J. Japan. Soc. Hort. Sci. 78 (3): 279–287. 2009.
Available online at www.jstage.jst.go.jp/browse/jjshs1

Development of a Marker-assisted Selection System for Columnar Growth Habit in Apple Breeding

Shigeki Moriya[1]*, Hiroshi Iwanami[1], Nobuhiro Kotoda[1], Sae Takahashi[1], Toshiya Yamamoto[2] and Kazuyuki Abe[1]**

[1]*Apple Research Station, National Institute of Fruit Tree Science, NARO, Shimokuriyagawa, Morioka 020-0123, Japan*
[2]*National Institute of Fruit Tree Science, NARO, Fujimoto, Tsukuba 305-8605, Japan*

To make progress in columnar apple breeding, we constructed genetic linkage maps of the *Co* genomic region and identified useful DNA markers that enable correct and efficient selection of columnar-type seedlings in a practical columnar apple breeding program. Linkage maps of three mapping populations were constructed on the apple linkage group 10, and the *Co* gene was mapped on two populations, 'Fuji' × 8H-9-45 and 'Fuji' × 5-12786. Closely linked marker genotypes of $SCAR_{682}$, $SCAR_{216}$, CH03d11, and Hi01a03 were determined on 33 columnar-type cultivars and/or selections, and non-columnar-type cultivars contained 7 ancestral cultivars of apple breeding in Japan. A 174 bp fragment for primer Hi01a03 was amplified in all columnar-type cultivars, selections, and 'McIntosh', but was not detected in other ancestral apples. Amplified products of 682 bp for $SCAR_{682}$ and 177 bp for CH03d11 were detected in almost all columnar genotypes except for one selection, and 'McIntosh', but the products were not found in other ancestral apples. A 177 bp allele of CH03d11, characteristic of a columnar phenotype, was detected in all 170 columnar-type plants obtained from 18 crosses, suggesting that it is one of the most tightly linked DNA markers to the *Co*. These results indicated that CH03d11 was the most reliable marker to distinguish columnar and non-columnar phenotypes for the MAS system. In our two most advanced breeding selections, the *Co* gene-associated alleles of the original columnar mutant ('Wijcik') on CH03d11 (177 bp) and Hi01a03 (174 bp) remained, indicating that using these two breeding selections as *Co* donors in the breeding program, CH03d11 and Hi01a03 enable the selection of seedlings with columnar phenotype.

Key Words: columnar-type apple breeding, linkage map, *Malus*, simple sequence repeat.

Introduction

Apples (*Malus* × *domestica* Borkh.) belong to the genus *Malus*, which is in the subfamily *Maloideae* of the family *Rosaceae*, and grow readily in all temperate climatic zones (Luby, 2003; O'Rourke, 2003). Worldwide commercial apple production was about 64 million tons in 2006 (FAOSTAT, Food and Agriculture Organization of the United Nations (FAO), http://faostat.fao.org, October 30, 2008). Tree architecture and shoot morphology traits influence the fruit yield through pruning or training. Great variability in tree characteristics has been demonstrated in apple cultivars, which have been qualitatively classified into several architectural types according to the tree growth pattern, distribution of branches, and fruiting position (Lauri et al., 1995; Lespinasse and Delort, 1986). The genetic backgrounds of tree forms are complicated and poorly understood.

The columnar growth habit in apple was initially discovered in a sport of 'McIntosh' (Fisher, 1970). This mutant, called 'Wijcik' or 'Wijcik McIntosh', has peculiar phenotypes, such as reduced branching and increased spurs on the lateral shoots and a trunk with short internodes, in contrast to 'McIntosh' and other major apple cultivars. These characteristics are considered to be important and advantageous for labor-saving cultivation because of the minimal pruning and training required by apple growers; therefore, 'Wijcik' is an important breeding source to develop columnar-type compact apple cultivars. Lapins (1976) revealed that a single dominant gene, *Co*, determined the columnar character; however, one or more modifiers, may be

Received; June 30, 2008. Accepted; December 18, 2008.
* Corresponding author (E-mail: moriyas@affrc.go.jp).
** Present address: Okitsu Citrus Research Station, National Institute of Fruit Tree Science, NARO, Shizuoka 424-0292, Japan.

involved in the expression of the character, because the percentage of plants with a columnar phenotype was slightly below that expected by the test cross study (Lapins, 1976).

Apple breeding programs for the columnar type were initiated at the National Institute of Fruit Tree Science (NIFTS, Japan) in 1987. The fruit characteristics of 'Wijcik' were unsuitable for apple growers and consumers in Japan because of severe preharvest drop, low sugar content and high acidity of fruit juice, soft fruit flesh, and short shelf life; therefore, the breeding objectives of a dessert columnar-type apple combined a columnar trait with improved fruit characteristics. To achieve these objectives, columnar apples have been crossed with major apple cultivars with good fruit quality, such as 'Fuji'. Since it takes many years to improve the fruit quality of 'Wijcik', some columnar-type selections introduced from Canada were used as donors of *Co* in the apple breeding program at NIFTS. In order to accelerate columnar apple breeding, early selection of columnar-type plants in their seedling stage is quite efficient, because at least 2-year cultivation in the nursery is required to select columnar-type seedlings based on their tree form. Long-term cultivation of many seedlings including the non-columnar type in the nursery is cost- and labor-intensive for such as fungicide, insecticide, and associated labor. To reduce these costs, the establishment of a marker-assisted selection (MAS) system would be valuable for columnar-type apple breeding.

Several sequence-tagged site (STS) markers linked to the *Co* gene have been reported. Hemmat et al. (1997) developed SSRco from the sequence of random amplified polymorphic DNA (RAPD) marker $OA11_{1000}$ by bulked segregant analysis (BSA). The SSRco was mapped on linkage group 10 (LG10) (Maliepaard et al., 1998). A RAPD marker, $WB82_{670}$, obtained from the mapping population of 'Fuji' × 'Tuscan', was converted into a sequence characterized amplified region (SCAR) marker, $SCB82_{670}$, by amplifying a product of 670 bp (Kim et al., 2003). In another BSA study, a RAPD marker, $S1142_{682}$, and an amplified fragment length polymorphism (AFLP) marker, E-ACT/M-CTA_{346}, from the mapping population of 'Spur Fuji' × 'Telamon' were also converted to SCAR markers, designated $SCAR_{682}$ (682 bp) and $SCAR_{216}$ (216 bp), respectively (Tian et al., 2005). Two inter simple sequence repeat (ISSR) markers linked to *Co* were developed by BSA from the mapping population of 'Fuji' × 'Telamon' (Zhu et al., 2007).

