Theor Appl Genet (2005) 110: 1410–1418
DOI 10.1007/s00122-005-1956-1

ORIGINAL PAPER

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CAPS markers improved by cluster-specific amplification for identification of octoploid strawberry (*Fragaria* × *ananassa* Duch.) cultivars, and their disomic inheritance

Received: 27 July 2004 / Accepted: 5 February 2005 / Published online: 21 April 2005

Abstract Cleavage amplified polymorphic sequence (CAPS) markers of strawberry (*Fragaria* × *ananassa* Duch.) can be useful for identifying mislabeled or patent-infringing cultivars in the marketplace. However, CAPS markers in octoploid strawberry tend to give unclear bands because multiple homologous sites are simultaneously amplified by the non-selective PCR. To overcome this problem, we used ''cluster-specific amplification'' based on the nucleotide sequences of PCR products and were able to improve the band clarity of 18 CAPS markers. By analyzing the marker segregation ratio, we demonstrated that 13 clarified markers were derived from single diploid loci that were transmitted to progeny in a manner consistent with Mendelian inheritance. We discuss the genomic structure of octoploid strawberry from the viewpoint of cluster and segregation analysis and suggest that it comprises independent genomes. We tested the utility of all of the markers we developed for cultivar identification and confirmed their ability to distinguish among 64 strawberry cultivars.

Introduction

As a result of growing consumer demand, increasingly more strawberry cultivars (*Fragaria* × *ananassa*) are entering the Japanese marketplace. As the market price of the most popular cultivars tends to be higher, the accurate and reliable labeling of cultivars is important not only to consumers but also to nursery growers and farmers. It is necessary to prevent the unregulated propagation and distribution of high-value strawberry cultivars in order to protect breeders' rights. In light of these concerns, the development of a practical technique for identifying strawberry cultivars is required.

Several types of DNA markers have been developed for *Fragaria* species with the aim of measuring genetic relationships (Harrison and Luby 1997; Degani et al. 2001), analyzing genetic linkage (Haymes et al. 1997; Lerceteau-Köhler et al. 2003; Hadonou et al. 2004), or identifying cultivars (Nehra et al. 1990; Congiu et al. 2000; Arnau et al. 2001; Tyrka et al. 2002). These DNA markers include random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR) markers. Having similar aims, we applied the cleavage amplified polymorphic sequence (CAPS) method to *F.* × *ananassa*. CAPS are usually amplified from known gene sequences and digested with an appropriate restriction enzyme (RE). The polymorphisms revealed by the combination of CAPS and RE (CAPS-RE) are CAPS markers. We previously developed six CAPS-REs for *F.* × *ananassa* and used these to identify 14 commercially available cultivars, thereby confirming that CAPS analysis yields very stable and reproducible results (Kunihisa et al. 2003). Because CAPS analysis is relatively inexpensive and straightforward, it might be useful for inspecting marketed strawberry fruits. However, our primary set of markers is no longer sufficient to distinguish all of the cultivars now in distribution because new cultivars are being released each year. Therefore, additional CAPS markers are needed to distinguish most of the commercial cultivars, including those that are in development.

Unlike the polymorphic bands detected by CAPS markers of diploid plants, those of octoploid strawberry are indistinct compared with non-polymorphic bands. This result is due to the low dose of alleles that generate polymorphic bands among the eight or more copies in a cultivar. If not enough PCR product is amplified, an error in judging whether polymorphic bands are present

Communicated by C. Möllers

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or not can result. Although DNA markers in polyploid plants occasionally exhibit such a dose problem (Zhang et al. 2003), allele-specific PCR markers developed in hexaploid wheat (Mohler and Jahoor 1996; Helguera et al. 2000) or ancestral-genome-specific markers in allopolyploids (Vanichanon et al. 2003) do not.

The development of genome-specific markers in *F.* × *ananassa* is difficult because the composition of the genome has not yet been resolved. However, two main hypotheses have been proposed: (1) allopolyploidy (AAA′A′BBB′B′), based on isozyme segregation patterns (Bringhurst 1990) and (2) allo-autopolyploidy (AAA′A′BBBB), based on mapping (Lerceteau-Köhler et al. 2003). Both hypotheses indicate that *F.* × *ananassa* is a complex polyploid with multiple independent genomes. Under this theory, the PCR products amplified from one gene of a single cultivar would show a mixture of alleles in the same genome and homologous genes in different genomes—i.e., they would reflect multiple loci. It might therefore be possible to separate these mixed products into each genome group by means of cluster analyses based on their nucleotide sequences and to develop clear genome-specific markers using cluster-specific amplification.

The objectives of the investigation reported here were: (1) to identify all strawberry cultivars currently marketed in Japan through the development of a sufficient number of CAPS markers; (2) to improve the process of developing CAPS markers so that they generate clearer polymorphic bands by means of cluster-specific amplification; (3) to clarify the inheritance of cluster-specific markers and determine whether they are genome-specific.

Materials and methods

Plant material

Sixty-four strawberry cultivars, 55 from Japanese sources and nine others, were used for DNA extraction and CAPS analysis (Table 1). When determining the polymorphism types of the cultivars, we evaluated two to seven ramets per cultivar. Each ramet had been maintained at a different laboratory. To investigate marker segregation in the progeny, we prepared four self-crossed populations: 96 individuals each derived from Sachinoka and Tochihime, and 48 individuals each derived from Nyoho and Cesena. We also used 112 progeny of a Sachinoka × Tochiotome cross to analyze two markers that could detect multiple alleles (APX-*Mlu*I and PYDA- *Hae*III) and to compensate for insufficient data provided by PYDB-*Hae*III in the test of self-crossed populations.

