Journal of Horticulture
ISSN: 2376-0354

Kikuchi et al., J Hortic 2017, 4:4
DOI: 10.4172/2376-0354.1000211

Research Article Open Access

Practical DNA markers to Estimate Apple (*Malus* × *domestica* Borkh.) Skin Color, Ethylene Production and Pathogen Resistance

Teppei Kikuchi#, Ichiro Kasajima#, Mizuki Morita and Nobuyuki Yoshikawa*

Faculty of Agriculture, Iwate University, Morioka, Iwate, Japan
#These authors equally contributed to this work.

Abstract

Determination of agronomically important traits with DNA markers in genetically segregating populations is a key to high-throughput breeding of apple trees. In the present report, we first estimated usefulness of four PCR-based DNA markers of apple in a set of 22 apple cultivars, which include popular cultivars in Japan such as 'Fuji', 'Orin', and 'Shinano Gold'. The four DNA markers are reported to determine fruit storage life (ethylene production), fruit skin color, Alternaria resistance, and scab resistance. The target genes/locus of these DNA markers are *MdACS1* gene, *MdMYB1* gene, *MdAlt* locus, and *MfVf* gene. The types of these DNA markers are SSLP, dCAPS, allele-specific, and gene-specific, thus the markers were designated MdACS1-SSLP, MdMYB1-dCAPS(BstEII), MdAlt-AS, and MfVf-GS, respectively. DNA band patterns after gel electrophoresis were reasonable and consistent with previous reports for MdACS1-SSLP and MfVf-GS markers. On the contrary, band patterns of the MdAlt-AS marker were not necessarily consistent with Alternaria tolerance of the apple cultivars. It was also quite difficult to determine genotypes from the band patterns of the *Bst*EII-based dCAPS marker MdMYB1-dCAPS(BstEII). Attachment of DS6 adaptor sequence to the forward primer improved genotype determinations, by making clear difference between the sizes of allelic DNA bands. Further improvement of this DNA marker was also achieved by the MdMYB1-CAPS(PmlI) marker, which is a *Pml*I-based CAPS marker. *MdMYB1* genotypes determined by the MdMYB1-CAPS(PmlI) marker was consistent with apple skin colors and their genetic segregations.

Keywords: *Alt*; CAPS marker; DS4 adaptor; *MdACS1*; *MdMYB1; Vf*

Abbreviations: ALSV: Apple Latent Spherical Virus; bp: Base Pairs; CAPS: Cleaved Amplified Polymorphic Sequence; CTAB: Cetyltrimethylammonium Bromide; dCAPS: Derived Cleaved Amplified Polymorphic Sequence; DS6: Dissimilar Synthetic DNA Sequence 6; EDTA: Ethylenediaminetetraacetic Acid; kb: Kilo-Bases; PCI: Phenol-chloroform-isoamylalcohol; PCR: Polymerase Chain Reaction; PVPP: Polyvinyl Poly Pyrrolidone; SNP: Single Nucleotide Polymorphism; SSR: Simple Sequence Repeat; Tm: Melting Temperature; Tris: Tris(hydroxymethyl) aminomethane.

Introduction

New apple cultivars are usually bred by crossing between existing apple cultivars. New apple cultivars are also often selected from apple seeds generated by random pollination, whose male parents are not clear during breeding process. Simple selection of progeny plants showing favorable traits from segregating populations would have been usually adopted, but this is somewhat inefficient way of breeding. For example, wide area of apple orchard is necessary if all the progeny apple plants are grown and their phenotypes, including fruit qualities, are tested. The screening process also takes great time and labor, considering the long juvenile phase (5-12 years) of apple seedlings [1,2]. Estimation process of the phenotypes may also need time, labor, or cost, depending on the types of estimated phenotypes. This is why DNA markers which are genetically linked to agriculturally important traits are developed in many crops, including apple. By using DNA markers, breeders can select promising cultivars at the juvenile phase, then further estimate these restricted number of plants in the second round of screening [3].

As being one of the most produced fruits in the world, relatively large number of agronomically important DNA markers is reported in apple, among the other fruit trees (e.g. http://www.naro.affrc.go.jp/genome/database/kaju/ringo/index.html, in Japanese). For example, the SSR (simple sequence repeat) -type DNA marker NZmsEB119405 for *Cg* locus estimates crown gall resistance of apple rootstock cultivars [4]. Another SSR-type DNA markers such as Mdo.chr10.14 for the *Co* locus is genetically linked to the columnar tree shape of apple scion cultivar 'Wijcik' [5]. We have also recently developed a sequencing-based DNA marker *APPLid* for determination of apple *S* alleles [6]. In the present study, we focused on other four DNA markers, because these four markers seem to be genetically linked to agricultural traits of any apple scion cultivars. These DNA markers target *MdACS1* gene, *MdMYB1* gene, *MdAlt* locus, and *MfVf* gene, respectively. Linkage between marker genotypes and phenotypes observed in 22 apple cultivars were re-estimated in the present study, to verify applicability of these markers in apple breeding, especially in Japanese apple cultivars.

MdACS1 gene encodes 1-aminocyclopropane-1-carboxylic acid synthase which catalyzes ethylene production in apple fruit [7-9]. In comparison with the functional *MdACS1-1* allele, a 138-bp (base pairs) insertion is observed in the promoter region of the non-functional *MdACS1-2* allele, which may strongly inhibit transcription of this gene. Thus apple, a typical climacteric (ethylene-regulated) fruit, enjoys low level of ethylene production and long storage life, when produced on cultivars harboring homozygous *MdACS1-2* alleles such as 'Fuji' [10-12].

***Corresponding author:** Nobuyuki Yoshikawa, Laboratory of Plant Pathology, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka, Iwate 020-8550, Japan, Tel/Fax: +81-19-621-6150; E-mail: yoshikawa@iwate-u.ac.jp

Received October 09, 2017; **Accepted** October 21, 2017; **Published** October 30, 2017

Citation: Kikuchi T, Kasajima I, Morita M, Yoshikawa N (2017) Practical DNA markers to Estimate Apple (*Malus* × *domestica* Borkh.) Skin Color, Ethylene Production and Pathogen Resistance. J Hortic 4: 211. doi: 10.4172/2376-0354.1000211

Apple skin colors (red or non-red) are regulated by *MdMYB1* gene, encoding R2R3-type MYB transcription factor [13]. Gene expressions in the pathway of anthocyanin synthesis and anthocyanin accumulation in the skin are high when the strong allele *MdMYB1-1* is possessed by apple cultivars such as 'Fuji', but they are low when only weak alleles *MdMYB1-2* and *MdMYB1-3* are possessed by apple cultivars such as 'Golden Delicious'. The strong *MdMYB1-1* allele is experimentally discriminated from the other alleles by using a dCAPS (derived cleaved amplified polymorphic sequence) marker, based on the restriction enzyme *Bst*EII.

Resistance to the diseases, 'Alternaria leaf blotch' and 'scab', can be estimated by PCR (polymerase chain reaction) amplification of *MdAlt* locus or *MfVf* gene, respectively. The *MdAlt* locus was detected by genetic comparison between a susceptible cultivar 'Starking Delicious' and a resistance cultivar 'Jonathan' [14]. The dominant allele *Alt* causes susceptibility to fungus *Alternaria alternata* (apple pathotype) to cause the disease Alternaria leaf blotch, whereas the recessive allele *alt* does not cause susceptibility (resistant). These dominant and recessive alleles are discriminated by the DNA marker DR033892: a set of three primers generates a 692-bp DNA fragment from *alt* allele, and a 429-bp fragment from *Alt* allele [15]. The *MfVf* gene (usually described '*Vf*') is derived from a crabapple species *Malus floribunda* '821' [16,17]. *MfVf* is a receptor-like gene, and the most popular genetic resource for resistance to fungus *Venturia inaequalis*, causing scab, among the other resistance genes. Possession of *MfVf* gene in apple cultivars like 'Prima' can be checked by gene-specific PCR amplification of a 469-bp DNA fragment using the marker ACS-9 [18].

When these four PCR markers were tested and re-examined for genomic DNAs of randomly selected 22 apple cultivars, the markers for *MdACS1* and *MfVf* amplified clear DNA fragments, with consistent band patterns. We also found that the band pattern of the *MdAlt* locus was not necessarily consistent with Alternalia resistance, whose reasons will be discussed. Both amplification of DNA fragments and discrimination between alleles were difficult with the reported primer set for *MdMYB1* gene, then we developed improved primer sets which clearly determined apple skin colors.

Materials and Methods

Plant materials

Twenty-two cultivars of apple (*Malus* × *domestica* Borkh.) were used in this study. Young grafted plants of 'Fuji', 'Orin' and 'Haruka' were purchased from plant supplier and grown in open field. Young expanding leaves of 'Golden Delicious', 'Indo', 'Starking Delicious', 'Ralls Janet', 'Delicious', 'Senshu' and 'Prima' were obtained from Iwate Agricultural Research Center (Kitakami city, Iwate, Japan). Young expanding leaves of 'Sansa', 'Tsugaru', 'Jonagold', 'Akane', 'Aori-9', 'Kitaro', 'Santaro', 'Shinano Sweet', 'Shinano Gold', 'Jonathan', 'Beniiwate' and 'Aikanokaori' were obtained from the Field Science Center of Iwate University (Takizawa city, Iwate, Japan).