On the other hand, for the last several years, significant progress in the molecular genetics of apple has been made, mainly in the development of multi-allelic simple sequence repeat (SSR) markers and highly informative genetic linkage maps. The first reference map of apple was constructed by Maliepaard et al. (1998), including 67 multi-allelic markers (RFLPs, SSRs, and isozyme) and comprising 17 linkage groups. Liebhard et al. (2002, 2003) and Silfverberg-Dilworth et al. (2006) developed approximately 300 SSR markers and mapped them on a saturated reference map covering 85% of the genome. In the report of Silfverberg-Dilworth et al. (2006), 29 SSR markers were mapped on the LG 10; however, it has been problematic that an integrated genetic linkage map of these SSR markers and closely linked markers for important traits, such as *Co*, has not been available to date; evaluation and identification of the most efficient marker in MAS are needed to make progress in columnar apple breeding.

The objectives of our study were 1) to construct genetic linkage maps of the *Co* genomic region for columnar apple genotypes important as breeding materials by using SSR markers and published SCAR markers and 2) to identify useful DNA markers that will enable correct and efficient selection of columnar-type seedlings in a practical columnar apple breeding program in Japan. These DNA markers will be used optically in the MAS system of columnar-type apple breeding.

Materials and Methods

Plant materials and phenotypic evaluation of growth characteristics

Thirty-one columnar-type cultivars and/or selections, 10 normal-type cultivars, and 21 F_1 populations obtained from their crosses were used for the phenotypic evaluation of growth characteristics and genetic mapping, and the evaluation of DNA markers associated with the columnar type of apple (Tables 1, 2, and 3). The growth pattern was visually categorized into columnar or non-columnar (normal) in the dormant season (Fig. 1). Plants possessing a main shoot with little difference in diameter between the top and base, and secondary branches with a narrow angle, almost parallel to the main shoot, were scored as columnar according to the description by Hemmat et al. (1997). In the F_1 progenies of 187 individuals of three crosses (55 plants

Table 1. Mapping populations for the *Co* gene and phenotypic evaluation of growth characteristics.

Maternal parent	Paternal parent	Tree age at phenotypic evaluation/root system	Number of plants	Columnar	Non-columnar	*P* value χ^2
Fuji	8H-9-45[z]	5 years/self rooting	55	47	8	0.000[y]
Fuji	5-12786	2 years/JM1	68	30	38	0.332

[z] Underlined cultivars and/or selections were the donors of *Co*.
[y] Statistically significant difference from the 1 : 1 segregation ratio.

Table 2. Marker genotypes of columnar cultivars and/or selections and normal-type cultivars of major ancestors of Japanese apple cultivars and offspring of 'McIntosh' in Japan.

Cultivars and/or selections	Phenotype	Origin	Marker genotype			
			SCAR682 (682 bp)	CH03d11	Hi01a03	SCAR216 (216 bp)
Wijcik	Columnar	Bud sport of McIntosh	+	126/177[y]	167/174	+
McIntosh	Non-columnar	Chance seedling	+	126/177	167/174	+
Tuscan	Columnar	Wijcik × Greensleeves	+	118/177	174/211	+
Trajan	Columnar	Golden Delicious × Wijcik	+	118/177	174/211	+
Telamon	Columnar	Wijcik × Golden Delicious	+	118/177	174/211	+
8H-1-13	Columnar	8N-24-57[z] × 8N-24-49[z]	+	118/177	174/211	+
8H-2-11	Columnar	8N-24-57[z] × 8N-24-49[z]	+	118/177	174/211	+
8H-2-16	Columnar	8N-24-57[z] × 8N-24-49[z]	+	118/177	174/211	+
8H-2-26	Columnar	8N-24-57[z] × 8N-24-49[z]	+	118/177	174/211	+
8H-4-27	Columnar	Unknown	+	118/177	174/211	+
8H-5-3	Columnar	8N-24-50[z] × 8N-25-23[z]	+	118/177	174/211	+
8H-5-29	Columnar	8N-24-50[z] × 8N-25-23[z]	+	118/177	174/211	+
8H-6-4	Columnar	8N-24-50[z] × 8N-25-23[z]	+	118/177	174/211	–
8H-6-5	Columnar	8N-24-50[z] × 8N-25-23[z]	+	118/177	174/211	+
8H-7-15	Columnar	8N-24-50[z] × 8N-25-23[z]	+	118/177	174/211	+
8H-9-1	Columnar	Wijcik × Alkmene	+	118/177	174/211	–
8H-9-45	Columnar	Unknown	+	118/177	174/211	+
8H-12-50	Columnar	Unknown	+	118/177	174/211	+
8C-7-20	Columnar	Wijcik × Empire[z]	+	177/177	174/174	+
8C-7-42	Columnar	Wijcik × Empire[z]	+	118/177	174/211	+
8C-9-111	Columnar	Wijcik × Cox' Orange Pippin	+	177/177	174/211	+
8C-14-8	Columnar	8N-24-59[z] × Wijcik	+	177/177	174/174	+
8S-60-74	Columnar	Unknown	+	118/177	174/211	+
5-128	Columnar	8H-1-14 open.	+	122/177	174/174	+
5-263	Columnar	8H-6-4 open.	–	118/118	174/174	–
5-296	Columnar	8H-6-4 open.	+	118/177	174/187	–
5-305	Columnar	8H-6-4 open.	+	122/177	174/211	–
5-306	Columnar	8H-6-4 open.	+	118/177	174/211	–
5-375	Columnar	8H-12-50 open.	+	122/177	174/187	+
5-400	Columnar	8H-6-5 open.	+	177/177	174/174	+
5-8246	Columnar	TSR29T219 × 8H-9-1	+	114/177	174/190	+
5-12786	Columnar	Fuji × 8H-2-26	+	118/177	174/187	+
Ralls Janet	Non-columnar	Chance seedling	–	118/118	187/211	–
Jonathan	Non-columnar	Chance seedling	–	122/122	187/187	+
Delicious	Non-columnar	Chance seedling	–	118/122	187/211	–
Golden Delicious	Non-columnar	Chance seedling	–	118/118	211/211	–
Indo	Non-columnar	Chance seedling	–	122/122	187/211	–
Akane	Non-columnar	Jonathan × Worcester Pearmain	–	118/122	187/211	–
Kitakami	Non-columnar	(Worcester Pearmain × McIntosh) × RedGold	+	122/177	174/187	+
Morioka #61	Non-columnar	Tsugaru × Kitakami	–	118/122	187/211	–
Shinano Red	Non-columnar	Tsugaru × Vista Bella	–	118/177	174/211	+