CAPS analysis

DNA was extracted from the leaves of cultivars, the four self-crossed populations, and the Sachinoka × Tochiotome cross using a DNeasy Plant Mini kit (Qiagen, Germany). CAPS analysis followed the procedure of Kunihisa et al. (2003). The annealing temperature of the PCR amplification was 55°C, except for three markers, which were annealed at 57°C or 58°C (Table 2).

Development of CAPS markers

Following the same procedure as described in Kunihisa et al. (2003), we searched the *Fragaria* nucleotide sequences (including introns) deposited in the DNA Data Bank of Japan (http://srs.ddbj.nig.ac.jp/index-e.html) for potential CAPS marker sites. Eighteen potential genes were randomly selected, and we designed primer pairs that would amplify 400- to 650-bp fragments in each gene (data not shown). Each gene was analyzed as follows. Fragments of intended length were amplified from cvs. Nyoho, Tochiotome, Sachinoka, Red Pearl, and Cesena. Because these cultivated strawberries are octoploid, the fragments amplified by one primer pair from one cultivar can contain various sequences derived from up to eight chromosomes (Kim et al. 2001). Therefore, it is necessary to consider several sequences from each target gene in each cultivar in order to correctly predict polymorphisms from the sequence data. We subcloned the amplified fragments, sequenced

Table 1 The cultivars used in this study and their origins

Cultivar name	Origin	Cultivar name	Origin
Aiberry	Japan	Malach	Israel
Aiko	USA	Meiho	Japan
Aistro	Japan	Maehyang	Korea
Akanekko	Japan	Miyoshi	Japan
Akasyanomitsuko	Japan	Nohime	Japan
Akihime	Japan	Nyoho	Japan
Asuka Ruby	Japan	Oishishikinari No.2	Japan
Asuka Wave	Japan	Pajaro	USA
Benihoppe	Japan	Pechika	Japan
Berrystar	Japan	Peastro	Japan
Belle Rouge	Japan	Red Pearl	Japan
Cesena	Italy	Reiko	Japan
Donner	USA	Rindamore	Japan
Echigohime	Japan	Sachinoka	Japan
Elsanta	Belgium	Sagahonoka	Japan
Everberry	Japan	Sanchigo	Japan
Fukuba	Japan	Satsumaotome	Japan
Fukuoka S6 go	Japan	Sawaberry	Japan
Harunoka	Japan	Serenata	UK
Haruyoi	Japan	Shizuchikara	Japan
Hatsukuni	Japan	Shizunoka	Japan
Himesodachi	Japan	Shizutakara	Japan
Himiko	Japan	Summerberry	Japan
Hinomine	Japan	Suruga-ace	Japan
Hogyoku	Japan	Syuko	Japan
Hokowase	Japan	Tioga	USA
Hottawonder	Japan	Tochihime	Japan
Keiki wase	Japan	Tochinomine	Japan
Kita-ekubo	Japan	Tochiotome	Japan
Kitanokagayaki	Japan	Tonehoppe	Japan
Koju	Japan	Toyonoka	Japan
Kunowase	Japan	Yakumo	Japan

five random clones from each of the five cultivars, and then compared these 25 sequences to identify restriction endonuclease recognition sites that would be polymorphic among these five cultivars. We then selected the restriction enzymes that generated visibly different electrophoresis patterns in an actual trial. Such a combination of products amplified by a primer pair and a single endonuclease was referred to as one CAPS-RE. We named each combination using the abbreviated amplified gene name and the endonuclease name. GENETYX software (Genetyx, Japan) was used to design the primer pairs and to search for restriction sites, referring to 117 endonucleases in its database.

Classification of sequenced clones and cluster-specific amplification

To classify the 25 sequenced clones from each gene, we used the unweighted pair group method with arithmetic mean (UPGMA) cluster analysis (GENETYX software). From the results of the classification, we selected a target cluster for amplification and designed primers homologous to cluster-specific sequences in order to amplify only the clones of this cluster. We designed several primers and tested up to six primer combinations for each targeted cluster (data not shown). The primer pair producing the clearest polymorphic bands was chosen for the improved CAPS marker.

Results

Development of new CAPS markers

We analyzed 18 potential genes to obtain new CAPS markers. Of 180 combinations of restriction endonucleases and genes predicted to generate polymorphic bands among the five tested cultivars (Nyoho, Tochiotome, Sachinoka, Red Pearl, and Cesena), 14 combinations raised from eight genes yielded recognizable polymorphisms and were selected as functional CAPS-REs. Of the total 20 CAPS-REs (14 new and six previously reported, data not shown), 16 identified a single polymorphism each. As an example of one of these 16 CAPS-REs, MSR-*Alu*I, which was found in 14 cultivars, is shown in Fig. 1 (top panel, a). Four other CAPS-REs identified two independent polymorphisms each [e.g., Fig. 1, bottom panel B-a, C-a. The primers for these CAPS-REs were designed to anneal to conserved regions. Therefore, they amplified all loci of the targeted gene in the octoploid genome. Because the alleles generating polymorphic bands were minor among the amplified products, the polymorphic bands on the electrophoresis gels were relatively unclear compared with the non-polymorphic ones. Moreover, homozygosis and heterozygosis were indistinguishable by these CAPS markers, unlike in diploid plants.