DNA extraction

The same DNA stocks as in the analysis of *S* alleles [19] were also used in the present study. Immediately after sampling apple leaves, approximately 50 mg (45-55 mg) were weighed out, and kept in deep freezer (−80°C) for at least 1 h. DNA was extracted following the previously reported protocols with partial modifications as follows [19,20].

Frozen leaves were crushed by using Micro Smash MS-100R (Tomy, Tokyo, Japan) at 2500 rpm for 30 s, and 500 µL of 'alkaline PVPP buffer' with high salt (Tris-HCl, pH 9.5, 50 mM; EDTA, 10 mM; NaCl, 4 M; CTAB, 1%; PVPP, 0.5%; β-mercaptoethanol, 1%) was added to the samples. Here, β-mercaptoethanol was added to the buffer immediately before use. 'Tris' represents tris(hydroxymethyl) aminomethane, 'EDTA' represents ethylenediaminetetraacetic acid, 'CTAB' represents cetyltrimethylammonium bromide, and 'PVPP' represents polyvinylpolypyrrolidone. Samples were crushed again at 3000 rpm for 30s by using Micro Smash. Crushed samples were heated at 80°C in a heating block for 30 min. Sample tubes were briefly shaken with hands, two or three times during eating.

Sample tubes were then centrifuged at 14000 rpm for 10 min at 4°C. Supernatant (400 µl) was recovered to new tubes. PCI (phenol-chloroform-isoamylalcohol, 25:25:1, v/v/v; 200 µl) was added to tubes, and vigorously mixed by vortex. After centrifugation at 14000 rpm for 5 min at 4°C, water phase (350 µl) was recovered to new tubes. Again, PCI (200 µl) was added to samples, vortexed, and centrifuged at 14000 rpm for 5 min at 4°C. Water phase (300 µl) was recovered to new tubes, and the same volume (300 µl) of sterilized ion-exchanged water was added to samples, to avoid salt precipitation in the next step. The same volume to the sample (600 µl) of isopropanol was added, mixed well by inverting tubes, placed at −80°C for at least 15 min, and then centrifuged at 14000 rpm for 60 min at 4°C. All supernatant was carefully removed from tubes, then the precipitate was dried by using hair drier. RNA was digested with 1 µg of RNase A in 100 µl of sterilized deionized water at 37°C for 5 min. A 100 µl of SDS buffer (Tris-HCl, pH 7.5, 200 mM; EDTA, 25 mM; NaCl, 250 mM; SDS (sodium dodecyl sulfate), 0.5%) was added to the solution, and mixed with 50 ml of PCI solution. After centrifugation at 14000 rpm for 5 min at 4°C, 180 µl of upper (water) phase was recovered to new tubes. Two volumes (360 µl) of ethanol was added to the tube, mixed well by inverting tubes, frozen at −80°C for at least 15 min, and DNA was precipitated by centrifugation at 14000 rpm for 1 h at 4°C. All supernatant was removed from the tube, and the precipitate was dried by using hair drier. Dried precipitate was dissolved in 50 µl of sterilized deionized water, so that DNA extract approximately equivalent to 1 mg fresh weight of apple leaf was dissolved every 1 µl of water (1 mgFW-eq μl^{-1}; [20].

The amount of DNA extracted from 5 mg of apple leaf was approximately quantified and qualified by agarose gel electrophoresis. Similar to our previous report on DNA extraction from other plants such as cyclamen by using alkaline PVPP buffer [20], DNA yield from apple leaves was relatively high, and DNA fragmentation was limited.

Primer sequences and primer design

Primer sequences are listed in Table 1. Some primers were newly designed in this study (such as MdMYB1-Pm-F). Primers for the following PCR markers are the same as the references in parentheses: MdACS1-SSLP [10,11], MdMYB1-dCAPS (BstEII) [13], MdAlt-AS [15], MfVf-GS [18]. Some of the primer names are the same as the previous reports (such as 'DR033892-F5'). When specific primers names were not provided in the original report or the primers were designed in the present study, new primer names were generated in the present study (such as 'MdMYB1-d-F' and 'MdMYB1-Pm-F').

Three new primers ('DS6-MdMYB1-d-F', 'MdMYB1-Pm-F', and 'MdMYB1-Pm-R') were designed in this study. DS6-MdMYB1-d-F primer was designed just by attaching a 68-base DS6 adaptor sequence, which is a partial sequence of the DS4 adaptor [21], to the MdMYB1-d-F primer sequence. Here, 'DS6' represents 'dissimilar synthetic DNA

Gene/Locus	PCR marker	Primer name	Primer sequence
MdACS1	MdACS1-SSLP	ACS1-5'F	5'-AGAGAGATGCCATTTTTGTTCGTAC-3'
		ACS1-5'R	5'-CCTACAAACTTGCGTGGGGATTATAAGTGT-3'
MdMYB1	MdMYB1-dCAPS(BstEII)	MdMYB1-d-F	5'-CCTGAACACGTGGGAACCGGCCCGTTGGTAAC-3'
		MdMYB1-d-R	5'-GTGAAGGTTGTCTTTATTAGTGACGTG-3'
	MdMYB1-dCAPS(BstEII)-DS6	DS6-MdMYB1-d-F	5'-ATCCGAAGTCACGATGATTCAGCGGCCTATAGC ACATCGGCGCATAGTTACGCGGTTAGTCGGATCCC CCTGAACACGTGGGAACCGGCCCGTTGGTAAC-3'
		MdMYB1-d-R	5'-GTGAAGGTTGTCTTTATTAGTGACGTG-3'
	MdMYB1-CAPS(PmlI)	MdMYB1-Pm-F	5'-GGATTTTGGGTGTTTGCTGT-3'
		MdMYB1-Pm-R	5'-CTCAAAGTTGGAGGGACCAG-3'
MdAlt	MdAlt-AS	DR033892-F5	5'-ATGGAGTGGTAGATTTATCATATTT-3'
		DR033892-R	5'-TGGTGAAGAAACAAGAAAATGC-3'
		DR033892-S-R2	5'-TTCAACAGCATAACCGGC-3'
MfVf	MfVf-GS	ACS9-F	5'-ACATGGAAGATGAAGGAGAAGGAG-3'
		ACS9-R	5'-GATAAATTGAGTGACTGCAAAGCG-3'

Table 1: List of PCR markers and primers.

sequence 6'. Length of the DS6-MdMYB1-d-F primer is 100 bases, which can be ordered at an ordinary oligo-DNA synthesis scale (Fasmac, Atsugi, Japan). MdMYB1-Pm-F and MdMYB1-Pm-R primers were designed by using Primer3 software [22,23]. The designed Tm (melting temperature) values of these primers were around 60°C, but test PCR amplification at a series of different annealing temperatures (from 48°C to 68°C, at 4°C intervals) showed that the annealing temperature of 68°C is the most stable PCR condition with these primers.

PCR amplification

The successful conditions of PCR amplifications were tested by using *Taq* DNA polymerase (Ex Taq, Takara, and Kusatsu, Japan) and KOD DNA polymerase (KOD Plus Neo, Toyobo, Osaka, Japan) separately. Primer concentrations in the PCR reactions were 0.5 µM each for Ex Taq, and 0.2 µM each for KOD Plus Neo, unless otherwise described. Gradient PCR amplifications were performed at a series of different annealing temperatures (48°C to 68°C, at 4°C intervals) following manufacturers' instructions. Extension was 1 min, and the number of PCR cycles was 40. One µl of 100-times diluted DNA solutions of control apple cultivars (such as 'Fuji' for *MdMYB1* primers) was added to 20-µl PCR solutions. PCR solutions were mixed well by vortex before PCR reaction. 10 µl of PCR products was loaded onto 2% agarose gels and electrophoresed at 100 V for approximately 30 min, until the bromophenol blue dye moved 80% of the gels. Gels were stained with ethidium bromide for 1 h after electrophoresis, and DNA bands were visualized by UV illumination.

PCR amplifications shown in the figures were performed at the optimum PCR conditions, as were determined in the present study, of each primer set (PCR marker) as follows. MdACS1-SSLP and MfVf-GS markers were amplified with KOD Plus Neo polymerase at the annealing temperature of 68°C (so that this is the 'two-step' PCR cycle, with the same annealing/extension temperatures). Extension was 1 min, and the number of PCR cycles was 40, for all PCR markers. Other PCR conditions followed manufacturer's instructions, or followed the conditions which are shown above. The sizes of DNA fragments generated by MdACS1-SSLP merker are 655 bp and 517 bp, for *acs1* and *ACS1* alleles respectively. The size of DNA fragment generated by MfVf-GS marker is 469 bp.

MdAlt-AS marker was amplified with KOD Plus Neo polymerase at the annealing temperature of 60°C. Concentrations of DR033892-F5 and DR033892-R primers in the PCR reactions were 1 µM, and the concentration of DR033892-S-R2 primer was 0.15 µM. The sizes of DNA fragments generated by MdAlt-AS marker are 692 bp and 429 bp, for *alt* and *Alt* alleles respectively.

