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Development of Expressed Sequence Tag (EST)-Based Cleaved Amplified Polymorphic Sequence (CAPS) Markers of Tea Plant and Their Application to Cultivar Identification

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ABSTRACT: To develop cleaved amplified polymorphic sequence (CAPS) markers for cultivar identification of the tea leaf, 5 primer pairs designed on the basis of genes that encode proteins related to nitrogen assimilation and 26 primer pairs based on expressed sequence tag (EST) sequences of the root of tea plant were screened. From combinations of primer pair and restriction enzyme that showed polymorphism among tea plants, 16 markers were selected and applied to DNA fingerprinting of Japanese tea cultivars. Sixty-three cultivars, except for a bud sport (Kiraka) and its original cultivar (Yabukita) and a pair that was the progeny of the same crossing parent (Harumoegi and Sakimidori), were distinguished from one another. By combining the 16 markers with previously developed CAPS markers and observing the physical appearance, 67 cultivars were distinguishable. The cultivars involve approximately 95% of total tea cultivating area in Japan; therefore, about 95% of tea leaves produced in Japan can be authenticated by labeling their cultivars.

KEYWORDS: *Camellia sinensis*, tea plant, fingerprinting, cultivar identification, CAPS

INTRODUCTION

Tea is a beverage widely consumed throughout the world. Many types of tea are made from young shoots of the tea plant [*Camellia sinensis* (L.) O. Kuntze]. They are divided broadly into three types: deeply fermented tea (black tea), which is mainly produced from one of two varieties of tea plant; *assamica*, semifermented tea; and nonfermented tea (green tea). Another variety of the tea plant, *sinensis*, is mainly used for semifermented and green tea production. Green tea is mainly produced and consumed in East Asia and recently has gained attention as a healthy beverage[1−3] in regions such as the United States and European countries, where green tea was not previously popular. In fact, the export of Japanese green tea to these countries increased approximately 2−3 times in the 5-year period from 2003 to 2008 [Trade Statistics of Japan, Ministry of Finance, Japan (http://www.customs.go.jp/toukei/info/index_e.htm)]. Interest in Japanese green tea is increasing around the world.

The content and composition of bioactive compounds often vary among cultivars,[4,5] and the characteristic aroma and taste of Japanese green tea are dependent on the cultivars.[6] Accordingly, consumers are interested in information on particular tea cultivars. Because deceptive labeling of cultivars may be used to drive sales of particular products, authenticating the characteristics of Japanese green tea is important. DNA fingerprinting is a good method for cultivar identification of commercial tea leaves because it is almost impossible to identify the original cultivars of processed tea leaves by appearance. For cultivar identification of green tea leaves, methods using cleaved amplified polymorphic sequence (CAPS) and simple sequence repeat (SSR) markers have been developed, and it was reported that they were applicable in cultivar identification of processed tea leaves.[7−11] However, more DNA markers are required to accommodate increasing new cultivars because limited elite cultivars and their progeny have been used recently for cross-breeding. Such new cultivars have similar genotypes in DNA markers. For example, one of the newly developed cultivars, Harumidori, shows the same fingerprint pattern as Toyoka using previous CAPS markers. At least 11 cultivars have been newly developed without fingerprinting using these markers. It is necessary to identify each cultivar component in mixed samples of Japanese green tea leaves. Blending is an essential process in the production of Japanese green tea. Commercial green teas in Japan are generally produced by a two-stage process (crude tea and refined tea production). In refined tea production, some monovarietal crude teas are blended to meet the required quality standards of each manufacturer.

Although SSR is an excellent DNA marker and is highly variable, the amplification efficiency of each allele is frequently unequal when a mixture of multiple cultivars is analyzed. Therefore, analysis by SSR marker is not necessarily suitable for mixed samples of green tea. On the other hand, CAPS markers possess the potential to be converted to more high-resolution markers such as single-nucleotide polymorphism (SNP) and structural variation, and these markers will be effective for mixed samples.

In this study, as a countermeasure for newly registered cultivars and cultivars that are indistinguishable by previously

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Table 1. Japanese Cultivars Used for Fingerprinting and Their Origin or Parents

