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Complete List of Authors:	Sato, Hideto; Nagoya University, Graduate School of Bioagricultural Sciences Otagaki, Shungo; Nagoya University, Graduate School of Bioagricultural Sciences Ono, Yuma; Nagoya University, Graduate School of Bioagricultural Sciences Shiratake, Katsuhiro; Nagoya University, Graduate School of Bioagricultural Sciences Matsumoto, Shogo; Nagoya University, Graduate School of Bioagricultural Sciences
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Abstract:	Type 2 red-fleshed apples are attractive to consumers due to their sweet taste, which is not present in type 1 apples, in addition to their vivid color and the accumulation of health-promoting polyphenol compounds to a greater degree than in white apples. Although the red pigmentation of type 2 apples is caused by the expression of *MdMYB110a* at coloring initiation, the endogenous factors that induce the expression of *MdMYB110a* are poorly understood. In this study, we investigated the effect of two phytohormones, ethylene and abscisic acid (ABA), on *MdMYB110a* expression and flesh pigmentation in type 2 red-fleshed apples. Although induction of the expression of the ethylene biosynthetic gene *MdACO1* coincided with the onset of flesh pigmentation, no obvious correlation was found between treatment with ethylene or its inhibitor 1-methylcyclopropene and the expression pattern of *MdMYB110a*. However, homologues of ABA-induced genes in apple were differentially expressed between pigmented and nonpigmented flesh derived from a single fruit, and ABA treatment in type 2 red-fleshed fruits led to the upregulation of *MdMYB110a* expression and the enhancement of anthocyanin production. These results strongly suggest that ABA, not ethylene, induced upregulation of *MdMYB110a*, the gene responsible for the type 2 red-flesh trait.

Upregulation of *MdMYB110a* is responsible for ABA-mediated coloration of type 2 red-fleshed apples

H. Sato[+], S. Otagaki[+], Y. Ono, K. Shiratake and S. Matsumoto*

Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

[+]These authors contributed equally to this work.

(e-mail: shogo@agr.nagoya-u.ac.jp)

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*Author for correspondence.

ABSTRACT

Type 2 red-fleshed apples are attractive to consumers due to their sweet taste, which is not present in type 1 apples, in addition to their vivid color and the accumulation of health-promoting polyphenol compounds to a greater degree than in white apples. Although the red pigmentation of type 2 apples is caused by the expression of *MdMYB110a* at coloring initiation, the endogenous factors that induce the expression of *MdMYB110a* are poorly understood. In this study, we investigated the effect of two phytohormones, ethylene and abscisic acid (ABA), on *MdMYB110a* expression and flesh pigmentation in type 2 red-fleshed apples. Although induction of the expression of the ethylene biosynthetic gene *MdACO1* coincided with the onset of flesh pigmentation, no obvious correlation was found between treatment with ethylene or its inhibitor 1-methylcyclopropene and the expression pattern of *MdMYB110a*. However, homologues of ABA-induced genes in apple were differentially expressed between pigmented and nonpigmented flesh derived from a single fruit, and ABA treatment in type 2 red-fleshed fruits led to the upregulation of *MdMYB110a* expression and the enhancement of anthocyanin production. These results strongly suggest that ABA, not ethylene, induced upregulation of *MdMYB110a*, the gene responsible for the type 2 red-flesh trait.

Apple (*Malus* x *domestica* Borkh.) is one of the most widely planted fruit trees, and 77,573,743 metric tons were produced worldwide in 2016–17 (USDA, http://apps.fas.usda.gov/psdonline/). Although procyanidins and other polyphenols in apple possess health benefits for chronic diseases (Eberheadt et al., 2000; Wolfe et al., 2003), both growers and consumers desire new cultivars of higher quality. Red-fleshed apples may answer this demand because of their vivid new color caused by the high accumulation of the anthocyanin cyanidene-3-*0*-galactoside, as well as high contents of health-promoting phenolic compounds such as chlorogenic acid, phloridzin, quercetin, catechin, and epicatechin in the flesh (Sato et al., 2017). Anthocyanin possesses antioxidative activity (Butelli et al., 2008; Toufektsian et al., 2008), and is mainly present in the skin of white-fleshed apples. However, Japanese consumers peel their apples before eating.

Two types of red-fleshed apples exist: type 1 and 2. The coloring of type 1 is caused by the *MdMYB10* gene located on chromosome (ch.) 9, while the coloring of type 2 is caused by the *MdMYB110a* gene located on ch. 17. *MdMYB10* is an allele of *MdMYBA*/*MdMYB1*, which are responsible for the coloration of fruit skin (Lin-Wang et al., 2010), and possesses five tandem MdMYB10 binding sites in its promoter region. Because *MdMYB10* (*R_6:MdMYB10*) positively autoregulates its own transcription through this minisatellite-like structure, red coloration occurs not only in the flesh of type 1 apples but also in the fruit skin and vegetative tissues, including leaves and stems (Espley et al., 2009). However, *MdMYB110a* expression is observed only at the cortex, and the red coloration in type 2 apples is independent of the color of the fruit skin or vegetative tissues (Chagné et al., 2013; Umemura et al., 2013). In Japan, type 2 red-fleshed apple cultivars with low acidity and high sugar contents, including ‘Irodori’

('Jonathan' x 'Pink pearl'), 'Enbu' ('Irodori' x 'Fuji'), 'Nakano Shinku' ('Irodori' x 'Fuji'), 'Nakano no Kirameki' ('Irodori' x 'Orin'), 'Moonrouge' ('Irodori' x 'Fuji'), 'Ruby Sweet' (JP114069 x 'Fuji'), and 'Rose Pearl' ('Fuji' x 'Pink Pearl'), have recently been registered or submitted for registration. Type 2 apples are superior to type 1 when breeding for flesh use because a quantitative trait loci for astringent taste is tightly linked with the *R_6:MdMYB10* locus of type 1 apples (Volz et al., 2013).

The red coloration of apple skin has been shown to be affected by sunlight irradiation. UV-B irradiation of the red-skinned cultivar 'Tsugaru' induced the upregulation of *MdMYBA*, whereas MdMYB1 protein was degraded through a ubiquitin-dependent pathway under dark conditions (Ban et al., 2007; Li et al., 2012). However, although anthocyanin biosynthesis in red-fleshed apples was inhibited by a double-layered paper bagging treatment that almost completely shielded out light, some accumulation of anthocyanin was still observed in the flesh (Umemura et al., 2011; Honda et al., 2016). These results suggest that sunlight does not trigger the initiation of anthocyanin biosynthesis in red-fleshed apples.