Because stable and clear amplification of MdMYB1-dCAPS(BstEII) and MdMYB1-dCAPS(BstEII)-DS6 markers were not observed when PCR was performed with Ex Taq or KOD Plus Neo polymerases, these markers were amplified with Platinum Taq polymerase (Thermo Fisher Scientific, Waltham, USA), the same polymerase as the original report. Annealing temperature was 55°C for MdMYB1-dCAPS (BstEII) and 60°C for MdMYB1-dCAPS (BstEII)-DS6. MdMYB1-CAPS (PmlI) marker was amplified with KOD Plus Neo polymerase, at the annealing temperature of 68°C (two-step cycle). A bit weaker but similar quality of DNA bands were also amplified with Platinum Taq polymerase, at the annealing temperature of 60°C. The sizes of DNA fragments generated by MdMYB1-dCAPS (BstEII) marker are 291 bp and 263 bp, for *myb1* allele and *MYB1* allele respectively. The sizes of DNA fragments generated by MdMYB1-dCAPS (BstEII)-DS6 marker are 359 bp and 263 bp, for *myb1* allele and *MYB1* allele respectively. In the case of MdMYB1-CAPS (PmlI) marker, a 324-bp DNA fragment is generated for *myb1* allele, whereas two DNA fragments (208 bp and 116 bp) are generated for *MYB1* allele.

Treatment with restriction enzymes

PCR products of the three markers MdMYB1-dCAPS (BstEII), MdMYB1-dCAPS (BstEII)-DS6, and MdMYB1-CAPS (PmlI) were digested by restriction enzymes as described below. Each PCR product (20 µl) was mixed well with 40 µl of ethanol by vortex, frozen at −80°C for at least 15 min, and centrifuged at 14000 rpm for 1 h at 4°C to precipitate DNA. All supernatant was removed by pipetting, and the precipitate was dried by using hair drier. These dried DNA samples were dissolved in 10 µl of enzyme mix, containing CutSmart buffer and 10 U of restriction enzymes: *Bst*EII for MdMYB1-dCAPS(BstEII) and MdMYB1-dCAPS(BstEII)-DS6 markers, and *Pml*I for MdMYB1-CAPS(PmlI) marker. Restriction enzymes were purchased from New England Biolabs (Ipswich, USA). The enzyme mixes containing amplified DNA were held at 37°C for 16 h in a thermal cycler. After reaction, samples were diluted with the same volume (10 µl) of sterilized deionized water, and 15 µl of the diluted samples were electrophoresed in 2% agarose gel.

DNA sequence alignment

Partial promoter sequences of *MYB1*, *myb1-1*, and *myb1-2* alleles (GenBank accessions DQ886414.1, DQ886415.1, and

DQ886416.1) were first organized by using Sequence Assistant software (Haruta's tool box; http://www2s.biglobe.ne.jp/~haruta/; released at Vector Inc., Tokyo, Japan; http://www.vector.co.jp/soft/win95/edu/se480631.html, in Japanese). DNA sequences were aligned with Clustal W [24] and further organized by Box Shade (http://www.ch.embnet.org/software/BOX_form.html) with default setting of the parameters. Output data was edited by using Acrobat software (Adobe Systems, San Jose, USA).

Apple skin colors

Photographs of apple cultivars were obtained from a website 'From a rural district in blue forest' (http://aomori.my.coocan.jp/, in Japanese) with permission by the authors. The same dataset is also published in a book (Sugiyama and Sugiyama, 2015). Apple photographs for each cultivar were used for colorimetric calculations. Colorimetric values were gathered at 10 different points on the photograph of each cultivar.

Colorimetric calculations

Apple colors in photographs were converted to colorimetric values and coordinates on color circle (the *round* diagram), based on RGB color matching functions [25-27]. Calculation of colors in digital photographs is also described in these papers. Briefly, standardized RGB colors (I_R, I_G, and I_B) were first obtained from photographs using Adobe Photoshop software. Hue (H^{RGB}), lightness (L^{RGB}), and saturation (S^{RGB}) were then calculated from I_R, I_G, and I_B. Coordinates on color circle (r and d values) were also calculated from S^{RGB2} (the same as S^{RGB}, in the case of digital photographs) and H^{RGB} values.

Results

Genotyping MdACS1 gene of apple cultivars

Alleles of *MdACS1* gene were amplified with the same primer set (Table 1) as the previous studies. The two alleles of *MdACS1* are originally described as *ACS1-1* and *ACS1-2*, where *ACS1-1* is a functional, semi-dominant, and ethylene-producing allele. For ease of understanding, these two alleles are designated *acs1* and *ACS1* respectively, in the present study. Thus, compared with the genomic sequence of the *ACS1* allele, *acs1* allele possesses a 138-bp insertion at 894 bases upstream from the adenine residue of the start codon of the *MdACS1* gene (Figure 1A).

The DNA marker to detect *MdACS1* alleles is designated 'MdACS1-SSLP' in the present study (Table 1). PCR amplification of the MdACS1-SSLP marker of 22 apple cultivars gave quite clear DNA band patterns, which discriminate *MdACS1* alleles of apple cultivars as homozygous for *ACS1* allele, homozygous for *acs1* allele, or heterozygous (Figure 1B).

Genotyping MdMYB1 gene of apple cultivars

Alleles of *MdMYB1* gene were first amplified with the same primer set as the previous study. This marker in the previous study is designated 'MdMYB1-dCAPS(BstEII)' in the present study (Table 1). There are three alleles of *MdMYB1*, which are originally described as *MYB1-1*, *MYB1-2*, and *MYB1-3*, where *MYB1-1* is a functional, dominant, and anthocyanin-accumulating allele causing red skin. The *MYB1-1* allele is designated *MYB1*, and *MYB1-2* and *MYB1-3* alleles

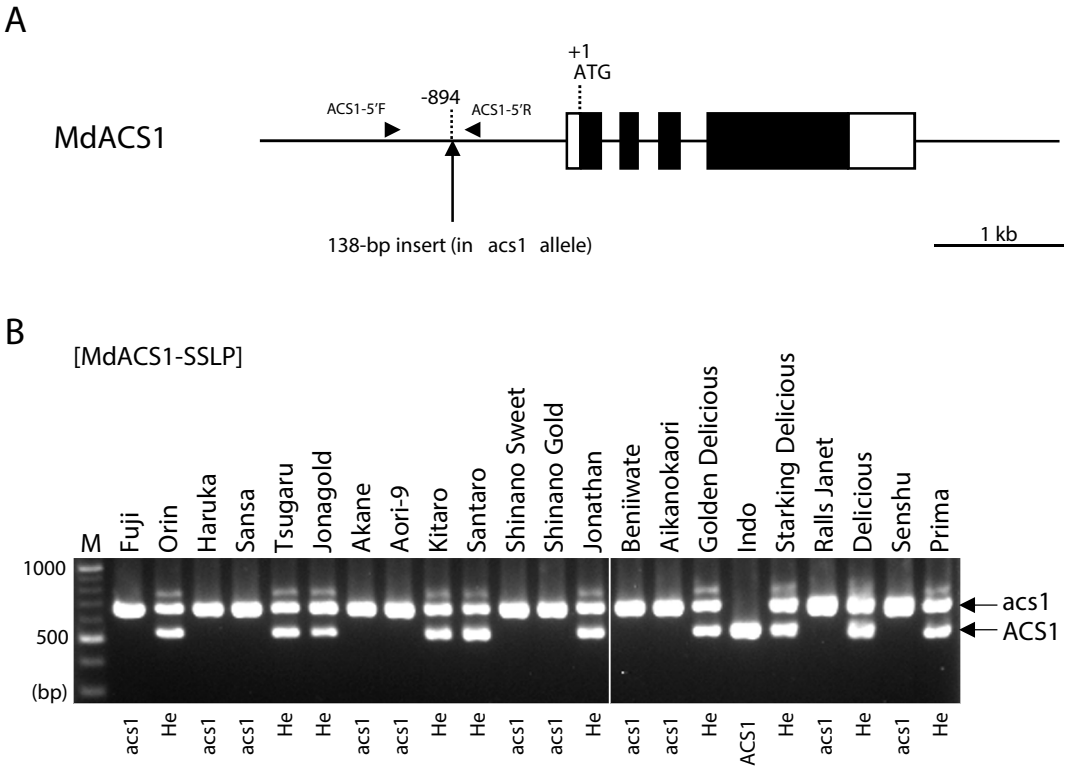

Figure 1: The SSLP marker for *MdACS1* gene. (A) Schematic representation of the *MdACS1* gene. The approximate positions and directions of the primers for MdACS1-SSLP marker are indicated by arrowheads. The target sequence of *Bst*EII digestion is underlined. (B) Amplification of the MdACS1-SSLP marker in 22 apple cultivars. Estimated apple genotypes by this PCR analysis are indicated below the gel photograph: *ACS1*, homozygous for *ACS1* allele; *acs1*, homozygous for *acs1* allele; He, heterozygous. M, molecular weight marker BRG-100-02 (Watson, Tokyo, Japan). kb, kilo-bases. bp, base pairs.

are designated *myb1-1* and *myb1-2* respectively, in the present study. DNA fragment including a single nucleotide polymorphism (SNP) at 1659 bases upstream from the adenine residue of the start codon of the *MdMYB1* gene is PCR amplified by dCAPS primers to generate the recognition sequence (5'-GGTNACC-3') of a restriction enzyme *Bst*EII, at the tail of the forward primer MdMYB1-d-F, then the DNA fragment is treated with *Bst*EII. DNA fragment derived from *MYB1* allele is digested by *Bst*EII, whereas DNA fragments derived from *myb1* alleles (*myb1-1* and *myb1-2*) are not digested by *Bst*EII due to the SNP (Figure 2A).

