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ORIGINAL PAPER

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Dot-blot-SNP analysis for practical plant breeding and cultivar identification in rice

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Abstract We report dot-blot hybridization with allele-specific oligonucleotides for single nucleotide polymorphisms (SNPs) analysis to be applicable for practical plant breeding and cultivar identification. Competitive hybridization of a digoxigenin-labeled oligonucleotide having the sequence of a mutant allele (or a wild-type allele) together with an unlabeled oligonucleotide having the sequence of a wild-type allele (or a mutant allele) was highly effective to reduce background signals in dot-blot hybridization. All 100 tested genes (200 alleles) in rice having SNPs or insertions/deletions were detected in an allele-specific manner. Genotypes of 43 rice cultivars were identified by this technique, and eight SNP markers were found to be sufficient for distinguishing all the cultivars from each other. Dot-blot analysis was also applied to genotyping of *Wx* and *Sd1* of F_4 plants in a conventional breeding program. Since dot-blot analysis with competitive hybridization provides a highly reliable, simple, and cost-effective technique for SNP analysis of a large number of samples, this technique is expected to realize the practical use of a novel breeding method, in which plants or breeding lines are selected by SNP analyses of many genes in a laboratory.

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Introduction

Genome studies of various organisms have determined the nucleotide sequences of a large number of genes. In plants, the genome sequences and many expressed sequence tag (EST) sequences of *Arabidopsis thaliana* and rice, *Oryza sativa*, have been determined and published in the database (The Arabidopsis Genome Initiative 2000; Asamizu et al. 2000; http://www.arabidopsis.org/; International Rice Genome Sequencing Project 2005; Wu et al. 2002; http://www.rgp.dna.affrc.go.jp/). The number of genes in Arabidopsis and rice have been estimated to be ca. 25,000 and 38,000 (The Arabidopsis Genome Initiative 2000; International Rice Genome Sequencing Project 2005), respectively. Although, the functions of most of genes with published nucleotide sequences are unknown, data on functional analyses of genes will rapidly accumulate, and many mutant genes controlling phenotypic variations will be identified. Mutant alleles can be distinguished from wild-type alleles by analyses of SNPs and indels (insertions/ deletions).

Single nucleotide polymorphism (SNP) analyses are useful for the distinction of cultivars. The most extensively used DNA polymorphism analysis for the distinction of individuals or cultivars at present is simple sequence length polymorphism (SSLP) of microsatellite regions, because such regions are highly polymorphic as to length (Saghai Maroof et al. 1994). However, mutant cultivars cannot be distinguished from their original cultivars by SSLP analysis. The semi-dwarf rice cultivar 'Reimei', which is a mutant of 'Fujiminori', has a transversion mutation in *Sd1*, a gene participating in gibberellin synthesis (Sasaki et al. 2002). The low-amylose rice cultivar 'Milky Queen', a mutant of 'Koshihikari', has a transition mutation in *Wx*, a gene encoding granule-bound starch synthase I (Sato et al. 2002). These mutant genes have been incorporated into a crossbreeding program in Japan. Only SNP analyses will enable distinction of these cultivars from their original cultivars.

Various methods of SNP analysis have been developed, i.e., heteroduplex analysis by density gradient gel electrophoresis (DGGE) (Fischer and Lerman 1983), single strand conformation polymorphism (SSCP) analysis (Orita et al. 1989), CAPS analysis using primers having modified sequences to create restriction sites (dCAPS) (Michaels and Amasino 1998), mismatch cleavage analysis with various mismatch cleavage enzymes (Till et al. 2004), mass-spectrometry using MALDI-TOF MS (Ross et al. 1997; Stoerker et al. 2000), primer extension analysis using fluorescence-labeled ddNTP (Nasu et al. 2002; Jain et al. 2003), allele-specific PCR using mismatched primers (Latorra et al. 2003; Zhou et al. 2004), and microarray technology (Wang et al. 2005). Most of these methods are labor-intensive or require expensive instruments.

Dot-blotting using allele-specific oligonucleotide has been reported to be usable for detecting SNPs in β-globin and HLA-DQα loci (Saiki et al. 1986). Although this method is simple, and is expected to be suitable for analyses of a large number of samples with low cost, it has not been adopted so much in plant genome studies. In the present study, we improved the dot-blotting technique for SNP analysis (dot-blot-SNP hereafter) using competitive hybridization with unlabeled oligonucleotides. One hundred SNP markers for distinction between *japonica* rice cultivars were produced and used for the identification of rice cultivars and selection of F_4 plants in a conventional crossbreeding program.

Materials and methods

Plant materials

Thrity-nine *japonica* paddy-rice cultivars, including 25 of the top 30 cultivars of Japan in 2004, two upland rice cultivars, i.e., 'Owarihatamochi' and 'Senshou', and two *indica* cultivars, i.e., 'Kasalath' and 'Habataki', were used for genotyping. These 39 *japonica* paddy-rice cultivars were kindly provided by rice-breeding research stations (Supplementary Table 1), where the cultivars had been developed. F_4 plants from a cross between an F_3 plant of 'Iwanan 25'/'Etsunan 190' and an F_4 plant of 'Xin Qing Ai 1'/'Ta Hung Ku' were used for SNP analyses of *Sd1* and *Wx*.

Preparation of probes

A set of two oligonucleotide probes of 17 nucleotides was used for each marker. The set of the probes for detection of a 'Nipponbare' allele was an oligonucleotide labeled with digoxigenin at the 5′-end having the sequence of the 'Nipponbare' allele and an unlabeled oligonucleotide having the sequence of a variant allele. The other set for the detection of the variant allele was an unlabeled oligonucleotide having the 'Nipponbare' sequence and a digoxigenin-labeled oligonucleotide having the variant sequence. SNPs of ESTs (Shirasawa et al. 2004), intergenic regions (Nasu et al. 2002), and reported mutant genes (Sasaki et al. 2002; Sato et al. 2002; Doi et al. 2004; Teranishi et al. 2004; Umemoto et al. 2004) observed between *japonica* cultivars were used for the production of the dot-blot-SNP markers. Our unpublished SNP data were also used.

