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Discrimination among rice varieties based on rapid detection of single nucleotide polymorphisms by a newly developed method, mass spectrometric cleaved amplified polymorphic sequence (MS-CAPS) analysis

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Abstract

The method described here discriminates among rice cultivars based on single nucleotide polymorphisms (SNPs). The method is rapid (less than 1 hour), and combines PCR and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). We obtained sequence data from genome databases, and identified SNPs that could be used to distinguish among rice cultivars after digestion with restriction enzymes or urasil-DNA glycosylase (UDG). A crude extract was prepared by vortexing rice grains in a tube, and this was used as the template for PCR without further purification of genomic DNA. The PCR primers were designed based on the sequence around the SNP. The PCR was performed with a short extension time (2 s) and the amplicons were treated with restriction enzymes or UDG. Single-stranded DNA (ssDNA) obtained by alkali denaturation was analyzed by MALDI-TOF MS. This method, which we have used previously to identify transgenic genes in plants, successfully discriminated among rice cultivars based on SNPs. We added an asymmetric PCR to obtain greater quantities of ssDNA and a fast PCR step to increase the speed of amplification.

Keywords: mass spectrometric cleaved amplified polymorphic sequence (MS-CAPS); matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS); PCR; Rice; single nucleotide polymorphisms (SNPs).
Abbreviations used: CAPS: cleaved amplified polymorphic sequence, gDNA: genomic DNA, MS-CAPS: mass spectrometric CAPS, MALDI-TOF MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry, SNP: single nucleotide polymorphism, ssDNA: single-stranded DNA, StAv-MB: streptavidin-coated magnetic bead, UDG: uracil-DNA glycosylase.

Introduction

There are several methods to detect single nucleotide polymorphisms (SNPs) (Giancola et al., 2006). One of them, the GOOD assay (Sauer et al., 2000; Sauer et al.; 2003, Smylie et al., 2004), is useful for analysis of genes that include SNPs, and a commercial system to detect SNPs based on this assay is now available (Pusch et al., 2001). However, genotyping using the GOOD assay is time consuming and labor intensive, because it requires two PCRs and special primers that are cleaved by ultraviolet radiation to decrease the mass of the amplicon for analysis. The first PCR in the GOOD assay requires purified genomic DNA (gDNA) because a relatively long sequence must be amplified to produce the template for the second PCR. There are several other approaches to analyze differences at the SNP level using gel electrophoresis, including cleaved amplified polymorphic sequence (CAPS) analysis (Neff et al., 1998) and derived CAPS analysis (Konieczny and Ausubel, 1993). Although these techniques are useful for analyzing differences among cultivars, their disadvantages are the time required to prepare purified gDNA for PCR and for gel electrophoresis to detect amplicons (Table 1). Also, it is difficult to analyze many samples simultaneously because amplicons must be analyzed manually by gel electrophoresis. In certain commercial settings, e.g., markets and customs, rapid methods for gene identification have been requested for on-site analysis of agricultural products, especially for identification of genetically modified plants and patented agricultural brands (Auer, 2003). A rapid method to detect bacteria and/or viruses would also be useful for quarantine stations (Walcott et al., 2006). Previously, we reported a method combining short PCR and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis to detect the transgenic gene in plant material using rice grains as the experimental material (Fig. 1) (Kajiwara, 2011). SNP detection is important for plant breeding, because SNPs are related to the characteristics of each cultivar. For those purposes, we developed a method combining PCR and MALDI-TOF MS, designated as mass spectrometric cleaved amplified polymorphic sequence (MS-CAPS) analysis, for rapid analysis of genes. In this method, PCR products were amplified using a biotinylated primer, and then digested by a restriction enzyme or treated with uracil-DNA glycosylase (UDG). The digested DNA was then purified using streptavidin-coated magnetic beads (StAv-MB) and analyzed by MALDI-TOF MS. Crude extracts containing gDNA could be used as the experimental material without further purification. In this study, we used the MS-CAPS method to discriminate among rice cultivars based on SNPs. We further improved the method by introducing asymmetric PCR (Saiki et al., 1986) and fast PCR (Sullivan et

al., 2006; Yap and McGee, 1991). Although the peak derived from the unreacted primer reported previously (Kajiwara, 2011) was still evident, the modifications to the method described in this report decreased the size of this peak. Therefore, the use of asymmetric PCR resulted in the product peak becoming the major peak in the mass spectrum. We decreased the total reaction time for PCR by adding a fast PCR method into the MS-CAPS analysis.

