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Development of New CAPS/dCAPS and SNAP Markers for Rice Eating Quality

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Rice eating quality traits are very complex and essential to be evaluated not only through physicochemical analysis and sensory test but also by PCR-based marker approach. To date, simple markers based on single nucleotide polymorphism (SNP) discovery to evaluate eating quality of cooked rice are still limited. Thus, the aims of this study were to develop PCR-based markers, called SNAP (single nucleotide amplified polymorphism) as alternative markers of cleaved amplified polymorphic sequence/derived cleaved amplified polymorphic sequence (CAPS/dCAPS). Four primer pairs specific to targeted alleles (CAPS/dCAPS and SNAP) of four loci were successfully designed based on the discovered SNPs according to the eating quality-QTL and searching genomic database. The primer pairs were able to identify alleles corresponding loci among indica and japonica varieties with diverse palatability (overall eating quality). There was consistent allele pattern produced by SNAP and CAPS/dCAPS for the same base mutation. The SNAP marker for rice eating quality trait could be easily assayed by standard agarose gel electrophoresis, allowing to increase the advantage of genotyping methods. Moreover, the SNAP markers together with our previous developed markers which were recommended as applicable marker set for evaluation of rice eating quality, will facilitate as marker-assisted selection for rice breeding program.

Key words: CAPS, dCAPS, SNAP, SNP, rice eating quality

INTRODUCTION

Evaluation of rice eating quality by the application of sensory test and physicochemical properties has long been performed. Nowadays, we detect palatability as a direct trait of overall eating quality through the glossiness of cooked rice in order to speed up and simplify measurement compared to sensory test. The sensory test requires a large amount of samples and well-trained panelists. Physicochemical traits are an indirect method to estimate the rice eating quality which also depends on the mental condition of panelist. Many researchers have worked on relationships between eating quality and physicochemical properties. However, due to the genetically complicated eating quality traits (Matsue & Ogata 1998), to estimate accurately the eating quality is difficult. A rapid, simple, and more accurate method to evaluate eating quality need to be developed, that will be very useful for both the producer and the consumer of rice.

The complex characters related to genetic and environmental factors influence the difficulty of rice eating quality estimation. QTL analysis has been used as a useful method to detect loci/genes controlling complex characters in rice. The use of molecular markers has facilitated the understanding of quantitative trait loci (QTLs) and marker-assisted selection (MAS). Many QTL analyses for grain quality of rice have been reported (Wan *et al*. 2004; Wada *et al*. 2008). QTLs related to palatability of cooked rice were identified at different loci in high quality japonica (Li 2004; Wada *et al*. 2008) and indica rice (Yong *et al*. 2006; Sabouri 2009). Thus, the allelic effect of QTLs associated with rice eating quality could possibly be used as markers.

Single nucleotide polymorphisms (SNPs), defined as single-base change at a specific nucleotide position, are highly abundant and distributed through out the rice genome (McCouch *et al*. 2010). These SNPs have recently been developed into genetic markers. The abundance of these polymorphisms leads the SNP marker system to be a more attractive tool than any other marker system, including for developing marker for certain gene of interest or within target gene regions. Since SNPs are highly stable markers that often contribute directly to a phenotype, they can serve as a powerful tool for MAS.

PCR molecular markers based on SNPs which are cleaved amplified polymorphic sequences (CAPS;

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Konieczny & Ausubel 1993) and derived CAPS (dCAPS; Michaels & Amasino 1998; Neff *et al*. 1998) are widely used for genotyping. CAPS markers detect polymorphisms that occur in restriction sites and dCAPS makers are created during PCR amplification by introducing a restriction site at an SNP site using specific primers. SNPs can also be detected using allele-specific PCR primers correspond to the site of the SNP (Ugozzoli & Wallace 1991). The other various methods based on allele-specific hybridization, primer extension, oligonucleotide ligation, endonuclease cleavage, or allelic-specific PCR (Gupta *et al*. 2001) for SNP marker have also been developed. Among many SNP genotyping methods, allele-specific PCR largely satisfies due to its simplicity. The products can be resolved on a standard agarose gel. However, allele-specific PCR has constrain, because a single-base pair change at the 3'-end is often not sufficient to ensure reliable discrimination between the two SNP alleles. To increase specificity, nucleotide-amplified polymorphism (SNAP) markers were developed. It is used the modified allele-specific primers with a mismatched base pair within four bases of the 3' end in addition to the 3'-end base complementary to the SNP site (Drenkard *et al*. 2000).