Phytohormones are endogenous factors that also affect fruit ripening and coloring. In general, an overall decrease in auxin, gibberellin, and cytokinin, as well as a simultaneous increase in abscisic acid (ABA) and ethylene, are observed at the maturation and ripening stages of fruits (McAtee et al., 2013). Exogenous ethylene or ABA treatment in tomato, a climacteric plant like apple, accelerates color change from green to orange or red (Su et al., 2015; Mou et al., 2016). Using the green-skinned, white-fleshed apple cultivar 'Granny Smith', Lara & Vendrell (2000) revealed that both ABA and the ethylene biosynthetic enzyme ACC start to accumulate around 160 days after full bloom (DAFB) in the flesh, with peaks occurring at approximately 10 days

before commercial harvest for ABA and at commercial harvest for ACC. However, whether these two phytohormones contribute to triggering the induction of *MdMYB110a* expression and onset of pigmentation in type 2 red-fleshed apples remains unknown.

In the present study, we revealed that ethylene does not contribute to the induction of flesh pigmentation in type 2 red-fleshed apples through treatment with ethylene and its inhibitor 1-methylcyclopropene (1-MCP). In contrast, genes induced by ABA were more highly expressed in pigmented flesh than in unpigmented flesh, and exogenous ABA treatment induced the upregulation of *MdMYB110a* expression and increased anthocyanin accumulation. These results suggest that ABA, not ethylene, causes the coloring of apple flesh and upregulation of *MdMYB110a*.

MATERIALS AND METHODS

Plant material

Samples of the type 2 red-fleshed cultivars 'Nakano Shinku' and 'Nakano no Kirameki' were taken from collections at the Nagano Fruit Tree Experimental Station, Japan, and samples of 'Ruby Sweet' and 'Rose Pearl' were taken from collections at the Apple Research Center of the National Institute of Fruit Tree Science, Japan. Fruits at 60, 90, 120, 133, 150, and 180 DAFB during the 2013 and 2015 growing seasons were used for expression analyses of *MdMYB110a*, *MdACO1*, and *MdNECD1*. For the ethylene and 1-MCP treatments, fruits of 'Nakano Shinku' and 'Nakano no Kirameki' were collected at 90, 105, and 130 DAFB at Yoshiie Orchard, Japan during the 2014 growing season. 'Nakano no Kirameki,' which shows red and white colors on different sides, were harvested at 130 DAFB at Yoshiie Orchard, Japan during the 2014 growing

season and used for differential display analysis. For the ABA treatment, fruits of 'JPP35' and 'Nakano no Kirameki' were collected at 90 DAFB at Yoshiie Orchard, Japan during the 2016 growing season.

Total RNA extraction and cDNA synthesis

The cortices of peeled apple fruits were subjected to total RNA extraction, essentially as described in Chang et al. (1993). Total RNA extractions were performed on three independent apple fruits. cDNA was synthesized from 500 ng of total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) kit (TaKaRa Bio Inc., Japan) according to the manufacturer's protocol. Complete elimination of the genomic DNA from cDNA was confirmed by PCR using an *MdActin* primer pair spanning the intron sequence.

Quantitative real-time PCR (qPCR) expression analyses of MdMYB110a, MdACO1, and MdNECD1

Quantitative real-time PCR (qPCR) for quantification of the transcripts for *MdMYB110a*, *MdACO1*, *MdNECD1*, *MdMYC2*, and *MdJAZ2* was conducted using the SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Bio Inc., Japan) and Thermal Cycler Dice Real Time System TP800 (TaKaRa Bio Inc., Japan). Gene expression was detected using the primers from An et al. (2015, 2016), Hamada et al. (2015), Kondo et al. (2012), and Yang et al. (2013) (described in Supplemental Table S1). qPCR conditions were as follows: preheating for 30 s at 95 ^{o}C, followed by 50 cycles of 5 s at 95 ^{o}C, 10 s at 60 ^{o}C, and 15 s at 72 ^{o}C. The data were analyzed with Thermal Cycler Dice Real Time System Software Ver.3.00D (TaKaRa Bio Inc., Japan). The relative

expression of each sample was normalized to expression of the *MdActin* gene. qPCR expression analysis was performed on three independent fruits (three biological replicates) per stage, and data are shown as means ± SE. PCR efficiency was determined for each gene by analyzing serial dilutions of cDNA. All PCRs displayed efficiencies between 93% and 100%.

Ethylene and 1-MCP treatment

Fruits of 'Nakano Shinku' and 'Nakano no Kirameki' at 90, 105, and 130 DAFB were harvested in 2013 and 2015. All of the fruits were kept for 24 hours at room temperature, except for those in the 1-MCP treatment. In the 1-MCP treatment, SmartFresh^{SM} (Rohm and Haas, Japan) was sprayed on the fruits, which were left to stand for 24 hours at room temperature under 1000 ppb 1-MCP. After 24 hours, both control and 1-MCP treatment fruits were sprayed with water and left to stand for 48 hours in sealed containers at 17 ^{o}C under dark conditions. Fruits in the ethylene treatment were sprayed with Ethephon (Wako Pure Chemical Industries, Ltd., Japan) and left to stand for 48 hours under 1000 ppm ethylene. After each treatment, all fruits were kept at 17 ^{o}C under dark conditions, and flesh at 10, 25, and 40 days after treatment was analyzed. This experimental design is also summarized in Supplemental Fig. S1.

Differential display analysis

Differential display analysis was conducted using a GeneFishing^{TM} DEG Premix Kit (Seegene, Inc., Korea). A 3 µg total RNA sample from each part was mixed with 2 µl dT-ACP1 [5'CTGTGAATGCTGCGACTACGATXXXXX$(T)_{18}$-3'] to a final

concentration of 2.11 µM and adjusted to 9.5 µl with RNase-free water. Each tube stood for 3 min at 80 °C, cooled on ice for 2 min, and was then brought to a final volume of 20 µl with a final concentration of 1x RT buffer, 0.5 mM dNTP, 20 U RNaseOUT (Invitrogen, USA), 5 mM DTT, 1 µM dT-ACP1, and 200 U SuperScript® III Reverse Transcriptase (Invitrogen). The reaction was performed for 90 min at 42 °C before heating at 94 °C for 2 min to inactivate the reaction. First-strand cDNAs were chilled on ice for 2 min before the addition of 80 µl RNase-free water.