Cluster analyses and cluster-specific amplification

To design primers for genome-specific markers, we needed to identify unique sequences in the target genome. To do this, we used cluster analysis of the 25 sequenced clones of each target gene to divide these clones into hypothetical clusters based on sequence similarity. We analyzed the 12 genes that gave rise to the 20 CAPS-REs that were developed [eight genes for 14 new CAPS-REs, and four genes for the six CAPS-REs developed previously (Kunihisa et al. 2003)].

The various clones of each gene were divided into three or four major clusters, and these cluster showed no relationship with cultivar (e.g., dendrogram in Fig. 2). For each marker, we determined the cluster containing the clones with the restriction sites that produced the polymorphic bands—referring to the dendrogram of the targeted gene. When the CAPS-REs identified a single polymorphism, all of the clones generating polymorphic bands were in a single cluster. For MSR-*Alu*I, for example, the three clones indicated in Fig. 2 by double asterisks (S1, N2, N3) possessed an *Alu*I restriction site producing polymorphic bands, and all of these clones belonged to cluster III of this MSR dendrogram. We then designed primers to amplify only the clones belonging to this cluster (Fig. 3). The same applied to the four CAPS-REs that identified two independent polymorphisms; that is, all of the clones that produced the same polymorphic bands belonged to a single cluster. However, it depended on the CAPS-RE whether the two different polymorphisms were derived from a single cluster or from two different clusters. In APX-*Mlu*I (Fig. 1, bottom panel, B-a), the clones generating the 512-bp band and the clones generating the 341-bp band were included in cluster I of the APX dendrogram (data not shown). In contrast, for PYD-*Hae*III (Fig. 1, bottom panel, C-a), the clones that produced the 412-bp band belonged to cluster II, but the clones that produced the 302/281-bp bands belonged to cluster III of the PYD dendrogram (data not shown). When the two polymorphisms came from different clusters, a cluster-specific primer pair was designed for each cluster.

Improved CAPS markers

We designed cluster-specific primer pairs (Table 2) for improving the markers generated by the CAPS-REs we had developed. APX, APX3, and APX4 (shown in Table 2) are products amplified by different cluster-specific primer pairs designed for different clusters of the *APX* gene. PGPA/PGPB and PYDA/PYDB are products derived from two different clusters, but they arise from a single gene (*PGP* and *PYD*, respectively). In every other case, only one CAPS could be amplified from one gene. (Homologous CAPS-REs could not be obtained.)

MSR-*Alu*I (Fig. 1, top panel) is an example of an improved marker. Following improvement, extraneous products were not amplified, and polymorphic bands