To complement sensory test and physicochemical analysis to evaluate rice eating quality, we developed a number of molecular markers for rice eating quality. These comprised microsatelite, indel (insertion/deletion), STS (sequence tagged-site) as well as SNP markers related to rice palatability, amino sugar, amino acid and starch physicochemical properties. Some of the developed markers combined with the validated markes from previous studies have successfully evaluated japonica rice eating quality (Lestari *et al*. 2009). In this study, we focused on the conversion of SNPs to CAPS/dCAPS and SNAP markers. For this objective, identification of SNP residing in the sequence of the genes likely corresponding to eating quality among japonica and indica varieties was conducted. The identified SNP was used to develop CAPS/dCAPS and the alternative marker, namely SNAP marker. The markers were to conduct genotyping and allow direct MAS for selection of rice according to eating quality traits at early growth stage.

MATERIALS AND METHODS

Plant Materials and DNA Extraction. A total of 10 japonica rice varieties, mostly bred in Korea, and 10 indica varieties originated Indonesia were used in this study (Table 1). These varieties were chosen because they represent diverse palatability scores (hedonic score) among japonica and indica rice.

Table 1. List of 20 rice varieties used in this study and their origin countries

Japonica rice	Origin country	Indica rice	Origin country
Koshihikari	Japan	Rojolele	Indonesia
Samgwang	South Korea	Ciliwung	Indonesia
Hwacheong	South Korea	Cisokan	Indonesia
Dobong	South Korea	Cibodas	Indonesia
Gopum	South Korea	Jatiluhur	Indonesia
Palkong	South Korea	Kalimutu	Indonesia
Dongjin	South Korea	Cirata	Indonesia
Samnam	South Korea	Memberamo	Indonesia
Giho	South Korea	Ciherang	Indonesia
Chucheong	South Korea	Sintanur	Indonesia

All rice varieties were grown in a light- and temperature-controlled greenhouse until the tillering stage. Only young and healthy leaves were harvested and collected for DNA extraction. Genomic DNA was extracted using the modified cetyltrimethylammonium bromide (CTAB) method (Murray & Thompson 1980). The quality of DNA was estimated using NanoDrop2000 on 260/280 and 260/230 wave length ratios. The DNA was migrated on 1% agarose and stained in ethidium bromide, then visualized on UV transilluminator.

Primer Design and PCR Amplification. Target regions were selected either close to or within the genes linked to interesting QTLs (Lestari *et al*. 2009) for rice eating quality traits. Analysis of nucleotide polymorphism of the sequences among japonica varieties was performed to develop primers for eating quality. To design primers, Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3) (Rozen & Skaletsky 2000) was used. PCR amplification of markers was carried out in a PTC-200 Peltier Thermal Cycler (MJ Research, Inc.) in a total volume of 50 µL with the following genotyping PCR reagents: 2 µL of DNA at 20 ng/µL, 2 µL of 10 x buffer containing 25 mM $MgCl_2$, 1 µL of 2.5 mM dNTPs, 1 unit of ExTaq Polymerase (TaKaRa, Japan), and 1 µL each of forward and reverse primers (10 µM). Amplified PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide. When the PCR product from each variety showed a single band, each of them was purified using PCR-Purification Kit (Intron Biotechnology, Korea).