Second-strand cDNA synthesis was performed in a reaction mixture containing 3 µl of the first-strand cDNAs, 0.5 µM arbitrary ACP (one of the 10 arbitrary ACPs provided in the GeneFishing™ kit), 0.5 µM dT-ACP2 [5'CTGTGAATGCTGCGACTACGATXXXXX$(T)_{15}$-3'], 1x SeeAmp™ACP™ Master Mix, and 4 µl of distilled water, for a final volume of 20 µl. PCR conditions were as follows: preheating at 94 °C for 5 min, 50 °C for 3 min and 72 °C for 1 min for a single cycle, followed by 40 cycles of 94 °C for 40 s, 65 °C for 40 s, and 72 °C for 40 s, after which extension was performed at 72 °C for 5 min.

The PCR products were then electrophoresed on 2.0% agarose gel and stained with 1 µg/ml ethidium bromide. Potential differentially expressed genes (DEGs) were identified and excised from the agarose gels with a clean scalpel and purified using MagExtractor™ -PCR & Gel Clean up- (TOYOBO CO., LTD., Japan). The amplified potential DEG inserts were cloned into the T-vector pMD20 (TaKaRa Bio. Inc., Japan), and their sequences were determined by performing a dideoxy chain termination with M13M4 or M13RV primer on an Applied Biosystems 3130-Avant Genetic Analyzer (Life Technologies Japan Co., Ltd., Japan) using the BigDye Terminator v3.1Cycle Sequencing Kit (Life Technologies Japan Co., Ltd.).

Clone identity was determined according to sequence homology with the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/). The BLASTX algorithm was used for similarity searching against the translated amino acid sequences in the database.

Expression analyses (RT-PCR)

RT-PCR was performed using Ex Taq HS (TaKaRa Bio. Inc., Japan). The primer sets for the expression analysis are listed in Table 1. *MdActin* was used as a housekeeping gene to calculate the relative expression. PCR conditions were as follows: initial heating at 98 ^{o}C for 1 min, followed by 28 cycles for *MdMYB1R1*, 36 cycles for *MdNECD1*, 30 cycles for *MdMYB110a*, and 30 cycles for *MdActin*, each consisting of 98 ^{o}C for 10 s, 60 ^{o}C for 10 s, and 72 ^{o}C for 20 s.

ABA treatment

Fruits of 'JPP35' and 'Nakano no Kirameki' were harvested at 90 DAFB in 2015 and 2016. All of the fruits were cut into two portions: one was used as the control, and the other was used for ABA treatment. For ABA treatment, the cut surface of side was dipped into a mixture of 2.5 mM ABA (Sigma, USA) solution containing 0.6% methanol and 1% (v/v) Tween 20 and 2.5 mM fluridone containing 10% methanol and 1% (v/v) Tween 20. The sample was then left to stand for 96 hours at 17 ^{o}C under dark conditions. A 2-mm cut of flesh from the dipped surface was removed, and a 3-mm cut of flesh from the cut section was used for analysis.

RESULTS AND DISCUSSION

Ethylene and ethylene inhibitor treatment do not induce either MdMYB110a expression or flesh pigmentation in type 2 red-fleshed apples

Previously, we have clarified that pigmentation in the cortex of type 2 red-fleshed apples begins during the immature stage and increases with fruit maturity (Umemura et al., 2011). The red flesh pigmentation of type 2 apples progresses without ultraviolet rays, which are essential for the development of red skin color by upregulation of *MdMYB1* (Umemura et al., 2011). Because increases in ethylene and ABA occur during fruit maturation for climacteric and non-climacteric fruit, respectively, ethylene synthesis is essential for apple fruit ripening. Therefore, we first focused on ethylene as a trigger for upregulation of *MdMYB110a*.

We investigated the expression pattern of *MdACO1*, responsible for ethylene production, and *MdMYB110a* to determine the correlation between ethylene production and *MdMYB110a* upregulation. For all of the analyzed type 2 red-fleshed apples, except for 'Rose Pearl', both genes expressed in the flesh at some point between 90 and 120 DAFB, likely just before coloring time (Supplemental Fig. S2). In case of 'Rose Pearl', the expression of *MdACO1* was earlier than that of *MdMYB110a*. To confirm the upregulation of *MdMYB110a* by ethylene, we conducted ethylene treatment using 'Nakano Shinku' and 'Nakano no Kirameki' fruits at 90 and 105 DAFB. Both fruit pigmentation and *MdMYB110a* expression were not observed in fruits at 90 DAFB (results not shown). For fruits at 105 DAFB, expression of *MdMYB110a* associated with that of *MdACO1* was observed in both the control and ethylene treatment conditions (Fig. 1 A, B and D, E), but fruit pigmentation was not observed. Moreover, expression of *MdMYB110a* without *MdACO1* expression under the 1-MCP treatment was detected

in 'Nakano no Kirameki' flesh at 10 days after 1-MCP treatment (Fig. 1 F), and little to no induction of *MdMYB110a* was observed at 40 days after 1-MCP treatment, at which point ethylene production may restart (Fig. 1 C and F). Although we analyzed the contribution of exogenous ethylene against the upregulation of *MdMYB110a*, the correlation between ethylene production and *MdMYB110a* expression was unclear.

Cloning of genes using chimeric colored fruits through the differential display method

Because no pigmentation of flesh and unclear upregulation of *MdMYB110a* by ethylene were observed in the previous experiments, ethylene appeared not to be a clear trigger for flesh pigmentation. We then used a fruit of 'Nakano Shinku,' which has red and white flesh color on opposite sides, to isolate the genes responsible for pigmentation besides *MdMYB110a* (Fig. 2A).