cultivar	origin/parents
Asagiri	selection, from Japanese local variety
Asahi	selection, from Japanese local variety
Asanoka	Yabukita × Ka-Cn1
Asatsuyu	selection, from Japanese local variety
Benifuji	Benihomare × C19
Benifuki	Benihomare × MakuraCd86
Benihikari	Benikaori × Ka-Cn1
Benihomare	progeny of *assamica* variety
Fujikaori	Shizu-Inzatsu131 × Yabukita
Fujimidori	unknown
Fukumidori	Yabukita × (Sayamamidori × Yabukita)
Fushun	Z-1 × Kanayamidori
Gokou	selection, from Japanese local variety
Harumidori	Kanayamidori × Yabukita
Harumoegi	NN27 × ME52
Hatsumomiji	Ai2 × Nka05
Himemidori	selection, from Japanese local variety
Hokumei	Sayamamidori × Sai5507
Izumi	progeny of Benihomare
Kanayamidori	S-6 × Yabukita
Karabeni	progeny of Chinese local variety
Komakage	selection, from Japanese local variety
Koushun	Kurasawa × Kanayamidori
Kurasawa	progeny of Yabukita
Kuritawase	selection, from Japanese local variety
Makinoharawase	selection, from Japanese local variety
Marishi	progeny of Sugiyamayaeho
Meiryoku	Yabukita × Z-1
Minamikaori	Yabukita × Miya A-11
Minamisayaka	Miya A-6 × NN27
Minekaori	Yabukita × Unkai
Miyamakaori	Kyouken283 × Sayamamidori
Musashikaori	Yabukita × 27F1-73
Natsumidori	selection, from Japanese local variety
Oiwase	Yaeho × Yabukita
Okuhikari	Yabukita × ShizuCy225
Okumidori	Yabukita × Shizuzai16
Okumusashi	Sayamamidori × Yamatomidori
Okuyutaka	Yutakamidori × NN8
Ryoufu	Houryoku × Yabukita
Saemidori	Yabukita × Asatsuyu
Sainomidori	Sayamamidori × Saitama7gou
Sakimidori	NN27 × ME52
Samidori	selection, from Japanese local variety
Sawamizuka	Yabukita × Fujimidori
Sayamakaori	progeny of Yabukita
Sayamamidori	selection, from Japanese local variety
Shunmei	Yutakamidori × NN8
Sofu	Yabukita × Shizu-Inzatsu131
Surugawase	progeny of Yabukita
Tadanishiki	progeny of *assamica* variety
Takachiho	selection, from Japanese local variety
Tamamidori	selection, from Japanese local variety
Toyoka	Sayamamidori × Yabukita
Tsuyuhikari	Shizu7132 × Asatsuyu
Ujihikari	selection, from Japanese local variety
Yaeho	selection, from Japanese local variety
Yamakai	progeny of Yabukita
Yamanami	progeny of Chinese local variety
Yamanoibuki	progeny of Yabukita
Yamatomidori	selection, from Japanese local variety
Yutakamidori	progeny of Asatsuyu
Kiraka	bud sport of Yabukita
Yabukita	selection, from Japanese local variety
Shizu7132	progeny of Yabukita
Shizu-Inzatsu131	hybrid between *sinensis* and *assamica* varieties
Z-1	progeny of Takachiho

reported methods, we developed CAPS markers based on genes related to nitrogen assimilation and expressed sequence tag (EST) sequences from the root of the tea plant. These markers were applied to fingerprinting Japanese tea cultivars to authenticate labeling of green tea leaves produced in Japan.

MATERIALS AND METHODS

Plant Materials. DNA was extracted from fresh leaves of tea plants collected at Kanaya (Shizuoka, Japan) and Makurazaki (Kagoshima, Japan) Tea Research Stations of the National Institute of Vegetable and Tea Science (NIVTS) using the CTAB method.[12] Fourteen tea plants, including 7 Japanese cultivars that are used for commercial tea production (Asatsuyu, Benifuki, Sayamakaori, Shizu7132, Shizu-Inzatsu131, Yabukita, and Z-1), 4 mating parents of some commercial cultivars (KanaCk17, ME52, Shizuzai16, and Ka-Cp1), and 3 genetic resources (Taiwan-yamacha95, Ak1699, Makura1gou) stored at Makurazaki Tea Research Station of NIVTS, as well as 2 *Camellia* species [*C. japonica* (L.) and *C. chrysantha* (Hu) Tuyama], were used for marker development. For fingerprinting, 60 additional commercial Japanese cultivars were used. Names and origins or parents of the 67 cultivars used for fingerprinting in this study are shown in Table 1.

Primers. To develop CAPS markers, 5 primer pairs designed on the basis of genes encoding three glutamine synthetases and two ammonium transporters of the tea plant, and 26 randomly selected primer pairs for EST sequence of the tea plant root were tested. A report on the construction and analysis of the EST library by Taniguchi and Tanaka is in progress. In brief, a cDNA library was constructed from poly(A) RNA purified from the roots of 2-week-old seedlings of the tea plant. Sequence data of both strands were acquired from each clone, and a unigene set was developed from the sequence data. The derived amino acid sequences were used for BLASTX search over the GenBank nonredundant database to assign functions of each sequence by using Blast2GO.[13]