Cloning, Sequencing, and SNP Identification. The purified PCR products were TA-cloned into pGEM-T Easy vectors and transformed into *Eschericia coli DH5á* competent cells prepared

according to the protocol of Sambrook and Russell (2001). Plasmids were isolated using the DNA-spin Plasmid DNA Purification Kit (Intron Biotechnology, Korea) and sequenced with an ABI-3700 DNA sequencer following the manufacturer's instructions (Applied Biosystems, Inc.). To identify SNPs, sequence results were aligned using the CLUSTALW program (Larkin *et al*. 2007) from EMBL European Bioinformatics Institute (http://www.ebi.ac.uk/tools), with assistance from Codoncode Aligner 2.0.6 (CodonCode Corporation, Dedham, MA) as well as BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Designing CAPS/dCAPS Primers. To detect one bp substitution in a specific fragment, a CAPS/dCAPS primer was designed, facilitated by dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps) (Neff *et al*. 2002). The primer corresponding to opposite site was generated by Primer3 program. Initially, the resulted sequences corresponding to loci were multiple aligned among rice varieties against reference japonica rice, Nipponbare from database (www.gramene.org) to identify SNPs. The two identical sequences existing an SNP in the middle with approximately 25 nucleotides on each side were entered for CAPS/dCAPS program. No more than 60 nucleotides of reference (SNP existed in Nipponbare) and the alternate SNP should be entered in the box with A, C, G, and T substitution facilitated in dCAPS Finder 2.0 program. In the dCAPS Finder 2.0 box for entered sequence, sequence of a reference allele and sequence of an alternate allele were denoted as wild type and mutant, respectively. A number of mismacthes started from zero and increased depending on the generated output. The output from zero mismatches showed whether a CAPS marker was present. If a CAPS marker was not generated, one mismatch was entered to search for a dCAPS marker. The number of mismatches was increased in each run until a potential dCAPS marker has been identified. The dCAPS primer included the necessary mismatches 5‘ of the mutation, but not include the SNP being analyzed. One mismatch produced several optional primers output for forward and reverse both on the wild type and the mutant as shown in the dCAPS Finder 2.0 program. This program only revealed one direction of primer (forward or reverse), thus the other direction of primer should be designed using the original sequence which exhibited the targeted SNP using Primer3 program. Finally one pair primer for dCAPS for AcPh was successfully developed.

Designing SNAP Primers. In addition to CAPS/dCAPS primer, the identified SNP could be converted to SNAP primer. A primer specific to the identified SNP was designed by entering the segment sequence approximately 500-800 bp containing the SNP site into the Web-available SNAPER program (http://ausubellab.mgh.harvard.edu/) with default option. In SNAPER program, items of PCR product with optimum size range of 325-375 bp and PCR product absolut ranged 300-500 bp were chosen. While other primer criteria followed SNAPER instruction. After submission, optional primers output with reference and alternate alleles could be seen on display. Around 16 output of combination of primer pairs corresponding to the SNP appeared. Candidate of primers pairs with high stability were selected and tested using optimum PCR reaction and program as SNAPER recommended. Finally, a pair of primer specific to corresponding allele with single band and consistent to the SNP existed in rice varieties could be used as SNAP marker.

PCR was performed in a total volume of 20 µl, which contained 10-100 ng of template DNA, 5 µM each of the forward and reverse primers, 100 µM of each dNTP, 1.5 mM $MgCl_2$, 1 X· reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), and 1 U of Ampli Taq Gold polymerase (Applied Biosystems). Template DNA was initially denatured at 94 °C for 5 min according to Drenkard *et al*. (2000), followed by 28 or 38 cycles for PCR amplification, using the following conditions: denaturation at 94 °C for 30 s and annealing and extension at 64 °C for 1 min, and then final extension at 72 °C for 10 min on a PTC-225 Peltier Thermal Cycler (MJ Research). Amplified products were separated on a 1.5% agarose gel to estimate each allele in the SNP site as presence or absence of a band.

RESULTS

SNP Identification. The analyses of QTL for rice eating quality allow to select candidate genes underlying the QTL regions (Lestari *et al*. 2009), including *sucrose synthase 3* (S3c, clone AP004988), *UDPN-acetylglucosamine pyrophosphorylase* (AcPh, clone AP003875), and *phosphoserine phosphatase* (PP, clone AP003727). In addition, a candidate gene involved in lipo-pholysacaharide biosynthesis, *6-phosphofructokinase-2* (PFruc, clone AP002743) contributing to starch physicochemical properties was selected (Table 2). A basic primer pair designed on the basis on highly

variable region from each locus was used to identify SNPs (Table 2). Sequence alignment analysis among japonica rice varieties showed that base changes were detected in the segment sequences of the genes positioned in intron and exon. All fragments of each locus belong to different contig in rice genome.