As shown in Fig. 2B, the expression level of *MdMYB110a* in the red part was significantly higher than that in the white part. From this result, we expected that the expression level of other genes related to flesh coloring would also be upregulated, and we conducted differential display analysis to isolate the genes specifically expressed in the red part of the flesh. We succeeded in isolating four DEG bands of 250 bp, 500 bp, 600 bp, and 500 bp, which corresponded to transcription factor MYB1R1-like (XM_008362597), protein MIZU-KUSSEI 1-like (XM 008353781), vacuolar protein sorting-associated protein 2 homolog 1 (XM 008390315), and enoyl-[acyl-carrier-protein] reductase [NADH], chloroplastic-like (XM 008352051), respectively. We investigated the relationships of the expression pattern of *MdMYB1R1* with those of the gene for 9-cis-epoxycarotenoid dioxygenase (*MdNECD1*) responsible for ABA biosynthesis, and *MdMYB110a*, because the genes of MYB1R1-like and

protein MIZU-KUSSEI 1-like are known to be induced by ABA (Shin et al., 2011; Miyazawa et al., 2012). As shown in Fig. 3, expression of all genes was detected only in the red part of the fruits. Moreover, we found an ABRE motif, an ABA responsive element, at 1.4 kbp upstream, likely in the promoter region of *MdMYB110a* (Table 1). Based on these results, we investigated the degree of upregulation of *MdMYB110a* by ABA treatment.

Change of color and RNA expression levels of MdMYB110a and biosynthetic genes responsible for phenolic compounds in the cortex of type 2 red-fleshed apples by ABA treatment

After we succeeded in isolating ABA response genes using the differential display method, we focused on ABA as a trigger of pigmentation. As shown in Fig. 4, upregulation of *MdMYB110a* occurred in the flesh of ABA treatment fruits, and anthocyanin accumulation was higher than in the control, suggesting that ABA may induce *MdMYB110a* expression. The elevated expression pattern of *NCED1*, which catalyzes the first step in the ABA biosynthesis pathway, from 90 to 120 DAFB in the flesh of 'Nakano Shinku' and 'Nakano no Kirameki' fruits was in accordance with that of *MdMYB110a* (Fig. 5). ABA is responsible for the ripening and/or pigmentation of both non-climacteric and climacteric fruits, such as tomato and peach (Sun et al., 2012a, b; Zhang et al., 2009a). However, its role in apple is unclear, despite the ABA accumulation in white-fleshed apples that occurs from late maturity to harvesting time (Vendrell & Buesa, 1989; Lara & Vendrell, 2000). In the case of type 2 red-fleshed apples, our experimental data suggest that expression of *MdMYB110a* may be induced by ABA; previous research has found that the expression of *PacMYB10* and *LcMYB*,

responsible for the pigmentation of cherry flesh and lychee skin, respectively, is induced by ABA (Lai et al., 2014; Shen et al., 2014).

In the climacteric fruits tomato and banana, ABA has been shown to increase before the increase of ethylene, and exogenous application of ABA has been shown to induce ethylene through the biosynthesis genes (Jiang et al., 2000; Zhang et al., 2009b). However, because no upregulation by ethylene treatment was observed in red-fleshed apples, *MdMYB110a* upregulation induced by ABA may not occur through the ethylene pathway. Instead, jasmonic acid (JA) may be related to anthocyanin accumulation through the upregulation of *MdMYB110a*. Previous research has shown that ABA treatment of discs from the pulp of 'Tsugaru' apples at the climacteric stage results in increased JA concentration (Kondo et al., 2001). In addition to the ABA motif, the JA response motif was also found in the 5′ upstream region of *MdMYB110a* (Table 1), and we observed increased and decreased expression levels of *MdMYC2* and *MdJAZ2*, respectively, after ABA treatment of the flesh of 'Nakano no Kirameki' (Fig. 6). The results seemed to reflect JA accumulation by ABA in type 2 flesh because *MdMYC2* transcripts, which act as a master regulator of JA signaling, have been shown to be induced by methyl jasmonate treatment, and MdJAZ2 protein acts as a repressor protein in the JA signaling pathway (Kondo et al., 2001; An et al., 2016). To prove that *MdMYB110a* is upregulated by JA, an experiment with JA treatment is necessary.

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DISCLOSURE STATEMENT

No potential conflict of interest is reported by the authors.

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REFERENCES

An, J.P., Li, H.H., Song, L.Q., Su, L., Liu, X., You, C.X., Wang, X.F. & Hao, Y.J. (2016). The molecular cloning and functional characterization of MdMYC2, a bHLH transcription factor in apple. *Plant Physiology and Biochemistry*, *108*, 24–31. doi:10.1016/j.plaphy.2016.06.032

An, X.H., Tian Y., Chen, K.Q., Liu, X.J., Liu, D.D., Xie, X.B., Chen, C.G., Cong, P.H. & Hao, Y.J. (2015). *MdMYB9* and *MdMYB11* are involved in .,the regulation of the JA-induced biosynthesis of anthocyanin and proanthocyanidin in apples. *Plant & Cell Physiology*, *56*, 650–662. doi:10.1093/pcp/pcu205

Ban, Y., Honda, C., Hatsuyama, Y., Igarashi, M., Bessho, H. & Moriguchi, T. (2007). Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. *Plant & Cell Physiology*, *48*, 958-970. doi:10.1093/pcp/pcm066

Butelli, E., Titta, L., Giorgio, M., Mock, H.–P., Matros, A., Peterek, S., Schijlen, E.G.W.M., Hall, R.D., Bovy, A.G., Luo, J. & Martin, C. (2008). Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. *Nature Biotechnology*, *26*, 1301–1308. doi:10.1038/nbt.1506

Chagné, D., Lin-Wang, K., Espley, R.V., Volz, R.K., How, N.M., Rouse, S., Brendolise, C., Carlisle, C.M., Kumar, S., De Silva, N., Micheletti, D., McGhie, T., Crowhurst, R.N., Storey, R.D., Velasco, R., Hellens, R.P., Gardiner, S.E. & Allan, A.C. (2013). An ancient duplication of apple MYB transcription factors is responsible for novel red fruit-flesh phenotypes. *Plant Physiology*, *161*, 225–239. doi: 10.1104/pp.112.206771

Chang, S., Puryear, J. & Cairney, J. (1993). A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter*, *11*, 113–116.

Eberhardt, M.V., Lee, C.Y. & Liu, R.H. (2000). Antioxidant activity of fresh apples. *Nature*, *405*, 903–904.