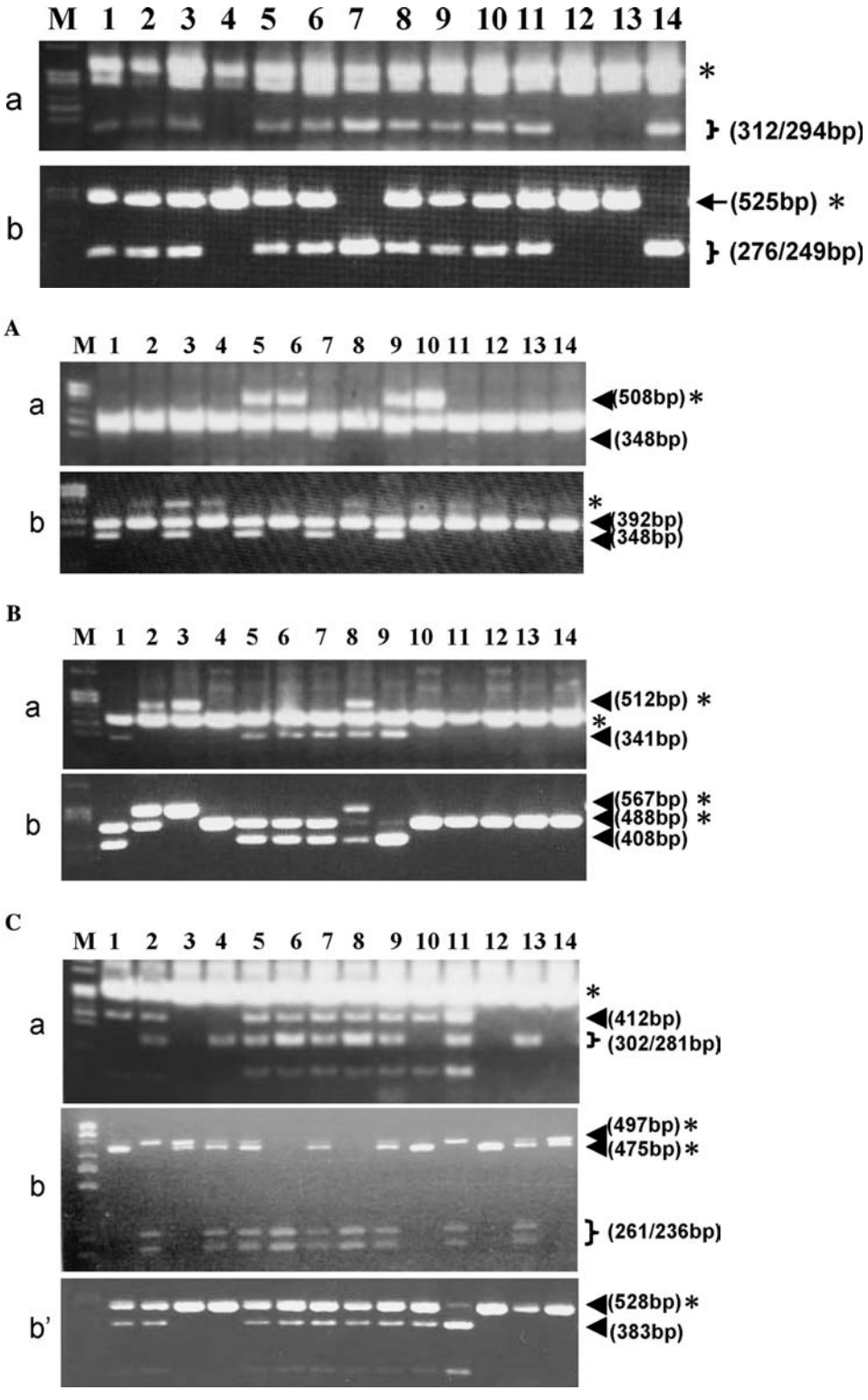

Fig. 1 **Top panel** Comparison of electrophoretic patterns produced by original and improved MSR-*Alu*I markers in 14 strawberry cultivars. **A** Original MSR-*Alu*I marker, **B** Improved marker. *Arrows* and *brackets* indicate the polymorphic bands, and *asterisks* show the positions of amplified products before restriction digestion. Polymorphic band sizes are indicated by *arrows*, and *brackets* were calculated based on sequence data. *Lanes*: *1* Toyonoka, *2* Nyoho, *3* Tochiotome, *4* Akihime, *5* Sachinoka, *6* Aiberry, *7* Redparl, *8* Nohime, *9* Sanchigo, *10* Pistro, *11* Aistro, *12* Benihoppe, *13* Kekiwase, *14* Kurume IH-1 go, *M* size markers (lambda-*Hin*dIII/*Hin*cII fragments). **Bottom panel** (A, B, C) Examples of CAPS marker improvement by cluster-specific amplification in 14 strawberry cultivars. **A** APX3-*Dra*I markers: **a** Original, **b** improved. A polymorphic band that was scarcely detectable with the original marker became obvious with the improved marker (348 bp). However, a distinct polymorphism at 508 bp disappeared. *Asterisks* show original positions of amplified products. **B** APX-*Mlu*I markers: **a** Original, **b** improved. The original version detected two independent polymorphisms, which both belonged to the same cluster (by cluster analysis). **C** PYD-*Hae*III markers: **a** Original, **b** improved, PYDA-*Hae*III, **b′** improved, PYDB-*Hae*III. The original version detected two polymorphisms that derived from different clusters (by cluster analysis). Cluster-specific amplification was carried out by cluster

were clearer. Moreover, the band pattern resembled that of a marker detecting a single locus in a diploid. The improved marker clearly discriminated the cultivars (e.g., Fig. 1, top panel, lanes 6, 7), which were indistinguishable using the original marker. Consequently, we can conclude that the marker became more effective for cultivar identification.

Fig. 1 illustrates various patterns of improvement. For APX3-*Dra*I (Fig. 1, bottom panel, A), the 348-bp polymorphic band that had been difficult to detect

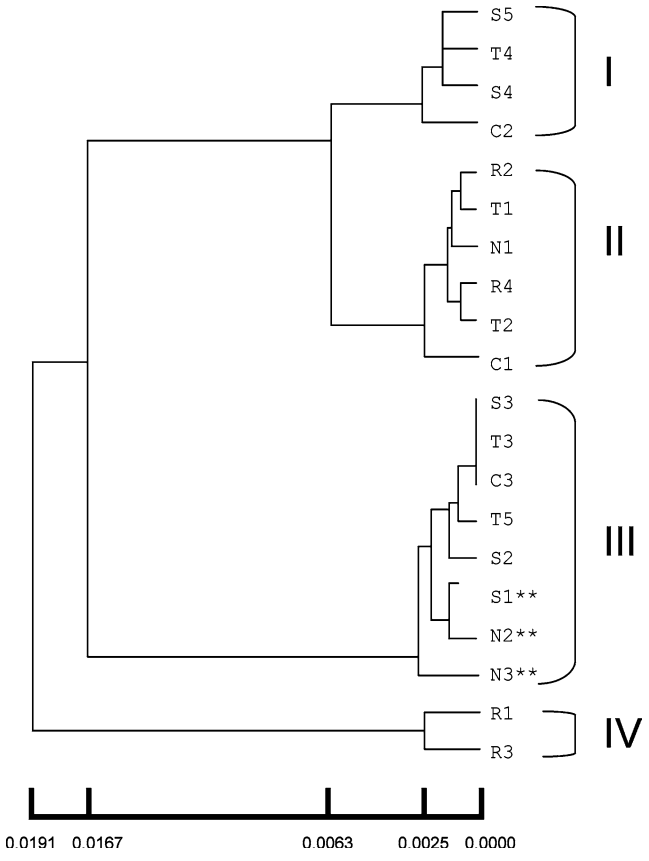

Fig. 2 Classification of clones amplified from the methionine sulfoxide reductase (MSR) gene from five cultivars based on their sequences (604–618 bp). *S1–S5* Clones from Sachinoka, *T1–T5* clones from Tochiotome, *N1–N3* clones from Nyoho, *R1–R4* clones from Red Pearl, *C1–C3* clones from Cesena. We used the UPGMA method to perform this classification, separating the clones among clusters I through IV. *Double asterisks* indicate the clones assumed to produce polymorphic bands following *Alu*I digestion