Results and Discussion

Selection of regions for amplification by PCR

For these analyses, one of the primers was biotinylated at the 5'-end for collection by StAv-MB after PCR (Fig. 1 and Table 2). In addition, a new restriction site and/or a cleavage site had to be created in the amplified DNA by PCR to show differences among some rice cultivars. After amplification, a restriction enzyme or UDG was added to cleave the amplicons at a specific sequence (Table 2). Therefore, when designing primers to discriminate among cultivars, the cleavage site recognized by a restriction enzyme or UDG must be considered. DNA fragments were collected by binding of the biotin at the 5'-end to StAv-MB using a magnetic stand. Double-stranded DNA was denatured under alkali conditions to generate single stranded DNA (ssDNA), and ammonia solution was added to dissociate the bond between biotin and StAv-MB. ssDNA was collected by lyophilization for subsequent analysis by MALDI-TOF MS.

Use of asymmetric PCR

As reported previously (Kajiwara, 2011), some unreacted biotinylated primer remained in the mixture at the end of the reaction period because the PCR amplification had reached a plateau. Therefore, the unreacted primer with biotin at its 5'-end also bound to StAv-MB along with the amplicon digested by enzymes. This resulted in signals from both the amplicon and the unreacted primer in the same mass spectrum, making its analysis rather complicated. In a conventional PCR, excess primer is used for the reaction. Consequently, the amount of unreacted primer exceeds that of reacted primer. In many cases, digested DNAs were longer than the unreacted primer (Table 2). In those cases, the peak height of the amplicon was smaller than that of the unreacted primer, even if there were equal amounts of amplicon and unreacted primer in the mixture. Altering the amount of PCR primers and the length of amplification times were not effective methods for decreasing the peak of the unreacted primer in the MS spectrum (data not shown). Therefore, we used asymmetric PCR (Saiki et al., 1986) to increase the peak height of the amplicon (Fig. 2). Asymmetric PCR is normally used to produce an excess of the strand complementary to the primer, and it is used in DNA sequencing and Southern hybridization methods. Here, the objective of using asymmetric PCR was to react all of the biotinylated primer so that no unreacted biotinylated primer remained at the end of the reaction. The molar ratio of the biotinylated primer to normal primer was adjusted to 1:3, 1:7, and 1:13, while the total molar concentration of primers remained constant (Fig. 3). No restriction enzyme was added in this experiment. At the end of the reaction time, the unreacted primer in the reaction solution was observed in the mass spectrum. The biotinylated primer used for primer extension was not observed in this mass spectrum because its molecular mass was too high; that is, it was out of range in the MALDI-TOF MS analysis. There was a peak from the unreacted biotinylated primer in the mass