[z] 8N-24-49 = 'Golden Delicious' × 'Wijcik'; 8N-24-50 = 'Golden Delicious' × 'Wijcik'; 8N-24-57 = 'Golden Delicious' × 'Wijcik'; 8N-24-59 = 'Golden Delicious' × 'Wijcik'; 8N-25-23 = 'Golden Delicious' × 'Wijcik'; 'Empire' = 'McIntosh' × 'Delicious'.

[y] Underlined alleles associated with *Co*. 8C-14-8 and 5-400 weren't able to confirm as heterozygous for the columnar locus by marker genotypes and phenotype segregation data of their offsprings wasn't available.

of 'Fuji' × 8H-9-45, 68 plants of 'Fuji' × 5-12786, and 64 plants of 'Fuji' × 'Tuscan'), the growth pattern was evaluated as shown in Table 1, and used to construct genetic maps of LG10. Weak selections were performed twice until investigation of the population of 'Fuji' × 8H-9-45. At the first selection at the end of the first growing season in 2001, 93 plants with a columnar-type tree form that grew vigorously were selected from 380 seedlings in the glass house, and then planted in the nursery. As a result, the selected plants included both columnar and

Table 3. Marker genotypes in the progenies of columnar-type plants.

Cross combination	Number of columnar-type plant	$SCAR_{682}$		CH03d11		Hi01a03	
		+	–	177 bp	Others	174 bp	Others
Fuji × 8H-6-5[z]	7	7	0	7	0	7	0
Sansa × 8H-7-15	11	11	0	11	0	11	0
Jonathan × 8H-7-15	5	5	0	5	0	5	0
Senshu × 8H-7-15	6	6	0	6	0	5	1
Fuji × 8H-7-15	9	8	1	9	0	8	1
Golden Delicious × 8H-9-1	16	16	0	16	0	16	0
Fuji × 8H-9-1	10	10	0	10	0	9	1
Golden Delicious × 8S-60-74	6	6	0	6	0	5	1
Senshu × 8S-60-74	17	17	0	17	0	17	0
Ralls Janet × 8S-60-74	2	2	0	2	0	2	0
Jonathan × 8S-60-74	7	6	1	7	0	7	0
Jonathan × 8H-2-26	9	9	0	9	0	9	0
Senshu × 8H-2-26	7	7	0	7	0	7	0
Fuji × 8H-2-26	15	15	0	15	0	15	0
Sansa × 8H-6-4	14	14	0	14	0	14	0
Jonathan × 8H-6-4	5	5	0	5	0	5	0
Golden Delicious × 8H-6-4	5	5	0	5	0	4	1
Senshu × 8H-6-4	19	19	0	19	0	19	0
Total	170	168	2	170	0	165	5

[z] Underlined breeding selections were donors of *Co*.

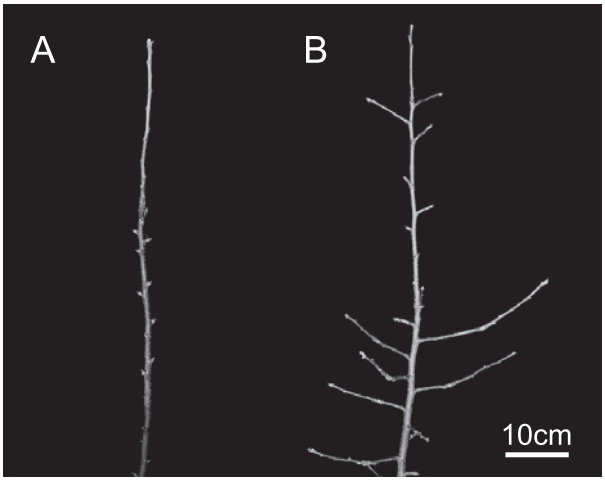

Fig. 1. Growth pattern of 5-year-old plants of (A) a columnar plant and (B) a non-columnar plant in the progenies of 'Fuji' × 8H-9-45.

non-columnar plants, because the tree pattern is unstable in one-year-old plants. In the mid-growing season in 2005, 55 plants that grew vigorously were chosen by second selection. The other two populations did not undergo any selection. A total of 170 representative F_1 columnar-type plants of 18 crosses were selected from intact populations at 4 years old in 1999 and then grafted onto dwarfing rootstock 'JM7' or 'JM1', and their growth pattern was re-evaluated in 2006 to evaluate the utility of DNA markers (Table 3).

DNA isolation

Genomic DNA was extracted from young leaves using the DNA-isolating system PI-50α (Kurabo Co., Ltd., Osaka, Japan). One hundred milligrams of frozen young leaves were crushed using a machine, SH-48 (Kurabo Co. Ltd.) and incubated with 1 mL of an isolation buffer (10% PEG#6000, 100 mM Tris-HCl (pH 8.0), 350 mM sorbitol, and 50 mM EDTA (pH 8.0)) for 30 minutes. Genomic DNA was then extracted by PI-50α according to the manufacturer's instructions.

SSR analysis

Ten SSR markers located on the LG 10 in the map constructed by Silfverberg-Dilworth et al. (2006) were used in this study. These SSR markers were AU223548-SSR, CH01f12, CH02a10, CH02c11, CH03d11, Hi01a03, Hi01b01, Hi03c04p, and Hi22f04, developed by Liebhard et al. (2002) and Silfverberg-Dilworth et al. (2006). The tenth SSR marker was COL, which was first developed by Hemmat et al. (1997) as SSRco and was converted by Gianfranceschi et al. (1998). Since SSR markers Hi01a03 and Hi01b01 contained an identical repeat sequence and its flanking region according to the HiDRAS web site (High-quality Disease Resistant Apples for a Sustainable Agriculture (HiDRAS), http://users.unimi.it/hidras/, October 30, 2008), and their observed PCR products differed only in size, Hi01a03 represented these two markers in this study.