Point mutation was determined by using japonica rice genome of Nipponbare as reference and the other alternate allele on other variety. Finally, base changes of T/G, T/G, A/G, and A/G on the consensus sequence of S3c, AcPh, PP, and P-Fruc were identified respectively, as shown in Figure 1. Deletion of 4 bp

Table 2. Selected candidate genes based on QTL for rice eating quality and searching genomic data base and their designed primers as sources for SNP marker development

Basic primer name	Candidate gene	Chr	Contig	QTL position	Position of product at genome (bp)		QTL source	Primer sequences	Estimated product size (bp)	SNP
					Start	End				
S3c	Sucrose synthase 3 (LOC_Os07g42490)	7	AP004988	RM234-RM47	25427790	25428733	Kwon *et al*. (2007)	F: 5'-GCAAACCTG CCAAGAAAGAG-3' R:5'-ACATTGCCAT GACATTTGGA-3'	963	T/G
AcPh	UDP-N-Acetylglucosamine pyrophosphorylase (LOC_Os08g10600)	8	AP003875	RM547-RM72	6233 988	6234836	Kwon *et al*. (2007)	F:5'-CGCCTTCGTTA TTGTTGGAG-3' R:5'-TGGTAACTGG GCACAGGATT-3'	849	T/G
PP2	Phosphoserine phosphatase (LOC_Os12g31820)	12	AP003727	RM1246	19119527	19120418	Wada *et al*. (2008)	F:5'-TTTGAATAGGT CCACTGCTTTAG-3' R:5'-CCATGCATCTCAT TAGTCAAAGT-3'	981	A/G
PFruc	6-Phosphofructokinase 2 (LOC_Os01g09570)	1	AP002743	-	4902825	4903704	Genomic data base	F:5'-CTTCTTCTTCGG GTGTCTCG-3' R:5'-TGTTAAGTCCAG GGCAGAGG-3'	880	A/G

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PP
Koshihikari  61 ATTTCAAAAAAATTGGAAGAACTAAGGAAGGTTGTGGCAAGAAATATTT G ATCCATATTT 120
Samgwang     61 ATTTCAAAAAAATTGGAAGAACAAAGGAAGGTTGTGGCAAGAAATATTT G ATCCATATTT 120
Hwaseong     61 ATTTCAAAAAAATTGGAAGGACAAAGGAAGGTTGTGGCAAGAAATATTT A ATCCATATTT 120
Nipponbare   61 ATTTCAAAAAAATTGGAAGGACAAAGGAAGGTTGTGGCAAGAAATATTT A ATCCATATTT 120
Dobong       61 ATTTCAAAAAAATTGGAAGAACAAAGGAAGGTTGTGGCAAGAAATATTT G ATCCATATTT 120
Gopum        61 ATTTCAAAAAAATTGGAAGAACAAAGGAAGGTTGTGGCAAGGAATATTT A ATCCATATTT 120
                                                                  *

S3c
Nipponbare  121 TCATTC T GTTTATTCACTGAAAACATTTGTCCATTCAATGGACTCATAAACTGTCTGTGT 180
Gopum       121 TCATTC T GTTTATTCACTGAAAACATTTGTCCATTCAATGGACTCATAAACTGTCTGTGT 180
Koshihikari 121 TCATTC T GTTTATTCACTGAAAACATTTGTCCATTCAATGGACTCATAAACTGTCTGTGT 180
Dobong      121 TCATTC T GTTTATTCACTGAAAACATTTGTCCATTCAATGGACTCATAAACTGTCTGTGT 180
Palkong     121 TCATTC T GTTTATTCACTGAAAACATTTGTCCATTCAATGGACTCATAAACTGTCTGTGT 180
Dongjin     121 TCATTC G GTTTATTCACTGAAAACATTTGTCCATTCAATGGACTCATAAACTGTCTGTGT 180
                      *