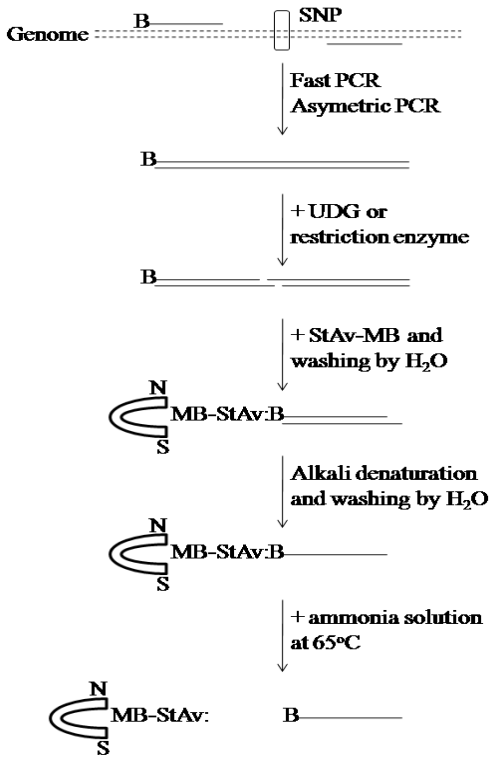

Fig 1. Scheme of MS-CAPS. A gene locus containing a SNP is selected for PCR amplification. The SNP should lie between the primers, which are designed based on the sequences around the SNP. The amplicons are digested by a restriction enzyme that produces different products from the amplicons; that is, digested or not, or restriction fragments that differ in length or composition. Otherwise, if there is a T (or A) near the SNP, then U can be substituted in place of the T by adding dUTP as a substrate to the PCR amplification, and the amplicons can be distinguished after UDG treatment. One of the primers must be biotinylated (B) at its 5'-end. Streptavidin-coated magnetic beads (StAv-MB) are mixed with the solution by pipetting after enzyme treatments. Then, single-stranded DNA is obtained by an alkali treatment, and then the complementary single-stranded DNA is removed. Ammonia solution is added to dissociate StAv-MB and biotin, and biotinylated ssDNA and StAv-MB in the ammonia solution are separated using a magnetic stand. Biotinylated ssDNA is obtained by lyophilization.

spectrum. The peak intensity obtained using the 1:3 molar ratio of primers was 30,000. [a.u], but was significantly lower using the 1:7 ratio (8,000 [a.u]). When the molar ratio of primers was 1:13, most of the biotinylated primer was used for the primer extension during PCR, and the peak derived from the unreacted biotinylated primer decreased to the background noise level.

Detection of SNPs

Table 1. compares the MS-CAPS method including MALDI-TOF MS analysis with the CAPS method including gel electrophoresis. Both methods can be used to distinguish differences in SNPs among varieties, cultivars, or lines. In the first case, the two lines have different nucleotide sequences; one has a region in the genomic DNA (gDNA) that matches the

Table 1. Comparison of CAPS method using gel electrophoresis with MS-CAPS method using MALDI-TOF MS to detect SNPs.

	CAPS	MS-CAPS
Template for PCR	Purified DNA	Crude extract*
Extraction of template for PCR	1-2 h	3 min
Total time for PCR**	2-3 h	20-40 min
Detection	Staining/imaging	MALDI-TOF MS
Time for detection	30 min	10 s
Automation	Difficult	Easy
Costs for consumables***	$12/sample	$2/sample
Fast PCR	Possible	Possible
Asymmetric PCR	Possible	Possible
Genome sequence required	No	Yes
Detection of differences:		
Region amplified or not-amplified by PCR	Possible	Possible
Presence of restriction site in amplicon	Possible	Possible
Length of restriction fragment	> 20–30 bp	> 1 bp
Mass of restriction fragments	Impossible	Possible
Use of UDG	Impossible	Possible
Application of dCAPS method	Possible	Possible

* Centrifugation is not required for rice seed extracts. Crude extracts from leaves and roots of various plants can be used directly as PCR templates (data not shown).
** Depends on annealing temperature and apparatus used for PCR amplification.
*** Costs of consumables do not include tips and tubes. Labor costs are not included.