PCR amplification of the MdMYB1-dCAPS(BstEII) marker of 22 apple cultivars, followed by treatment with *Bst*EII, gave only ambiguous DNA band patterns, from which *MYB1* and *myb1* alleles are not clearly distinguishable (Figure 2B).

Adaptor-joined dCAPS marker for genotyping MdMYB1 gene

Poor discrimination between *MYB1* and *myb1* alleles will be partly because the sizes of DNA fragments for *MYB1* and *myb1* are not so much different from each other. As a matter of fact, sizes of these DNA fragments are different just by 30 bases. This situation cannot be helped for dCAPS markers in general, because the target sequences of restriction enzymes are always at the tail of one of the PCR primers in dCAPS markers. Then, we noticed a possible solution to this problem with dCAPS markers. That is, random nucleotide sequence is joined to the upstream of the forward primer of the MdMYB1-dCAPS(BstEII) marker, to enlarge the size of non-digested DNA fragment for *myb1* alleles, without changing the size of digested DNA fragment for *MYB1* allele.

Random DNA sequence joined to the forward primer was a 68-base DS6 adaptor (Figure 2C). This dCAPS marker joined by the DS6 adaptor is designated 'MdMYB1-dCAPS(BstEII)-DS6' (Table 1). PCR amplification of the MdMYB1-dCAPS(BstEII)-DS6 marker of 22 apple cultivars, followed by treatment with *Bst*EII, gave much clearer DNA band pattern than the MdMYB1-dCAPS(BstEII) marker (Figure 2D). However, the DNA band pattern obtained by this adaptor-joined marker could not completely discriminate alleles of *MdMYB1* genes. This could be partly because DNA fragment is not successfully amplified from apple genomes (e.g. cultivar 'Prima'), and partly because non-specific DNA fragment with a similar size to the target sequence is amplified with this primer set (e.g. cultivar 'Akane'; Figure 2D).

CAPS marker for genotyping MdMYB1 gene

To design a new DNA marker from the beginning, we checked DNA sequences of the *MdMYB1* gene alleles, including the sequences around the target site of above dCAPS markers (Figure 3A). There are several SNP sites and one insertion/deletion site between *MdMYB1* alleles within the genomic region shown in Figure 3A. An SNP (C/A/A: *MYB1*/*myb1-1*/*myb1-2*) is the target of *Bst*EII digestion in the dCAPS markers. Please note that a T to G mutation is introduced by the forward PCR marker of the dCAPS markers, generating the target sequence of *Bst*EII (5'-GGTNACC-3') in the PCR-amplified DNA fragment from *MYB1* allele. Only 21 bases upstream from this SNP, another SNP (G/A/T) is also seen. Nucleotide sequences of *MYB1* allele around this upstream SNP includes a 6-base palindrome (5'-CACGTG-3'), which is a typical target sequence of restriction enzymes. An investigation on the catalog of restriction enzymes actually identified a list of isoschizomers which digest this palindrome sequence: *Acv*I, *Bbr*PI, *Eco*72I, *Pma*CI, *Pml*I, and *Psp*CI, among which *Pml*I is provided by New England Biolabs. A CAPS (cleaved amplified polymorphic sequence) marker targeting this *Pml*I site will provide equivalent genetic information on *MdMYB1* alleles, with the dCAPS markers targeting the *Bst*EII site.

Forward and reverse PCR primers were designed around the *Pml*I site (Figure 3B). The binding sites of the primers were chosen so that SNP or insertion/deletion site between *MdMYB1* alleles are not included, to certify even amplification of the alleles. This CAPS marker is designated 'MdMYB1-CAPS(PmlI)' (Table 1). PCR amplification of the MdMYB1-CAPS(PmlI) marker of 22 apple cultivars, followed by treatment with *Pml*I, gave clear DNA band pattern (Figure 3C). Alleles of *MdMYB1* (*MYB1* homozygous, *myb1* homozygous, or heterozygous) in all 22 examined cultivars can be now determined from these DNA bands (Figure 3C and Table 2).

Colorimetric estimation of apple skin colors

The alleles of *MdMYB1* are known to regulate apple skin color (red or yellow), but relationship between *MdMYB1* alleles and apple skin colors have not been examined in Japanese apple cultivars, except for 'Fuji', the most popular apple cultivar both in Japan and in the world. Judgement of skin colors have been also performed just by seeing photographs, without colorimetric measurements and calculations. Then we measured apple skin colors, and compared these colorimetric values with *MdMYB1* genotypes which were determined in Figure 3C.

The set of 22 apple cultivars examined in Figure 3C includes red-skin cultivars such as 'Fuji', and yellow-skin cultivars such as 'Shinano Gold' (Figure 4A). Colorimetric values of apple skins were calculated from apple photographs, based on RGB color system (Table 2). Here, H^{RGB} values represent hues such as 'yellow' and 'red'. H^{RGB} values (degrees) are evenly distributed around the color circle, for example pink (330°), red (0°), orange (30°), yellow (60°), and lawn (90°; yellow-green). The 'color' of each apple cultivar is also described in Table 2, such as 'red-pink' and 'yellow-orange'. 'Red-pink' is an intermediate between red and pink, but closer to red. In the same manner, 'yellow-orange' is an intermediate between yellow and orange, but closer to yellow. S^{RGB} values represent saturation, or deepness/vividness of the color (the S^{RGB} value of the fully saturated colors is 1). L^{RGB} values represent lightness, or relative brightness of the color (the highest value is 1).

Coordinates of apple skin colors on the color circle (or the '*round* diagram') were also calculated from colorimetric values above, and apple skin colors were plotted on the color circle (Figure 4B). In Figure 4B, different symbols are used to indicate apple cultivars with different *MdMYB1* alleles (homozygous for *MYB1*, heterozygous, or homozygous for *myb1*). Hue angles of both apple cultivars harboring *MYB1*-homozygous genotype and apple cultivars harboring heterozygous genotype are around 0° (red), ranging from −10.9° ('Jonathan') to 9.7° ('Delicious'). The range of hue angles was relatively wide for apple cultivars harboring *myb1*-homozygous genotype, ranging from 17.2° ('Indo') to 62.6° ('Orin'), although the range did not overwrap with those of *MYB1*-homozygous or heterozygous genotypes.

Genotyping MdAlt locus and MfVf gene of apple cultivars

Finally, two DNA markers estimating pathogen resistance were examined. Alleles of *MdAlt* locus were amplified with the same primer set (Table 1) as the previous studies. The dominant allele of this locus, *Alt*, causes susceptibility to Alternaria leaf blotch, and the *MfVf* gene introduced from *Malus floribunda* adds scab resistance, as already described.

The DNA marker to detect *MdAlt* alleles is designated 'MdAlt-AS' in the present study, where 'AS' represents 'allele-specific' (Table