Amplification of CAPS Fragments and Detection of Polymorphism. A polymerase chain reaction (PCR) mixture contained 50−150 ng of DNA, 0.25 μM of each primer, 200 μM of dNTPs, 2 mM magnesium chloride, 1× *Ex Taq* buffer, and 0.025 U of *TaKaRa Ex Taq* DNA polymerase (Takara, Shiga, Japan) and brought up to a 15 μL final volume with distilled water. PCR conditions were the same as for other CAPS markers in tea plants, except for annealing temperature.[8] For primer pairs designed to amplify genes for nitrogen assimilation, the annealing temperature was 58 °C; for primer pairs based on EST sequences, the annealing temperature was 60 °C. PCR was performed

Table 2. Primer Sequences and Polymorphic Restriction Enzymes of the 16 CAPS Markers

fragment (accession no.)	gene name or annotation (gene name in database, plant name, accession no., *E* value)	primer sequence (5′−3′)	annealing temperature (°C)	amplified fragment size (bp)	enzyme
AMT1[a] (AB117640)	ammonium transporter	forwardCCAGCGCTTCCAACACAAACC reverse CCTTCTGAAAGTGGTTTGAACAGAGC	58	1358 1076	*Dde* I *Rsa*I
AMT2 (AB114913)	ammonium transporter	forwardACCTCCGGCGCCCTCTTGTT reverse GCTGATACGTAGGAAGTTTGACTCTG	58	1014	*Alw*26I
GS1A (AB115183)	glutamine synthetase	forwardTTTGAGGTCATCAAAAAGGCCATT reverse ACACAAAGGTTGGGTTGGGATTATG	58	919	*Alw*26I *Rsa*I
GS1C[a] (AB115184)	glutamine synthetase	forwardTGGTGAAGGCAACGAGCGTC reverse AAAGATGATAATAAAATTAACCCCACCA-TATTGT	58	1320[c] 1140 1095 1073 874	*Hin*fI *Rsa*I
GS2B[a] (AB117934)	glutamine synthetase	forwardGCTTATGGAGAAGGCAATGAGCG reverse AACAGCCAAACATACCATGATGAATTTAC	58	1420 1142	*Hin*fI *Taq*I
3B02S[b] (FS951215)	pathogenesis-related protein (pathogenesis-related protein 10, *Solanum virginianum*, AAU00066, 1.95e−43)	forwardACCCACAATCAACTCATTCTCATT reverse TACTTGGTTCAAGCATAAACATCAGA	60	682	*Hae*III
C5S22[b] (FS951237)	nucleic acid binding protein (nucleic acid binding protein, putative, *Ricinus communis*, XP_002511182, 1.23e−35)	forwardCCTTCTCTCCTCACCACCTT reverse AAAATTTAAGGTCGAAATTTTTGC	60	558	*Dde*I *Msp*I
C10S38[b] (FS 951245)	tubulin β chain (tublin β chain, putative, *Ricinus communis*, XP_002271992, 6.77e−83)	forwardCAGCCAGTGACCATTTTTAGC reverse TTGAGTGGATCCCCAACAAT	60	551	*Hin*fI *Msp*I
E3S12[b] (FS951271)	pathogenesis-related protein (pathogenesis-related protein 10, *Solanum virginianum*, AAU00066, 1.95e−43)	forwardTCATTTTGGTTTTACATGCTTCA reverse TTGATGTTGAGAACTATAAGTGCAA	60	556	*Alw*26I *Hin*fI

[a] They showed ALP in tea plant. [b] These sequences were selected from EST of the tea plant root. [c] This fragment was detected in MAKURA1gou only.

with a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) and Mastercycler gradient (Eppendorf, Tokyo, Japan). Three microliters of PCR reaction mix was used for restriction digestion. Polymorphism was detected using 1.5−2.0% agarose gel electrophoresis stained with ethidium bromide.

DNA Sequencing. For DNA sequencing, CAPS fragments were amplified for polymorphism detection and purified with a QIAquick PCR Purification Kit (Qiagen, Tokyo, Japan). Purified CAPS fragments were ligated to pT7Blue T-Vector (Novagen, available from Merck KGaA, Darmstadt, Germany), and the vectors were used to transform *Escherichia coli* HB101. Insert size was checked by direct PCR amplification from colonies grown on an LB agar plate containing 80 μg/mL ampicillin. The components of the PCR reaction mix were the same as for polymorphism detection, except for the absence of template DNA solution. Positive samples were purified with a QIAquick PCR Purification Kit. The nucleotide sequence of purified products was determined using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI310 genetic analyzer (Applied Biosystems).