Espley, R.V., Brendolise, C., Chagné, D., Kutty-Amma, S., Green, S., Volz, R., Putterill, J., Schouten, H.J., Gardiner, S.E., Hellens, R.P. & Allan, A.C. (2009). Multiple repeats of a promoter segment causes transcription factor autoregulation in red apples. *The Plant Cell*, *21*, 168–183. doi:10.1105/tpc.108.059329

Hamada, Y., Sato, H., Otagaki, S., Okada, K., Abe, K. & Matsumoto, S. (2015). Breeding depression of red flesh apple progeny containing both functional *MdMYB10* and *MYB110a_JP* genes. *Plant Breeding*, *134*, 239–246. doi:10.1111/pbr.12255

Honda, C., Iwanami, H., Naramoto, K., Maejima, T., Kanamaru, K., Moriya-Tanaka, Y., Hanada, T. & Wada, M. (2016) Thinning and bagging treatments and the growing

region influence anthocyanin accumulation in red-fleshed apple fruit. *The Horticulture Journal*, *advance publication*. doi:10.2503/hortj.OKD-010

Jiang, Y., Joyce, D. C. & Macnish, A.J. (2000). Effect of abscisic acid on banana fruit ripening in relation to the role of ethylene. *Journal of Plant Growth Regulation*, *19*, 106–111. doi:10.1007/s003440000011

Kondo, S., Tsukada, N., Niimi, Y. & Seto, H. (2001). Interactions between jasmonates and absisic acid in apple fruit, and stimulative effect of jasmonetes on anthocyanin accumulation. *Journal of the Japanese Society for Horticultural Science*, *70*, 546–552.

Kondo, S., Sugaya, S., Sugawa, S., Ninomiya, M., Kittikorn, M., Okawa, K., Ohara, H., Ueno, K., Todoroki, Y., Mizutani, M. & Hirai, N. (2012). Dehydration tolerance in apple seedlings is affected by an inhibitor of ABA 8-hydroxylase CYP707A. *Journal of Plant Physiology*, *169*, 234–241. doi:10.1016/j.jplph.2011.09.007

Lai, B., Li, X.–J., Hu, B., Qin, Y.–H., Huang, X.–M., Wang, H.–C. & Hu, G.–B. (2014). *LcMYB1* is a key determinant of differential anthocyanin accumulation among genotypes, tissues, developmental phases and ABA and light stimuli in *Litchi chinensis*. *PLoS One*, *9*, e86293. doi:10.1371/journal.pone.0086293

Lara, I. & Vendrell, M. (2000). Changes in abscisic acid levels, ethylene biosynthesis, and protein patterns during fruit maturation of 'Granny Smith' apples. *Journal of the American Society for Horticultural Science*, *125*, 183–189.

Li, Y.Y., Mao, K., Zhao, C., Zhao, X.Y., Zhang, H.L., Shu, H.R. & Hao, Y.J. (2012) MdCOP1 ubiquitin E3 ligases interact with MdMYB1 to regulate light-induced anthocyanin biosynthesis and red fruit coloration in apple. *Plant physiology*, *160*, 1011-1022. doi:10.1104/pp.112.199703

Lin-Wang, K., Bolitho, K., Grafton, K., Kortstee, A., Karunairetnam, S., McGhie, T.K., Espley, R.V., Hellens, R.P. & Allan, A.C. (2010). An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. *BMC Plant Biology*, *10*, 50. doi:10.1186/1471-2229-10-50

McAtee, P., Karim, S., Schaffer, R. & David, K. (2013). A dynamic interplay between phytohormones is required for fruit development, maturation, and ripening. *Frontiers in Plant Science*, *4*, Article 79 (1-7). doi:10.3389/fpls.2013.00079

Miyazawa, Y., Moriwaki, T., Uchida, M., Kobayashi, A., Fujii, N. & Takahashi, H. (2012). Overexpression of *MIZU-KUSSEI1* enhances the root hydrotropic response by retaining cell viability under hydrostimulated conditions in *Arabidopsis thaliana*. *Plant & Cell Physiology*, *53*, 1926–1933. doi:10.1093/pcp/pcs129

Mou, W., Li, D., Bu, J., Jiang, Y., Khan, Z.U., Luo, Z., Mao, L. & Ying, T. (2016). Comprehensive analysis of ABA effects on ethylene biosynthesis and signaling during tomato fruit ripening. *PLoS One*, *11*, e0154072. doi:10.1371/journal.pone.0154072

Sato, H., Otagaki, S., Saelai, P., Kondo, S., Shiratake, K. & Matsumoto, S. (2017). Varietal differences in phenolic compounds metabolism of type 2 red-fleshed apples. *Scientia Horticulturae*, *219*, 1–9. doi:10.1016/j.scienta.2017.02.041

Shen, X., Zhao, K., Liu, L., Zhang, K., Yuan, H., Liao, X., Wang, Q., Guo, X., Li, F. & Li, T. (2014). A role for PacMYBA in ABA-regulated anthocyanin biosynthesis in red-colored sweet cherry cv. Hong Deng (*Prunus avium* L.). *Plant & Cell Physiology*, 55, 862–880. doi:10.1093/pcp/pcu013

Shin, D., Moon, S.–J., Han, S., Kim, B.–G., Park, S.R., Lee, S.–K., Yoon, H.–J., Lee, H.E., Kwon, H.–B., Baek, D., Yi, B.Y. & Byun, M.–O. (2011). Expression of

StMYB1R-1, a novel potato single MYB-like domain transcription Factor, increases drought tolerance. *Plant Physiology*, *155*, 421–432. doi:10.1104/pp.110.163634

Su, L., Diretto, G., Purgatto, E., Danoun, S., Zouine, M., Li, Z., Roustan, J.P., Bouzayen, M., Giuliano, G. & Chervin, C. (2015). Carotenoid accumulation during tomato fruit ripening is modulated by the auxin-ethylene balance. *BMC Plant Biology*, *15*:114. doi:10.1186/s12870-015-0495-4

Sun, L., Sun, Y., Zhang, M., Wang, L., Ren, J., Cui, M., Wang, Y., Ji, K., Li, P., Li, Q., Chen, P., Dai, S., Duan, C., Wu, Y. & Leng, P. (2012a). Suppression of 9-cis-epoxycarotenoid dioxygenase, which encodes a key enzyme in abscisic acid biosynthesis, alters fruit texture in transgenic tomato. *Plant Physiology*, *158*, 283–298. doi:10.1104/pp.111.186866

Sun, L., Yuan, B., Zhang, M., Wang, L., Cui, M., Wang, Q. & Leng, P. (2012b). Fruit-specific RNAi-mediated suppression of *SlNCED1* increases both lycopene and ß-carotene contents in tomato fruit. *Journal of Experimental Botany*, *63*, 3097–3108. doi:10.1093/jxb/ers026

Toufektsian, M.–C., de Lorgeril, M., Nagy, N., Salen, P., Donati, M.B., Giordano, L., Mock, H.–P., Peterek, S., Matros, A., Petroni, K., Pilu, R., Rotilio, D., Tonelli, C., de Leiris, J., Boucher, F. & Martin, C. (2008). Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-rerefusion injury. *The Journal of Nutrition*, *138*, 747–752.