Dot-blot-SNP analysis

Genomic DNAs of the 43 cultivars and those of 382 F_4 plants of the breeding line were isolated from 10 mg of leaf tissue by the methods of Edwards et al. (1991) and Wang et al. (1993), respectively, and dissolved in 1 ml 1× TE buffer. DNA fragments of ca. 300 bp covering the SNP sites were amplified by PCR using primers listed in Table 1. Ten microliters of the reaction mixture contained ca. 10 ng of the plant DNA, 10 pmol of each primer, 1× *ExTaq* buffer, 2 nmol of each dNTP, and 0.5 U of *Taq* DNA polymerase (*ExTaq*, Takara Biomedicals, Japan). Thermal cycling conditions were as follows: 1-min denaturation at 94°C; 40 cycles of 30-s denaturation at 94°C, 30-s annealing at 58°C, 30-s extension at 72°C; and a final 1-min extension at 72°C, unless otherwise specified (Table 1). The amplified DNA was denatured in a solution of 0.4 N NaOH and 10 mM EDTA, and dot-blotted onto a nylon membrane (Nytran, Schleicher & Schuell, Germany) by Multi-pin Blotter (Atto, Tokyo, Japan). The probe mixture containing 0.5 μM of the digoxigenin-labeled oligonucleotide and 2.5 μM of unlabeled counterpart oligonucleotide was hybridized at 50°C. After hybridization, the membranes were washed twice in 0.1× SSC (NaCl 0.877 g/l, sodium citrate 0.441 g/l, pH 7.0) containing 0.1% SDS at 50°C for 20 min. Signals were detected with a DIG Nucleic Acid Detection Kit (Roche, Switzerland). SSCP analysis of DNA fragments was carried out according to Shirasawa et al. (2004).

Results

Improvement of dot-blot-SNP analysis

The SNPs of three genes were analyzed by dot-blot hybridization using oligonucleotide probes. PCR products of about 300 bp were dot-blotted after denaturation in 0.4 N NaOH. Hybridization of only digoxigenin-labeled oligonucleotide probes having the sequences of wild type detected wild-type alleles, but weaker signals of mutant alleles, which result in ambiguous genotyping, were also observed in some genes (Fig. 1). The addition of unlabeled oligonucleotides having mutant sequences to the hybridization buffer containing the digoxigenin-labeled wild-type probes enabled specific detection of the wild-type alleles. In tests of the ratio of the unlabeled oligonucleotide to the labeled probe, i.e., 0:1, 1:1, 2:1, 5:1, and 10:1, twofold to tenfold amounts of the

Table 1 Sequences of primer pairs and oligonucleotide probes of SNP markers and the conditions of PCR, hybridization and washing