AcPh
Nipponbare  361  ATTATTTTATTACTTATTTAGTACTATATAGCTAAC T TACTAAACTTTAAAGAAAAGGAC 420
Koshihikari 361  ATTATTTTATTACTTATTTAGTACTATATAGCTAAC T TACTAAACTTTAAAGAAAAGGAC 420
Hwaseong    361  ATTATTTTATTACTTATTTAGTACTATATAGCTAAC T TACTAAACTTTAAAGAAAAGGAC 420
Dobong      361  ATTATTTTATTACTTATTTAGTACTATATAGCTAAC T TACTAAACTTTAAAGAAAAGGAC 420
Samnam      361  ATTATTTTATTACTTATTTAGTACTATATAGCTAAC T TACTAAACTTTAAAGAAAAGGAC 420
Gopum       361  ATTATTTTATTACTTATTTAGTACTATATAGCTAAC G TACTAAACTTTAAAGAAAAGGAC 420
                                                    *

PFruc
Giho         121 TTT-CCCGCTCTTTTGGGGATGTTTTTTTGGCCTGTGTTTGGTTCAGGTCATCCC A ATTC 179
Nipponbare   121 TTT-CCCGCTCTTTTGGGGATGTTTTTTTGGCCTGTGTTTGGTTCAGGTCATCCC A ATTC 179
Dobong       121 TTT-CCCGCTCTTTTGGGGATGTTTTTTTGGCCTGTGTTTGGTTCAGGTCATCCC A ATTC 179
Chucheong    121 TTT-CCCGCTCTTTTGGGGATGTTTTTTTGGCCTGTGTTTGGTTCAGGTCATCCC A ATTC 179
Koshihikari  121 TTTTCCCGCTCTTTTGGGGATGTTTTTTTGGCCTGTGTTTGGTTCAGGTCATCCC G ATTC 180
Gopum        121 TTTTCCCGCTCTTTTGGGGATGTTTTTTTGGCCTGTGTTTGGTTCAGGTCATCCC G ATTC 180
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Figure 1. Partial sequence alignments showing exact location of a base substitution among japonica rice varieties at 110, 127, 397, and 176 bp of consensus sequences derived from loci of PP, S3c, AcPh, and PFruc, respectively. The reference allele was determined on Nipponbare and the alternate allele was noted by bold letter. The sites of single nucleotide polymorphisms (SNPs) were indicated as bold black asterisks.

at S3c was proved to be potential for indel marker based on the full sequence of this gene (Lestari *et al*. 2011) and included in the marker set to estimate japonica rice palatability (Lestari *et al*. 2009). The nucleotide substitutions for four loci were subjected to design SNP primers to evaluate rice eating quality. To validate the markers, rice variety of mapping parent for linkage genetic analysis (Kwon *et al*. 2007) as source of candidate genes selection, also was used as genetic material for application of these markers developed in our study.

Development of CAPS/dCAPS Marker. Once SNP is identified, it is likely to convert in developing primer of CAPS/dCAPS as demonstrated in the examples of this study. Several optional outputs of primers either forward and/or reverse depending on restriction enzyme site of each sequence, could be used as primer candidates. Importantly, number of mismatch contributed to reveal primer type, CAPS or dCAPS. In case with nol mismatch, it seemed to be easy to convert SNP existed in PP locus leading for CAPS primer design. Conversely, the other three loci were preferentially developed as dCAPS primers since at least one mismatch should be entered to produce primer candidate.

Finally, a total of four molecular markers were successfully generated on the basis of SNPs, comprising one CAPS primer for PP locus, and three dCAPS primers for S3c, AcPh and PFruc loci (Table 3). For development of specific CAPS/dCAPS primers in this study, only the commonly used restriction enzymes were selected to be complement with the designed primers. For instance, *Mse*I, *EcoR*I, and *Taq*I. The dCAPS method could potentially use any sequences with identified SNP for the development of a PCR-based marker. Application of these markers for genotyping showed polymorphism of the allele belonging to each locus among varieties with diverse palatability (Table 4). This result demonstrates different genetic background of both japonica and indica rice varieties. Loci of PP and S3c revealed dominant alleles of G and T on

Table 3. Sequences primers of CAPS/dCAPS and SNAP with the amplification condition for SNAP primers