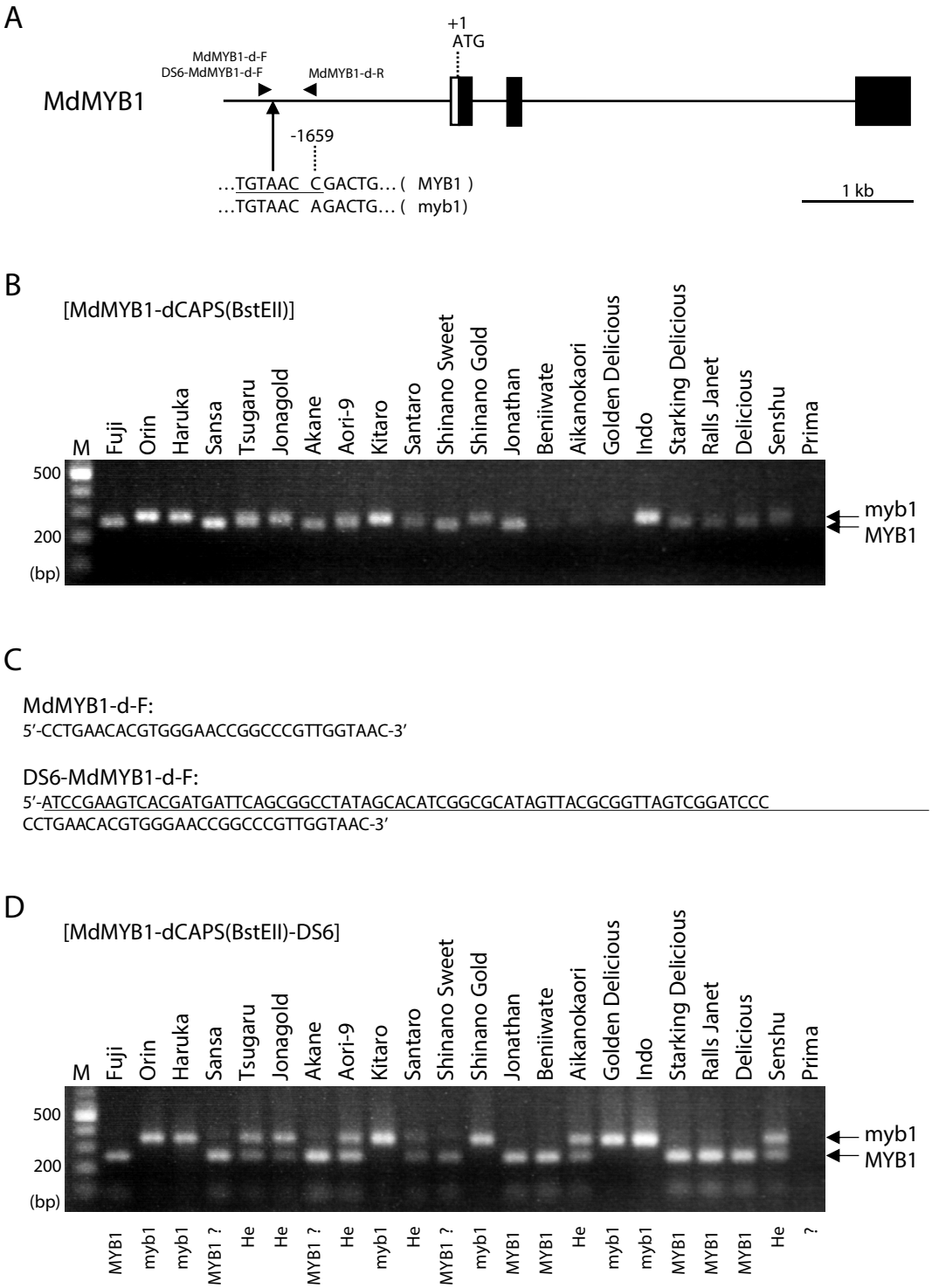

Figure 2: The two dCAPS markers for *MdMYB1* gene. (A) Schematic representation of the *MdMYB1* gene. The approximate positions and directions of the primers for MdMYB1-dCAPS(BstEII) and MdMYB1-dCAPS(BstEII)-DS6 markers are indicated by arrowheads. (B) Amplification of the MdMYB1-dCAPS(BstEII) marker in 22 apple cultivars. (C) Comparison between the structures of forward primers for MdMYB1-dCAPS(BstEII) and MdMYB1-dCAPS(BstEII)-DS6 markers. DS6 adaptor sequence is underlined. (D) Amplification of the MdMYB1-dCAPS(BstEII)-DS6 marker in 22 apple cultivars. Estimated apple genotypes by this PCR analysis are indicated below the gel photograph: *MYB1*, homozygous for *MYB1* allele; *myb1*, homozygous for *myb1* allele; He, heterozygous. Unclear results are indicated by question marks. M, molecular weight marker BRG-100-02.

Cultivar	MdMYB1-CAPS(PmlI)	H^{RGB} (degrees)		Color	S^{RGB}		L^{RGB}	
	genotype	av	SD		av	SD	av	SD
Jonathan	*MYB1*	-10.9	1.8	Red-Pink	0.86	0.10	0.27	0.03
Starking Delicious	He	-5.2	2.5	Red-Pink	0.53	0.09	0.16	0.05
Aori-9	He	-4.5	3.4	Red-Pink	0.62	0.09	0.36	0.05
Akane	*MYB1*	-2.7	2.3	Red-Pink	0.89	0.12	0.23	0.04
Sansa	*MYB1*	-2.5	2.3	Red-Pink	0.60	0.10	0.37	0.06
Santaro	He	-1.7	2.0	Red-Pink	0.54	0.06	0.34	0.03
Beniiwate	*MYB1*	-1.6	1.8	Red-Pink	0.38	0.07	0.30	0.03
Senshu	He	-1.4	2.7	Red-Pink	0.59	0.10	0.36	0.05
Aikanokaori	He	3.1	8.9	Red-Orange	0.45	0.07	0.39	0.08
Jonagold	He	3.7	4.6	Red-Orange	0.70	0.12	0.35	0.12
Ralls Janet	*MYB1*	3.7	10.4	Red-Orange	0.41	0.08	0.45	0.03
Fuji	He	3.9	4.9	Red-Orange	0.37	0.05	0.33	0.04
Shinano Sweet	*MYB1*	5.2	4.8	Red-Orange	0.52	0.05	0.40	0.07
Tsugaru	He	8.1	6.0	Red-Orange	0.42	0.09	0.45	0.10
Delicious	He	9.7	15.7	Red-Orange	0.67	0.25	0.41	0.19
Indo	*myb1*	17.2	12.4	Orange-Red	0.42	0.04	0.47	0.09
Kitaro	*myb1*	30.5	16.7	Orange-Yellow	0.45	0.06	0.58	0.14
Shinano Gold	*myb1*	45.8	3.0	Yellow-Orange	0.67	0.05	0.61	0.02
Haruka	*myb1*	48.6	0.7	Yellow-Orange	0.63	0.04	0.59	0.01
Golden Delicious	*myb1*	52.5	3.9	Yellow-Orange	0.40	0.04	0.79	0.04
Orin	*myb1*	62.6	4.7	Yellow-Lawn	0.53	0.12	0.55	0.06

Note: av: average; SD: standard deviation; He: heterozygous.

Table 2: Apple skin colors.

1). PCR amplification of the MdAlt-AS marker of 22 apple cultivars gave clear DNA band patterns, which discriminate *MdAlt* alleles of apple cultivars as homozygous for *Alt* allele, homozygous for *alt* allele, or heterozygous (Figure 5A). The band patterns of control cultivars ('Jonathan' and 'Starking Delicious') were consistent with the previous report [15].

The DNA marker to detect *MfVf* gene is designated 'MfVf-GS' in the present study, where 'GS' represents 'gene-specific' (Table 1). PCR amplification of the MfVf-GS marker of 22 apple cultivars detected the *Vf* gene only from the positive control cultivar 'Prima' (Figure 5B).

Discussion

Deduced levels of ethylene production from the DNA band patterns

Genotypes of the *MdACS1* gene have already been reported in many apple cultivars [10-12]. We also determined *MdACS1* genotypes in the present study (Figure 1B). Among the analyzed cultivars, *MdACS1* genotypes of 13 already reported cultivars ('Fuji', 'Orin', 'Sansa', 'Tsugaru', 'Jonagold', 'Akane', 'Jonathan', 'Golden Delicious', 'Indo', 'Starking Delicious', 'Ralls Janet', 'Delicious', and 'Prima') were completely consistent with the previous results, demonstrating stable and reproducible amplification of *MdACS1* alleles by MdACS1-SSLP marker.

MdACS1 genotypes of the other 9 cultivars will have been clarified for the first time in the present study. These cultivars were relatively recently bred in Japan. Two of these cultivars ('Kitaro' and 'Santaro') are heterozygous for *MdACS1* alleles, then these cultivars are expected to have moderate levels of ethylene production. The other seven cultivars ('Haruka', 'Aori-9', 'Shinano Sweet', 'Shinano Gold', 'Beniiwate', 'Aikanokaori', and 'Senshu') are all homozygous for *acs1* allele. These cultivars are expected to have low levels of ethylene production, and have long storage lives.

Both parentage and *MdACS1* genotypes of 21 apple cultivars examined in the present study, except for 'Prima', are shown in Figure 6. Although this is comparison between just 21 apple cultivars, there seems to have been preference for *acs1*-homozygous genotypes during selection of new apple cultivars. These cultivars are deduced to have been selected by growing hundreds of candidate plants for years, and estimating fruit qualities. If *acs1*-homozygous genotype, and its accompanying trait of long storage life, is always required for new apple cultivars, selection of this genotype by MdACS1-SSLP marker will greatly reduce the labor of the selection process. Another promising and high-throughput way to breed long storage-life apple is just crossing between *acs1*-homozygous cultivars, such as 'Fuji', 'Shinano Gold', 'Haruka', and 'Beniiwate', to generate next-generation seedlings for selection.

Effective use of DS6 adaptor for improvement of a dCAPS marker

Amplification and discrimination of MdMYB1-dCAPS(BstEII) marker was not clear in our experiments (Figure 2B). We tried amplification of this marker repeatedly. We even performed PCR amplification using the same DNA polymerase with the reference paper, but we did never obtain clear DNA band patterns for this dCAPS marker. Such problem with this DNA marker was partly solved in MdMYB1-dCAPS (BstEII)-DS6 marker (Figure 2D). DS6-MdMYB1-dCAPS (BstEII) marker is different from MdMYB1-dCAPS (BstEII) marker only in that the forward primer is attached with DS6 adaptor.