Marker name	Chr.	cM	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Probe sequence of Nipponbare allele (5′–3′)	Probe sequence of variant allele (5′–3′)	PCR conditions (annealing temperature)	Hybridization temperature	Second washing buffer	Washing temperature
Dot-blot-SNP markers used for genotyping of the breeding lines										
Wx-mq			GTTCTTGATCATCGCATTGG	CTGAAACGCATCTGGTTGTC	CTACAAGCgTGGAGTCG	CTACAAGCaTGGAGTCG	58	50	0.1× SSC/0.1%SDS	50
sd1-r			GCACTACCCGGACTTCACCTG	AGCCAGCGCGTGAAGGCGTC	CCTGGGCCgACCTCATG	CCTGGGCCcACCTCATG	58[b]	50/65	0.1× SSC/0.1%SDS	50
Dot-blot-SNP markers used for identification of the 43 cultivars										
S13157a	1	5.6	GCATGCATTCATACAGATGAAG	AAGCTCTGCATCGTTGGATT	CATCTGTGtGACTGTCT	CATCTGTGcGACTGTCT	56	50	0.1× SSC/0.1%SDS	50
S13157b[a]	1	5.6	GCATGCATTCATACAGATGAAG	CGAATGCGGAGAATTCAAGC	GCATACtatccatcac	GCATACatgacaagaa	58	50	1× SSC/0.1%SDS	50
C60656	1	10.9	GTTCGACGAGCCAAGTTCTC	GCTGCTACAAACACGCCATA	TCGGGTAGtGGAATGTA	TCGGGTAGcGGAATGTA	58	50	0.1× SSC/0.1%SDS	50
C30013	1	25.4	ACGAACCCTAGCCACATGAC	CTCCCCTCTCTCTCTTGTTCC	CCATCCAAcACGCCAAA	CCATCCAAtACGCCAAA	58	50	0.1× SSC/0.1%SDS	50
S4655	1	36.9	TATCCGGCACTTGTTTGTCAC	GCCTCATAAAGCCCAAGATG	GAGACATCaTGCAACTA	GAGACATCgTGCAACTA	58	50	0.1× SSC/0.1%SDS	50
S3813a	1	95.7	CCTCTTTAGTACCACCTCGACAG	GCAGCAGCCTGTTATCCTTC	CTCGCCTAcCCTGCCTC	CTCGCCTAtCCTGCCTC	58	50	0.1× SSC/0.1%SDS	50
S3813b[a]	1	95.7	CCTCTTTAGTACCACCTCGACAG	CTGCAAGACAGTTTCGTACC	AACTGAAAtatcaaat	AACTGAAAagacatct	58	40/50[c]	1× SSC/0.1%SDS	50
S13155	1	118.9	ACATCGCTGGTGGATCTCTT	ATGGTTGTATTCCAAGCGTCG	CAATCTGAaCTTGCAGC	CAATCTGAgCTTGCAGC	58	50	0.1× SSC/0.1%SDS	50
S13781	1	139.9	AGTTGAGCATTGTCCTTTGT	CAAGCAACAATAATGACAGCAG	ATGACAATaTAAGCCCA	ATGACAATtTAAGCCCA	58	50	0.1× SSC/0.1%SDS	50
S10925	1	142.9	TATTGCCGGACATTCACAAA	GGTACTGCCAGGATTTGCAT	AAACTAGAgTTCAAGTA	AAACTAGAaTTCAAGTA	58	50	0.1× SSC/0.1%SDS	50
C727A	1	145.3	CCAAACACCAACCTCTCCTA	CAGATCGTTGTGAGGGGAAT	TCCTCATTcTCTTGCTA	TCCTCATTtTCTTGCTA	58	50	0.1× SSC/0.1%SDS	50
C62963	1	147.2	GACAAACTTCCCCTCTGCTG	AGACACTGCTGGAGAAAGATCAG	ATCCATGCtATCACCCT	ATCCATGCcATCACCCT	58[b]	50	0.1× SSC/0.1%SDS	50
C11876	1	154.6	CAGGCTGAGGCAAACTATCA	TTCAGGTCTTTCTTCCTTCTGC	ATCACACA–GCTATATGT	ATCACACAcaGCTATAT	58	50[d]	1× SSC/0.1%SDS	50
R3203	1	159.6	GCAAGGGATTTCATCAAGGA	CCTCGATCTTGGCCTGTAGA	AACTTCTCaAGGACTGG	AACTTCTCgAGGACTGG	58	50	0.1× SSC/0.1%SDS	50
C112	1	181.8	CCTTGATGACACGCTGTATCCG	AGTTCCATACTGCTTGTAGAGG	CGGTGACCtCCGGCATT	CGGTGACCaCCGGCATT	58	50	0.1× SSC/0.1%SDS	50
S0633	2	15.0	ACGAAGAGCAAAGCAGCAAC	ACCGGGAGATTTCAAGGATT	ATTCGCTCtCTGACATG	ATTCGCTCaCTGACATG	58	50	0.1× SSC/0.1%SDS	50
R2542	2	28.0	ACTTCATGACTAGGAAGCAAGCA	CATAGGCAGACAAGATGGTTCAT	AGCACAGCaATTGACTG	AGCACAGCgATTGACTG	58	50	0.1× SSC/0.1%SDS	50
C53722	2	57.3	GCAGGGGCTCAGATTTCTTT	TAATGGGCAGATCACGACAG	GAGGGCAAaGAGCTGGA	GAGGGCAAgGAGCTGGA	58	50	0.1× SSC/0.1%SDS	50
R1843	2	57.6	CACGTATGCATTTTCTACCAGC	GCCTGCTGACAATCATCTCTTC	AAATATTCgATGTTCTT	AAATATTCaATGTTCTT	58	40	1× SSC/0.1%SDS	40
S20768	2	95.2	CCTGTGTACCTCGAAGAGTCAA	TAGCTGATGTGCCACGTGAA	TCTGTACCaTGGTAAAT	TCTGTACCtTGGTAAAT	58	50	0.1× SSC/0.1%SDS	50
C63726	2	95.2	TTGGTCATCCTTGGGTCCTA	CTGGATCGTACGAGCAACCT	TTACCGTTtTTTAAAGC	TTACCGTTgTTTAAAGC	58	50	0.1× SSC/0.1%SDS	50
R1906	2	96.6	CCTTGCATCCCCACCTCTTGT	GCAACTGGAATCCAAGCATTG	GTGCAATAtGCATCTTT	GTGCAATAgGCATCTTT	58	50	0.1× SSC/0.1%SDS	50
S10844	2	98.2	CTCTTCTACGCCAGGTTCCAA	GCACAGTAACCAGATCAACAG	CAGTAGAGcTGTGGAGA	CAGTAGAGtTGTGGAGA	58	50	0.1× SSC/0.1%SDS	50
R2559	2	99.6	GCAATTGTGCCTTATACCGAG	GAGCTTCTGTCTTTGGTAACACG	ATATGTGGtTGGGTTaG	ATATGTGGcTGGGTTgG	58	50	0.1× SSC/0.1%SDS	50
E4169	2	99.6	ACTATTCTTGCAGGCCAGTG	CGGGAATGCTTTTCAACAGT	ATGTATGTgTTGCTTTA	ATGTATGTtTTGCTTTA	58	50	0.1× SSC/0.1%SDS	50
C12187	2	99.6	AAGGTAAGAACTCCACACCTGA	TACAGTGACTCTGCGTGGTACAT	TCTTGGTAaTGCCCATG	TCTTGGTAtTGCCCATG	58	40	1× SSC/0.1%SDS	40
E3295	2	101.2	ACGTACGTCACACAACATCTGTC	GCTGCAGTTCGTGATATGGA	TGCAAGCGcCGTCGTGA	TGCAAGCGaCGTCGTGA	58	50	0.1× SSC/0.1%SDS	50
C12409	2	157.9	GCAGGTGTGTCACAATCTTCA	CTTCGAATAGCCCTGTTGCTTA	AACAGGAAgCCTGACAG	AACAGGAAaCCTGACAG	58	50	0.1× SSC/0.1%SDS	50
S0040	3	20.3	TCTGCTGCCTCTGCACATAC	CAAAAGCAACCAGAGCCAAT	AGTACTAGaTCAGTCTT	AGTACTAGtTCAGTCTT	58	50	1× SSC/0.1%SDS	50
R1862	3	49.3	GCACGAGGGGAAATTACTAAGAT	CGTCAAAGATAACCAGGTTGGTA	GGATATCCaTGACCGTG	GGATATCCgTGACCGTG	58	50	0.1× SSC/0.1%SDS	50
S0279	3	69.2	AGCACCAGAATCGTACTTCCT	GCGAACGAGATCAAGTCACA	ACGAAGATaAGTGGATG	ACGAAGATgAGTGGATG	58	50	0.1× SSC/0.1%SDS	50
S14262	3	101.6	CCTCCACTGCTTAGTCACCA	CAGTGAACTAATGGGAACAGCAG	AATTCTGTtATACAAGT	AATTCTGTaATACAAGT	56	50	0.1× SSC/0.1%SDS	50
C11223	3	149.1	ATTGTACCCTGACGATCGAA	CTGTCTTTCCAGACAGAAGAACC	CGCTTGACaGTGAATGG	CGCTTGACgGTGAATGG	58	50	0.1× SSC/0.1%SDS	50
S0738	4	30.8	AGCTGTCAGGCGTATCAAGC	GGGAGCATTATTCCCAGTTG	CATAAGGGaTGAGGTGA	CATAAGGGcTGAGGTGA	58	50	0.1× SSC/0.1%SDS	50
C62054	4	57.5	TGATGCACCGTCCTCAATTA	TGTTGGAAGGGAACATGTCA	AAACTTGTgACCAATGA	AAACTTGTcACCAATGA	58	50	0.1× SSC/0.1%SDS	50
C11882	4	108.2	ATTGTGATGTCCCAGGAAGC	CACAGTGCTACATGTGACATTCC	TGACAAAAtTGAGAACT	TGACAAAAcTGAGAACT	58	50	0.1× SSC/0.1%SDS	50
E30565	4	108.2	GTTCGGACGAAATGAGTGTATCC	TGGCTCATGCTGGTATGGTA	ATATTTTAaAACGAAGA	ATATTTTAgAACGAAGA	58	40	1× SSC/0.1%SDS	40
R2376	4	111.0	GGCTGCTTGGTGTAGTTAGT	GACTAGGTAAAAAGACGGCCAAG	TGATCATAcGCTTGATA	TGATCATAtGCTTGATA	58	50	0.1× SSC/0.1%SDS	50
S0703	5	20.6	TGGATCATTCCTGCTTATCC	CACCACCCATTCCAATTCA	CTCAATTGgTTGCTGAT	CTCAATTGtTTGCTGAT	58	50	0.1× SSC/0.1%SDS	50
S10091	5	50.2	CGACAATGTGATGCCAACAT	ACCAAAACAATGGGCAGCTA	GTTACCATcGCATCTGG	GTTACCATtGCATCTGG	58	50	0.1× SSC/0.1%SDS	50
S0994	5	95.8	GGATTGCAAACAACCAAACG	TGGAGTGCTACAGCTGGCTA	CTATAGTAcCAGGAGTA	CTATAGTAaCAGGAGTA	58	50	0.1× SSC/0.1%SDS	50

Table 1 (Contd.)

