Note

Utilization of the CAPS/dCAPS Method to Convert Rice SNPs into PCR-based Markers

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Linkage maps of rice have been constructed based on restriction fragment length polymorphism (RFLP) markers (Kurata *et al.* 1994, Tsunematsu *et al.* 1996, Harushima *et al.* 1998). The mapped RFLP markers have been widely used in various fields from fundamental research to practical breeding programs (Sasaki 2001, Yano 2001), and genotype data by RFLP analysis have accumulated. For example, the results of an RFLP survey among sixteen rice varieties and RFLP genotyping data of 212 doubled-haploid lines derived from the cross between Akihikari and Koshihikari have been published by the Rice Genome Research Program (RGP) (<http://rgp.dna.affrc.go.jp>). However, because the RFLP analysis includes time-consuming steps and requires a large amount of purified DNAs, conversion to PCR-based markers has been desired.

The cleaved amplified polymorphic sequence (CAPS) method (Konieczny and Ausubel 1993) has made it possible to develop PCR-based markers that can be rapidly and reliably analyzed. The existence of nucleotide sequence polymorphism generating a restriction site difference between varieties/lines to be analyzed is essential for developing a CAPS marker. Although single nucleotide polymorphism (SNP) is one of the most common classes of DNA polymorphism in many organisms including rice (Nasu *et al.* 2002, Hayashi *et al.* 2004), the majority of single-base changes generate no restriction site difference and thus seem useless for developing CAPS markers. However, recent research (Michaels and Amasino 1998, Neff *et al.* 1998) demonstrated that single-base changes generating no restriction site difference could be utilized for the development of PCR-based markers by the derived CAPS (dCAPS) method in *Arabidopsis thaliana*. The dCAPS method was proved to be robust for developing PCR-based markers adequate for the genetic analysis in rice whose genome is about four times

larger than that of *A. thaliana* (Komori and Nitta 2003, Komori *et al.* 2003, Yamanaka *et al.* 2004).

The recent availability of numerous rice genome sequences has promoted the survey of SNPs between varieties (Shen *et al.* 2004). Because SNP genotyping is useful for map-based cloning, marker-assisted breeding, seed purity tests and so on, several new techniques including pyrosequencing (Ahmadian *et al.* 2000, Alderborn *et al.* 2000), TaqMan (Livak 1999), and fluorescence energy transfer (Chen *et al.* 1998) methods, for example, have been recently developed to genotype SNPs. These techniques, however, require specialized detection instruments and/or labeled oligonucleotides. In contrast, CAPS/dCAPS markers can be analyzed by simple PCR followed by restriction enzyme treatment and agarose gel electrophoresis, and therefore are still preferred in many laboratories including breeding stations.

RGP converted 171 RFLP markers that detected sequence polymorphisms between Nipponbare (*japonica* rice variety) and Kasalath (*indica* rice variety) into 165 CAPS markers and six dCAPS markers and released the information on the developed markers (<http://rgp.dna.affrc.go.jp>). In a preliminary study, some of the 171 PCR-based markers were used to analyze Asominori (*japonica* rice variety) and IR24 (*indica* rice variety), and nearly half of the tested markers did not show any clear polymorphisms with respect to the gel electrophoresis profiles, which strongly suggested that further development of PCR-based markers is necessary to more efficiently perform a genetic analysis for a wide range of variety/line combinations.

In this study, the genome sequences corresponding to 53 RFLP probes were compared between Asominori and IR24, and 33 PCR-based markers consisting of nine CAPS markers, 21 dCAPS markers and three amplicon length polymorphism (ALP) markers have been developed. The successful conversion from RFLP markers into PCR-based markers demonstrated the propriety of the strategy. The effect of mismatch positions in designing primers by the dCAPS method is also described.

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Development of 33 PCR-based markers based on RFLP markers

Total DNA was extracted from the green leaves of Asominori and IR24 by the CTAB method (Murray and Thompson 1980). Using the total DNA as templates, PCR was performed with sequence tagged site (STS) primers that were designed on the basis of the end sequences of 53 RFLP probes. As regards the 53 RFLP markers, STS information was obtained mainly from the RGP website (<http://rgp.dna.affrc.go.jp>), but CAPS/dCAPS information was unavailable or inadequate for clear discrimination between Asominori and IR24. These RFLP markers were selected without performing RFLP analysis between Asominori and IR24. After electrophoresis of the PCR products on agarose gel, the DNA fragments of interest were excised and purified with SUPREC™-01 (TaKaRa). End nucleotide sequences were determined with the STS primers using dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) and an automatic DNA sequencer (Prism 310, Applied Biosystems). Sequence analyses were conducted with Genetyx-Mac version 10.0 (Software Development).