SSR analysis was carried out using two methods with slight modifications: the direct fluorescent primer and the T7-tailed primer methods (Schuelke, 2000; Ziegle

et al., 1992). In CH02c11, CH03d11, Hi03c04p, and COL, the former method was carried out as follows. For each SSR marker, the forward primer was synthesized and then conjugated to fluorescent dye, D2 or D3 or D4 (Sigma-Aldrich, Tokyo, Japan), at the 5' end. PCR amplification was performed in a 12 μL solution of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each dNTP, 10 pmol of each fluorescent-labeled forward primer and unlabeled reverse primer, 10 ng genomic DNA, and 0.25 units *Taq* DNA polymerase (Invitrogen Corp., CA, USA). Amplification was performed in 35 cycles at 94°C for 1 min (denaturing), 52–55°C for 1 min (annealing), and 72°C for 2 min (primer extension). In AU223548-SSR, CH01f12, CH02a10, Hi01a03, Hi01b01, and Hi22f04, using the latter method, based on a forward primer for each SSR primer set, a primer with a 5' tail was designed. The 5' tail has the partial T7 promoter sequence, 5'-AATACGACTCACTATAG-3'. A third T7 primer (5'-AATACGACTCACTATAG-3') carries the fluorescent label (D2, D3, or D4) at the 5' end that binds to the first PCR amplicons during the second PCR amplification. The first PCR amplification was performed in a 12 μL solution as in the former method, except for the primer concentration: solutions contained 2 pmol of each of the T7-tailed forward primer and 10 pmol of each unlabeled reverse primer. Amplification was performed in 10 cycles at 94°C for 1 min, 52–55°C for 1 min, and 72°C for 2 min. After the first amplification, 1 μL of 10 pmol/μL of the fluorescent-labeled T7 primer was added to each PCR tube. A second amplification was then performed in 35 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min. PCR products amplified by both methods were separated and detected using the auto sequencer CEQ 8000 (Beckman-Coulter Inc., Fullerton, CA, USA). The sizes of the amplicons were determined on the basis of an internal standard DNA (DNA size standard kit-400, Beckman-Coulter Inc.) with a CEQ Genetic Analyzer (Beckman-Coulter Inc.). The allele sizes obtained by the T7-tailed primer method contained about 17–18 bp of tailed sequences.

SCAR marker analysis

Three SCAR markers, designated as $SCB82_{670}$, $SCAR_{682}$, and $SCAR_{216}$ (Kim et al., 2003; Tian et al., 2005), previously found to be associated with *Co*, could generate amplified products for all of the test plants. The SCAR markers were amplified according to the method described by Kim et al. (2003) and Tian et al. (2005). The PCR products were separated on a 1.5% agarose gel with a 0.5 × TBE buffer, stained with ethidium bromide, and visualized with ultraviolet light.

The nucleotide sequences of the amplified products of $SCB82_{670}$ were determined by direct sequencing using a DTCS Quick Start Kit for Dye Terminator Cycle Sequencing (Beckman-Coulter Inc.) and the auto sequencer CEQ 8000 (Beckman-Coulter Inc.) in combination with Exo-SAP IT (GE Healthcare UK Ltd., Bukinghanshire, England) to purify PCR products according to the supplier's protocol.

Linkage analysis

A pseudo-testcross mapping strategy was applied to linkage analysis (Grattapaglia and Sederoff, 1994). JoinMap ver. 3.0 (Van Ooijen and Voorrips, 2001) was used to identify linkage between the columnar trait and DNA markers and to construct a genetic linkage map of the columnar parent. A logarithm of odds (LOD) score of 5.0 was used to define the linkage group. The Kosambi mapping function was used to convert recombination units into genetic distances.

Results

Phenotypic evaluation of growth pattern

One-hundred and twenty-three individual progeny of the population of 'Fuji' × 8H-9-45 and 'Fuji' × 5-12786 were clearly categorized into columnar or non-columnar growth patterns (Table 1). No plants showed the intermediate-type growth habit. The segregation ratio for the phenotypes fitted the ratio of 1 columnar: 1 non-columnar for single dominant inheritance in the F_1 population of 'Fuji' × 5-12786 ($\chi^2 = 0.9$, $P = 0.33$). The segregation ratio for the phenotypes, however, did not approximate to 1 columnar: 1 non-columnar in the population of 'Fuji' × 8H-9-45 ($\chi^2 = 27.6$, $P < 0.01$). The distorted segregation in this population could be due to the selection of columnar-like plants from the intact progeny of 'Fuji' × 8H-9-45 described in Materials and Methods. The one-year-old population of 'Fuji' × 'Tuscan' was too young for a phenotypic evaluation to be made in 2007 and did not show significant differences regarding the growth pattern. The segregation data for the growth pattern in the progeny 'Fuji' × 5-12786 was consistent with the expected ratio, in which the single dominant gene *Co* controlled the columnar habit and the genotype of 5-12786 was heterozygous, *Co*/*co*.

Mapping of the Co gene with SCAR and SSR markers

As a result of linkage analysis, linkage maps were constructed on LG 10, and the *Co* gene was mapped on two populations, 'Fuji' × 8H-9-45 and 'Fuji' × 5-12786 (Fig. 2A, 2B). In 'Fuji' × 'Tuscan', the map was also constructed on LG 10, but it did not contain the *Co* gene because phenotypic data on the growth pattern were not available for this population (Fig. 2C). Six DNA markers, $SCAR_{682}$, $SCAR_{216}$, CH02a10, CH03d11, Hi01a03, and COL, were successfully mapped on all linkage maps. The other markers were not mapped on all three linkage maps since their amplified alleles were homozygous in specific columnar parent(s) or the amplified product could not be detected. In the populations of 'Fuji' × 8H-9-45, the *Co* gene co-segregated with $SCAR_{682}$ and the 177 bp allele of CH03d11 and, in the population of 'Fuji' × 5-12786, the *Co* gene was mapped between $SCAR_{682}$/