Marker name	Chr.	cM	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Probe sequence of Nipponbare allele (5′–3′)	Probe sequence of variant allele (5′–3′)	PCR conditions (annealing temperature)	Hybridization temperature	Second washing buffer	Washing temperature
C1272	6	13.5	TGCGTGAATTGGAAACTTGAC	TGTTGCATCCTTTCATGTCG	GGTAACCTtCATATGCG	GGTAACCTgCATATGCG	58	50	0.1× SSC/0.1%SDS	50
C62866	6	13.5	CTCGTCATGTCTAACTTCCCTTTC	CGCACCCCGTACTACATTCT	TCTAAATTgTAACAATG	TCTAAATTaTAACAATG	58	50	0.1× SSC/0.1%SDS	50
C0226	6	13.5	GCACAGTCGCAAGCTAGCTC	CATCAGATACAGGGAGAACATCC	CAGCAGACgACCCAGAA	CAGCAGACtACCCAGAA	58	50	0.1× SSC/0.1%SDS	50
SSIIa	6	37.8	GTGGGGTTCTCGGTGAAGAT	GGCGGACATGGTCTCTTCAC	GGGACACCaTGTCGGCG	GGGACACCgTGTCGGCG	58[b]	50	0.1× SSC/0.1%SDS	50
R2171	6	51.0	GCACATTGGCATTACACAAGTAG	ATGTAGACCTGCTCTTTCTGACC	CTTTACAAtTATGTAGT	CTTTACAAgTATGTAGT	58	40	1× SSC/0.1%SDS	40
C235	6	54.1	AGCGAGGAGTCAAGGAG	TGATGCACAATCTGCAAACC	ATGTAATTtCAGAGGAA	ATGTAATTgCAGAGGAA	58	40/50[c]	1× SSC/0.1%SDS	40
S0567	6	79.0	CCTTTGAACTGGGGCATCTA	TGTCGGCAAGCAAAGTGTAG	GCGCTACAaTAGTACCG	GCGCTACAgTAGTACCG	58	50	0.1× SSC/0.1%SDS	50
S0497	6	100.0	AAGATGTTGGGGTGCTTCAC	CCTATCATCCGAGTGCCATT	GTGTGATGtAGACAAAT	GTGTGATGcAGACAAAT	58	50	0.1× SSC/0.1%SDS	50
S0500	7	0.8	TTTACGCAAAACGAGGTGGT	GGCGTTAATCCTGCAAAACT	ATCTTAATtTTCAATCC	ATCTTAATcTTCAATCC	58	40	1× SSC/0.1%SDS	40
R565	7	24.2	TCTCCAGGATGGAAATGTTC	AATGGCCCAAATGCAGATAG	CACTGTTGcTCTCATCA	CACTGTTGtTCTCATCA	58	50	1× SSC/0.1%SDS	40
E31015	7	24.8	AAAGGAGCAGCTTCCAGTAGG	CACACTGATACCGTTGTCACCT	GAATGATGaGCAAGAAT	GAATGATGgGCAAGAAT	58	50	0.1× SSC/0.1%SDS	50
C60064	7	35.7	CTACAAGCTTGCCAGCATCA	TCCAGAACACCATCGTGAAG	TGCTGGCCcTTATTTGA	TGCTGGCCtTTATTTGA	58	50	0.1× SSC/0.1%SDS	50
C1467	7	73.2	CAATCGTCTGGAGAGCTCTATG	AAGCAAATCGGAAGAACAGG	AATTCATGaAAACAGTT	AATTCATGgAAACAGTT	58	50	0.1× SSC/0.1%SDS	50
R2394	7	80.5	CTACTATTGAAAAGCCATAGTTAGG	GCTTGAAAATGTGGTGGTTC	TTTGGAGCcTTCTTAAT	TTTGGAGCtTTCTTAAT	58	50	0.1× SSC/0.1%SDS	50
NK10[a]	7	80.5	CGAGGTTCCCTAATGACCAA	CTTGTACTTCGCCCCTCTTG	agaaggcaGCTGTGTG	catctcacGCTGTGTG	58	50	1× SSC/0.1%SDS	50
R2561	7	81.1	ACATCCGTCTTATCAGGCAAGTA	CTAAAGCAGTTGGGAGAGGG	ATTCCTTTgCTGGATCA	ATTCCTTTtCTGGATCA	58	50	0.1× SSC/0.1%SDS	50
C847	7	91.7	GGAAAGCATCGAGTACAATA	CTGCAAAAAGCATACCCACA	ATTTCCCAaCTCATTTT	ATTTCCCAgCTCATTTT	58	50	0.1× SSC/0.1%SDS	50
E50066	8	25.2	ATGCAGCAGTTTAGGCATGA	CTGAAGACCAGGGGATAGGG	AAGTTAGCgCTAAGCCT	AAGTTAGCcCTAAGCCT	58	50	0.1× SSC/0.1%SDS	50
E61986	8	44.6	AAGGACAATTGTGTGGCTGT	CTCTCAAGGGCTAGAAATTACCAC	TTGCCTAGtGGTAAATA	TTGCCTAGaGGTAAATA	58	50	0.1× SSC/0.1%SDS	50
S20045	8	58.1	TATTTCTCCCTCTATCTTCTCC	GTATCGATTGATCGGTGTTGCAC	TAAAAGATgTGGAGGAA	TAAAAGATaTGGAGGAA	58	40	1× SSC/0.1%SDS	40
C52335	8	58.4	CCCATCATATCACCCATTCC	AAGCCCTTGTTCCTTATCCAAC	AAACAATAtATGCATGA	AAACAATAgATGCATGA	58	50	0.1× SSC/0.1%SDS	50
R80	8	73.2	GGAGTTCGCGACGTTCCGCCCT	ACTTGTCTTTGTGGGGCAAG	AGAAGCCTgGGCCAGCC	AGAAGCCTaGGCCAGCC	56	50	0.1× SSC/0.1%SDS	50