before improvement was prominent after cluster-specific amplification. An additional polymorphic band (508 bp) was no longer amplified because it came from a different cluster. APX-*Mlu*I (Fig. 1, bottom panel, B) identified two polymorphisms simultaneously following improvement. This phenomenon occurs when both polymorphisms are derived from the same cluster. The three types of band generated by this CAPS-RE appeared to be equivalent multiple alleles because no cultivar displayed all three bands simultaneously.

Whereas PYD-*Hae*III (Fig. 1, bottom panel, C) also detected two polymorphisms in its original version, the cluster-specific improvement separated these two polymorphisms into PYDA-*Hae*III (Fig. 1, bottom panel, C-b) and PYDB-*Hae*III (Fig. 1, bottom panel, C-b′). As these polymorphisms were derived from two different clusters, the sequences of each cluster had to be amplified separately. PYDA-*Hae*III generates three kinds of polymorphic bands (497-, 475-, and a 261/236-bp pair). A very slight band always appeared at 497 bp in the samples, that were heterozygous for the 475-bp and 261/236-bp bands (lanes 4, 5, 7, 9, and 13). This band is thought to be a non-specific band, but its cause is unknown. PYDB-*Hae*III also retained a faint, non-specific band. Although the specificity of amplification was not perfect, the complete separation of two polymorphisms indicates that our cluster-specific primer pairs amplified two different loci of the *PYD* gene.

Of the 20 CAPS-REs subjected to improvement, we successfully converted 15 of them into 18 CAPS-REs that were more distinct. This increase in the number of CAPS-REs was due to the separation of polymorphisms, such as that of PYD-*Hae*III into PYDA- and PYDB-*Hae*III. Of the 18 improved CAPS-REs, 13 appeared to detect a single diploid locus, and the other five generated clearer polymorphic bands. Our failure to clarify the remaining five CAPS-REs was due to insufficient sequence differentiation among the clusters at the targeted genes, whcih

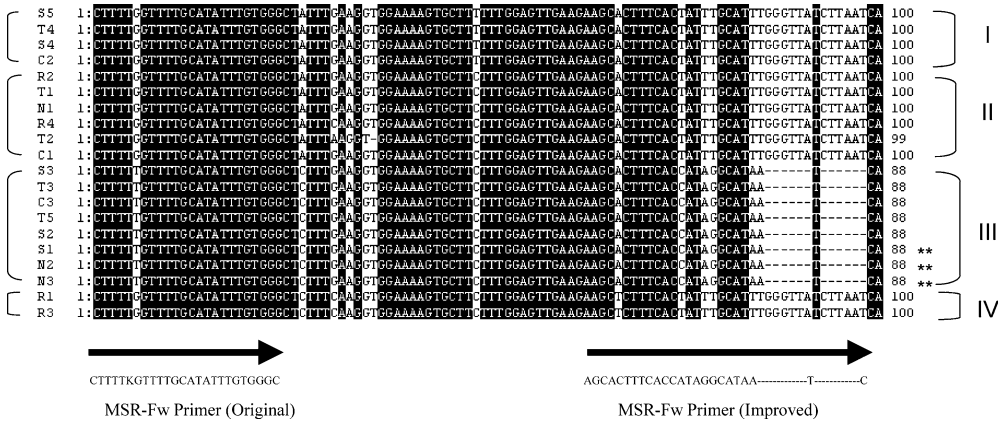

Fig. 3 Design of the forward primer for cluster III-specific amplification of the MSR-*Alu*I marker. *S1–S5* Clones from Sachinoka, *T1–T5* clones from Tochiotome, *N1–N3* clones from Nyoho, *R1–R4* clones from Red Pearl, *C1–C3* clones from Cesena. *Double asterisks* indicate those clones assumed to produce polymorphic bands following *Alu*I digestion. *Arrows* parallel to the sequences show the positions of the primers. The original primer was designed to amplify all clones (regardless of cluster). The improved primer was designed to amplify only cluster III clones

Table 2 CAPS-RE names, primer sequences, and restriction endonucleases used for marker improvement