As a result of the sequence comparisons between Asominori and IR24, some degree of sequence polymorphism was observed in 33 RFLP probe regions out of 53. Insertions/deletions (InDels) consisting of more than 17 nucleotides were identified in three regions (S11114, C797 and R887), and ALP markers were developed utilizing the InDels. Although only SNP(s) and/or small InDels consisting of less than nine nucleotides were identified in 30 RFLP probe regions, nine CAPS markers and 21 dCAPS markers were developed utilizing SNPs in these regions. The dCAPS method was employed mainly when it was difficult to employ the CAPS method because of the lack of adequate SNP and/or bothersome restriction site(s) adjacent to a potential target SNP. In addition, the dCAPS method was strategically used in some cases in order to develop reliable PCR-based markers, as described below by the example of C239 *Mbo*I. The PCR-based markers developed in this study are listed in Table 1.

Marker analysis was conducted in the following way. PCR was carried out in 30 µl reactions that consisted of 15 ng total DNA, 0.2 mM of each dNTP, 0.2 µM of each primer, 1 × Ex Taq Buffer with Mg²⁺ (TaKaRa) and 1.5 U TaKaRa Ex Taq™ (TaKaRa). After preheating at 94°C for 2 min, 35

Table 1. List of PCR-based markers developed in this study

Marker	Type	Forward primer (5' to 3')	Reverse primer (5' to 3')	Enzyme	Fragment size after digestion (bp)		Chromosome
					Asominori	IR24	
C970 <i>Bcl</i> I	dCAPS	CAATTATAAGTGCTTCTGGAGCAATCC	GAGGGGATAGGTTGAACAGAAATGATC	<i>Bcl</i> I	154	127, 27	1
C955 <i>Tsp</i> 509I	CAPS	TGGATCCTGCAAAACAAAGGCCTC	TCTACACAGAACAGAGGCATCTC	<i>Tsp</i> 509I	161, 158	318	1
S2523 <i>Hha</i> I	dCAPS	CTCTACATGAAGAGGTATCCAGCC	TGATGACAGCGAAGAAGAGCGcGGGTG	<i>Hha</i> I	231, 19	177, 54, 19	1
S10581 <i>Alu</i> I	CAPS	GAGAGATTGCCTGGAGGAGTAACC	GAGAGCGCCTTCACATCGTAAAGG	<i>Alu</i> I	317, 193, 72, 31	510, 72, 31	1
S10012 <i>Msp</i> I	dCAPS	AGTGACCTCCCTCTGGGTCTCAGA	GAAATGTCAACAAACACTGTCAATTcCG	<i>Msp</i> I	146	118, 29	1 ¹⁾
R1843 <i>Bst</i> II	dCAPS	TAGGTAAAGAAATCAAATCCAACACCG	GACTTGCCCATTTTGTATACcGCTAG	<i>Bst</i> II	110, 24	83, 27, 24	2
C1496 <i>Dra</i> I	dCAPS	TGAGGCATGTCTTGTTATCTTTGTTA	CCACAGTTGTAGTTTGTCTCCAGG	<i>Dra</i> I	121, 26	147	2 ²⁾
R3361 <i>Nde</i> I	CAPS	GTACGGTAATGGAGGTGGAATGC	CAATATCTTTGCTCATAGGACTCC	<i>Nde</i> I	276	216, 60	3
C582 <i>Alu</i> I	dCAPS	TTTTGTGCCTCCACCACAGCAAGG	TCCTTTTGCAAGGAACATTCaGCTGC	<i>Alu</i> I	98, 47, 23	145, 23	3
R273 <i>Hha</i> I	CAPS	AGGCTGCCTTAGGTGGTCTTCTCG	TCGCTCCTCACGATGTGCGGAGAG	<i>Hha</i> I	129, 32	99, 32, 30	3