Original primer	Sequence	Primer type	SNAP primer name	Sequence	PCR condition of SNAP primer
AcPh	F:AGTTGTGGTT TAAGCATAG	dCAPS/*Mse*I	AcPh-T	F:TCAGGTATGCCTGC ATATGTTTACTTT	94 °C 5min
	R:ATTGTCCTTTTCT TTAAAGTTTATTA			R:CACACAATTGTCCTTTT CTTTAAAGTTTACTAA	94 °C 30sec 63 °C 1min } 28 cycles 72 °C 10min
PFruc	F:CTTCTTCTTCG GGTGTCTCG	dCAPS/*Eco*RI	PFruc-A	F:GGCCTGTGTTTGGT TCAGGTCATCCTA	94 °C 5min
	R:TGTTAAGTCCAG GGCAGAGG			R:ACGAGGAGAATTCTTAC TTGACGACTGAATAAGC	94 °C 30sec 62 °C 1min } 28 cycles 72 °C 10min
S3cII	F:TTCCATGATGTG CCACTCTC	dCAPS/*Taq*I	S3cII-T	F:CATCCAAATTCTGTTTAT TTATTCAAAAATCATCCT	94 °C 5min
	R:GGACAAATGTTTT CAGTGAATAAAT			R:AGTAAATGCACTCTTTAT CCAGCTAACATCAGCA	94 °C 30sec 64 °C 1min } 28 cycles 72 °C 10min
PP2	F:TTTGAATAGGTC CACTGCTT	CAPS/*Mse*I	PP2-A	F:AAGAACAAAGGAAGGT TGTGGCAAGAAATATTAA	94 °C 5min
	R:CCATGCATCTCA TTAGTCAA			R:CAGCAACATGGCAGAGT GGAGAGTTAAAA	94 °C 30sec 63 °C 1min } 28 cycles 72 °C 10min

Table 4. Allele variation produced by the 4 designed SNP markers observed on japonica and indica rice varieties

Japonica rice	Hedonic score*	AcPh	S3cII	PP2	PFruc	Indica rice	Hedonic score*	AcPh	S3cII	PP2	PFruc
Koshihikari	4.3	T	T	G	G	Rojolele	4.1	G	T	G	A
Samgwang	4.5	G	G	A	A	Ciliwung	3.9	T	T	G	A
Hwacheong	3.4	G	T	G	G	Cisokan	3.45	G	T	G	G
Dobong	2.3	T	G	G	A	Cibodas	3.6	G	T	G	G
Gopum	4.2	T	G	G	A	Jatiluhur	2.2	G	T	G	G
Palkong	3.1	T	G	G	A	Kalimutu	3.6	T	G	A	A
Dongjin	3.9	T	T	G	A	Cirata	3.4	G	T	G	G
Samnam	2.9	T	T	G	A	Memberamo	4.0	G	T	G	A
Giho	3.5	T	T	G	A	Ciherang	3.9	G	T	G	A
Chucheong	4.0	T	T	G	A	Sintanur	3.1	G	T	G	G

*The higher score represents the higher palatability.