Umemura, H., Shiratake, K., Maejima, T., Komatsu, H. & Matsumoto, S. (2011). Practical breeding of red-fleshed apple: cultivar combination for efficient red-fleshed progeny production. *HortScience*, *46*, 1098–1101.

Umemura, H., Otagaki, S., Wada, M., Kondo, S. & Matsumoto, S. (2013). Expression

and functional analysis of a novel *MYB* gene, *MdMYB110a_JP*, responsible for red flesh, not skin color in apple fruit. *Planta*, 238, 65–76. doi: 10.1007/s00425-013-1875-3

Vendrell, M. & Buesa, C. (1989). Relationship between abscisic acid content and ripening of apples. *Acta Horticulturae*, *258*, 389–396.

Volz, R.K., Kumar, S., Chagné, D., Espley, R., McGhie, T.K. & Allan, A.C. (2013). Genetic relationships between red flesh and fruit quality traits in apple. *Acta Horticulturae*, *976*, 363–368.

Wolfe, K., Wu, W. & Liu, R.H. (2003). Antioxidant activity of apple peels. *Journal of Agricultural and Food Chemistry*, *51*, 609–614.

Yang, X., Song, J., Campbell-Palmer, L., Fillmore, S. & Zhang, Z. (2013). Effect of ethylene and 1-MCP on expression of genes involved in ethylene biosynthesis and perception during ripening of apple fruit. *Postharvest Biology and Technology*, *78*, 55–66. doi:10.1016/j.postharvbio.2012.11.012

Zhang, M., Leng, P., Zhang, G. & Li, X. (2009a). Cloning and functional analysis of 9-*cis*-epoxycarotenoid dioxygenase (NCED) genes encoding a key enzyme during abscisic acid biosynthesis from peach and grape fruits. *Journal of Plant Physiology*, *166*, 1241–1252. doi:10.1016/j.jplph.2009.01.013

Zhang, M., Yuan, B. & Leng, P. (2009b). The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *Journal of Experimental Botany*, *60*, 1579–1588. doi:10.1093/jxb/erp026

Figure legends

FIG. 1 Expression profiles of *MdMYB110a* and *MdACO1* on 'Nakano Shinku' (A, B, C) and 'Nakano no Kirameki' (D, E, F) flesh after ethylene (B, E) and 1-MCP (C, F) treatment. Control of no treatment was shown on A and D. DAT, days after treatment.

FIG. 2 A: Fruits of 'Nakano Shinku' used for differential display analysis. B: Expression level of *MdMYB110a* at red and white parts of 'Nakano Shinku'. Error bars indicate the standard deviations (n = 3).

FIG. 3 Expression analyses of *MdMYBR1*, *MdNECD1* and *MdMYB110a* at red and white parts of 'Nakano Shinku' flesh. MdActin was used as control.

FIG. 4 Corelation between antyocyanin accumulation and *MdMYB110a* expression by ABA treatment. Flesh of 'JPP35' (A) and 'Nakano no Kirameki' (B) were kept 96 hours at 17^{o}C with (circle) or without (square) ABA treatment.

FIG. 5 Expression profile of *MdNECD1* and *MdMYB110a* on flesh of 'Nakano Shinku' and 'Nakano no Kirameki' at 90, 120, 150 and 180 DAFB.

FIG. 6 Expression of *MdMYC2* and *MdJAZ2* on 'Nakano no Kirameki' after ABA treatment. Flesh of 'Nakano no Kirameki' was kept 96 hours at 17^{o}C with (ABA treatment) or without (control) ABA treatment.

Table 1 Motifs related to ABA and MeJA found in 1.5 kb up-stream region of *MdMYB110a*

Motif	Position	Strand	Sequence	Function
ABRE	-632	-	CACGTG	**abscisic acid**-responsive element
CGTCA-motif	-635	+	CGTCA	**MeJA**-responsive element
CGTCA-motif	-162	+	CGTCA	**MeJA**-responsive element

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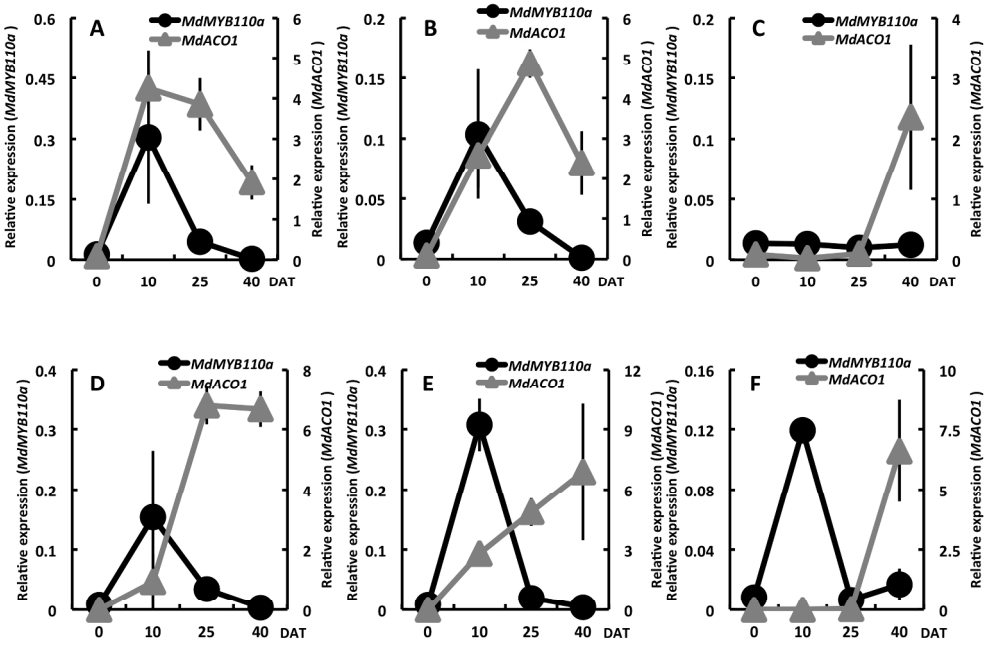

FIG. 1

FIG. 1. Expression profiles of *MdMYB110a* and *MdACO1* on 'Nakano Shinku' (A, B, C) and 'Nakano no Kirameki' (D, E, F) flesh after ethylene (B, E) and 1-MCP (C, F) treatment. Control of no treatment was shown on A and D. DAT, days after treatment.