primer, and the other does not (Table S1). Therefore, PCR will amplify a product from one line, but not the other. It is simple to distinguish the two lines in this case, because the absence of the amplicon is clearly visible in the mass spectrum. In this case, the CAPS method can also detect the SNP because of the different electrophoretic patterns. In the second case, amplicons are obtained from both lines, but one of them contains a restriction enzyme site and the other does not. Thus, the PCR product with a restriction site would produce restriction fragments, but the other would not. This is readily detected in MS-CAPS analysis and also in CAPS analysis. Although ssDNAs could be obtained both from digested and non-digested DNAs, the ssDNA derived from the sequence without a restriction site would be out of range in the MALDI-TOF MS analysis. Thus, only the amplicon that was cleaved by a restriction enzyme would show a mass peak in the MS-CAPS analysis (Fig. 3). In the third case, both strains produce amplicons with restriction sites, but the lengths of the restriction fragments differ because of inserted or deleted nucleotides. In this case, there is a difference in mass because of the different number of nucleotides. This can be detected by MS-CAPS analysis even if only one nucleotide is inserted or deleted. CAPS analysis can only detect such differences if there is a greater number of insertions or deletions (>20–30). In the fourth case, the lengths of the restriction fragments are the same, but their nucleotide composition differs (Fig. 4 and Fig. 5). CAPS analysis using gel electrophoresis would not detect differences in nucleotide composition, but MS-CAPS analysis is sensitive enough to distinguish such differences. The mass difference between nucleosides is summarized in Table S2. Most differences are large enough to be detected easily by MALDI-TOF MS analysis. However, the difference between A and T (mass difference of 9.02) may be difficult to detect using low-grade MS instruments. In this case, a 'T' in the sequence of one amplicon can be replaced by a U by adding dUTP as a substrate to the PCR amplification. Then, the amplicons can be treated with UDG to yield products that are distinguishable by MALDI-TOF MS analysis. Figure 4 shows an example of the second case, in which the amplicon contained a restriction site. The amplicon obtained from *Akitakomachi* contained an EaeI restriction site, whereas that obtained from *Himenomochi* did not (Table 2). Thus, digestion of the *Akitakomachi* amplicon with EaeI yielded a product 2-bp shorter than primer E20943FB. Theoretically, there should be no peak in *Himenomochi*, but a small peak was observed at *m/z* 5687 in the mass spectrum. The reason for this was unclear, but it is possible that there was some contamination from *Akitakomachi* during the sample preparation process. There was also a peak from the unreacted biotinylated primer at *m/z* 6570. This peak was greater than that of the product derived from PCR and EaeI digestion. The origins of the peaks at *m/z* 7782 in *Akitakomachi* and *m/z* 7458 in *Himenomochi* are unknown, and further research is required to clarify this point. The peaks observed at *m/z* 3619, *m/z* 3737, *m/z* 3752, and *m/z* 4186 originated from StAv-MB (data not shown). Figure 4 shows an example of the fourth case, in which sequences from the two lines were the same length but differed in their nucleotide composition. Both *Himenomochi* and *Akitakomachi* yielded amplicons in the PCR, and Tsp509I was used to digest the amplicons (Table 2). The amplicon from *Himenomochi* contained a G, whereas that from *Akitakomachi* contained a C. The mass of G is larger than that of C (Δ40.03) (Table S2). The peaks obtained from *Himenomochi* and *Akitakomachi* were *m/z* 8735.504 and *m/z* 8694.644, respectively, and the observed difference between *Himenomochi* and *Akitakomachi* was 40.860. In this experiment, Tsp509I cleaved the amplicons at a recognition site that was already present in their nucleotide sequence. There were two minor peaks in spectra from both *Himenomochi* and *Akitakomachi* (Fig. 4 and Fig. 5). Every analysis using the matrix 2',4',6'-trihydroxyacetophenone showed these minor peaks (vertical arrows), and they were considered to be degradation products of the main peaks (*m/z* 8735.504 in *Himenomochi* and *m/z* 8694.644 in *Akitakomachi*). That is, the main products were degraded by laser shots. Further research will be useful to identify a better matrix for DNA analysis by MALDI-TOF MS. Amplicons can be subjected to UDG treatment in the MS-CAPS method, and the reaction products can be detected by MALDI-TOF MS analysis (Fig. 5). UDG cuts at the nucleotide U, which is substituted in place of T by adding dUTP as a substrate to the PCR amplification. UDG cannot be used in CAPS analysis because the products after UDG digestion are too small to separate by gel electrophoresis (Table 1). However, MALDI-TOF MS is sensitive enough to detect differences in the digestion products after UDG