RESULTS AND DISCUSSION

Development and Selection of CAPS Markers. To amplify five genes related to nitrogen metabolism, the primers were designed to amplify regions of approximately 1 kb including introns by comparison with the genes of other plant species. Among 26 primer pairs for EST sequences, 18 pairs gave amplification products and the remaining 8 pairs did not yield product. Five of 18 showed larger (approximately 2.5−4 kb) amplification fragments than expected (500 bp). These five primer pairs were eliminated because long fragments would be difficult to amplify, and the cleaved fragment pattern was also

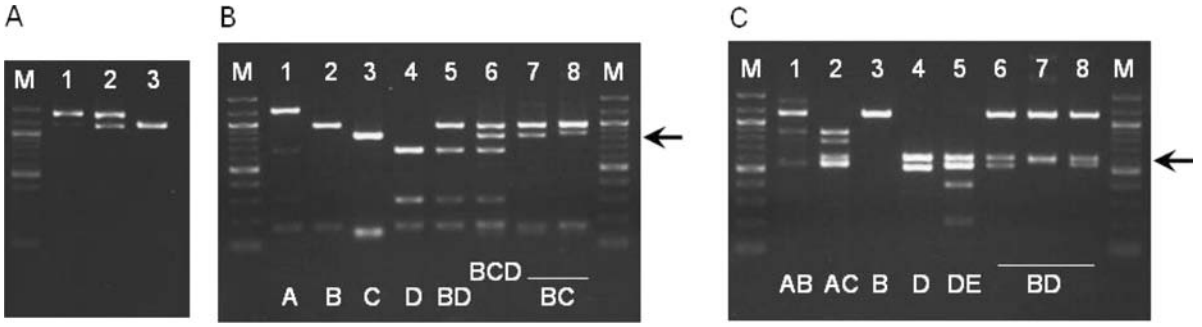

Figure 1. ALP of GS2B and representative cleaved fragment patterns of GS2B/*Hin*fI and GS2B/*Taq*I: (A) ALP detected in GS2B (lanes: M, 100 bp DNA ladder (NEB); 1, longer amplified fragment only; 2, both longer and shorter amplified fragments; 3, shorter amplified fragment only); (B) cleaved fragment patterns of GS2B/*Hin*fI (lanes: M, 100 bp DNA ladder (NEB); 1, fragmentation type A; 2, fragmentation type B; 3, fragmentation type C; 4, fragmentation type D; 5, combination of fragmentation types B and D; 6, combination of fragmentation types B, C, and D; 7 and 8, combination of fragmentation types B and C); (C) cleaved fragment patterns of GS2B/*Taq*I (lanes: M, 100 bp DNA ladder (NEB); 1, combination of fragmentation types A and B; 2, combination of fragmentation types A and C; 3, fragmentation type B; 4, fragmentation type D; 5, combination of fragmentation types D and E; 6−8, combination of fragmentation types B and D). Cleaved fragments show small size difference caused by SSR (B and C, indicated by arrow); however, these size differences are not counted because the exact size difference could not be detected by agarose gel electrophoresis.

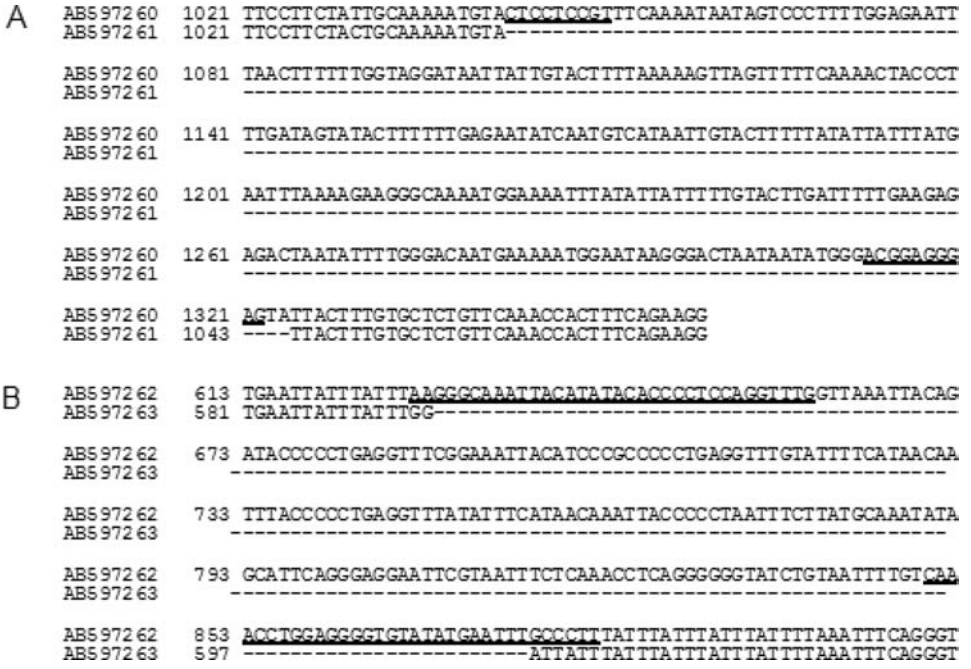

Figure 2. Sequences of inserted fragments in longer amplicon of AMT1 (A) and GS2B (B). In these fragments, difference of amplified fragment length is caused by insertion of small fragments into original fragments. The inserted fragments contain a short region that has innverted repeat sequences at their termini though mutation (insertion of one nucleotide into putative inverted repeat) occurring in inverted repeat sequences of both fragments.