R374 <i>Bst</i> UI	dCAPS	AATCTGTGATTTACTGTGATGAGCcG	TCCTGCGAGCGGCATTTGAGAACC	<i>Bst</i> UI	45, 27, 25	72, 25	4
R3166 <i>Bst</i> II	dCAPS	GCTGTTGCCCAAGAAAGAACAcCACCG	TTTTTGGCAGCAGCATTCcAGAGGAC	<i>Bst</i> II	106, 28	84, 28, 22	5
R1838 <i>Dde</i> I	dCAPS	ATTATCATCAAAATGAATGCTTCcTGA	GAACATGCATAACAGACGGGGcTGAGC	<i>Dde</i> I	92, 25, 24	116, 25	5
C235 <i>Bst</i> PI	dCAPS	ATGTTTCACTGCTATAAGTTgGTGAC	TGCGAGATCCTTAACACAATGAGG	<i>Bst</i> PI	83, 22	105	6
R2123 <i>Rsa</i> I	dCAPS	CATGGGAACAATGTTGATTGATTGC	GCAGCAAGATTATCAGCTTGGCAAGT	<i>Rsa</i> I	137	110, 27	6
C81 <i>Eco</i> RI	dCAPS	GGTACCAGTAACATGCATGATGCgAATT	CGGATGACAGATTAACACGAGCTC	<i>Eco</i> RI	106	83, 23	6
S10324A <i>Msl</i> II	dCAPS	ATGGGTTTATAGTGTACGGAGATC	ATTATAAACAAAGATAGAAACaCCTGAG	<i>Msl</i> II	117	93, 24	6
R2829 <i>Tsp</i> 509I	dCAPS	CCGATGAAGACGGTTCATGGCATGAaA	ACAGATTGACTACCGCTTGCAaTTGGC	<i>Tsp</i> 509I	139, 25, 24	164, 24	7
C1467 <i>Bsr</i> GI	dCAPS	GTGATTTTGTCTTGGTGAATAaIGTAC	GGGAATAAACAAAGGATAGCTTCGTC	<i>Bsr</i> GI	72, 23	95	7
R2561 <i>Alu</i> I	CAPS	AAGTGCAAAGGGTGTCCACCAGC	GATGAAGAAATGACCTCCTTGTGG	<i>Alu</i> I	146, 69, 23	215, 23	7
C847 <i>Tsp</i> 509I	dCAPS	GCGATCATGTTTCATAATGAcATTGACTC	CACAAACCACCTTCCATACAGCA	<i>Tsp</i> 509I	156, 31	118, 38, 31	7
C924 <i>Tsp</i> 509I	CAPS	GAAGCTCAGTTGATTGATGTTCCG	CCAAGTTCCAAACTGTGCAAGAGG	<i>Tsp</i> 509I	408, 79	254, 154, 79	7
R1394A <i>Hin</i> II	dCAPS	TGGATTGCATTCTGTTTTGGCAGC	TTTCTGCGTCCCTGATATGTGATAgAT	<i>Hin</i> II	158	130, 28	8
S11114 ALP	ALP	TGCTGGTGAGAAAATTACTGGCGG	ATGATAGCACAAAAACACACAAGC	—	159	141	8
R2638 <i>Tsp</i> 509I	CAPS	AAGGTATGCGTTCCTCTGGTTGCT	TCGCCAGGATTTCTGATCCCGCTG	<i>Tsp</i> 509I	90, 35	125	9
C913A <i>Xba</i> I	dCAPS	TTCAACAGCTCTTAGGATTGAGG	CCGATGATATTCACCATCAAGTcTAG	<i>Xba</i> I	86, 27	113	10
C8 <i>Tsp</i> 509I	dCAPS	GTCTCTGGCGAGTCATCTTCCTCC	GGGTTTGGTCTTGTGTATATGAGaAT	<i>Tsp</i> 509I	106	77, 28	10
C797 ALP	ALP	ATGAAGGTTCACAAAGAGAGGGG	CATATTTCTTTGCCATTGGCGAC	—	158	130	10
C239 <i>Mbo</i> I	dCAPS	TCCAGATgGAGACCGCCAAATcATCCTC	ATCATCTCATCGGACCGTGcAGATCCG	<i>Mbo</i> I	152, 25	123, 29, 25	10
C1116B <i>Tsp</i> 509I	CAPS	TAAAGTGTATGATGATTACACGGG	AACCAATAGAAACCGTAGAAGCTAC	<i>Tsp</i> 509I	126, 77	77, 67, 59	11
C950 <i>Bst</i> II	dCAPS	GACACTGATGTTTTATCTATcCAGTCC	CAACATCCATAACAAGCAACAGAT	<i>Bst</i> II	122	95, 27	11
R887 ALP	ALP	GGATTTGATGAAACTTGCTACGGG	GATTACGCCATTACAAAATGCC	—	120	142	12
C454 <i>Tsp</i> 509I	CAPS	CATGGTCTTCTCAAGTTAGTTGC	TCAGCGTAGGAAAGAGGAaCTTGC	<i>Tsp</i> 509I	134, 28	110, 28, 24	12

¹⁾ Original RFLP marker S10012 is located on Chromosome 7 (Harushima *et al.* 1998).