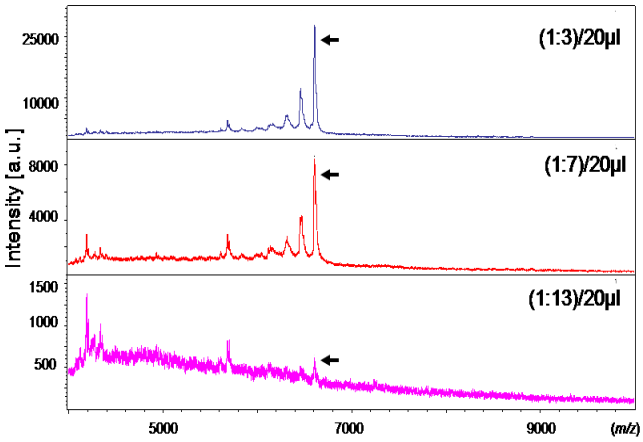

Fig 2. MALDI-TOF MS analysis of amplicons obtained by asymmetric PCR using different amounts of primer. Molar ratio of biotinylated primer to normal primer was 1:3, 1:7, and 1:13. Total primer concentration was 2.0 nmol/ml in the 20-µl reaction mixture. Horizontal arrow shows unreacted primer.

treatment. The sequence of the amplicon from *Heiseimochi* contains an A in the position occupied by a G in the *Nipponbare* sequence (Table 2). Thus, the theoretical mass difference is 16.00 (Table S2). *Heiseimochi* and *Nipponbare* showed peaks at *m/z* 6976.991 and *m/z* 6961.424, respectively. The peak in *Heiseimochi* was 15.576 higher than that from *Nipponbare*. Therefore, the mass difference observed here was consistent with the theoretical difference. The resolution of the MALDI-TOF MS analysis was sufficient to distinguish the mass difference of 16.00.

Comparison of MS-CAPS and CAPS

MS-CAPS analysis has several advantages over CAPS methods using gel electrophoresis (Table 1). In the MS-CAPS method, crude extracts are easily prepared from rice grains. These extracts, which contain some gDNA, can be used as templates for PCR amplification without further purification because the length of the amplicon is very short (Kajiwara, 2011). Longer PCR products (>70–80 bp) were not obtained from crude extracts from rice seed grains. Starch and sugars in seeds did not inhibit the PCR amplification (data not shown). Phytochemicals, probably phytic acids in rice seeds, may inhibit the DNA polymerase because low concentrations of phytic acids inhibited PCR amplification (data not shown). In the CAPS method, extraction of gDNA takes 1–2 h, and includes steps to powder rice seeds and remove contaminants. In the method described here, crude extracts are prepared simply by vortexing a rice grain. Because the amplicon is very short, the primer extension time can be decreased to 2 s. If better PCR apparatus was used for these experiments, the total PCR time could be further decreased because most of the PCR time was allocated for increasing and decreasing temperatures. A fast PCR method was introduced into the MS-CAPS method (Fig. 3-5), decreasing the time for the PCR amplification step to less than 20 min if the PCR annealing temperature was relatively high (data not shown). Conventional PCR requires 1–3 h for amplification because longer amplicons require longer primer extension times. In CAPS analysis, after PCR amplification, amplicons digested by a restriction enzyme are separated by gel electrophoresis and stained using a dye such as ethidium bromide. This requires 1–2 h, plus approximately 30 min in dye solution before photographing the gel. In MS-CAPS analysis, the peak for one sample was detected in approximately 10 s. The entire MS-CAPS process could easily be automated. In fact, a robotic system for transferring the solution from a tube to a 96-well PCR plate already exists. In addition, we have used a mechanized system to spot the solution onto the target plate for MALDI-TOF MS (data not shown). Full automation of the MS-CAPS method would significantly decrease labor costs. At present, the approximate cost of consumables for CAPS analysis (including the gel, electrophoresis buffer, DNA polymerase, *etc*.) is US $12 per sample, compared with US $2 per sample for the MS-CAPS method. The cost per sample could be further reduced to less than US $1 per sample by decreasing the amount of DNA polymerase, StAv-MB, and other reagents. Fast PCR and asymmetric PCR methods can be incorporated in the MS-CAPS method. The disadvantage of the MS-CAPS method is that the nucleotide sequence of the region of interest must be already known to design primers and to predict the reaction products after PCR and enzymatic treatments. If there is no nucleotide sequence information available, the peaks in the mass spectrum cannot be interpreted. Recently, genome information for many species and varieties has been added to public databases, making it easy to detect differences in SNPs among cultivars. At present, the requirement for genome information for MS-CAPS is a disadvantage, but this may not be the case in the near future as the amount of genome information is increasing on a daily basis.