expected to be complicated. Amplification fragments were digested with 13 restriction enzymes to test whether they showed polymorphism. For the test, eight four-cutter (*Rsa*I, *Hpa*II, *Hae*III, *Hha*I, *Alu*I, *Bsh*1236I, *Taq*I, *Tru*1I) and five five-cutter (*Hin*fI, *Dde*I, *Alw*26I, *Ava*II, *Tsp*RI) enzymes were used. Among the 16 samples, *C. chrysantha* did not give any fragments from all primer pairs used in this study. Therefore, this sample was omitted from further analyses. All five primers designed for nitrogen metabolism genes gave amplification fragments in tea plants; however, *C. japonica* did not give AMT1 fragment. Two of 15 samples did not give amplification fragments of E3S12 (data not shown). Details about this fragment are mentioned below. For the application of the developed CAPS markers to cultivar identification, markers that have intraspecific polymorphism and clear fragment patterns were selected. As a result, 16 markers were used for DNA fingerprinting of tea cultivars. Information about primers and restriction enzymes that were selected for fingerprinting is shown in Table 2, and detected cleaved fragment patterns are shown in Table 3. Markers were named as fragment/restriction enzymes. To illustrate cleaved fragment patterns, the pattern of fragment GS2B is shown in Figure 1.

Amplified fragments of AMT1 and GS1C showed ALP. Amplified fragments of GS2B also showed ALP; in addition, an SSR of (TC)n was found in an intron region. This SSR caused a small size difference in the restriction fragment. For example, the repeat numbers were 13 and 20 in the shorter fragment (accession no. B597263) and longer fragment (AB597262) of Shizu7132, respectively, and repeat numbers were 12 and 31 in

Table 3. Detected Fragmentation Types in Each CAPS Marker

	original fragment size	size of cleaved fragments[a]	fragmentation type	accession no.
AMT1/ *Dde*I	1358	800 + 558	A	AB597260
	1076	1076	B	
	1076	800 + 276	C	AB597261
AMT1/ *Rsa*I	1358	437 + 413 + 116 + 91 + 74 + 70 + 66 + 62 + 29	A	AB597260
	1076	528 + 449 + 70 + 29	B	
	1076	449 + 437 + 91 + 70 + 29	C	AB597261
AMT2/ *Alw*26I	1014	931 + 83	A	
		719 + 212 + 83	B	
GS1A/ *Alw*26I	919	495 + 317 + 87 + 20	A	
		495 + 174 + 143 + 87 + 20	B	
GS1A / *Rsa*I	919	919	A	
		627 + 292	B	
GS1C/ *Hin*fI	1320	755 + 271 + 188 + 74 + 32	A[b]	AB597264
	1140	755 + 197 + 188	B	
		755 + 197 +188	C	AB597265
	1095	710 + 197 + 188	D	AB597266
		548 + 197 + 188 + 159	E	
	1073	755 + 164 + 154	F	AB597267
	874	497 + 189 + 188	G	AB597268
GS1C/ *Rsa*I	1320	609 + 578 + 133	A[b]	AB597264
	1140	1007 + 133	B	
		578 + 429 + 133	C	AB597265
	1095	579 + 429 + 31 + 56	D	AB597266
		576 + 429 + 31 + 56	E	
	1073	578 + 362 + 133	F	AB597267
	874	753 + 121	G	AB597268
GS2B/ *Hin*fI	1420	1252 + 168	A	AB597262
	1142	974 + 168	B	AB597263
		830 + 168 + 142	C	
		674 + 299 + 170	D	
Gs2B/ *Taq*I	1420	870 + 550	A	AB597262
	1142	1143	B	
		612 + 537	C	
		597 + 545	D	AB597263
		545 + 389 + 206	E	
3B02S/ *Hae*III	682	682	A	
		612 + 70	B	
C5S22/ *Dde*I	558	558	A	
		291 + 267	B	
C5S22/ *Msp*I	558	491 + 67	A	
		372 + 119 + 67	B	
C10S38/ *Hin*fI	551	551	A	
		434 + 117	B	
C10S38/ *Msp*I	551	551	A	
		497 + 54	B	
E3S12/ *Alw*26I	564	446 + 118	A	
		385 + 118 + 61	B	
E3S12/ *Hin*fI	564	298 + 196 + 70	A	
		196 + 149 + 149 + 70	B	

[a] The total fragment size of each type possibly does not correspond to the original fragment size because of insertion/deletion of single or several nucleotides. [b] This fragmentation type was not detected in commercial Japanese cultivars.

the fragments of Yamatomidori. However, the size difference could not be detected exactly by agarose gel electrophoresis (Figure 1). Therefore, we confined the polymorphism of this marker solely to the ALP easily detectable by agarose gel electrophoresis and the existence or nonexistence of restriction site. In a longer fragment of AMT1 (AB597260), a small fragment that had short inverted repeats in its terminus was inserted into 3′-UTR (Figure 2A). In a longer fragment of GS2B, insertion of a small fragment, which had short inverted sequences in its terminus, was found in the intron region of the fragment (Figure 2B).