²⁾ Original RFLP marker C1496 is located on Chromosome 6 (Harushima *et al.* 1998).

PCR cycles (94°C for 0.5 min, 58°C for 0.5 min and 72°C for 0.5 min) were performed. The last cycle was followed by a final 2-min incubation at 72°C. For CAPS and dCAPS markers, aliquots (10 µl) of the PCR products were digested for 3 h in 20 µl total volume with 5–15 units of the appropriate restriction enzyme. Then, the reactions were separated by electrophoresis on 3% MetaPhor™ Agarose (FMC BioProducts)

gel in Tris-acetate EDTA (TAE) buffer and detected by ethidium bromide staining. The pBR322 DNA-*MspI* digest (New England BioLabs) was used as a DNA size marker.

All 33 markers showed clear polymorphism between Asominori and IR24 with respect to the gel electrophoresis profiles, and were subjected to mapping using 71 recombinant inbred lines (RILs) derived from the cross between

Fig. 1. Position effect of the introduced mismatch. (A) Strategy to convert an RFLP marker C81 into a PCR-based marker. The polymorphic site between Asominori and IR24 is shown in upper-case bold type. Five forward primers (F1 to F5) containing a mismatch shown in lower-case bold type were designed together with a reverse primer (R). The mismatch was expected to generate an *EcoRI* site (GAATTC) only in the PCR products from the IR24 DNA. (B) Agarose gel electrophoresis profiles. PCR was conducted with five primer combinations. The PCR products (left panel) and their digests with *EcoRI* (right panel) were separated on a 3% MetaPhor™ Agarose gel in TAE buffer. M: DNA size marker (pBR322/*MspI* digest), 1: Asominori, 2: IR24. (C) Sequence analysis of the PCR products. The sequence of each PCR product was determined by direct sequencing.

Asominori and IR24, as previously described (Tsunematsu *et al.* 1996). Genotype data of the 71 RILs for 375 markers were obtained from a web site of RGP (<http://rgp.dna.affrc.go.jp>). Using this mapping population and the genotype data, a linkage analysis was performed with MAPMAKER version 2.0 (Lander *et al.* 1987) to locate the developed PCR-based markers. Among the 33 markers, 31 markers were mapped on the same chromosomal location as their corresponding RFLP marker, while two dCAPS markers (S10012 *MspI* and C1496 *DraI*) were mapped on a chromosome different from that containing the locus of their corresponding RFLP marker. The change in the mapped location probably occurred because the dCAPS markers and the RFLP markers detected different genomic regions sharing the high sequence homology. The fact that two or three bands were detected by RFLP analysis of S10012 and C1496 by RGP (<http://rgp.dna.affrc.go.jp>) supports this hypothesis. This change has also been observed for some CAPS markers that were developed based on RFLP markers by RGP (<http://rgp.dna.affrc.go.jp>). Thus, it is essential to confirm the map position of a newly developed PCR-based marker prior to its use in genetic analysis.

Of 53 RFLP probe regions where the SNP search was conducted in this study, no SNP was found in 20 regions. Because only end sequences were analyzed, SNP(s) might be found by further sequencing. If there is no SNP in the entire RFLP probe region, sequence comparison in the outer regions is necessary. Once a SNP is identified, it is highly possible to convert it into a CAPS/dCAPS marker, as demonstrated by the 30 examples in this study. It is in contrast with the allele-specific PCR method by which only 33 SNPs of 49 could be used to reliably discriminate between two alleles (Hayashi *et al.* 2004).