S0641	8	105.7	CACGCTCAGCAAAAATGTCA	GGCCTCATCGGATGTTCTAA	TTAAAATTcTTCATCGT	TTAAAATTaTTCATCGT	58	40	1× SSC/0.1%SDS	40
S1872	9	6.8	CCATCACCTCCAATTGTGCT	AGCTAGAAGCTCCCCCAAAC	TAATATTGcGACACGCT	TAATATTGtGACACGCT	58	50	1× SSC/0.1%SDS	50
S0313	9	30.6	GGTGAATTCATGGCACTGGT	GGAACGATGTGACGGAAAAG	AATTCTACaTGTATGCT	AATTCTACgTGTATGCT	58	50	0.1× SSC/0.1%SDS	50
S1580	9	33.0	GGGCTCCCCCACTTATATCT	GCCCGTATAGGACACCAATG	CGGCCCACaGTCCATCA	CGGCCCACgGTCCATCA	58	50	0.1× SSC/0.1%SDS	50
C30515	9	55.3	CCTGCAAGAACAACCTGATG	ACTATTACCTTGGTCCGAAGAGC	TTGACAGCaACCATCAC	TTGACAGCtACCATCAC	58	50	1× SSC/0.1%SDS	40
S11615	9	55.9	CTGCAGCAAACTCACTCACTCTA	ACAACTACTGCTGGGGCTTC	AGCACGGTgGTGGCTCT	AGCACGGTaGTGGCTCT	58	60/50[c]	1× SSC/0.1%SDS	40
S1974S	9	55.9	TTGTTCGCCTCCGCTTCTTC	AAAACCACTTCCCCCAATTC	TTTGATTCcTCCAAGCT	TTTGATTCaTCCAAGCT	58	50	0.1× SSC/0.1%SDS	50
S0708	9	93.5	GTTAGCATGCATCGGTAAATTC	GCCATGCTGGTTTCAACAGT	ATATATATgTCTAGATT	ATATATATaTCTAGATT	58	50/40[c]	1× SSC/0.1%SDS	40
E50836	10	5.5	GTACACACCGTCGCTGTTCTT	TGGCCTGCTTTTTCTTCACT	CATCACGAgGGCGGCGA	CATCACGAtGGCGGCGA	58	50	0.1× SSC/0.1%SDS	50
OsPHR	10	10.9	GTCTTGCGAAATGTGACGTG	CCTGCATTCGAAGAATTCCA	GCTGGTGCgGCGCCTTG	GCTGGTGCaGCGCCTTG	58[b]	50	0.1× SSC/0.1%SDS	50
C913A[a]	10	10.9	GCTTCTTAGGATTGAGGAGGAG	AGAACACGCAAGCTCAGAAC	atgctgtcTTCACCAT	gcatgataTTCACCAT	58	50	0.1× SSC/0.1%SDS	50
C148	10	17.9	CCTGCAAAGTGCAAGAACAA	CAACGAGCAGGAAGTTGTGA	AAGATATGtCGAGAATC	AAGATATGcCGAGAATC	58	50	0.1× SSC/0.1%SDS	50
Ehd1	10	44.3	GAGGATCGAAGAGCTGAGCA	CTTCTTTGGAACTGCCTCTGC	ACCACCTCgGAGAAGAC	ACCACCTCaGAGAAGAC	58	50	1× SSC/0.1%SDS	50
S20925	10	53.9	TGGTTGCAAATCTGGAATACCT	GAGAAAGCACATGCCAACTTGT	GCCAATAGcTAATAATA	GCCAATAGtTAATAATA	58	50	0.1× SSC/0.1%SDS	50
C16	10	71.4	ACATCTTGAGGATTGGGAGC	GAGCGTCTTCTTGGCAATCT	TGTATGGAaAAGTTAAT	TGTATGGAgAAGTTAAT	58	50	0.1× SSC/0.1%SDS	50
R0835	10	72.8	GTCCGTCCGGTATCAAGAGT	ACCGGATAAAATCAGCCACA	TAAACCTAcTATTAAAT	TAAACCTAaTATTAAAT	58	30	1× SSC/0.1%SDS	40
S10616	11	20.3	CACCCTTGTGGTCACACTTG	CGGTGAACACCAGGTTCATT	GATCCGCTgACGGAGGG	GATCCGCTaACGGAGGG	58	50	0.1× SSC/0.1%SDS	50
E30139a	11	57.3	ACCTATTGGCTGGTCTGCAT	GAGACTCAAAATCAGTCGCGTA	GGCATGAAcTTTTGTTG	GGCATGAAtTTTTGTTG	58	50	0.1× SSC/0.1%SDS	50
E30139b	11	57.3	TCAATAGTGCGTCCGGAAAT	GGTGCACAACTCATGTACAACT	ACATACGAtCCAATTGT	ACATACGAcCCAATTGT	58	50	0.1× SSC/0.1%SDS	50
S723	11	85.7	GTGGGATTTAGGAGGACAAG	AGGCCTGTATGTGCCAATCG	GTGTTCGCaAACAAGCA	GTGTTCGCgAACAAGCA	58	50	0.1× SSC/0.1%SDS	50
C1172	11	85.7	CAGGAAGCTCTGGCATTGAG	GTACAAGAAACCCGGCTGTG	TATCTATAgTCAGCAAG	TATCTATAaTCAGCAAG	58	50	0.1× SSC/0.1%SDS	50
C11589	11	91.4	TCTTGCAGGTCACTACAAGCAT	GGTCAGCTATATGCATCAGGGTA	GAATTCAAaCAACAGGC	GAATTCAAgCAACAGGC	58	50	0.1× SSC/0.1%SDS	50
E3876	11	91.4	AGCATTTGGCTCGTAGCAAT	ACAAGTCGCCTCCAATATGC	TCCTGAGCgACAAGCCT	TCCTGAGCaACAAGCCT	58	50	0.1× SSC/0.1%SDS	50
C12965	11	99.2	TCTGTTACGCAGGTGTCCAG	CTTGCCAACATTGCCAGATA	TACAATTTtGATGCGTG	TACAATTTgGATGCGTG	58	50	1× SSC/0.1%SDS	50