Fingerprinting of the Japanese Tea Cultivars Using CAPS Markers. Next, we used the developed CAPS markers for fingerprinting Japanese tea cultivars. Sixty-seven cultivars used in this study and their fragmentation patterns at each marker are shown in Table 4. The cultivars involve about 95% of the total tea-cultivating area in Japan.

Gokou, Kuritawase, Natsumidori, and Ujihikari did not give the E3S12 fragment because of a null allele. In AMT1, 11 of 67 cultivars (Asatsuyu, Kuritawase, Natsumidori, Okumusashi, Samidori, Sayamamidori, Takachiho, Tamamidori, Tsuyuhikari, Ujihikari, and Z-1) showed longer amplified fragment (fragmentation type A). In GS2B, five cultivars (Minamikaori, Minekaori, Surugawase, Tsuyuhikari, and Shizu7132) showed longer amplified fragment (fragmentation type A). Accordingly, AMT1 and GS2B could be used as screening markers for Tsuyuhikari. The quality of tea leaf made by Tsuyuhikari is excellent, and its growing area increased 1.8 times between 2006 and 2007. For the other cultivars, except Asatsuyu and Minamikaori, the growing area did not change or decreased during the same period.

Several cultivars showed disordered fingerprint patterns in marker GS2B/*Hin*fI. Although cultivars used in this study with the exception of Makinoharawase are diploid (n = 15), three fragmentation types (B, C, and D) were detected in these cultivars. This is because GS2B might be multilocus, or a similar sequence that can be amplified by primers designed for GS2B

