*. 1993, Girard *et al*. 1995). On the molecular biological basis, two genes related to production of major volatiles have been isolated and characterized in apple so far. One is (*E*,*E*)-α-farnesene synthase (AFS), which catalyzes the final step in sesquiterpene biosynthesis, converting farnesyl diphosphate to (*E*,*E*)-α-farnesene (Pechous and Whitaker 2004, Green *et al*. 2007). The corresponding gene (*MdAFS1*) from apple is significantly up-regulated in response to ethylene (Pechous and Whitaker 2004). The other is alcohol acyltransferase (AAT), which catalyzes the final step in ester formation by linkage of an acyl moiety from acyl-CoA to the appropriate alcohol. Apple AAT (*MdAAT*), whose expression is also up-regulated by ethylene (Li *et al*. 2006), is expressed late in fruit ripening (Defilippi *et al*. 2005, Souleyre *et al*. 2005). On the other hand, a novel R2R3-type MYB transcription factor, *ODORANT1* (*PhODO1*), that regulates the coordinate expression of genes encoding enzymes involved in diurnal fluctuation of volatiles emission in petunia, has been recently identified (Verdonk *et al*. 2005). Thus, a regulatory factor other than biosynthetic genes such as *AFS* and *AAT*, should be also considered to elucidate the molecular basis of regulation of volatile biosynthesis. Therefore, we performed an isolation and expression analysis of a putative *PhODO1* homologous gene, *MdMYBB*.

Apple (*Malus domestica* Borkh.) cv. Tsugaru fruits were harvested at 16, 37, 60, 79, 102, and 116 d after full bloom (DAFB) along with the leaf, shoot, root, flower (balloon stage), and fruit flesh at 116 DAFB from the orchard of the National Institute of Fruit Tree Science in Morioka, Japan, in 2005. All the tissues and the entire skin,

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Abbreviations: AAT - alcohol acyltransferase, AFS - (*E*,*E*)-α-farnesene synthase, DAFB - days after full bloom, DAT - days after treatment, DIG - digoxigenin, 1-MCP - 1-methylcyclopropene, RACE - rapid amplification of cDNA ends, RT-PCR - reverse transcription-PCR.
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including 1 mm of the cortical tissue, were collected, immediately frozen in liquid nitrogen, and stored at -80 °C until needed for RNA isolation. Total RNA was isolated from various tissues and from the fruit skins using the hot borate method (Wan and Wilkins 1994). To isolate the cDNA of the apple *MYB* transcription factor gene, total RNA from fruit skin 116 DAFB was used. A partial cDNA fragment of the *MYB* transcription factor gene was amplified by reverse transcription-PCR (RT-PCR) as described by Kobayashi *et al*. (2002). Forward (5'-GGV AAG AGY TGY MGR YTS MSD TGG-3') and reverse (5'-GTK CCA IWM RTT YTT DAT YTC RTT-3') degenerate primers designed from a highly conserved region of plant R2R3-type MYB transcription factors were used for RT-PCR. The PCR product was cloned into the pCR2.1 vector using a TA cloning kit (*Invitrogen*, San Diego, CA, USA) and sequenced. To isolate the full-length cDNA of the apple *MYB* transcription factor gene, the 5'-/3'- rapid amplification of cDNA ends (RACE) was carried out using a *SMART RACE* cDNA amplification kit (*Clontech*, Palo Alto, CA, USA) with 5' and 3' gene-specific primers that were designed on the basis of the partial sequence of the *MYB* transcription factor gene. The cDNA ends were ligated into pCR-Blunt (*Invitrogen*) and sequenced. The resultant sequences were aligned to obtain full-length cDNA. The full-length gene was designated as *MdMYBB* and deposited in the DDBJ database under the accession number AB370230. For Northern blotting, total RNA (10 µg) isolated from various tissues and from the fruit skins was electrophoresed on a 1.2 % agarose/formaldehyde gel and blotted onto a nylon membrane (*Hybond-N*, *Amersham Biosciences*, Piscataway, NJ, USA) by capillary. The partial cDNA of the *MdMYBB* (positions 423-937 in the nucleotide sequence) was labeled with digoxigenin (DIG)-dUTP (*Roche Diagnostics*, Mannheim, Germany) and used as a probe. Prehybridization, hybridization, washing and detection were carried out according to Ban *et al*. (2007). For 1-methylcyclopropene (1-MCP, *SmartFresh*™, *AgroFresh*, Spring House, PA, USA) treatment, apple fruits were harvested at the pre-climacteric stage (3 d before commercial harvest) in 2007. 1-MCP treatment was carried out according to the method described by Tatsuki and Endo (2006). All samples were used for ethylene measurement, and the entire skin was collected and stored as noted above for RNA isolation.