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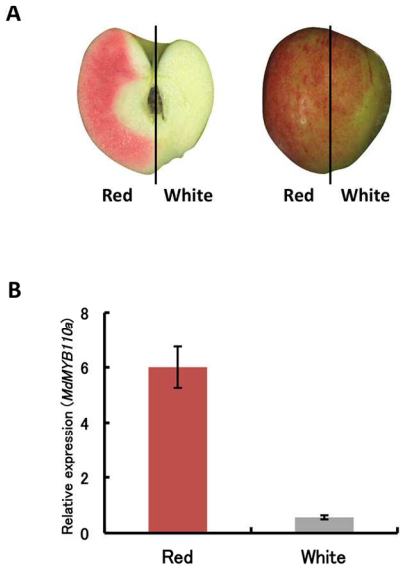

FIG. 2

Fig. 2. Fruits of 'Nakano Shinku' used for differential display analysis. B: Expression level of *MdMYB110a* at red and white parts of 'Nakano Shinku'. Error bars indicate the standard deviations (n = 3).

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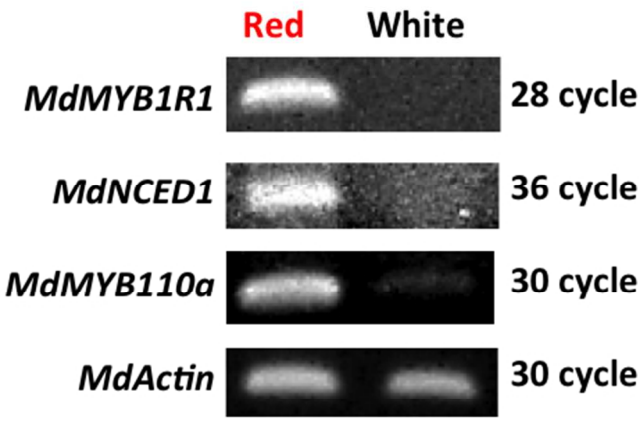

FIG. 3

FIG. 3. Expression analyses of *MdMYBR1*, *MdNECD1* and *MdMYB110a* at red and white parts of 'Nakano Shinku' flesh. *MdActin* was used as control.

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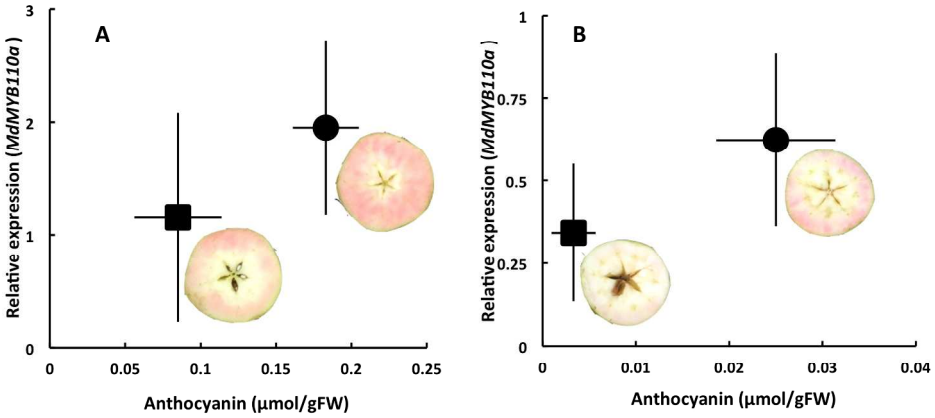

FIG. 4

FIG. 4. Corelation between antyocyanin accumulation and *MdMYB110a* expression by ABA treatment. Flesh of 'JPP35' (A) and 'Nakano no Kirameki' (B) were kept 96 hours at 17°C with (circle) or without (square) ABA treatment.

952x714mm (72 x 72 DPI)

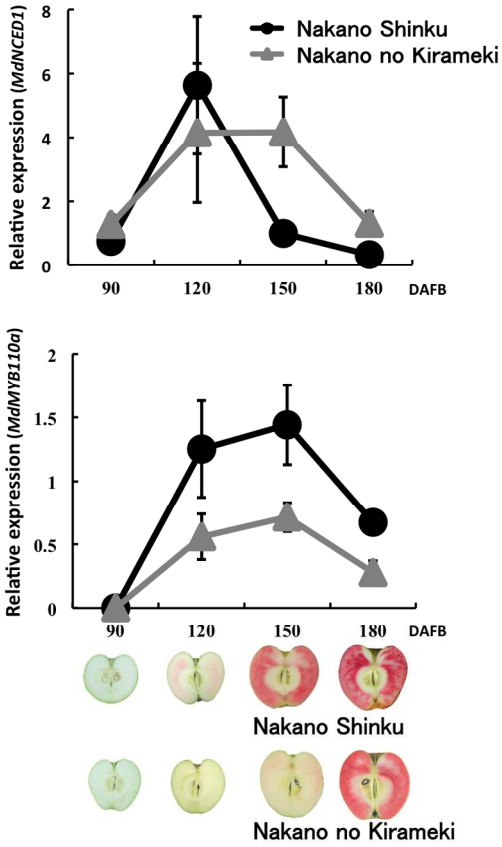

FIG. 5

FIG. 5. Expression profile of *MdNECD1* and *MdMYB110a* on flesh of 'Nakano Shinku' and 'Nakano no Kirameki' at 90, 120, 150 and 180 DAFB.