Position effect of the introduced mismatch

In the process of developing C81 *EcoRI* in Table 1, five forward primers (F1 to F5) that contained a mismatch in different positions were designed so that the mismatch would generate a unique *EcoRI* site for IR24 (Fig. 1A). A reverse primer (R) was located approximately 60 bp apart from the forward primers and contained no mismatch. Each of the forward primers was combined with the reverse primer, and PCR was carried out using the total DNA of Asominori and IR24 as templates. Figure 1B shows agarose gel electrophoresis profiles of the PCR products and their digests with *EcoRI*. When F3, F4 or F5 was used as a forward primer, the target fragments of approximately 110 bp were efficiently amplified, and their *EcoRI* digests showed the expected pattern of electrophoresis profiles; the PCR products from IR24 DNA were cleaved with *EcoRI*, and those from Asominori DNA were not. In contrast, when F1 or F2 was used as a forward primer, the target fragments were less efficiently amplified, and/or, the majority of the PCR products from IR24 DNA were tolerant to *EcoRI*.

To clarify the reason for the unexpected results for F1 and F2, we determined the nucleotide sequences of the PCR

products by direct sequencing. As shown in Figure 1C, the result demonstrated that the mismatch introduced into F1 and F2 was at least partially repaired during the PCR amplification, while the mismatch introduced into F3, F4 and F5 remained in the PCR products.

The effect of the position of a primer mismatch was studied further for some other cases (data not shown). In some cases, the dCAPS method worked out independent of the mismatch positions. In other cases, it worked out for the primer carrying a mismatch at the third nucleotide from the 3' end, but not for the primer carrying a mismatch at the first or the second nucleotide from the 3' end, presumably due to the repair of the mismatch as well as in the case of C81. Therefore, it was concluded that mismatches should be introduced at the third or more distant nucleotide from the 3' end, if possible. It should be noted that the present results were inconsistent with a previous report (Michaels and Amasino 1998) that concluded that the mismatches at the first or the second nucleotide from the 3' end are preferable to avoid the generation of unexpected digestion-resistant PCR products. The difference might be due to the PCR conditions, especially the polymerase used. In fact, the results obtained with Y14824 *MboI* (Komori and Nitta 2003), one of the dCAPS markers developed by locating a mismatch at the second nucleotide from the 3' end, varied with the polymerase (Fig. 2). When TaKaRa Taq™, which lacks proofreading activity, is used, the marker shows the expected pattern of electrophoresis profiles. On the contrary, when TaKaRa Ex

Fig. 2. The effect of proofreading activity of DNA polymerase on the agarose gel electrophoresis profiles of Y14824 *MboI*, a dCAPS marker developed by Komori and Nitta (2003). PCR was performed with TaKaRa Taq™ (left) or TaKaRa Ex Taq™ (right). The PCR products were digested with *MboI* and separated on a 3% MetaPhor™ Agarose gel in TAE buffer. M: DNA size marker (pBR322/*MspI* digest), 1: Asominori, 2: IR24.

Fig. 3. Example of advanced applications of the dCAPS method. (A) Strategy to develop C239 *Mbo*I. The polymorphic site between Asominori and IR24 is shown in upper-case bold type. The forward primer contains two mismatches shown in lower-case bold type to disrupt the *Mbo*I sites (GATC). The reverse primer contains a mismatch shown in lower-case bold type to create an *Mbo*I site. (B) Agarose gel electrophoresis profiles. The PCR products and their digests with *Mbo*I were separated on a 3% MetaPhor™ Agarose gel in TAE buffer. M: DNA size marker (pBR322/*Msp*I digest), 1: Asominori, 2: IR24.

Taq™, which has proofreading activity and a higher amplification efficiency than TaKaRa Taq™, is used, a considerable amount of digestion-resistant PCR products is observed. It is reminded that the electrophoresis profiles of the dCAPS markers could be changeable depending on the polymerase used.

Advanced application of the dCAPS method

The dCAPS method can potentially utilize any sequence polymorphism for the development of a PCR-based marker. In addition to the primary application, the dCAPS method provides advanced applications. One typical example is shown in Figure 3A, which illustrates the strategy to develop C239 *Mbo*I in Table 1. In this case, a SNP was found in the *Mbo*I site. However, it seemed difficult to detect the SNP by the CAPS method because of the existence of *Mbo*I sites in the vicinity of the polymorphic *Mbo*I site. To solve this problem, we introduced two mismatches into the forward primer so that the redundant *Mbo*I sites would be disrupted. Furthermore, a mismatch was introduced into the reverse primer to generate an *Mbo*I site for both alleles. The common *Mbo*I site is used to avoid mis-genotyping due to incompleteness of the restriction enzyme digestion; complete digestion can be confirmed by disappearance of the original PCR products (Fig. 3B).

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