CAPS-RE name[a,b]	Primer name[b]	Sequence	Endonuclease	Standard length of polymorphic bands after digestion (length of PCR product) in base pairs	Remarks
DFR-*Hha*I	DFR-Fw	5′-GAGACCCTGGTCCGTCG- 3′	*Hha*I	385 + 168 (553)	Not improved
	DFR-Rv	5′-CCTCCGAACTGTCTTTGCTTTGAG- 3′			
APX-*Mlu*I	APX-Fw	5′-GTGGTCACACCTTGGTGC- 3′	*Mlu*I	567/488/408 (567/488)	Specific to single diploid locus
	APX-Rv	5′-AGTATAATATTTAAGCAGAATGCAGACTTC- 3′			
APX2-*Dra*I	APX2-FwA	5′-CAGAGGCCTCATCGCCG- 3′	*Dra*I	508 (508)	
	APX2-Rv	5′-TCAGGTCCACCGGTGACC- 3′			
APX3-*Dra*I	APX2-FwB	5′-GAGGCCTCATCGCCGAG- 3′	*Dra*I	392/348 (505)	Specific to single diploid locus
	APX2-Rv	5′-TCAGGTCCACCGGTGACC- 3′			
APX4-*Taq*I	APX2-FwC	5′-GTCTCCGATCCCTATCTTTTCTTT- 3′	*Taq*I	445/313 + 132 (445)	Specific to single diploid locus. Anneaing temp: 58°C
	APX2-Rv	5′-TCAGGTCCACCGGTGACC- 3′			
CHI-*Pvu*II	CHI-Fw	5′-AGGAGTTGACAGAGTCGGTTG- 3′	*Puv*II	531/418 (531)	Specific to single diploid locus
	CHI-Rv	5′-GACTTGTGAGTATGATAGTCTGCTG- 3′			
F3H-*Nco*I	F3H-Fw	5′-AMCCTGTGGAAGGACCTTTCG- 3′	*Nco*I	359 + 256 (615)	Not improved (reported in 2003 (Kunihisa et al.)
	F3H-Rv	5′-GAGTTCACTACKGCCTGGTGATC- 3′			
F3H-*Ear*I			*Ear*I	385 + 230 (615)	Not improved
F3H-*Hpa*II			*Hpa*II	615 (615)	Not improved (reported in 2003 (Kunihisa et al.)
F3H-*Dde*I			*Dde*I	561 (615)	Not improved
F3H2-*Acc*I	F3H2-Fw	5′-TAATAGGGTCTAGGTGCGTGG- 3′	*Acc*I	390/311 (390)	Specific to single diploid locus
	F3H-Rv	5′-GAGTTCACTACKGCCTGGTGATC- 3′			
CTI1-*Hinf*I	CTI1-Fw	5′-TTCTAATGATCAACACCTACTTTCCC- 3′	*Hinf*I	500/432 (593)	Specific to single diploid locus
	CTI1-Rv	5′-GTAGCCCACCCGCCTG- 3′			
MSR-*Alu*I	MSR-Fw	5′-AGCACTTTCACCATAGGCATAATC- 3′	*Alu*I	525/276 + 249 (525)	Specific to single diploid locus. Annealing temp: 57°C
	MSR-Rv	5′-CCTTGAGCATAAATGAACTGGCA- 3′			
PGPA-*Acc*I	PGP-FwA	5′-CCTCACCTTCCTCGAGCTC- 3′	*Acc*I	467/355 (467)	
PGPA-*Rsa*I	PGP-RvA	5′-ACAATCTGGGTCGTCTTGTTCATC- 3′	*Rsa*I	294 + 173 (467)	
PGPB-*Rsa*I	PGP-FwB	5′-ACCTCACCTTCCTTGAGCTT- 3′	*Rsa*I	386 (497)	
	PGP-RvB	5′-GACAAGTCTATCCGATCAAAGTTCATA- 3′			
AUB-*Hha*I	AUB-Fw	5′-ATTCAAGTGGGTGTTTGTGAATTG- 3′	*Hha*I	538/430 (538)	Specific to single diploid locus
	AUB-Rv	5′-GCCTGTGTATCGGTGTGCC- 3′			
OLP-*Dde*I	OLP-Fw	5′-TGTGTCCAAAACCGATCAGTATTGC- 3′	*Dde*I	520/415 (520)	Specific to single diploid locus
	OLP-Rv	5′-TCTTTCAGAGTGGTACGTACCCC- 3′			
CT12-*Mbo*I	CT12-Fw	5′-GTCAAACCTCTCACGAAACCACT- 3′	*Mbo*I	312/201 + 111 (312)	Specific to single diploid locus
	CT12-Rv	5′-GTTRCTAAGAAAATGAAAGAGCTGATG- 3′			
CYT-*BsaB*I	CYT-Fw	5′-CTTTGGTGGTTGTGAGAGCATCG- 3′	*BsaB* I	499 (499)	
	CYT-Rv	5′-CCGTACTTGAGCCTATCTGACTGG- 3′			
PYDA-*Cfr*13I	PYD-FwA	5′-CTTTCAGGTAAGGAACATGATCAAG- 3′	*Cfr13* I	497/475/373 (497/475)	Specific to single diploid locus
PYDA-*Hae*III	PYD-RvA	5′-GTAAGAACTTAACAAAACCATAATCTCTCTA- 3′	*Hae*III	497/475/261 + 236 (497/475)	Specific to single diploid locus
PYDB-*Hae*III	PYD-FwB	5′-AGGTAAGGAACATGATCAACTTTGAG- 3′	*Hae*III	528/383 (528)	Specific to single diploid locus Anneaing temp: 58°C
	PYD-RvB	5′-ATCTGAAAAACCAAGTAGAAACTTACG- 3′			

[a]Polymorphisms revealed by the combination of CAPS and RE (CAPS-RE) are CAPS markers