CH03d11 and Hi01a03. In the population of 'Fuji' × 'Tuscan', segregation was detected between $SCAR_{682}$ and 177 bp of CH03d11, probably due to recombination between the two markers, and co-segregation between CH03d11 and Hi01a03 was detected. To confirm the order between markers and the *Co*, we drew a graphical genotype of all individuals of mapping populations, and no discrepancy was found (data not shown). From all apple cultivars, selections, and F_1 plants of the three mapping populations in the study, $SCB82_{670}$ was detected only in 'Tuscan', TSR29T219, 'Jonathan', its offspring, and 'Greensleeves', which was a parent of 'Tuscan' (data not shown). The nucleotide sequences of these amplified products were identical to the sequence of $SCB82_{670}$ reported by Kim et al. (2003) in 'Tuscan' and 'Greensleeves', and several single nucleotide polymorphisms (SNPs) were found in the sequence of 'Jonathan' (data not shown). In the population of 'Fuji' × 'Tuscan', the recombination frequency and LOD score between $SCB82_{670}$ and CH03d11 were 0.484 and 0.01, respectively. $SCB82_{670}$ did not show any significant correlation with the other DNA markers mapped on the LG 10. The results indicate that $SCB82_{670}$ was not located in LG 10; in other words, $SCB82_{670}$ was not linked to *Co*.

Establishing a system for marker-assisted selection using $SCAR_{682}$, CH03d11, and Hi01a03

To verify the utility of DNA markers mapped on the *Co* region, the marker genotype was determined on 33 columnar-type cultivars and/or selections and 7 non-columnar-type cultivars, which were ancestral cultivars of apple breeding in Japan and 3 non-columnar-type cultivars in Japan, which were offspring of 'McIntosh' (Table 2). Four markers, $SCAR_{682}$ and CH03d11 from the distal side and Hi01a03 and $SCAR_{216}$ from the basal side, were chosen from those mapped around the *Co* region. A 174 bp fragment of primer Hi01a03 was amplified in all columnar-type cultivars and selections and 'McIntosh' but not in ancestral non-columnar apples. The amplified products of 682 bp for $SCAR_{682}$ and 177 bp for CH03d11 were detected in almost all columnar genotypes except for one selection, 5-263, but the products were not found in ancestral non-columnar apples. On the other hand, a fragment of primer $SCAR_{216}$ was not detected in columnar-type selections of 8H-6-4, 8H-9-1, and their offspring 5-263, 5-296, 5-305, and 5-306, although it was found in most of the columnar apples. The fragment of primer $SCAR_{216}$ was amplified even in the non-columnar apple, 'Jonathan', in addition to 'McIntosh' and its offspring. These results indicate that three markers, $SCAR_{682}$, CH03d11, and Hi01a03, are candidates for the identification of columnar apple seedlings in a MAS system in apple breeding. In the offspring of 'McIntosh', 'Kitakami', and 'Shinano Red' showed the same alleles associated with *Co* for 4 and 3 markers, respectively. On the other hand, 'Morioka #61' did not show the same allele associated with *Co*. In 5-263, we confirmed that recombination events should occur between these markers and the *Co* gene because the *Co* gene was mapped between CH02c11 and COL in the F_1 population of 'Fuji' × 5-263 (data not shown); i.e., the *Co* gene was located on LG 10 of 5-263. Graphical genotyping of the *Co* region of several columnar apples is shown in Figure 3. The *Co* region derived from 'Wijcik' is the smallest on 5-263 of all tested cultivars/selections.

To investigate the performance of the markers $SCAR_{682}$, CH03d11, and Hi01a03 in MAS for columnar trait, 170 columnar-type plants obtained from 18 crosses were tested for their marker genotypes. A 177 bp allele of CH03d11, characteristic of the columnar phenotype,

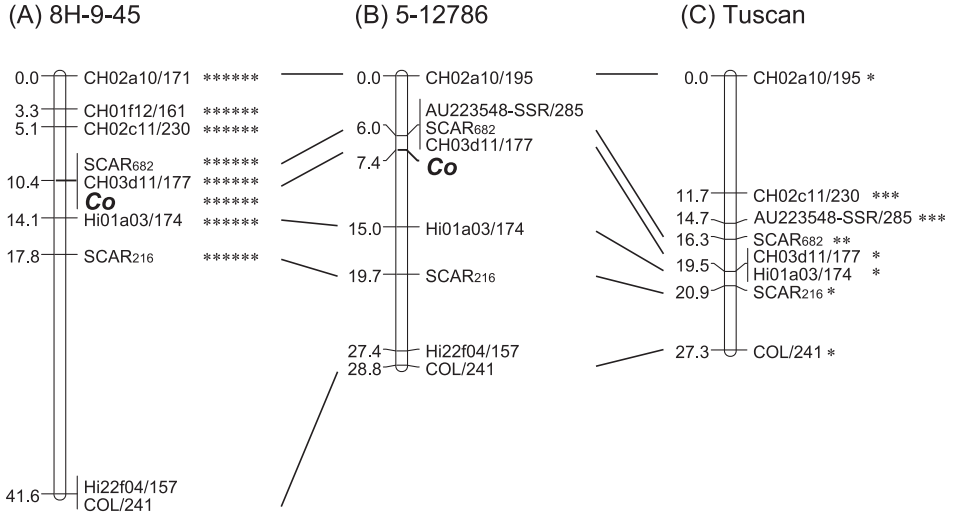

Fig. 2. Linkage maps of the regions around the *Co* gene for columnar-type cultivar or selections. The genetic maps of (A) 8H-9-45, (B) 5-12786, and (C) 'Tuscan' were aligned using microsatellite and SCAR markers successfully mapped on all maps. Map distances are shown on the left (cM). *Co* loci are listed in bold on the right. Observed fragment lengths are shown with SSR names as bp. Segregation distortion is indicated by significance level *P* of the χ^2 test: *: 0.05, **: 0.01, ***: 0.005, ******: 0.0001