Table 1 (Contd.)

Marker name	Chr.	cM	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Probe sequence of Nipponbare allele (5′–3′)	Probe sequence of variant allele (5′–3′)	PCR conditions (annealing temperature)	Hybridization temperature	Second washing buffer	Washing temperature
E50658	11	112.9	TGAGGATTCTATCACTGAAATCC	GAAAGACCAAAGTCCGCAAC	ACAAGAATaAGAATGAT	ACAAGAATtAGAATGAT	58	40	1× SSC/0.1%SDS	40
C10295	11	117.3	GGGTCAACCAGATCTTACGGTAG	GGATTGTGAAATGCCTGACC	CATGTATTgAAATCAGT	CATGTATTcAAATCAGT	58	50	0.1× SSC/0.1%SDS	50
S0479	12	27.6	CGAGCAATTTGCCTCATTTC	CGTTACATGGCTACAAGGTCA	TGAGACCTgTAAGGCTG	TGAGACCTaTAAGGCTG	58	50	0.1× SSC/0.1%SDS	50
S20454	12	29.2	TCGTGCTATTTTCTCGCTGT	ACATGCATTGTCCCAATGTG	ATTCATTTgGGCAAGTT	ATTCATTTtGGCAAGTT	58	50	0.1× SSC/0.1%SDS	50
E30254	12	29.2	TTCGTCTCGATGTCGATCTG	CGATGGCCAGGTATCTACTCTC	ACTTAAAAaGCTTAAGC	ACTTAAAAgGCTTAAGC	58	50	0.1× SSC/0.1%SDS	50
E10037	12	49.3	GCATCCTATATCCGGGTTTT	GTCAACAAACAAGAGGGGATGT	GGGTTTTTtCGATACCA	GGGTTTTTgCGATACCA	58	50	0.1× SSC/0.1%SDS	50
E60142	12	61.6	CGTATATCCTGCTCGGGTTC	CTTATCATTCAGGTGAACTCC	AAAGACCCaTTTGCAGA	AAAGACCCgTTTGCAGA	58	50	0.1× SSC/0.1%SDS	50
R1709	12	91.4	ATGTGCGTGCTTTGTTTG	CAAATCGTTTCCACACCTACAAG	AGAGAGATgCAAAATGC	AGAGAGATaCAAAATGC	58	50	0.1× SSC/0.1%SDS	50
E60101	12	100.9	ACAAAACCACCAAAGGATGG	CAGGATGCTTAAGTGAACAACG	CATTTTCTtGTTCTGAA	CATTTTCTcGTTCTGAA	58	50	0.1× SSC/0.1%SDS	50
S21125	12	100.9	GATGCATATTCAAACGTCCAGA	TGGCGTACCAGATTTTGATG	ATATTAATtGCACCtGC	ATATTAATcGCACCcGC	58	50	1× SSC/0.1%SDS	50
S14134	12	107.4	GAAAGAGGCCTAAAAGGCAGA	CCCAGATGATAGACTGATGAGTTG	TTGATCTAcTATCAGTG	TTGATCTAaTATCAGTG	58	50	0.1× SSC/0.1%SDS	50
S21168	12	108.2	GCATCATCATCATCCACTCTGT	CTATAATTGTGCCCTACAGTTCA	GTGCAGCCaTGAAAGCA	GTGCAGCCgTGAAAGCA	58	50	0.1× SSC/0.1%SDS	50
E60843	12	108.2	CTAGTTGTGGAGGCCACTGTAAA	CCTTTCCCTCCGATAGGTTC	ACAACCCAgGAATCAAG	ACAACCCAaGAATCAAG	58	50	0.1× SSC/0.1%SDS	50

[a] < 100 bp indel (Shirasawa et al. 2005)
[b] DMSO was added to the PCR reaction mixture (5% DMSO)
[c] The left of the slash is the hybridization temperture of Nipponbare-allele probe, and the right is that of variant-allele probe
[d] The ratio of the competitive probe to the dig-labeled probe is 10:1

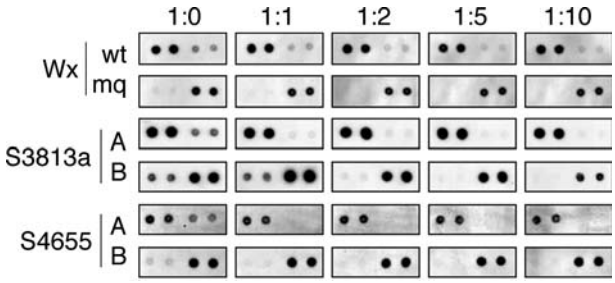

Fig. 1 Effect of competitive hybridization on background signals in dot-blot-SNP analysis. Three genes having SNPs, *Wx*, S3813a, and S4655, were analyzed by dot blotting with or without competitive hybridization. "wt" and "mq" are alleles of Wx, and "A" and "B" indicate alleles of S3813a and S4655. No competitive oligonucleotides (1:0) and one- (1:1), two- (1:2), five- (1:5), and tenfold (1:10) amounts of competitive oligonucleotides were added to hybridization mixture. Two dots were blotted for each allele. Addition of two-, five-, and tenfold amounts of competitive oligonucleotides reduced the background signals, giving clear allele-specific signals were detected

unlabeled oligonucleotide yielded clear allele-specific signals (Fig. 1). Therefore, a fivefold amount of the unlabeled oligonucleotide was used as the standard hybridization condition.

Although, alleles containing insertions have been detected using probes of insertion sequences in our previous study (Shirasawa et al. 2005), alleles with deletions have not. To detect both alleles, junction sequences of the insertion sites were used as probes in the present study. Competitive hybridization of a labeled probe for a mutant allele and a fivefold amount of an unlabeled oligonucleotide for a wild-type allele, which share a sequence from one end to the middle and have different sequences in the other half, detected only by the mutant allele (Fig. 2a).

In the analysis of the SNP in *Sd1*, an oligonucleotide probe for *sd1-r* detected no signal, while a probe for the wild-type allele of *Sd1* detected allele-specific signals. Since the probe for *sd1-r* contained a palindromic sequence, this probe was considered to create a self-annealed form. Raising the hybridization temperature to

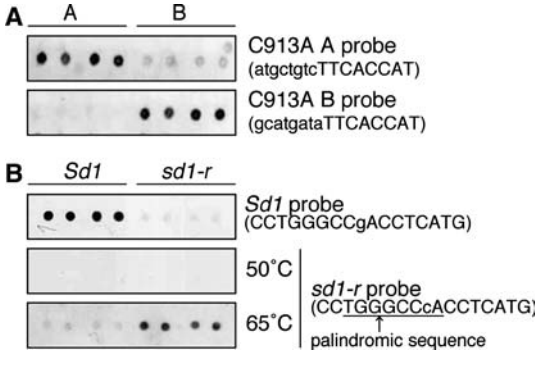

Fig. 2 Dot-blot-SNP analyses of C913 having indel (**a**) and *Sd1* (**b**). Both alleles of the gene having indel were detected in an allele-specific manner. Signals of the *sd1-r* allele were detected by raising the hybridization temperature to 65°C

65°C enabled specific detection of the *sd1-r* allele (Fig. 2b).

In the 100 dot-blot-SNP markers (= 200 probes) listed in Table 1, including 95 SNP markers, four < 100-bp-indel markers, and one 2-bp-indel marker, 154 probes detected allele-specific signals under the standard condition described in Sect. Materials and methods. Use of washing buffer with a high SSC concentration, i.e., 1× SSC/0.1% SDS, enabled the detection of allele-specific signals by 19 probes, and lowering the washing temperature to 40°C with 1× SSC/0.1% SDS washing also enabled the detection of allele-specific signals by three other probes. With hybridization at 40°C, allele-specific signals were detected by 18 probes and one probe with 1× SSC/0.1% SDS washing at 40 and 50°C, respectively. For a probe having high GC content, i.e., 11 GC bases, hybridization at 60°C yielded allele-specific signals, and for two probes having low GC content, i.e., two and three GC bases, hybridization at 30°C yielded allele-specific signals. In C11876, a marker for the 2-bp indel, an allele-specific signal was detected when the concentration of the unlabeled oligonucleotide in the hybridization buffer was increased to 5 μM. All the 200 probes (100 SNP markers) produced in the present study detected allele-specific signals under the optimized conditions of the hybridization and the washing (Table 1).