Materials and methods

Materials

Laboratory chemicals and enzymes were purchased from Wako Pure Chemical Industries (Japan) or Nacalai Tesque (Japan) unless otherwise stated. Rice seed grains (*Oryza sativa* L., cv. *Akitakomachi*, *Heiseimochi*, *Himenomochi*, and *Nipponbare*) were used after removing the seed coats.

Primer design

For each cultivar, a specific DNA region was amplified using a combination of one unlabeled primer and one primer that was biotinylated at the 5'-end. The primers were synthesized by a commercial manufacturer (Hokkaido System Science Co., Ltd.) (Table 2). To discriminate among rice varieties, the gene loci E20943 (SNP position 259894 bp, Chromosome 7) and R1744 (16605526 bp, chromosome 5) were selected as examples of rice SNPs (Sato et al., 2010). Nucleotide sequences were obtained from the Rice Genome Annotation Project Database (http://rapdb.dna.affrc.go.jp/). There was a one-nucleotide difference between *Himenomochi* and *Akitakomachi* at gene locus E20943 (Table 2). The restriction enzyme EaeI digested the amplicon from *Akitakomachi*, but not that from *Himenomochi*. Therefore, these cultivars could be distinguished by their restriction fragments (Table S1). The mass spectrum contained a peak from the amplicon of *Akitakomachi*, but not from that of *Himenomochi* because the undigested single-stranded DNA (ssDNA) was out of range of detection in the MALDI-TOF MS analysis. After digestion with the restriction enzyme Tsp509I, both the amplicon and its digested products (gene locus E20943) were within the range of detection in the MALDI-TOF MS analysis (Table 2). Although the lengths of the digested products were the same, the

Table 2. Nucleotide sequences of gene locus and PCR primers. Gene locus was selected based on data from Sato et al. (2010) and database (http://rapdb.dna.affrc.go.jp/). Primers were synthesized by Hokkaido System Co. Ltd. Vertical arrow shows enzyme cleavage site. Position of SNP shown in red.

Gene locus and Primer	Nucleotide sequence
E20943	Cleavage site by EaeI
Himenomochi	↓ Cleavage site by Tsp509I
	-tctgttgg**G**caa↓ttgatat-
Akitakomachi	No site of EaeI
	Cleavage site by Tsp509I
	-tctgttgg**C**caa↓ttgatat-
E20943FB	Biotin-ggcaactatgtttctgttgg-3'
E20943R	5'-gtcatcaacagagtaacatatcaatt-3'
R1744	Cleavage site by UDG
Heiseimochi	-gtacatac**G**t↓tgtgtat-
Nipponbare	Cleavage site by UDG
	-gtacatac**A**t↓tgtgtat-
R1744FB	Biotin-catcaacctgttgtacatac-3'
R1744R	5'-acgacatgacctcatacaca-3'

nucleotide sequence (composition) differed between the two products. The *Himenomochi* product contained a G, whereas the *Akitakomachi* product contained a C (Table S1). Except for this single SNP, all of the other nucleotides were same in the products from *Himenomochi* and *Akitakomachi*. Therefore, the mass difference between the restriction fragments from *Himenomochi* and *Akitakomachi* was equal to the mass difference between G and C, 40.03 (Table S2). In the case of *Heiseimochi* and *Nipponbare*, we did not find a suitable restriction enzyme to cleave the sequence at the gene locus R1744 to recognize the SNP (Fig. 5 and Table 2). Instead, a U was substituted for a T in the sequence of the amplicon by adding dUTP as a substrate during PCR amplification. The amplicons from *Heiseimochi* and *Nipponbare* were treated with UDG, and the resulting fragments differed by one nucleotide, G and A (Table S1). Therefore, the peak obtained from *Heiseimochi* and *Nipponbare* differed by 16.00 because of the mass difference between G and A (Table S2).