[b]DFR, dihydroflavonol 4-reductase; APX, ascorbate peroxidase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; CTI1, chitinase 2–1; MSR, methionine sulfoxide reductase; PGP, polygalacturonase inhibitor protein; AUB, auxin-binding protein; OLP, osmotin-like protein; CTI2, chitinase 2–2; CYT, cystathionine gamma synthase; PYD, pyruvate decarboxylase

Table 3 Segregation ratio of heterozygotic CAPS markers in the progeny, and goodness-of-fit test for Mendelian inheritance using the chi-squared tests

Marker	Population[a]	Parental genotypes[b]	Progeny genotypes		
			Observed	Expected	*P*–value[c]
MSR-*Alu*I	Sachinoka-self	ab	28:51:17	1:2:1	0.24
	Tochihime-self	ab	19:52:25	1:2:1	0.49
	Nyoho-self	ab	13:25:10	1:2:1	0.8
	Cesena-self	ab	10:30:8	1:2:1	0.21
APX-*Mlu*I	Sachinoka-self	bc	31:38:27	1:2:1	0.11
	Tochihime-self	aa	96	1	–
	Nyoho-self	ab	11:24:13	1:2:1	0.92
	Cesena-self	ac	4:33:11	1:2:1	0.01*
	Sachinoka×Tochiotome	bc×aa	56:56	1:1	1
APX3-*Dra*I	Sachinoka-self	ab	28:44:24	1:2:1	0.6
	Tochihime-self	ab	18:59:19	1:2:1	0.08
	Nyoho-self	aa	48	1	–
	Cesena-self	aa	48	1	–
APX4-*Taq*I	Sachinoka-self	ab	16:47:33	1:2:1	0.05*
	Tochihime-self	bb	96	1	–
	Nyoho-self	ab	16:22:10	1:2:1	0.4
	Cesena-self	ab	11:20:17	1:2:1	0.24
OLP-*Dde*I	Sachinoka-self	ab	23:55:18	1:2:1	0.28
	Tochihime-self	ab	24:47:25	1:2:1	0.97
	Nyoho-self	ab	9:27:12	1:2:1	0.57
	Cesena-self	ab	16:26:6	1:2:1	0.11
PYDA-*Hae* III	Sachinoka-self	bc	17:51:28	1:2:1	0.24
	Tochihime-self	ab	28:46:22	1:2:1	0.63
	Nyoho-self	ac	10:29:9	1:2:1	0.35
	Cesena-self	bc	9:22:17	1:2:1	0.22
	Sachinoka×Tochiotome	bc×ab	27:26:28:31	1:1:1:1	0.92
PYDB-*Hae*III	Sachinoka-self	ab	12:53:31	1:2:1	0.01*
	Tochihime-self	aa	96	1	–
	Nyoho-self	ab	15:26:7	1:2:1	0.22
	Cesena-self	aa	48	1	–
	Sachinoka×Tochiotome	ab×aa	54:58	1:1	0.71

[a] -Self means the self-crossed population
[b] a, b, and c indicate multi-allelic types in the locus
[c] Results that deviate from the expected value at $P < 0.05$ are marked by an asterisk

made it difficult or impractical to design sequence-specific primers to amplify the clones of a particular cluster. Each of the 23 CAPS-REs in Table 2 identifies only one marker; therefore, we refer to these 23 markers by their corresponding CAPS-RE names.

Inheritance of cluster-specific markers

We investigated the segregation of the 13 improved CAPS markers that appeared to detect single diploid loci in order to test whether they were actually transmitted under disomic inheritance. All 13 markers were analyzed in four self-crossed populations. APX-*Mlu*I and PYDA-*Hae*III, which appeared to detect three multiple alleles each, were additionally analyzed in the Sachinoka × Tochiotome cross. This cross was also used for PYDB-*Hae*III because the data from self-crossed populations were not sufficient to determine the inheritance manner of this marker. For each marker, PCR amplification produced sufficient DNA from all of the tested progeny to detect bands on a gel.

Table 3 shows the results of the analysis of seven representative markers. The segregation of these markers was consistent with disomic Mendelian inheritance in most of the populations tested. The only three exceptions deviating at the $P < 0.05$ level were APX-*Mlu*I in the selfed progeny of Cesena, APX4-*Taq*I in those of Sachinoka, and PYDB-*Hae*III in those of Sachinoka. However, the segregation of these three markers fitted the expected value in all other tested populations. The remaining six markers also segregated in accordance with the expected ratio in all of the populations tested. Statistically, such accidental deviations in low frequency are possible in when analyses of a number of markers or populations are carried out. The average P value was $P = 0.41$, which is close to the statistically expected value of $P = 0.5$.

The analyses of APX-*Mlu*I and PYDA-*Hae*III in the Sachinoka × Tochiotome cross showed that the three kinds of bands in each marker identified equivalent multiple alleles under disomic inheritance. As was the case with analysis of octoploid *F. virginiana* (Ashley et al. 2003), there was no indication of tetrasomic or octosomic inheritance in the progeny of the Sachinoka × Tochiotome cross of *F.* × *ananassa*. For example, in the cross of genotypes bc × aa, the abc genotype that should have appeared if transmission were polysomic never appeared in the progeny, while in the bc × ab cross, none of the progeny exhibited all three bands.

Our results indicate that all 13 tested markers were consistent with disomic Mendelian inheritance and that they corresponded to alleles of single diploid loci.