DS6 adaptor is a partial sequence of the original DS4 adaptor [21]. DS4 adaptor was ligated to digest genomic DNA to perform genome walking [21,27]. Then attachment of DS5 adaptor was tried and successfully amplified apple *S-RNase* fragment [5]. Attachment of the DS5 adaptor to the forward primer slightly reduced PCR amplification of the target sequence, together with amplification of

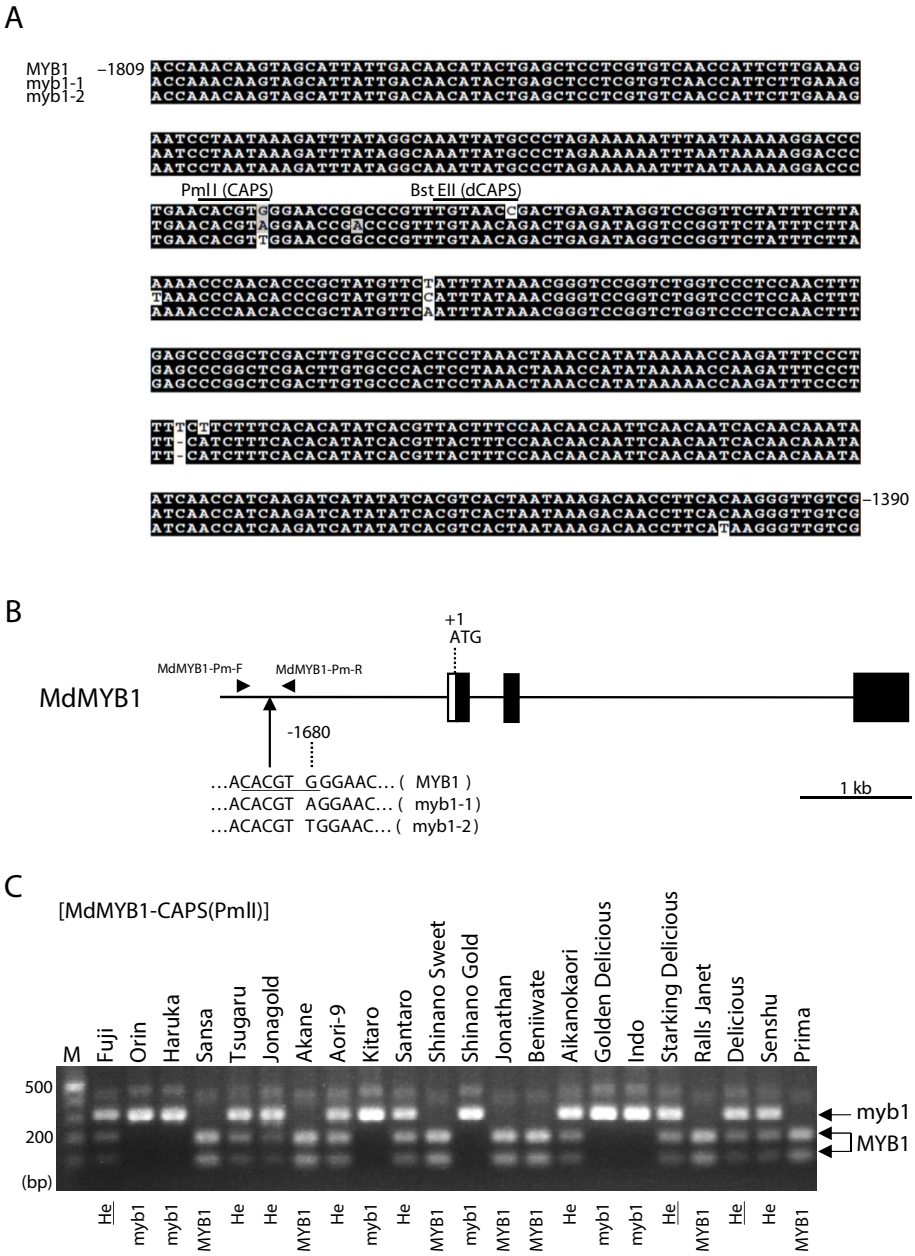

Figure 3: The CAPS marker for *MdMYB1* gene. (A) Alignment of the partial promoter sequences of *MdMYB1* alleles. The target sites (sequences) of *Bst*EII (in dCAPS markers) and *Pml*I (in CAPS marker) are indicated by black bars. (B) Schematic representation of the *MdMYB1* gene. The approximate positions and directions of the primers for MdMYB1-CAPS(PmlI) marker are indicated by arrowheads. (C) Amplification of the MdMYB1-CAPS(PmlI) marker in 22 apple cultivars. Estimated apple genotypes by this PCR analysis are indicated below the gel photograph: *MYB1*, homozygous for *MYB1* allele; *myb1*, homozygous for *myb1* allele; He, heterozygous. The estimated genotypes which are different from Figure 2D are indicated by underline. M, molecular weight marker BRG-100-02.

non-specific smear DNA band. On the contrary, attachment of the DS6 adaptor to the forward primer looks to strengthen amplification of the target sequence, when DNA bands in Figure 2B and 2D are compared. We have tested just these two examples at the moment, but the effects of DS4-related adaptors on PCR amplifications can be both positive and negative, depending on the target sequence and/or primer sequence. Attachment of DS4-related adaptors is at least advantageous for clear discrimination of DNA band sizes after PCR amplification, digestion with restriction enzymes, and electrophoresis of dCAPS markers.

Consistency of MdMYB1 genotypes determined by DNA markers

The *MdMYB1* genotypes of 22 apple cultivars were partly determined by MdMYB1-dCAPS(BstEII)-DS6 marker, as described above. The DNA band patterns were still somewhat ambiguous for part of the cultivars, and no amplification was obtained for 'Prima' (Figure 2D). An even better DNA band pattern was obtained in MdMYB1-CAPS(PmlI) marker (Figure 3C). Considering this result, there are some possible advantages of CAPS markers over dCAPS markers, even if dCAPS

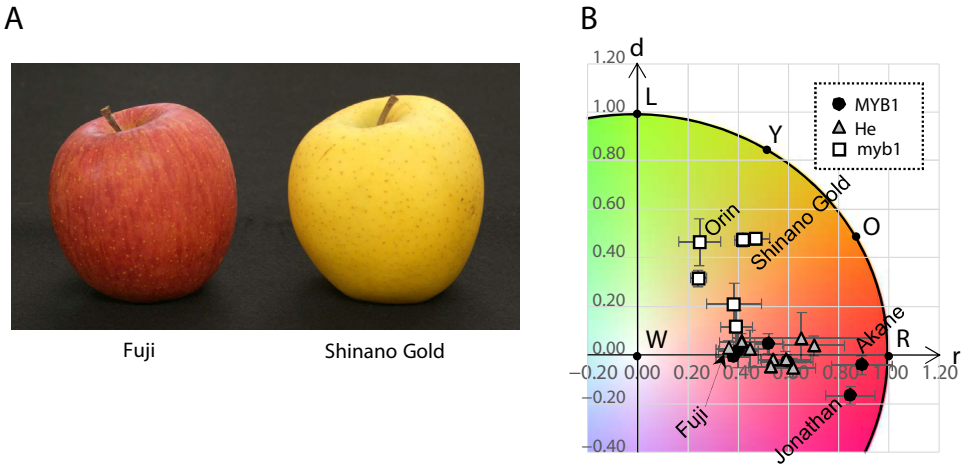

Figure 4: Apple skin colors. (A) Photograph of representative red and yellow apple cultivars 'Fuji' and 'Shinano Gold'. (B) Apple skin colors of 21 cultivars except for 'Prima', plotted on the color circle (*round* diagram). Cultivars are plotted with different symbols according to the *MdMYB1* genotypes, as indicated in the graph. Data represent means and standard deviations. R, O, Y, L, and W represent red, orange, yellow, lawn (yellow-green), and white.

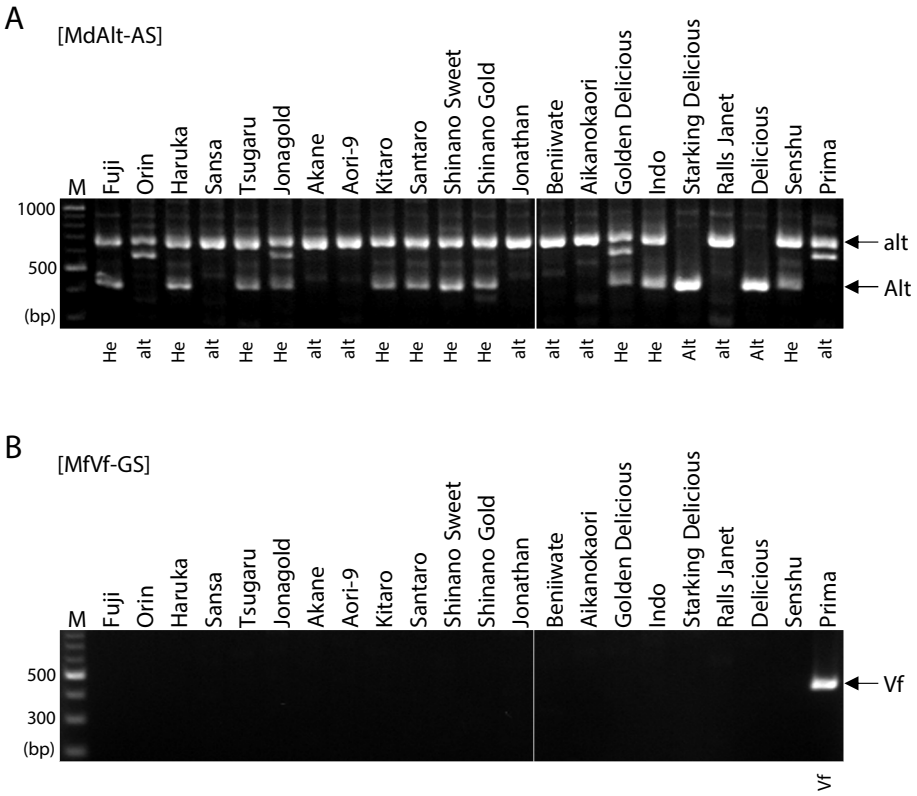

Figure 5: The DNA markers for *MdAlt* locus and *MfVf* gene. (A) Amplification of the MdAlt-AS marker in 22 apple cultivars. Estimated apple genotypes by this PCR analysis are indicated below the gel photograph: *Alt*, homozygous for *Alt* allele; *alt*, homozygous for *alt* allele; He, heterozygous. (B) Amplification of the MfVf-GS marker in 22 apple cultivars. Apple cultivar harboring *MfVf* gene, as estimated by this PCP analysis, is indicated by '*Vf* below the gel photograph. M, molecular weight marker BRG-100-02.

markers have been improved by attachment of DS4-related adaptors. That is, there is wider range of the choice of the target sequence of primers in CAPS markers. In the cause of dCAPS primers, one of the primers must be designed at the proximity of the polymorphic nucleotide. This condition limits design of preferable sequence of primers for successful PCR amplification. The forward primer of MdMYB1-dCAPS(BstEII) marker also included SNP sites within its target sequence. This may cause inferior and biased amplification of different alleles. Primers for CAPS markers can be more freely designed, so DNA amplification will be better in CAPS markers in general.