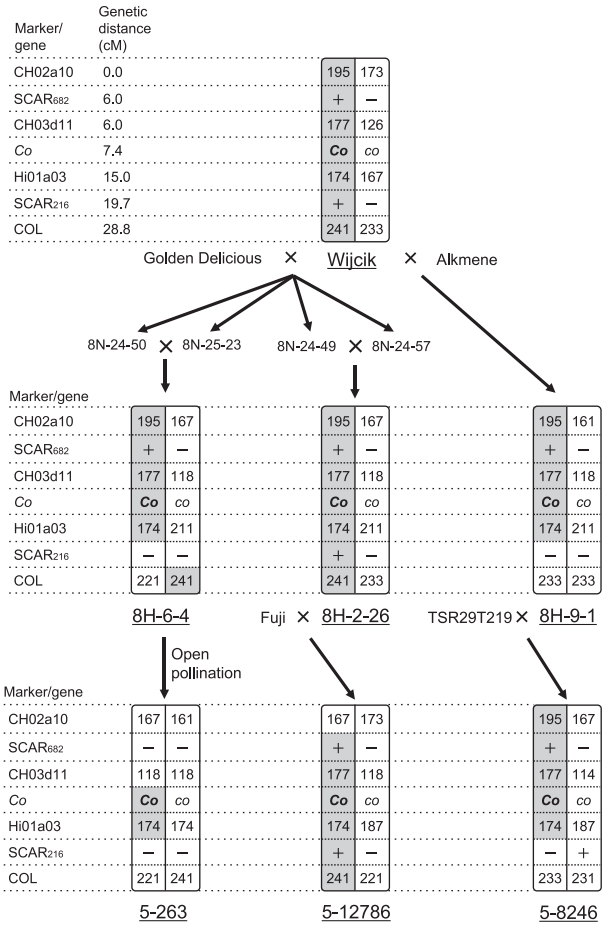

Fig. 3. Graphical genotyping around the *Co* gene on linkage group 10 in several columnar breeding materials. Underlined cultivars and/or selections showed columnar growth habit. It was not known whether 8N-24-49, 8N-24-50, 8N-24-57, and 8N-25-23 showed a columnar growth habit. Alleles of each SSR are shown in bp. The genetic distances were according to Figure 2B (5-12786).

was detected in all 170 plants (Table 3), suggesting that it is one of the most tightly linked DNA markers to *Co*. Fragments of $SCAR_{682}$ and 174 bp of Hi01a03 were amplified in 168 and 165 plants, respectively; recombination occurred between the markers and the *Co* gene in 2 plants for $SCAR_{682}$ and in 5 plants for Hi01a03. These 7 plants were independent of each other. The result showed that CH03d11 was the most reliable marker to distinguish columnar and non-columnar individuals for the MAS system in apple breeding. $SCAR_{682}$ and Hi01a03 could also ensure successful selection for columnar individuals when these markers were applied to MAS, although a small degree of error would be expected when distinguishing columnar plants.

Discussion

Relationship between the Co gene and DNA markers

In previous studies, phenotypic evaluation to determine whether the tree growth pattern was columnar was conducted using self-rooting plants that were more than two years old (Hemmat et al., 1997; Kim et al., 2003; Tian et al., 2005; Zhu et al., 2007); however, Kenis and Keulemans (2007) conducted a study in which they replicated two types of plants on the same genotype, which was self-rooted and grafted onto the 'M.9' rootstock. They revealed that grafted plants showed more distinct differences on the main axis traits than self-rooted plants and concluded that the root system significantly affects all the traits determining tree architecture. To evaluate the phenotype correctly, we conducted an evaluation using plants grafted onto the dwarfing rootstock of 'JM1' in the population of 'Fuji' × 5-12786 and well-established five-year-old rooting plants in the population 'Fuji' × 8H-9-45; we did not conduct a phenotypic evaluation of the one-year-old infant population 'Fuji' × 'Tuscan'. Thus, we considered that our phenotypic evaluation retained a high level of confidence for mapping the *Co* gene.

In the population of 'Fuji' × 8H-9-45 and 'Fuji' × 5-12786, we could not find the intermediate-type plants described by Hemmat et al. (1997) and Kim et al. (2003). On the other hand, in 'Fuji' × 'Tuscan', because all plants were young and small, unclear tree architecture, such as that of the intermediate type, was found. In this context, an intermediate phenotype could be due to its fragile growth.

To confirm the linkage of published DNA markers to the *Co* gene, we constructed three linkage maps of apple LG 10 (Fig. 2). The *Co* gene was successfully mapped between the investigated markers. In the report of Tian et al. (2005), $SCAR_{682}$ was mapped in the vicinity of *Co* than CH03d11; the distance was 2.9 and 3.9 cM, respectively. The inconsistency in the marker order between our map and the 'Telamon' map could be due to several reasons, such as the accuracy of phenotypic evaluation, unintentional mistake in marker genotyping, multiple recombinations occurred in adjacent regions, and differences in the software used in the linkage analysis.

$SCB82_{670}$, reported as the DNA marker most tightly linked to the *Co* gene (Kim et al., 2003), was not linked to the *Co* gene in our results; furthermore, it was not located on LG 10. Kim et al. (2003) developed $SCB82_{670}$ by bulked segregant analysis on the F_1 population of 'Fuji' × 'Tuscan'. We also produced the F_1 population of 'Fuji' × 'Tuscan' in this study and tried to confirm the linkage of $SCB82_{670}$ to the *Co* gene but failed to do so. In previous reports, $SCB82_{670}$ was detected only in 'Tuscan' (Fernandez-Fernandez et al., 2008; Kenis and Keulemans, 2007; Tian et al., 2005). Since the parentage of 'Tuscan' is 'Wijcik' as the maternal columnar-type parent and 'Greensleeves' as the paternal non-columnar parent, we tried to detect $SCB82_{670}$ on these cultivars. As a result, $SCB82_{670}$ was detected in 'Greensleeves' and 'Tuscan' but not in 'Wijcik' (data not shown), and their nucleotide sequences were identical to the original sequence of $SCB82_{670}$, suggesting that the locus

$SCB82_{670}$ was inherited by 'Greensleeves'.