Genotyping of rice cultivars using the dot-blot-SNP analysis

Genotypes of the 95 SNP markers and the five indel markers were analyzed in 43 rice cultivars (Supplementary Table 1). Although, four and nine DNA fragments of the markers were not amplified by PCR and hybridized with neither probe, respectively, data of the 417 combinations of cultivars and markers were obtained. The nine DNA fragments hybridized with neither probe showed band patterns different from those of 'Nipponbare' alleles and the variant alleles by SSCP analysis, indicating that these fragments have other SNPs. Twenty markers separated the 43 cultivars into two groups at about 50%:50%. These are considered to be useful markers for the identification of cultivars. The 43 cultivars were separated into 30–40%:70–60%, 20–30%:80–70%, 10–20%:90–80%, and 0–10%:90–100% by 23, 24, 28, and five markers, respectively. Analysis of eight markers, i.e., E30565, R0835, R1709, S10091, S10844, S11615, S13781, and S4655, was sufficient for distinguishing the 43 cultivars from each other (Table 2).

Both S723 and C1172 are DNA markers that are closely linked with *Pb1* (the panicle blast resistance gene) and *Stvb-i* (the rice stripe disease resistance gene), which were derived from *indica* cultivar 'Modan' (Fujii et al. 2000; Hayano-Saito et al. 1998). Four *japonica* cultivars, namely, 'Asahinoyume', 'Goropikari', 'Matsuribare' and 'Tsukinohikari', were found to have 'Modan'-type alleles of S723 and C1172, suggesting that these cultivars may have both *Pb1* and *Stvb-i*. *Ehd1* is a gene for

Table 2 The eight selected SNP markers for distinguishing the 43 rice cultivars

Marker name	Aki-hikari	Ake-bono	Matsu-ribare	Akita-komachi	Asahino-yume	Chura-hikari	Doman-naka	Nippon-bare	Nanatsu-boshi	Mutsu-homare	Men-koina	Donto-koi	Rei-mei	Sasa-nishiki	Fuku-hibiki	Some-wake	Fusa-otome	Taichung 65	Gohyaku-mangoku	Toyo-nishiki	Goro-pikari	Tsugaru-roman	Hae-nuki	Hana-echizen	Hino-hikari	Yamada-nishiki	Hitome-bore	Yama-houshi	Hoshino-yume	Yume-akari	Ita-daki	Yume-tsukushi	Kin-maze	Owari-hatamochi	Kinu-hikari	Kirara 397	Sen-shou	Koshi-hikari	Haba-taki	Koshi-ibuki	Kasa-lath
E30565	A	B	A	B	B	A	A	A	B	B	B	B	B	A	A	B	B	B	A	B	A	A	A	B	A	A	B	B	B	B	B	A	A	A	B	B	B	B	B	A	A
R0835	A	B	A	B	B	B	B	A	A	A	B	A	A	B	A	B	B	A	A	B	A	B	B	B	A	B	B	A	A	B	A	A	A	A	B	B	B	B	B	B	B
R1709	B	A	B	B	B	A	A	A	B	A	A	B	B	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	B	B	B	B	B	B	B	B
S10091	A	B	A	B	B	A	B	A	B	A	B	B	B	B	A	A	A	A	A	A	A	B	B	B	B	A	A	A	A	A	A	B	A	B	B	B	B	B	B	A	A
S10844	A	A	A	B	B	B	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	A	B	B	B	B	B	B	A	A	A	B	B	A	A	A	A	A
S11615	B	A	B	B	B	A	B	B	B	B	B	B	B	B	B	A	B	B	B	B	B	B	A	A	B	A	B	B	B	A	A	A	A	A	B	A	A	A	A	B	B
S13781	A	A	B	A	A	B	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	A	A	B	B	A	A	A	A	A	A	A	A	A	B	B	B	B	A	A
S4655	B	A	A	A	A	A	B	A	B	B	B	B	A	B	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	A	A	A	A	A	B	A	B	B	B	B	B

Marker name	Mana-musume	Matsu-ribare	Men-koina	Mutsu-homare	Nanatsu-boshi	Nippon-bare	Rei-mei	Sasa-nishiki	Some-wake	Taichung 65	Toyo-nishiki	Tsugaru-roman	Tsukino-hikari	Yamada-nishiki	Yama-houshi	Yume-akari	Yume-tsukushi	Owari-hatamochi	Sen-shou	Haba-taki	Kasa-lath
E30565	A	A	A	A	B	A	A	A	B	B	B	A	A	B	A	B	B	B	B	B	B
R0835	A	B	B	A	B	A	A	B	A	A	A	A	B	A	B	A	A	A	B	A	A
R1709	A	B	A	A	A	A	B	A	B	A	A	A	A	A	A	B	B	A	A	A	A
S10091	B	A	A	B	B	A	B	A	B	A	A	A	A	B	B	A	B	A	A	B	B
S10844	B	B	B	A	A	A	A	A	B	B	A	A	B	A	B	A	A	B	B	B	A
S11615	A	B	B	B	B	A	B	A	B	A	B	B	A	A	A	B	A	A	A	A	A
S13781	B	A	B	A	A	A	A	B	B	B	B	B	A	A	A	B	A	B	B	B	B
S4655	B	A	B	B	A	A	A	B	B	A	B	A	A	A	A	B	A	B	B	B	B

Nipponbare-alleles are shown as "A"

heading date, and the loss-of-function of *Ehd1* in 'Taichung 65' is due to one amino acid substitution (Doi et al. 2004). This mutation was not found in the other 42 cultivars. Five cultivars, namely, 'Dontokoi', 'Itadaki', 'Kinuhikari', 'Yumetsukushi', and 'Habataki', showed signals of *indica*-type alleles of C727A, C62963, C11876, and *sd1-d*, all of which are located on the long arm of chr. 1. These five cultivars are progenies of 'IR8' having the *sd1-d* allele (Sasaki et al. 2002). The chromosomal region containing C727A, C62963, C11876 and *sd1-d* is considered to be transmitted from 'IR8' to these cultivars. The 'Sasanishiki'-type allele of *OsPHR*, which strengthens UV-B resistance (Teranishi et al. 2004), was frequent in *japonica* cultivars (Supplementary Table 1).