Cultivar identification

We used 23 CAPS markers (Table 2) to identify 64 cultivars of strawberry (Table 1). We confirmed that all or almost all of the ramets of a cultivar, despite being gathered from different laboratories, showed exactly the same band pattern (which we defined as the typical polymorphic pattern of the cultivar). On rare occasions, a ramet with a very different band pattern was detected, possibly owing to mislabeling during maintenance of the cultivars (data not shown). On the basis of the polymorphisms that were determined during our analyses, the 64 cultivars could be distinguished using a minimum of nine markers.

Discussion

Application of the nine CAPS markers was sufficient to identify the 64 strawberry cultivars examined in the present study. These cultivars include almost all of those currently sold on the Japanese market. However, we have developed a total of 23 markers and, consequently, it is possible to select a smaller subset of markers that are appropriate for cultivar inspection at a given level of accuracy. It is often only necessary to use a few markers to judge whether a sample fruit has been labeled correctly. For example, a mere three markers were required to distinguish Sachinoka from the other 63 cultivars. Furthermore, the markers we developed successfully distinguished not only Japanese cultivars but also foreign cultivars that have diverse breeding backgrounds. This result indicates that our CAPS markers are suitable for identifying strawberry cultivars from around the world.

Most CAPS markers became more distinct and more stable following amplification specific to a single cluster. These improved markers increase the likelihood that polymorphisms can be detected correctly, even if the amount of amplified PCR products is low. A good example of stabilization is APX3-*Dra*I (Fig. 1, bottom panel, A), in which an almost undetectable polymorphic band (348 bp) was converted into a useful band as a marker. We believe that cluster-specific amplification in strawberry is very useful method for revealing masked polymorphic signals.

The significant effect of cluster-specific amplification is the generation of markers corresponding to alleles of single diploid loci. CAPS markers improved by cluster-specific amplification are likely to become disomic markers that will allow robust cultivar identification and other genetic analyses (in this study, 13 disomic markers were generated out of the 18 improved CAPS markers). To our knowledge, the creation of markers detecting only a single locus is rare in *F.* × *ananassa*. Although various microsatellite (SSR) markers have been shown to generate disomic inheritance in the wild octoploid strawberry *F. virginiana* (Ashley et al. 2003), they generated more than two bands in *F.* × *ananassa* because of non-selective amplification of multiple loci (Nourse et al. 2002). A further important point regarding cluster-specific amplification is the annealing temperature. We had three primer pairs that required annealing temperatures higher than 55°C (Table 2), because extra bands appeared at 55°C, confounding any possible analysis (data not shown). Suboptimal annealing temperatures lead directly to non-specific amplification, which negates the improving effect of cluster-specific amplification.

If we considering the results of the cluster analyses and marker segregations, a ''cluster-specific marker'' might be equivalent to a ''genome-specific marker''. Cluster analysis provides information on the genomic structure of *F.* × *ananassa*, which is still under investigation. All of the clones of each amplified gene tended to be divided among three or four clusters. So what does each cluster represent? There was no distinct relationship between clusters and cultivars, and the clones of any particular cultivar were scattered among multiple clusters. We speculate that the clusters reflect the construction of the genome of octoploid strawberry. In other words, the octoploid comprises three or four clusters, each of which corresponds to a different genome.

The segregation of ''cluster-specific markers'' in the progeny supports this genome-cluster hypothesis. Their disomic Mendelian inheritance suggests that these markers were derived from equivalent allelic pairs of single loci of the diploid genome. Furthermore, it is notable that two or three of the polymorphisms that came from different clusters of a particular gene could be independently separated by discrete cluster-specific amplifications. APX-*Mlu*I, APX3-*Dra*I, and APX4-*Taq*I are independent, disomically inherited markers from the *APX* gene, as is PYDA-*Hae*III and PYDB-*Hae*III from the *PYD* gene. This implies that cultivated strawberry has at least three independent diploid loci for the *APX* gene. When these three loci were placed in homoeologous chromosomes, there was a strong indication that these separate markers were derived from three different genomes, each of which was disomic. Therefore, the ''cluster-specific marker'' referred to in this report could be a ''genome-specific marker'' similar to that reported in wheat (Guyomarc'h et al. 2002).

Octoploid strawberry is generally considered to have arisen from the reduplication and interspecies crosses of several diploid wild strawberries. This hypothesis is based on cytological information obtained from observations on chromosome pairing behavior (Senanayake and Bringhurst 1967). In addition, several studies have suggested that *F.* × *ananassa* is highly or partly diploidized based on isozyme inheritance (Arulsekar et al. 1981) or genome mapping (Lerceteau-Köhler et al. 2003). The results of our DNA marker analyses also suggest that most genetic loci of cultivated strawberry are transmitted by disomic inheritance and that strawberry is composed of independent genomes. These results support conclusions drawn from previous research.

Acknowledgments We thank the Aichi-Ken Agricultural Research Center, the Agricultural Research Division of the Mie Prefectural Science and Technology Promotion Center, the Gifu Prefectural Agricultural Technology Research Center, the Nara Prefectural Agricultural Experiment Station, the Shizuoka Agricultural Experiment Station, and the Tochigi Prefectural Agriculture Experiment Station for kindly providing plant materials. This study was supported by grants from the National Agricultural Research Organization's funds for "Integrated Research Program for Functionality and Safety of Food toward Establishment of a Healthy Diet".

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