We confirmed that the order of SSRs on each LG 10 was reasonable by drawing graphical genotypes of all individuals. The order of SSRs on each LG 10 was consistent with the apple reference map constructed by Silfverberg-Dilworth et al. (2006) and N'Diaye et al. (2008). The SSR marker Hi01a03, which was developed by Silfverberg-Dilworth et al. (2006), has not been investigated to determine whether it links to the *Co* gene. In our results, 177 bp of CH03d11 and $SCAR_{682}$ were co-segregated in the populations of 'Fuji' × 8H-9-45 and 'Fuji' × 5-12786, and their map distances from the *Co* gene were 0 cM and 1.4 cM, respectively. On the other hand, in the population of 'Fuji' × 'Tuscan', 177 bp of CH03d11 was co-segregated with 174 bp of Hi01a03 mapped on the opposite side of the *Co* gene in the other two populations. Furthermore, marker genotypes in the progenies of columnar-type plants (possessing *Co*) showed co-segregation of 177 bp of CH03d11, and recombination between *Co* occurred for 2 and 5 independent plants in $SCAR_{682}$ and 174 bp of Hi01a03, suggesting that $SCAR_{682}$ and Hi01a03 are located on different sides of *Co* (Table 3). These results indicated that 177 bp of CH03d11 was linked to the *Co* gene more tightly than $SCAR_{682}$ and that Hi01a03 was the most tightly linked to *Co* on the other side. The reliability of these results will be increased by mapping *Co* on the population of 'Fuji' × 'Tuscan'; for this objective, further investigation is required. Fernandez-Fernandez et al. (2008) mapped *Co* between CH03d11 and CH02c11. This might be an artifact because of low marker density in this region, and accuracy in the marker genotyping and the phenotypic evaluation. Thus, the relationship between the *Co* and molecular markers was slightly different in the respective reports, causing confusion about the precise location of *Co*, which retards progress in advanced studies of *Co*, such as map-based cloning. To reveal the precise location of *Co*, it is important to develop unified criteria for phenotypic evaluation, increasing marker density in this region, and using a large number of plants.

MAS in columnar-type apple breeding

With regard to LG 10, some quantitative trait loci (QTL) have been identified in previous studies. QTL concerning fruit vitamin C content, harvest date, diameter, weight, firmness, and sugar content were detected in the vicinity of the region surrounding CH03d11, i.e., close to the locus of the columnar habit, in the population of 'Telamon' × 'Braeburn' (Davey et al., 2006; Kenis et al., 2008). In another study, QTL affecting fruit weight was also identified on LG 10 in the population of 'Fiesta' × 'Discovery' (Liebhard et al., 2003). On the other hand, the columnar-type apple, especially 'Wijcik', had various unsuitable characteristics, such as low sugar content and soft fruit flesh. These results suggested that LG 10 was concerned with diverse traits of apple, including tree architecture and fruit quality, and the chromosome region associated with the *Co* gene could be linked to such poor characteristics; therefore, to develop a columnar-type apple with high fruit quality, the replacement of the *Co* gene-associated chromosome region with that of cultivars having excellent fruit quality is considered to be a possible approach.

When we were trying to use DNA markers for MAS in a practical breeding program, it was an important step to develop markers not only linked tightly to useful genes, but marker alleles associated with useful traits are rare in breeding materials (Tanaka, 2006). The columnar-type-associated alleles of $SCAR_{682}$, CH03d11, and Hi01a03 were "+" (682 bp), 177 bp, and 174 bp, respectively. On the other hand, 'Delicious', 'Golden Delicious', 'Indo', 'Jonathan', 'Ralls Janet', and 'Akane', which were the ancestral parental cultivars used reproducibly as parents in apple breeding in Japan (Kitahara et al., 2005), did not have the same alleles (Table 2). In 170 columnar-type seedlings from 18 crosses, 177 bp of CH03d11 showed perfect co-segregation to the columnar trait, while recombination seemed to occur between $SCAR_{682}$ and the *Co* gene in two individuals (Table 3). These results suggest that CH03d11 is the most tightly linked DNA marker to the *Co* gene on the distal side and the best marker to employ for MAS in the columnar-type apple breeding program of Japan. On the basal side of the *Co* gene region, Hi01a03 is the most tightly linked DNA marker; however, based on the mapped position and results of the detection of Hi01a03 in 170 columnar-type seedlings obtained from 18 crosses, Hi01a03 seems to be slightly inferior to CH03d11 in the detection of the columnar trait in the MAS system of apple breeding.

When offspring of 'McIntosh' (non-columnar type) are used to cross with a *Co* donor, we should pay attention to their marker genotype in MAS system. 'Kitakami', e.g., had the same alleles associated with *Co* on the investigated markers (Table 2); however, the more advanced breeding selection 'Morioka #61' did not have such an allele, so we could apply 'Morioka #61' in MAS easily. 'Shinano Red' had the same alleles associated with *Co* on CH03d11, Hi01a03, and $SCAR_{216}$, but did not have $SCAR_{682}$, indicating that $SCAR_{682}$ enables the use of 'Shinano Red' in the MAS system. It was considered that the lack of $SCAR_{682}$ in 'Shinano Red' was the result of recombination between $SCAR_{682}$ and CH03d11 on the chromosome of 'McIntosh' in the breeding process.

In Figure 3, the chromosome structures of our most advanced breeding selections, 5-8246 and 5-12786, are shown. 5-8246, which is scab-resistant and has high fruit quality, replaced the chromosome on the basal side of the *Co* gene. 5-12786, which has a high sugar content, retained the 'Wijcik' chromosome around the *Co* region. In these two selections, *Co*-associated alleles of 'Wijcik'

on CH03d11 and Hi01a03 remained, indicating that, if 5-8246 or 5-12786 may be used as *Co* donors in the breeding program, the detection of CH03d11 and Hi01a03 will allow us to select seedlings expressing a columnar phenotype. On the other hand, a smaller chromosome region around *Co* was found in 5-263. In this material, homozygous alleles on tested SSR markers or no amplified fragments on SCAR markers were observed, indicating the requirement of closer genetic markers when using this material in a MAS system.

In addition, multiplex PCR of CH03d11 and Hi01a03 is an economical method that will achieve the precise selection of columnar seedlings, thus saving both time and money. The identification of the *Co* gene (i.e., the mutation point of 'McIntosh') and development of a DNA marker including that point will ultimately lead to an accurate selection method in MAS of columnar-type apple breeding. Map-based cloning is one way to identify the *Co* gene. In further studies, CH03d11, $SCAR_{682}$, and Hi01a03 could be DNA markers at the starting point of the positional cloning of *Co*.

Literature Cited

Davey, M. W., K. Kenis and J. Keulemans. 2006. Genetic control of fruit vitamin C contents. Plant Physiol. 142: 343–351.