PCR amplifications

One grain of brown rice (*Akitakomachi*, *Heiseimochi*, *Himenomochi*, and *Nipponbare*) was vortexed in 0.1 ml pure sterilized water in a tube for 3 min. Crude extracts (0.8 µl) were used for PCR amplifications without further purification of gDNA. The PCR was performed in a 20 µl reaction volume using *Tfi* DNA polymerase (Invitrogen, USA). The reaction was denatured for 1 min at 94°C, then thermocycled 30 times as follows: 2 s at 94°C, 2 s at 60°C, and 2 s at 72°C (Kajiwara, 2011) (2720 Thermal Cycler, Applied Biosystems, USA). For fast PCR (Yap and McGee, 1991; Sullivan et al., 2006), the thermocycling conditions were as follows: 5 cycles of 2 s at 94°C, 2 s at 60°C, and 2 s at 72°C, then 25 cycles of 2 s at 85°C, 2 s at 60°C, and 2 s at 72°C. dUTP was substituted for dTTP when UDG (New England Biolabs, USA) was going to be used to cleave the PCR products. Oligonucleotides (Table 2) were synthesized by a commercial manufacturer. For asymmetric PCR (Saiki et al., 1986), the ratio of the biotinylated primer and primer was altered to 1:3, 1:7, and 1:13 (Fig. 3). Electrophoresis was performed on 20% acrylamide gels in Tris-borate buffer (Sambrook et al., 1989).

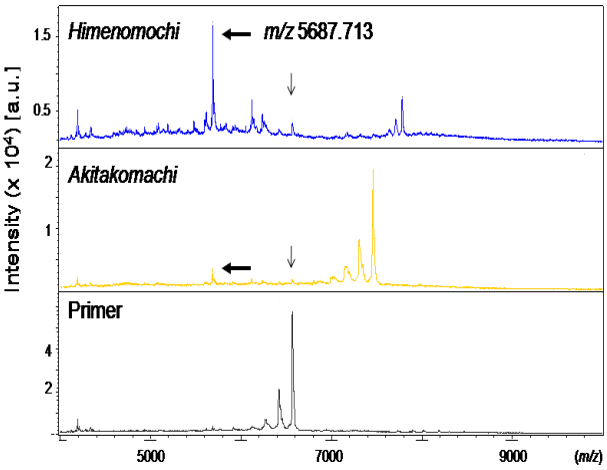

Fig 3. MALDI-TOF MS analysis of rice cultivars *Himenomochi* and *Akitakomachi*. Gene locus E20943 was selected for SNP detection and digested by EaeI. Peak at *m/z* 5687 differed significantly between *Akitakomachi* and *Himenomochi*. Peak at *m/z* 6570 was derived from unreacted biotinylated primer. Horizontal arrow shows peak of amplicon and vertical arrow shows peak of unreacted primer.

Digestion of amplicons and MALDI-TOF MS analysis

An aliquot of PCR product was incubated with restriction enzyme or with UDG for 10 min according to the manufacturer's instructions (New England Biolabs, USA). Digested DNA fragments were bound to 2 µl StAv-MB (Takara Bio Inc., Japan) and then collected after washing with 100 µl sterilized water using a magnetic stand according to the manufacturer's instructions (Promega, USA). Then, 50 µl denaturing solution containing 100 mM NaCl and 100 mM NaOH was added and the mixture was incubated for 5 min at