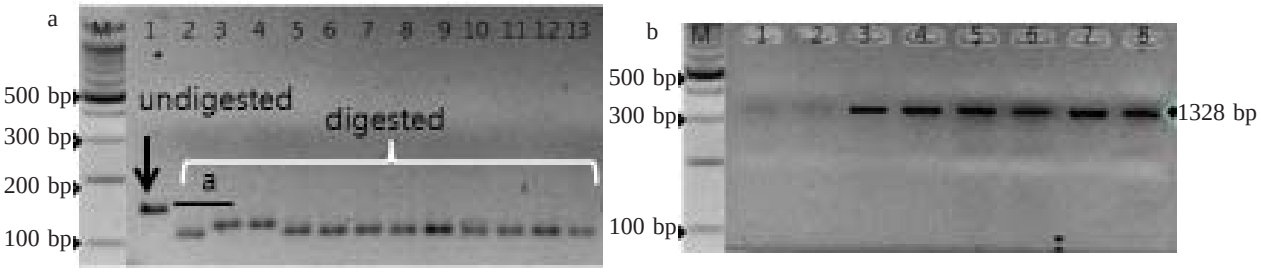

Figure 2. Products of digestion separated by gel electrophoresis. DNA banding pattern produced by dCAPS and SNAP primer corresponding to AcPh locus that shows a base change of T/G alleles observed on japonica and indica rice varieties. (a) SNP polymorphism of amplicon produced by dCAPS-AcPh primer. Undigested product with size of 161 bp shown in lane 1; lane 2-13: digested products in which, varieties in lane 3-4 have G allele while others have T allele (M. DNA ladder of 100 bp, lane 1. Koshihikari 2. Koshihikari, 3. Samgwang, 4. Rojolele, 5. Gopum, 6. Dobong, 7. Palkong, 8. Dongjin, 9. Samnam, 10. Giho, 11. Chucheong, 12. Ciliwung, 13. Kalimutu. (b) Analysis of specificity for single nucleotide amplified polymorphism (SNAP) primer of AcPh, the banding pattern was produced by T allele-specific primer (M. DNA ladder of 100 bp, 1. Samgwang, 2. Rojolele, 3. Gopum, 4. Koshihikari, 5. Dobong, 6. Dongjin, 7. Samnam, 8. Giho).

both japonica and indica, respectively, but not for the other loci. These alleles for each single marker have no significant association with rice palatability either indica or japoncia. Consequently, combination of these markers than single marker were preferentially applied. The designed dCAPS markers, both S3cII and AcPh were as components of two marker sets for each comprising 13 and 14 markers to evaluate japoncia rice eating quality (Lestari *et al*. 2009). Establishment of dominant SNP-based markers which included in these marker set detection will give simpler application.

Development of SNAP Marker. A simple and low-cost marker is more prospective and practical especially for selection of early breeding with abundant population. Therefore, another alternative of dCAPS marker that less expensive and simple was generated in this study. All the detected SNPs as modified into CAPS/dCAPS primers were converted into SNAP marker (Table 3). As an example, based on the identified a base change of T to G in AcPh locus, a SNAP marker specific to T allele was developed. SNAP primers were usually developed on specific allele, in which the base change type exhibited. The T-specific primer of AcPh (AcPh-T) showed a band in only variety possessed T allele in AcPh locus. The banding pattern of T/G allele of T-specific primers was the same as that of dCAPS-AcPh primer (Figure 2). SNAP primers to modify the dCAPS or CAPS primers were also performed on S3cII-T, PFruc-A, and PP2-A.

PCR program for SNAP primer is different from standard PCR commonly used. Annealing temperature and number of cycles during PCR amplification had a crucial role in SNAP marker development. All primers corresponding to total locus showed amplification by 28 cycles with annealing temperature ranged 62-64 ^{o}C were considered as optimum conditions for these SNAP markers during PCR amplification. Example of banding pattern generated by the AcPh-SNAP marker showed consistent results with that of AcPh-dCAPS primer (Figure 2). In specific case, T allele-specific marker only amplified genomic DNA on varieties that had a T allele at locus AcPh and vice versa for alternate allele.

DISCUSSION

A numerous variation of rice genome sequences has promoted the SNPs search between varieties. Since SNP genotyping is needed for many applications such as map based cloning, marker-assisted breeding, and seed purity test (Komori & Nitta 2005), many new techniques have been established. The techniques were to convert an identified SNP to molecular markers either on the basis of random total genome or emphasizing specific candidate genes related to target traits. This SNPs converted to functional molecular marker might be derived from a functionally characterized sequence motif of the loci studied here, which is superior to random DNA markers owing to its complete linkage with the target gene.

Based on analysis of QTL for rice eating quality (Kwon *et al*. 2007; Wada *et al*. 2008), three candidate genes encoding proteins, i.e. *sucrose synthase 3* (S3c), *UDPN-acetylglucosamine pyrophosphorylase* (AcPh), and *phosphoserine phosphatase* (PP) sequences underlying target QTL were successfully selected as a potential source for markers in this study. Searching gene involved in the rice pathway of lippo-polysaccharide biosynthesis

which is overlapped its physical position to the QTL region for rice starch physicochemical properties could also be a potential source as functional marker (Lestari *et al*. 2009). The *sucrose synthase 3* functions to synthesize sucrose maximally at filling grain in rice, while *UDPN-acetylglucosamine pyrophosphorylase* is well known to involve in amino sugar biosynthesis. *Phosphoserine phosphatase* seems to participate in amino acid metabolism, in contrast, *6-phosphofructokinase-2* focuses on the pathway of lippo-polysaccharides biosynthesis.