Fernandez-Fernandez, F., K. M. Evans, J. B. Clarke, C. L. Govan, C. M. James, S. Maric and K. R. Tobutt. 2008. Development of an STS map of an interspecific progeny of *Malus*. Tree Genet. Genom. 4: 469–479.

Fisher, D. V. 1970. Spur strains of McIntosh discovered in British Columbia. Can. Fruit Var. Hort. Digest. 24: 27–32.

Gianfranceschi, L., N. Seglias, R. Tarchini, M. Komjanc and C. Gessler. 1998. Simple sequence repeats for the genetic analysis of apple. Theor. Appl. Genet. 96: 1069–1076.

Grattapaglia, D. and R. Sederoff. 1994. Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: mapping strategy and RAPD markers. Genetics 137: 1121–1137.

Hemmat, M., N. F. Weeden, P. J. Conner and S. K. Brown. 1997. A DNA marker for columnar growth habit in apple contains a simple sequence repeat. J. Amer. Soc. Hort. Sci. 122: 347–349.

Kenis, K. and J. Keulemans. 2007. Study of tree architecture of apple (*Malus*×*domestica* Borkh.) by QTL analysis of growth traits. Mol. Breed. 19: 193–208.

Kenis, K., J. Keulemans and M. W. Davey. 2008. Identification and stability of QTLs for fruit quality traits in apple. Tree Genet. Genom. 4: 647–661.

Kim, M. Y., K. J. Song, J.-H. Hwang, Y.-U. Shin and H. J. Lee. 2003. Development of RAPD and SCAR markers linked to the *Co* gene conferring columnar growth habit in apple (*Malus pumila* Mill.). J. Hort. Sci. Biotechnol. 78: 512–517.

Kitahara, K., S. Matsumoto, T. Yamamoto, J. Soejima, T. Kimura, H. Komatsu and K. Abe. 2005. Molecular characterization of apple cultivars in Japan by *S*-RNase analysis and SSR markers. J. Amer. Soc. Hort. Sci. 130: 885–892.

Lapins, K. O. 1976. Inheritance of compact growth type in apple. J. Amer. Soc. Hort. Sci. 101: 133–135.

Lauri, P. E., E. Terouanne, J. M. Lespinasse, J.-M. Regnard and J.-J. Kelner. 1995. Genotypic differences in the axillary bud growth and fruiting pattern of apple fruiting branches over several years—An approach to regulation of fruit bearing. Sci. Hort. 64: 265–281.

Lespinasse, J. M. and J. F. Delort. 1986. Apple tree management in vertical axis: appraisal after ten years of experiments. Acta Hort. 160: 139–155.

Liebhard, R., L. Gianfranceschi, B. Koller, C. D. Ryder, R. Tarchini, E. Van de Weg and C. Gessler. 2002. Development and characterisation of 140 new microsatellites in apple (*Malus*×*domestica* Borkh.). Mol. Breed. 10: 217–241.

Liebhard, R., B. Koller, L. Gianfranceschi and C. Gessler. 2003. Creating a saturated reference map for the apple (*Malus*×*domestica* Borkh.) genome. Theor. Appl. Genet. 106: 1497–1508.

Luby, J. J. 2003. Taxonomic classification and brief history. p. 1–14. In: D. C. Ferree and I. J. Warrington (eds.). Apples: botany, production, and uses. CABI Publishing, Cambridge.

Maliepaard, C., F. H. Alston, G. v. Arkel, L. M. Brown, E. Chevreau, F. Dunemann, K. M. Evans, S. Gardiner, P. Guilford, A. W. V. Heusden, J. Janse, F. Laurens, J. R. Lynn, A. G. Manganaris, A. P. M. den Nijs, N. Periam, E. Rikkerink, P. Roche, C. Ryder, S. Sansavini, H. Schmidt, S. Tartarini, J. J. Verhaegh, M. V.-v. Ginkel and G. J. King. 1998. Aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers. Theor. Appl. Genet. 97: 60–73.

N'Diaye, A., W. E. Van de Weg, L. P. Kodde, B. Koller, F. Dunemann, M. Thiermann, S. Tartarini, F. Gennari and C. E. Durel. 2008. Construction of an integrated consensus map of the apple genome based on four mapping populations. Tree Genet. Genom. 4: 727–743.

O'Rourke, D. 2003. World production, trade, consumption and economic outlook for apples. p. 15–30. In: D. C. Ferree and I. J. Warrington (eds.). Apples: botany, production, and uses. CABI Publishing, Cambridge.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nat. Biotechnol. 18: 233–234.

Silfverberg-Dilworth, E., C. L. Matasci, W. E. Van de Weg, M. P. W. Van Kaauwen, M. Walser, L. P. Kodde, V. Soglio, L. Gianfranceschi, C. E. Durel, F. Costa, T. Yamamoto, B. Koller, C. Gessler and A. Patocchi. 2006. Microsatellite markers spanning the apple (*Malus*×*domestica* Borkh.) genome. Tree Genet. Genom. 2: 202–224.

Tanaka, J. 2006. Breeding system combining of juvenile selection using DNA markers and acceleration of generations, aming for the significant improvement of the tea breeding in Makurazaki Tea Research Station, NIVTS. Breed. Res. 8: 119–126 (In Japanese).

Tian, Y.-K., C.-H. Wang, J.-S. Zhang, C. James and H.-Y. Dai. 2005. Mapping *Co*, a gene controlling the columnar phenotype of apple, with molecular markers. Euphytica 145: 181–188.

Van Ooijen, J. W. and R. E. Voorrips. 2001. JoinMap 3.0, Software for the calculation of genetic linkage maps. Plant Research International, Wageningen, The Netherlands.

Zhu, Y. D., W. Zhang, G. C. Li and T. Wang. 2007. Evaluation of inter-simple sequence repeat analysis for mapping the *Co* gene in apple (*Malus pumila* Mill.). J. Hort. Sci. Biotechnol. 82: 371–376.

Ziegle, J. S., Y. Su, K. P. Corcoran, L. Nie, P. E. Mayrand, L. B. Hoff, L. J. McBride, M. N. Kronick and S. R. Diehl. 1992. Application of automated DNA sizing technology for genotyping microsatellite loci. Genomics 14: 1026–1031.