Table 4. Fragmentation Patterns of Japanese Tea Cultivars in Each CAPS Marker

	AMT1/ *Dde*I	AMT1/ *Rsa*I	AMT2/ *Alw*26I	GS1A/ *Alw*26I	GS1A/ *Rsa*I	GS1C/ *Hin*fI	GS1C/ *Rsa*I	GS2B/ *Hin*fI	GS2B / *Taq*I	3B02S / *Hae*III	C5S22/ *Dde*I	C5S22/ *Msp*I	C10S38/ *Hin*fI	C10S38/ *Msp*I	E3S12/ *Alw*26I	E3S12/ *Hin*fI
Asagiri	B	BC	A	AB	B	C	C	BC	D	A	AB	AB	B	A	B	A
Asahi	BC	BC	A	A	B	CE	CE	B	D	A	AB	AB	B	A	B	A
Asanoka	C	BC	A	AB	A	CE	CE	BCD	BD	A	AB	B	B	A	B	A
Asatsuyu	AC	AC	A	AB	B	CF	CF	BC	D	A	B	AB	B	A	B	A
Benifuji	BC	BC	A	AB	B	C	C	BC	D	A	AB	B	B	AB	B	A
Benifuki	BC	BC	A	AB	B	C	C	BC	D	A	A	B	B	AB	AB	A
Benihikari	C	BC	A	AB	B	CE	CE	BD	DE	A	AB	B	AB	A	B	A
Benihomare	BC	BC	A	AB	B	C	C	B	BD	A	A	B	B	B	B	A
Fujikaori	C	C	AB	AB	B	BE	BE	BCD	BD	A	A	B	B	AB	AB	A
Fujimidori	C	BC	A	AB	B	CF	CF	BCD	DE	A	B	AB	AB	A	B	A
Fukumidori	C	BC	A	AB	AB	CE	CE	BC	D	AC	A	B	B	A	B	A
Fushun	BC	BC	A	B	B	CF	CF	BC	D	A	AB	AB	B	A	B	A
Gokou	C	C	A	AB	AB	CE	CE	C	D	A	AB	B	B	A	—[a]	—[a]
Harumidori	C	C	AB	AB	AB	EF	EF	B	D	A	AB	AB	B	A	B	A
Harumoegi	BC	BC	AB	B	B	EF	EF	BCD	BD	A	A	B	B	A	B	A
Hatsumomiji	C	C	A	AB	B	C	C	BCD	DE	A	AB	B	B	AB	B	A
Himemidori	C	BC	A	B	B	F	F	BC	D	A	B	A	B	A	B	A
Hokumei	BC	BC	A	AB	AB	C	C	BC	D	A	A	B	B	A	B	A
Izumi	BC	BC	A	AB	B	CE	CE	BC	BD	A	AB	B	B	AB	B	A
Kanayamidori	C	C	AB	B	B	EF	EF	BC	D	A	AB	AB	B	A	B	A
Karabeni	C	BC	A	B	AB	CE	CE	C	D	A	AB	AB	B	AB	B	A
Komakage	C	BC	A	AB	B	E	E	BC	D	A	B	A	B	A	B	A
Koushun	BC	BC	AB	AB	B	E	E	BD	BD	AB	A	B	B	A	B	A
Kurasawa	BC	BC	A	A	AB	CE	CE	D	B	AB	A	B	B	AB	B	A
Kuritawase	AB	AB	A	B	B	E	E	BC	D	A	B	A	B	AB	—[a]	—[a]
Makinohara-wase	BC	BC	A	AB	AB	EF	EF	BC	D	A	B	A	B	A	B	A
Marishi	BC	BC	A	B	B	C	C	BCD	BD	A	AB	B	B	A	B	A
Meiryoku	BC	BC	AB	AB	B	DE	DE	BC	D	A	AB	AB	B	A	B	A
Minamikaori	C	B	B	AB	B	CE	CE	AD	AB	A	A	B	B	A	B	A
Minamisayaka	BC	BC	AB	AB	B	EF	EF	BC	D	A	A	B	B	A	B	AB
Minekaori	C	BC	AB	AB	AB	CE	CE	AB	AC	A	AB	B	B	A	B	A
Miyamakaori	C	BC	A	B	B	CE	CE	BD	BD	A	AB	AB	B	AB	B	A
Musashikaori	C	BC	AB	A	A	CE	CE	BCD	BD	A	A	B	B	A	B	A
Natsumidori	AC	AC	A	A	AB	CE	CE	BC	D	A	AB	AB	B	AB	—[a]	—[a]
Oiwase	BC	BC	AB	B	B	EF	EF	D	B	A	A	B	B	A	B	A
Okuhikari	C	BC	AB	A	AB	CE	CE	BC	D	A	A	B	B	A	B	A
Okumidori	C	BC	A	A	AB	CE	CE	BCD	BD	A	A	B	B	A	B	A
Okumusashi	AB	AB	A	AB	B	C	C	C	D	A	A	B	B	A	B	A
Okuyutaka	C	C	A	B	B	CF	CF	BC	D	A	B	AB	B	AB	B	A
Ryoufu	C	BC	A	AB	B	C	C	BD	BD	A	AB	AB	B	A	B	A
Saemidori	C	C	AB	AB	B	EF	EF	B	D	A	AB	AB	B	A	B	A
Sainomidori	C	C	AB	A	AB	CE	CE	BD	B	A	A	B	B	AB	B	A
Sakimidori	BC	BC	AB	B	B	EF	EF	BCD	BD	A	A	B	B	A	B	A
Samidori	AC	AC	A	AB	AB	CE	CE	BC	D	A	A	B	B	A	B	A
Sawamizuka	C	C	AB	A	AB	EF	EF	BCD	BD	A	AB	AB	AB	A	B	A
Sayamakaori	BC	BC	AB	AB	AB	CE	CE	B	BD	A	A	B	B	A	B	A
Sayamamidori	AB	AB	A	B	B	C	C	BC	D	A	A	B	B	A	B	A
Shunmei	C	C	A	B	B	CF	CF	C	D	A	AB	B	B	A	B	A
Sofu	C	BC	AB	B	B	BE	BE	BC	D	A	A	B	B	AB	AB	A
Surugawase	BC	BC	AB	B	B	E	E	AB	AC	A	AB	B	B	A	B	A
Tadanishiki	BC	BC	A	AB	AB	CF	CF	B	BD	A	AB	AB	B	A	B	A
Takachiho	AB	AB	A	B	B	CE	CE	C	D	A	AB	AB	B	A	B	A

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Table 4. Continued

	AMT1/ *Dde*I	AMT1/ *Rsa*I	AMT2/ *Alw*26I	GS1A/ *Alw*26I	GS1A/ *Rsa*I	GS1C/ *Hin*fI	GS1C/ *Rsa*I	GS2B/ *Hin*fI	GS2B / *Taq*I	3B02S / *Hae*III	C5S22/ *Dde*I	C5S22/ *Msp*I	C10S38/ *Hin*fI	C10S38/ *Msp*I	E3S12/ *Alw*26I	E3S12/ *Hin*fI
Tamamidori	AC	AC	A	A	AB	CE	CE	C	D	A	B	A	B	A	B	A
Toyoka	BC	BC	A	B	B	CE	CE	B	D	A	A	B	B	A	B	A
Tsuyuhikari	AC	AC	A	A	AB	CF	CF	AB	AC	A	B	A	B	A	B	A
Ujihikari	AC	AC	A	AB	AB	CF	CF	BC	D	A	AB	AB	B	A	—[a]	—[a]
Yaeho	BC	BC	A	B	B	CF	CF	BCD	BD	A	A	B	B	AB	B	A
Yamakai	C	BC	AB	AB	B	CE	CE	BD	BD	A	AB	B	B	A	B	A
Yamanami	C	BC	A	AB	AB	CD	CD	BC	D	AB	AB	AB	B	A	B	A
Yamanoibuki	BC	BC	A	B	B	CE	CE	BC	D	A	A	B	B	A	B	A
YamatomidoriBC		BC	A	AB	B	CG	CG	BCD	BD	A	A	B	B	AB	B	A
Yutakamidori	C	BC	A	B	B	C	C	BC	D	A	AB	B	B	AB	B	A
Kiraka	C	C	AB	AB	AB	E	E	BD	BD	A	A	B	B	A	B	A
Yabukita	C	C	AB	AB	AB	E	E	BD	BD	A	A	B	B	A	B	A
Shizu7132	C	C	A	A	AB	C	C	AB	AD	A	AB	AB	B	AB	B	A
Shizu-Inzatsu131	C	BC	A	AB	B	BC	BC	C	D	A	AB	AB	B	B	A	A
Z-1	AB	AB	A	AB	B	CD	CD	BC	D	A	B	A	B	AB	B	A