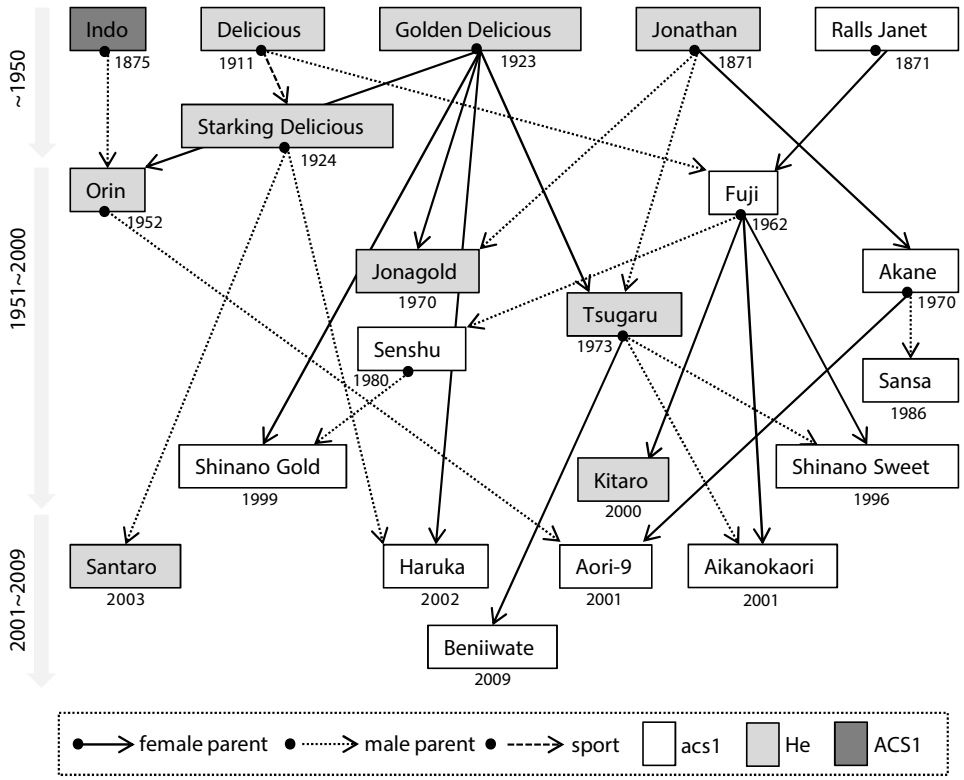

Figure 6: *MdACS1* genotypes and parentage of apple cultivars. *MdACS1* genotypes of 21 cultivars except for 'Prima' are indicated with different colors. Parentage between the cultivars are also indicated by different arrows. The years when each cultivar was released in Japan are written below the names of cultivars. Cultivars are placed according to the released years, from the top to the bottom.

A clear inconsistency between the results obtained with MdMYB1-dCAPS(BstEII)-DS6 marker and MdMYB1-CAPS(PmlI) marker is the genotypes of three cultivars ('Fuji', 'Starking Delicious', and 'Delicious'). Their genotypes were *MYB1*-homozygous with MdMYB1-dCAPS(BstEII)-DS6 marker, but heterozygous with MdMYB1-CAPS(PmlI) marker. The latter is correct, when considering parentage of 'Fuji' and 'Starking Delicious': these cultivars are parents of both red- and yellow-skin cultivars, respectively. Thus, 'Fuji' is the parent of both red-skin cultivars ('Senshu', 'Shinano Sweet', 'Aikanokaori', 'Aori-21', 'Akiakane', 'Akiyo', etc.) and yellow/green-skin cultivars ('Kitaro', 'Aori-15', 'Akita Gold', 'Koko', 'Seirin', and 'Aoi'). Similarly, 'Starking Delicious' is the parent of red-skin cultivars ('Priscilla', 'Komitsu', 'Santaro', 'Hirodai-1', 'Jupiter', and 'Hazen') and a yellow-skin cultivar 'Haruka'. 'Fuji' and 'Starking Delicious' can be the parents of both red-skin and yellow-skin cultivars, only because these are heterozygous for *MdMYB1* gene alleles. If so, the dCAPS markers will have failed to amplify *myb1* alleles due to their inferiority in even amplification of different alleles. Examination of *MdMYB1* genotypes of many more apple cultivars by using MdMYB1-CAPS(PmlI) marker will facilitate control of fruit skin colors in breeding programs.

Relationship between MdMYB1 genotypes and apple skin colors

As shown in Figure 4B and Table 2, *MdMYB1* genotypes are able to roughly discriminate apple skin colors: *MYB1*-homozygous and heterozygous cultivars produce red-pink to red-orange-skin apples, and *myb1*-homozygous cultivars produce orange-red to yellow-lawn-skin apples. There was no discrimination of the hues between *MYB1*-homozygous cultivars and heterozygous cultivars, but the two red-skin cultivars with highest saturation values ('Akane' and 'Jonathan') were both *MYB1*-homozygous. It is easily imaginable that homozygous *MYB1* alleles activate production of more anthocyanin in apple skin than heterozygous alleles (semi-dominancy), although all *MYB1*-homozygous apple fruits did not necessarily show such high saturation values. Possession of homozygous *MYB1* alleles may be prerequisite for high saturations values of red apple skins, together with some unknown genetic factor(s).

Both hues and saturations were variable within apple cultivars possessing homozygous *myb1* alleles. For example, skin colors of cultivars 'Indo' and 'Kitaro' were orange, rather than yellow. This seems to be because low levels of red anthocyanin pigment are generated in the skin, even with the recessive *myb1* alleles. Such spontaneous production of anthocyanin in yellow cultivars would be widely observed, whose genetic or physiological mechanisms are not understood. The cultivars 'Shinano Gold' and 'Haruka' enjoyed high saturation values. These deep (vivid) yellow colors will attract consumers and beneficial for sales. The cultivar 'Orin' is a bit greenish, which attaches this cultivar the unique identity as 'green apple'. Green coloration may be caused by enhanced chlorophyll synthesis or inhibition of chlorophyll degradation. The mechanism for green coloration in green apple cultivars should be also clarified.

Re-estimation of apple resistance to pathogens by DNA markers

Amplification of MdAlt-AS marker in 22 apple cultivars clarified their genotypes at the *Alt* locus (Figure 5A). Genotypes of the control

cultivars of this DNA marker, 'Jonathan' and 'Starking Delicious', were consistent with the previous report [14,15]. On the other hand, *MdAlt* genotypes were not necessarily consistent with the reported Alternaria resistance of the other cultivars [28]. For example, a sensitive cultivar to Alternaria, 'Orin', has homozygous *alt* alleles, which must be resistant to Alternaria. On the contrary, resistant cultivars including 'Haruka', 'Shinano Gold', and 'Golden Delicious' are heterozygous at the *MdAlt* locus. The genotypes of 'Sansa' and 'Akane' were homozygous for *alt* allele, which are consistent with their resistance to Alternaria. Thus, genotypes of the *MdAlt* locus are not correlated with resistance of apple cultivars to Alternaria leaf blotch. This DNA marker will be effective on the progeny plants generated by the cross between 'Jonathan' and 'Starking Delicious', but extensive chromosomal recombination seems to have taken place between *MdAlt* locus and the sensitivity gene to *Alternaria alternata* in the other cultivars. Alternatively, other sensitivity/resistance gene may regulate Alternaria resistance in the other cultivars.

We have also investigated existence of *MfVf* gene in 22 cultivars, with the expectation for identifying new scab-resistant cultivars. PCR amplification revealed that none of the cultivars excepting the positive control cultivar 'Prima' has the *MfVf* gene (Figure 5B). This result shows that introduction of the *MfVf* gene into major apple cultivars should be promoted by using the DNA marker MfVf-GS, to make scab-resistant cultivars widely cultivated and to reduce the use of agrichemicals.