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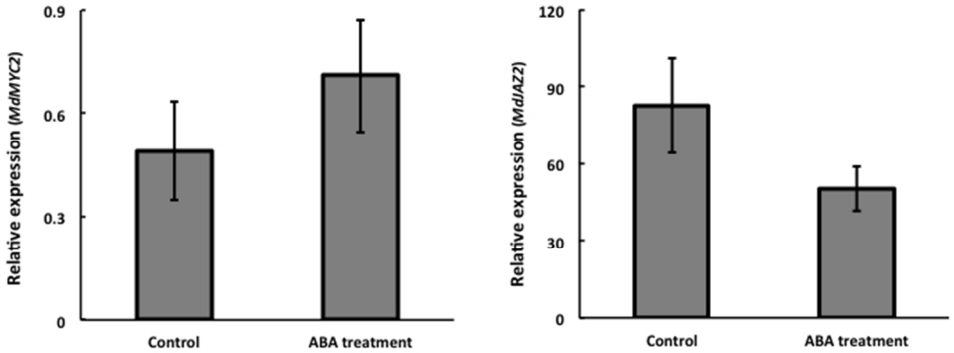


FIG. 6

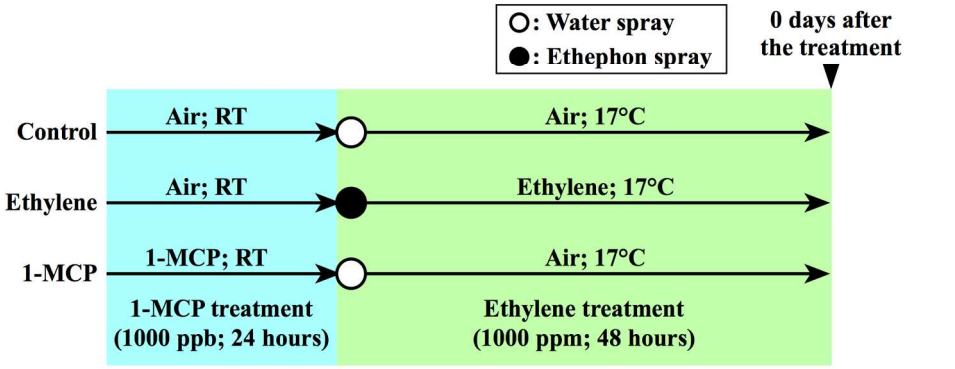
FIG. 6. Expression of *MdMYC2* and *MdJAZ2* on 'Nakano no Kirameki' after ABA treatment. Flesh of 'Nakano no Kirameki' was kept 96 hours at 17°C with (ABA treatment) or without (control) ABA treatment.

254x190mm (72 x 72 DPI)

Table S1 Primers used in this study

Gene name	Accession No.	Forward Primer	Reverse Primer	Reference
MdMYB110a	AB743999	AAGACCTTGTTAGAAGACGACGA	TGCCGACTGTTGCATATCAT	Hamada et al., 2015
MdACO1	DQ137850	ATCAATGATGCTTGTGAGAACTG	GGTCTTCTTGTAGTGATCCTTGG	Yang et al., 2013
MdMYB1R1	XM_008362597	AACTCAACGACGGACACATC	TCAAGCGACACTGATCATGC	This article
MdNCED1	AB593328	GTATCACGTCCAAATCACTGAAAC	ATTTGAGGTATGGCTTCTGAACG	Kondo et al., 2012
MdMYC2	NM_001328944	TTGGAGGTCGGTTCTTGGC	GGGCTTGGGTTCTCGGATAAG	An et al., 2016
MdJAZ2	KU179650	CGTGGAACAATGTCAAGAC	CAGCACTAGAGGAGTTAGAAC	An et al., 2015
MdActin	CN938023	CCTTCGTCTTCGGCTTGTTC	GGAGCATCATCACCAGCAAA	This article

254x366mm (72 x 72 DPI)



SUPPLE FIG. S1

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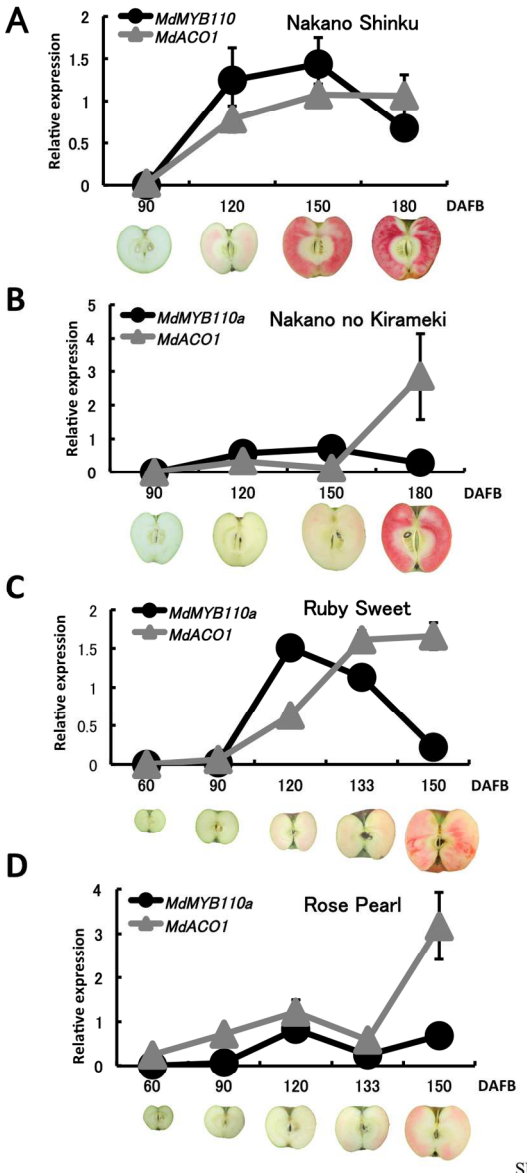

SUPPLE FIG. S2

549x793mm (72 x 72 DPI)

SUPPLE Figure legends

SUPPLE FIG. S1 Experimental design for the ethylene and 1-MCP treatment.

SUPPLE FIG. S2 Expression profile of *MdMYB110a* and *MdACO1* on flesh of 'Nakano Shinku' (A), 'Nakano no Kirameki (B), 'Ruby Sweet' (C) and 'Rose Pearl' (D) at (60), 90, 120, 150 and 180 DAFB.