[a] No amplification.

exists in tea genome. Furthermore, this pattern might be caused by a heteroduplex of fragments that show fragmentation types C and D in this marker.

Among 16 markers, 6 showed unique fingerprint patterns. In marker AMT1:1/*Dde*I, only Asagiri showed fragmentation type B exclusively. In marker AMT2/*Alw*26I, Minamikaori showed type B only. In marker C10S38/*Msp*I, Benihomare showed type B only. In marker E3S12/*Hin*fI, Minamisayaka showed both fragmentation types A and B, and this cultivar showed only type B among 67 Japanese cultivars used in this study. Minamisayaka has resistance against some serious pests,[14,15] and its growing area doubled between 2006 and 2007; this marker could be used to identify this emerging cultivar. In GS1C, fragmentation type D was detected in three cultivars (Meiryoku, Yamanami, and Z-1); type B also was detected in three cultivars (Fujikaori, Sofu, and Shizu7132), and type G was detected in Yamatomidori only. Fragmentation type A was not detected in commercial Japanese cultivars.

For the identification of tea cultivars, 47 Japanese cultivars had been identified using 6 of 13 CAPS markers[8] developed on the basis of three genes encoding enzymes in the flavonoid synthesis pathway.[7,11] However, Toyoka and Harumidori had been indistinguishable by these six markers because they showed the same fingerprinting patterns. These two cultivars were distinguished using CAPS markers developed in this study.

Using 16 CAPS markers constructed in this study, 63 of 67 tested Japanese tea cultivars could be distinguished from one another. Two pairs of cultivars, Harumoegi/Sakimidori and Yabukita/Kiraka, showed the same fingerprint patterns and could not be distinguished from each other by the CAPS markers used in this study. For Harumoegi and Sakimidori, it is because these two cultivars are progeny of the same mating pair. However, these cultivars can be distinguished using previously developed CAPS markers;[7] Harumoegi shows fragmentation types A, B, and AB at markers PALex1/*Hpa*II, CHSex2/*Rsa*I, and CHSex2/*Bsp*HI, respectively, whereas Sakimidori shows AB, AB, and B, respectively. Yabukita and Kiraka could not be distinguished using CAPS markers because Kiraka is the bud sport of Yabukita. To distinguish such cultivars by DNA, a different approach that compares a wide region of the genome will be needed.[16,17] However, Yabukita and Kiraka can be distinguished by their appearance: the shoots of Kiraka are yellowish-white to yellow in color, and the color of processed leaves is also yellow-tinged in comparison with the other 66 cultivars, including Yabukita. Therefore, although the new CAPS markers distinguish the 63 cultivars, in practical terms, 67 tested Japanese tea cultivars were distinguished from each other. This was accomplished by adding CAPS fingerprint patterns of previously reported markers to judgment based on appearance to the CAPS fingerprint patterns of the markers developed in this study.

In conclusion, using the selected 16 CAPS markers based on the genes related to nitrogen metabolism and EST sequence of the root of tea plant, 63 Japanese cultivars, except for 1 pair and 1 bud sport and its origin, were substantially distinguished from one another. For a pair from the same mating pair, they were distinguishable using other CAPS markers reported previously. The bud sport can be easily identified by appearance. The 63 cultivars contain 2 cultivars that had been indistinguishable by previously developed CAPS markers. By combined application of the 16 CAPS markers developed in this study with the previously developed CAPS markers and judgment by appearance, 67 cultivars involving about 95% of the total tea-cultivating area in Japan can be distinguished from one another. The cultivar labeling of approximately 95% of tea leaves produced in Japan can be authenticated using this method.

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■ ABBREVIATIONS USED

CAPS, cleaved amplified polymorphic sequence; SSR, simple sequence repeat; SNP, single-nucleotide polymorphism; EST, expressed sequence tag; PCR, polymerase chain reaction; ALP, amplicon length polymorphism.

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