Conclusion

In the present study, we re-estimated and partly improved four different DNA markers for breeding and characterization of agricultural traits of apple cultivars. The results in two markers for pathogen resistance did not fully conform to our expectations. *MdAlt* gene marker was effective only on specific cultivars, and we could not detect new cultivar possessing *MfVf* gene. Further improvement of DNA markers or further breeding of resistant cultivars will be necessary to improve apple tolerance to pathogens. Breeding of new apple cultivars by using DNA markers can be also accelerated by combination with early-flowering technology of apple seedlings: Our research group has already developed such method by using the vector of *Apple latent spherical virus* [29-34]. The results with the *MdACS1* gene marker were quite reproducible, and we also clarified *MdACS1* genotypes of new apple cultivars such as 'Haruka' and 'Shinano Gold'. We also noticed clear preference for *acs1*-homozygous genotypes in newly bred apple cultivars, supporting usefulness of the *MdACS1* gene marker for apple breeding. The *MdMYB1* gene marker was improved by attachment of DS6 adaptor to the forward primer. The marker was even more improved by designing new CAPS marker, with which consistent and clear DNA band patterns were obtained. *MdMYB1* genotypes can roughly determine apple skin colors (reddish or yellowish), but further analyses are necessary to predict precise colors of apple skins.

Acknowledgements

Photographs of apple cultivars were provided by the courtesy of Kaworu Sugiyama and Yasushi Sugiyama. We also appreciate Shigeki Moriya for providing information about the *MdAlt* marker. Apple leaves were kind gifts from Iwate Agricultural Research Center and Manabu Watanabe at Iwate University orchard. The experiments were performed with helpful assistances and technical advices by Tomiko Kawaguchi, and the other laboratory members. We also appreciate Noriko Yamagishi and Chunjiang Li for reviewing the manuscript.

Funding

This study was supported by the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry (Japan).

References

1. Crosby JA, Janick J, Pecknold PC, Korban SS, O'Connon PA, et al. (1992) Breeding apples for scab resistance: 1945-1990. Fruit Varieties J 46: 145-166.
2. Crosby JA, Janick J, Pecknold PC (1994) 'GoldRush' apple. Hort Science 29: 827-828.
3. Igarashi MY, Harada HT, Fukasawa-Akada T (2016) Biotechnology and apple breeding in Japan. Breed Sci 66: 18-33.
4. Moriya S, Iwanami H, Takahashi S, Kotoda N, Suzaki K, et al. (2010) Genetic mapping of the crown gall resistance gene of the wild apple *Malus sieboldii*. Tree Genet Genomes 6: 195-203.
5. Moriya SK, Haji OT, Yamamoto T, Abe K (2012) Fine mapping of *Co*, a gene controlling columnar growth habit located on apple (*Malus* × *domestica* Borkh) linkage group 10. Plant Breed 131: 641-647.
6. Kasajima I, Kikuchi T, Yoshikawa N (2017a) Rapid identification of apple (*Malus* × *domestica* Borkh.) *S* alleles using sequencing-based DNA marker *APPLid*. Plant Biotechnol 34: 97-106.
7. Dong JG, Kim WT, Yip WK, Thompson GA, Li L, et al. (1991) Cloning of a cDNA encoding 1-aminocyclopropane-1-carboxylate synthase and expression of its mRNA in ripening apple fruit. Planta 185: 38-45.
8. Lay-Yee M, Knighton ML (1995) A full-length cDNA encoding 1-aminocyclopropane-1-carboxylate synthase from apple (accession no. L31347) (PID: g606759). Plant Physiol 107: 1017-1018.
9. Harada T, Sunako T, Sakuraba W, Goto S, Senda M, et al. (1997) Genomic nucleotide sequence of a ripening-related 1-aminocyclopropane-1-carboxylate synthase gene (MdACS-1) in apple (accession no. U89156) (PGR 97-066) Plant Physiol 113: 1465.
10. Sunako T, Sakuraba W, Senda M, Akada S, Ishikawa R, et al. (1999) An allele of the ripening-specific 1-Aminocyclopropane-1-carboxylic acid synthase gene (*ACS1*) in apple fruit with a long storage life. Plant Physiol 119: 1297-1303.
11. Harada T, Sunako T, Wakasa Y, Soejima J, Satoh T, et al. (2000) An allele of the 1-aminocyclopropane-1-carboxylate synthase gene (*Md-ACS1*) accounts for the low level of ethylene production in climacteric fruits of some apple cultivars. Theor Appl Genet 101: 742-746.
12. Dougherty L, Zhu Y, Xu K (2016) Assessing the allelotypic effect of two aminocyclopropane carboxylic acid synthase-encoding genes *MdACS1* and *MdACS3a* on fruit ethylene production and softening in *Malus*. Hortic Res 3: 16024.
13. Takos AM, Jaffé FW, Jacob SR, Bogs J, Robinson SP, et al. (2006) Light-induced expression of a *MYB* gene regulates anthocyanin biosynthesis in red apples. Plant Physiol 142: 1216-1232.
14. Moriya S, Terakami S, Iwanami H, Haji T, Okada K, et al. (2013) Genetic mapping and marker-assisted selection of the gene conferring susceptibility to Alternaria blotch caused by *Alternaria alternata* apple pathotype in apple. Proceedings of the 13th Eucarpia Symposium on Fruit Breeding and Genetics. Evans KM (edn). Acta Hortic 976: 555-560.
15. Moriya S (2014) Development of early selection methods of apple seedlings harboring four important traits using DNA markers. Doctor thesis, Tohoku University.
16. Vinatzer BA, Patocchi A, Gianfranceschi L, Tartarini S, Zhang HB, et al. (2001) Apple contains receptor-like genes homologous to the *Cladosporium fulvum* resistance gene family of tomato with a cluster of genes cosegregating with *Vf* apple scab resistance. Mol Plant Microbe Interact 14: 508-515.
17. Belfanti E, Silfverberg-Dilworth E, Tartarini S, Patocchi A, Barbieri M, et al. (2004) The *HcrVf2* gene from a wild apple confers scab resistance to a transgenic cultivated variety. Proc Natl Acad Sci U.S.A. 101: 886-890.
18. Xu M, Huaracha E, Korban SS (2001) Development of sequence-characterized amplified regions (SCARs) from amplified fragment length polymorphism (AFLP) markers tightly linked to the *Vf* gene in apple. Genome 44: 63-70.
19. Kasajima I (2017a) Protocol for DNA extraction from any plant species. Protoc Exch.
20. Kasajima IK, Tanaka SY, Terakawa T, Ohtsubo N (2013) Large-scale extraction of pure DNA from mature leaves of *Cyclamen persicum* Mill and other recalcitrant plants with alkaline polyvinylpolypyrrolidone (PVPP). Sci Hortic 164: 65-72.
21. Kasajima I, Ohtsubo N, Sasaki K (2014) Faster, safer, and better DNA purification by ultracentrifugation using GelRed stain and development of mismatch oligo DNA for genome walking. Biosci Biotechnol Biochem 78: 1902-1905.

22. Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. Bioinformatics 23: 1289-1291.

23. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, et al. (2012) Primer3 - new capabilities and interfaces. Nucleic Acids Res 40: e115.

24. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680.

25. Kasajima I (2016) Alexandrite-like effect in purple flowers analyzed with newly devised round RGB diagram. Sci Rep 6: 29630.

26. Kasajima I, Fekih R (2017) Eating colors: a scientifically based perception of food colors. Jacobs J Food Nutr 4: 028.

27. Kasajima I, Ohtsubo N, Sasaki K (2017b) Combination of *Cyclamen persicum* Mill. floral gene promoters and chimeric repressors for the modification of ornamental traits in *Torenia fournieri* Lind. Hortic Res 4: 17008.

28. Iwanami AKH, Kotoda N, Moriya S, Sumiyoshi ST (2010) Evaluation of apple genotypes and *Malus* species for resistance to Alternaria blotch caused by *Alternaria alternata* apple pathotype using detached-leaf method. Plant Breed 129: 208-218.

29. Yamagishi N, Kishigami R, Yoshikawa N (2014) Reduced generation time of apple seedlings to within a year by means of a plant virus vector: a new plant-breeding technique with no transmission of genetic modification to the next generation. Plant Biotechnol J 12: 60-68.

30. Yamagishi N, Li C, Yoshikawa N (2016) Promotion of flowering by *Apple latent spherical virus* vector and virus elimination at high temperature allow accelerated breeding of apple and pear. Front Plant Sci 7: 171.

31. Yamagishi N, Sasaki S, Yamagata K, Komori S, Nagase M, et al. (2011) Promotion of flowering and reduction of a generation time in apple seedlings by ectopical expression of the *Arabidopsis thaliana FT* gene using the *Apple latent spherical virus* vector. Plant Mol Biol 75: 193-204.

32. Kasajima I, Ito M, Yamagishi N, Yoshikawa N (2017c) Apple latent spherical virus (ALSV) vector as a tool for reverse genetic studies and non-transgenic breeding of a variety of crops. pp: 513-539. In: N. Rajewsky, S. Jurga, and J. Barciszewski (eds). Plant Epigenetics. Springer International Publishing, Cham, Switzerland.

33. Kasajima I (2017b) Plotting colors on color circle: interconversion between XYZ values and RGB color system. Scientific Pages Anal Bioanal Chem 1: 1-8.

34. Sugiyama K, Sugiyama Y (2015) Apples in Aomori prefecture (revised edition). Apple association of Aomori prefecture, Hirosaki, Japan.