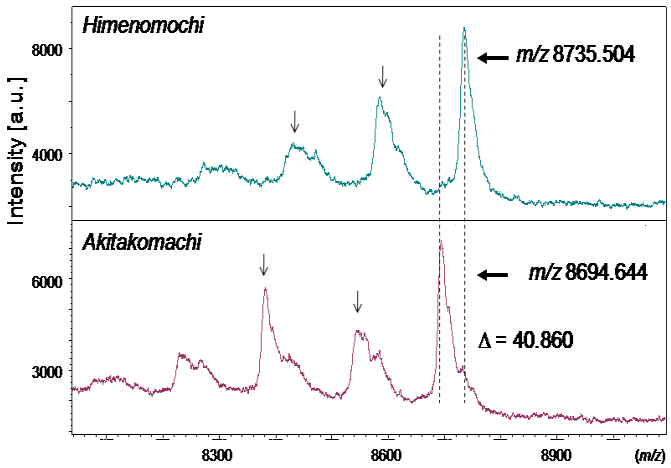

Fig 4. MALDI-TOF MS analysis of rice cultivars *Himenomochi* and *Akitakomachi*. Gene locus E20943 was selected for SNP detection and digested by Tsp509I. Theoretical mass difference between cv. *Himenomochi* and *Akitakomachi* was 40.03; observed difference in MS-CAPS analysis was 40.860. Horizontal arrow shows observed peak and vertical arrows show degradation products produced during MALDI-TOF MS analysis.

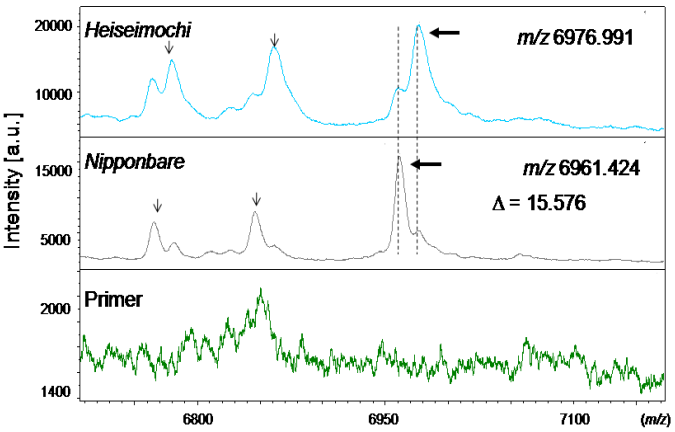

Fig 5. MS-CAPS analysis of amplicon treated with UDG. Biotinylated primer R1744FB and primer R1744R were used to amplify sequences from *Heiseimochi* and *Nipponbare*, and peaks in amplicons were detected at *m/z* 6976.991 and *m/z* 6961.424, respectively. PCR products after digestion by UDG and alkali treatment are shown. Difference detected by MS-CAPS analysis was 15.576 (theoretical difference between A and G = 16.00). Vertical arrows show degradation products produced during MALDI-TOF MS analysis.

room temperature. The beads were washed twice with 100 µl sterilized pure water using the magnetic stand, mixed with 50 µl 25% ammonia solution by pipetting several times, and then incubated at 65°C for 10 min. The solution was collected using the magnetic stand and lyophilized using a vacuum concentrator. ssDNA was dissolved in 2 µl 150 mM diammonium hydrogen citrate and spotted onto the target (Anchor Chip, Bruker Daltonics, Germany) and the same volume of 100 mg/ml 2',4',6'-trihydroxyacetophenone in 50% acetonitrile was added. Spectra were recorded by a MALDI-TOF MS (Ultraflex, Bruker Daltonics) in the linear positive ion mode (Kajiwara, 2011).

Conclusion

The method described here, designated as MS-CAPS, is a rapid method to discriminate among rice cultivars based on differences in SNPs. There are several advantages of using MS-CAPS/MALDI TOF-MS over CAPS methods using gel electrophoresis. Although this method was originally developed to rapidly detect the transgenic gene in genetically modified plants, it can also be used to discriminate among varieties based on differences in SNPs. The procedure we described previously was improved by adding asymmetric PCR and fast PCR methods.

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