The existence of SNP generating a restriction site difference between rice varieties on the PP locus is critical for CAPS marker development. However, the SNP generating no restriction site differences on AcPh, S3c and PFruc could be converted to dCAPS marker, as proved in *Arabidopsis thaliana* as well (Hayashi *et al*. 2004; Komori & Nitta 2005). Thus, by introducing 1-2 mismatches into forward or reverse primer could disrupt the redundant restriction sites (Komori & Nitta 2005) for dCAPS. The dCAPS is different from CAPS in using specific primers, which are designed with one or two mismatches (Drenkard *et al.* 2000). Through these mismatches, primers bring mutations into the target sequences during amplification and in conjunction with the identified SNP resulting in a unique restriction site. Moreover, the output of this program provides a set of suitable primer sequences along with appropriate restriction endonucleases (Hruba 2007). The primary application of CAPS/dCAPS method allows to advance application with restriction enzyme and was successful to detect the distinction of rice varieties based on the alleles corresponding to their loci as shown in our study. The simple PCR, treatment of restriction enzyme and observation on agarose gel of this marker bring it to be still preferentially applied in any laboratories (Michaels & Amasino 1998).

The SNP markers mainly developed are CAPS/dCAPS that need a large amount of restriction enzyme (Ayres *et al*. 1997; Liu *et al.* 2004; He *et al.* 2006). In some cases, a high cost of restriction enzyme might be a problem, especially for selection process using a large number of breeding population. Thus, the other allele specific primer, called SNAP could be an alternatively promising solution. When observed in both japonica and indica varieties, we found the same DNA banding patterns between CAPS/CAPS and SNAP markers corresponding to all locus. SNAP markers on the basis of SNP with easy and low cost will complement to the previous established markers for rice eating quality (Bao *et al*. 2006; He *et al*. 2006).

Several functional markers for targeted eating quality traits of rice with high association have been developed to be powerful markers as single marker (Bao *et al*. 2006; He *et al*. 2006; Wang *et al*. 2010; Ni *et al*. 2011) or marker set for marker assisted selection (Nakamura *et al*. 2004). These markers themselves may not have direct functions, but they can still relate to the variations in phenotypic traits, between the marker site and the functional domain of the target genes (Bao *et al*. 2006). These SNP-based primers investigated in this study virtually which were components of the marker sets as multiple interaction showed highly significant association to rice eating quality both on japonica and indica ($P < 0.001$) (Lestari *et al.* 2009). Relevan to this study, several markers for rice eating quality also have been developed based on universal primers, RAPD (random amplified polymorphic DNA) converted to SCAR (sequence characterized amplified regions) and made in marker set showing powerfully to evaluate rice premium with high palatability in Japan (Ohtsubo *et al*. 2002, 2003). In addition, this result was supported by other study that markers for palatability developed on the basis of japonica genome was able to identify Indonesian indica rice with high palatability (Lestari *et al.* 2012). No doubt that even the SNPs were discovered based on the japonica rice genome, those identified SNPs were also detected and could be applied in indica rice because of the wide range of genetic diversity between japonica and indica rice varieties. Wider diversities of landraces and breeding lines could be included for genotyping study to assure the allele variation patterns.

Due to the genetical complexity of rice eating traits, by combining with other functional markers that we developed, these SNAP markers are recommended to be used as marker set than single marker. These dominant markers will simplify the marker set performed for evaluation of rice eating quality (Lestari *et al*. 2009) and are useful for selection of rice eating quality with large samples. These SNP-based markers investigated in this study could be complement the other functional markers (STS, indel, and microsatelite) that we developed on the basis of loci/genes controlling rice starch physicochemical properties determining eating quality. These all SNP-based markers together with the other developed markers (Lestari *et al*. 2009) will expectedly use to establish more marker sets for evaluation of other physiochemical properties of both japonica and indica rice.

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