Using RT-PCR and 5'-/3'-RACE, we identified one gene (*MdMYBB*) that encodes a putative R2R3-type MYB protein. The 937-bp full-length *MdMYBB* cDNA contained an open reading frame encoding 251 amino acid residues with the first in-frame ATG at nucleotide position 27 and a stop codon at position 782 (data not shown). A phylogenetic tree for plant R2R3-type MYB transcription factors, including MdMYBB, was constructed by means of neighbor-joining methods using the full-length deduced amino-acid sequences (Fig. 1). MdMYBB formed a cluster with PhODO1 and PbMYB (Fig. 1) and shared 91 % homology with the R2R3 DNA-binding domain of PhODO1 (data not shown). Moreover, the R2R3 DNA-binding domain of MdMYBB did not contain an R/B-like bHLH binding motif ($[D/E]Lx_2[R/K]x_3Lx_6Lx_3R$; Zimmermann *et al*. 2004), which was also the same feature as PhODO1 (Verdonk *et al*. 2005). Southern blot analysis using a non-conserved region (outside of the R2 and R3 DNA-binding sites) that included the 3'-untranslated region of *MdMYBB* showed single bands after *Bam*HI, *Eco*RI, or *Xho*I digestion, indicating a single copy of *MdMYBB* in the genome of 'Tsugaru' (data not shown). When the expression level of *MdMYBB* was analyzed using the same probe as that in Southern blot analysis, the *MdMYBB* transcript was detected only in the skins at 102 and 116 DAFB the signal at 116 DAFB being stronger than that at 102 DAFB (Fig. 2*A*). To investigate the regulation of *MdMYBB* by ethylene, ethylene biosynthesis was inhibited by 1-MCP. Five days after treatment (DAT), the ethylene concentration of 1-MCP-treated fruits was about 1/20 times lower than that of 1-MCP-untreated fruits (data not shown). Thus, 1-MCP effectively inhibited ethylene production. Accordingly, *MdMYBB* expressions were almost completely inhibited by 1-MCP at 5 DAT (Fig. 2*B*).

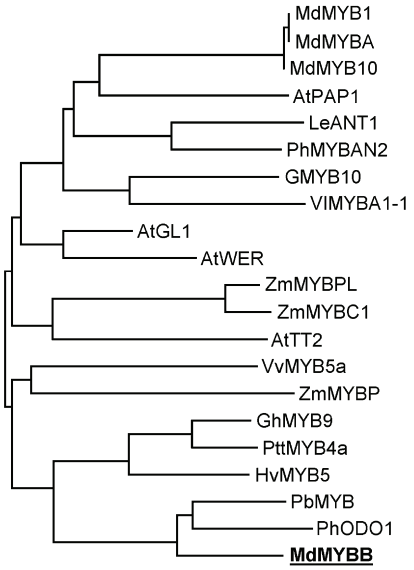

Fig. 1. A phylogenetic tree for plant R2R3-type MYB transcription factors, including the one isolated from apple in the present study, was constructed by Neighbor-joining methods using full-length deduced amino-acid sequences. Previously reported MYB transcription factor sequences were retrieved from the EMBL or GenBank databases (*M. domestica MdMYB1* [ABK58136], *M. domestica MdMYBA* [AB279598], *M. domestica MdMYB10* [ABB84754], *Gerbera hybrida GMYB10* [AJ554700], *Vitis labruscana VlMYBA1-1* [AB073010], *Lycopersicon esculentum LeANT1* [AAQ55181], *Petunia hybrida PhMYBAN2* [AF146702], *Arabidopsis thaliana AtPAP1* [AF325123], *A. thaliana AtGL1* [M79448], *A. thaliana AtWER* [AF126399], *Gossypium hirsutum GhMYB9* [AF336286], *Populus tremula × tremuloides PttMYB4a* [AJ567346], *Hordeum vulgare HvMYB5* [CAA50221], *Pimpinella brachycarpa PbMYB* [AF161711], *P. hybrida PhODO1* [AY705977], *Zea mays ZmMYBC1* [AF320614], *Z. mays ZmMYBPL* [L19496], *Z. mays ZmMYBP* [M73029], *A. thaliana AtTT2* [AJ299452], and *Vitis vinifera VvMYB5a* [AY555190]).

We first isolated an *MYB* transcription factor (*MdMYBB*) with high homology to *PhODO1*, a key regulatory gene for floral scent biosynthesis in petunia (Verdonk *et al*. 2005) from apple. In petunia, the expression level of *PhODO1* was positively correlated with the volatile emission, and its expression was detected in the corresponding tissues, where the volatile biosynthetic genes were expressed (Verdonk *et al*. 2005). The expression of *MdMYBB* was not detected in fruit flesh from ripened fruit at 116 DAFB but only in the skin from nearly ripened fruits. Since the expressions *pMdAFS1* and *pMdAAT* were detected in fruit flesh (data not shown) and exogenous volatiles from fruit flesh have been reported (Defilippi *et al*. 2005, Holland *et al*. 2005), the *MdMYBB* expression was expected in fruit flesh if this transcription factor is involved in the regulation of volatile biosynthesis. More directly, the T_0 generation of transgenic tobacco plants in which *MdMYBB* was constitutively expressed under the control of the cauliflower mosaic virus 35S promoter showed no significant differences from the wild-type lines in the endogenous volatile concentrations (data not shown). Considering the similarity of MdMYBB to PhODO1, we expected that *MdMYBB* could be involved in volatile biosynthesis of apple. However, the obtained results demonstrated that *MdMYBB* may not be involved in the regulation of apple volatile biosynthesis.

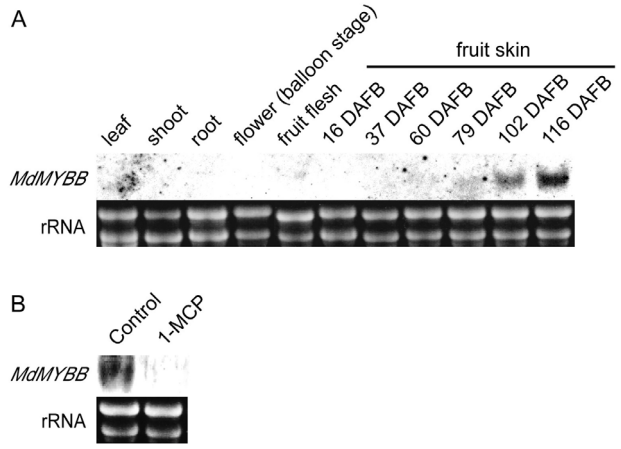

Fig. 2. *A* - Northern blot analysis of *MdMYBB* in several tissues and in fruit skins at different ripening stages. Each lane contains 10 μg of total RNA from each sample. *B* - Effect of 1-MCP treatment on *MdMYBB* expression in apple cv. Tsugaru. Each lane contains 10 μg of total RNA from each sample. In both (*A*) and (*B*), ethidium bromide staining showed equal loading of rRNA.

PhODO1 regulates the shikimate pathway: thus, biosynthesis of the compounds that located downstream in the pathway emerges as a target for regulation by *MdMYBB*. In apple, flavonols, condensed tannins, anthocyanins, dihydrochalcones, and hydroxycinnamic acids are detected as metabolites biosynthesized *via* the shikimate pathway (Treutter 2001, Takos *et al*. 2006). However, flavonols, condensed tannins, and dihydrochalcones are present in fruitlet skin, and hydroxycinnamic acids are detected in mature fruit flesh. Nevetheless, the transcripts of *MdMYBB* could not be detected in these tissues (Fig. 2*A*). In addition, it is known that anthocyanin biosynthesis in apple skin is regulated by *MdMYBA* (Ban *et al*. 2007), whose sequences are different from those of *MdMYBB*. Therefore, *MdMYBB* may not be involved in the regulation of the biosyntheses of these compounds. Since *MdMYBB* is expressed only in mature fruit skins and is regulated by ethylene (Fig. 2), it should be involved in the biosynthetic pathway of specific secondary compounds of mature skin other than the compounds noted above. Although extra work is needed to reveal the function of *MdMYBB*, the data herein could present a new insight on *R2R3-type MYB* transcription factors in apple.

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