DNA-based selection of rice breeding lines using dot-blot-SNP analysis

Dot-blot SNP analysis was applied to DNA-based selection of breeding lines in a conventional crossbreeding program in rice. *Wx* alleles of the 382 F_4 plants of bulk-population derived from the cross between an F_3 plant of 'Iwanan 25'/'Etsunan 190' having *sd1-r* and *Wx-mq* and an F_4 plant of 'Xin Qing Ai 1'/'Ta Hung Ku' having low-temperature germinability were analyzed by dot-blot-SNP analysis. To reduce the time needed for DNA preparation, the simple DNA preparation technique of Wang et al. (1993) was adopted for this analysis. The amounts of amplified DNA by PCR were variable, probably due to the inconstant purity of DNA. Densities of the allele-specific signals were compared with those of the combined signals detected by a mixture of the labeled probes for estimation of the amount of amplified DNA (Fig. 3). About three-fourths of the plants, 288, showed wild-type signals of densities comparable to the combined signals, and about one-fourth of the plants, 119, showed mutant signals comparable to the combined signals. In 39 of the 382 plants, both the wild-type signals and the mutant signals were as dense as the combined signals. These results suggest that there were 249 homozygotes of *Wx*, 80 homozygotes of *Wx-mq*, and 39 heterozygotes. Genotypes of the remaining 14 F_4 plants were not determined because of the small amounts of amplified DNA.

Sd1 alleles of the F_4 plants were analyzed. About half of the plants, 159, showed wild-type (*Sd1*) signals of the densities comparable to the combined signals of *Sd1* and *sd1-r*, and about the other half of the plants, 218, showed mutant (*sd1-r*) signals comparable to the combined signals. Thirty-nine plants exhibited dense signals with both the wild-type probe and the mutant probe, suggesting that these plants are heterozygotes of *Sd1*/*sd1-r*. In 44 plants, an insufficient amount of DNA was amplified. Genotypes of *Wx* and *Sd1* of the F_4 plants were successfully determined by dot-blot SNP analysis.

Discussion

Allele-specific detection was achieved by dot-blot-SNP analysis in all the tested genes having SNPs. Mutant alleles with indels were also distinguished from wild-type alleles. These results suggest the wide applicability of this technique to genotyping of various genes. Our dot-blot-SNP analysis is based on competitive allele-specific short oligonucleotide hybridization (CASSOH) reported by Matsubara and Kure (2003). Their technique does not require technical expertise, and results can be obtained within a very short time. However, the use of an immunochromatographic strip raises the cost of this analysis. Other techniques for SNP analysis reported to date are costly or laborious (Nasu et al. 2002; Jain et al. 2003), and some of them require expensive instruments (Ross et al. 1997; Stoerker et al. 2000; Wang et al. 2005). Although, dot-blot-SNP analysis requires some labor for the detection of the allele-specific signals, this method is suitable for a large number of samples because 864 samples can be blotted on one 8×12 cm^2 membrane. The most costly process in dot-blot-SNP analysis is PCR. However, less than 1 µl of the reaction mixture was sufficient for dot-blotting. Multiplex PCR using

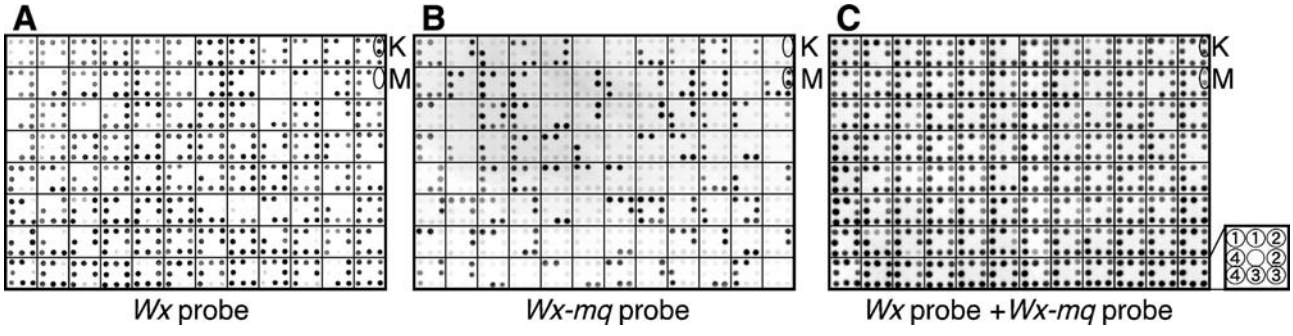

Fig. 3 Dot-blot-SNP analysis of the *Wx* alleles in F_4 plants of a crossbreeding program. Two dots were blotted for each plant as shown in the picture at the *right*. PCR products of 'Koshihikari' (K) having *Wx* and 'Milky Queen' (M) having *Wx-mq* were blotted as positive and negative controls at the *upper right corner* of each membrane. The pictures show hybridization with the labeled *Wx* probe and the unlabeled *Wx-mq* probe (**a**), with the labeled *Wx-mq* probe and the unlabeled *Wx* probe (**b**), and with a mixture of the labeled *Wx* probe and the labeled *Wx-mq* probe (**c**)

several primer pairs may be effective in reducing the cost and labor for PCR.

High reliability of the data is another advantage of dot-blot-SNP analysis. Specific amplification using allele-specific primers is a highly simple method for SNP analysis, and may be widely applicable (Latorra et al. 2003; Zhou et al. 2004). However, the efficiency of DNA amplification by PCR depends on the purity of the template DNA sample. In the analysis of a large number of samples, reducing the time and labor needed for DNA preparation by using simple techniques is important, but such techniques sometimes result in failure of the DNA amplification by PCR, leading to a misinterpretation of results. In dot-blot-SNP analysis, the dots without amplified DNA were not detected by either a wild-type probe or a mutant probe. The result of amplification failure can be abandoned without confusion of the data.

Since leading cultivars of rice, which differ in cooking quality, are highly similar to each other in appearance, establishment of a technique for cultivar identification is required not only by seed suppliers but also by food markets. Eight markers were found to be sufficient to differentiate the 43 cultivars used in the present study. Theoretically, all the cultivars can be distinguished from each other by SNP analyses, and dot-blot-SNP analysis can be used as a simple technique for cultivar identification. Furthermore, dot-blot-SNP analysis of each grain enables detection of the contaminated seeds and estimation of the percentage of each cultivar in blended grains. One problem of this method for cultivar identification is that its ability to distinguish lines is too high. Since lines in F_9 to F_{10} generations are generally released as new cultivars in conventional rice breeding, the rice cultivars are heterozygous at some loci. Propagation of the cultivars in different places may produce different genotypes in one cultivar. To avoid a misidentification of cultivar, heterozygosity of the SNP markers should be recorded using the original seeds released from research stations, as was done in the present study (Supplementary Table 1).

Marker-aided selection using DNA markers closely linked to a gene controlling an important agronomical trait can be applied to conventional crossbreeding of various crops. However, selection using the linked DNA markers may sometimes result in the loss of the target alleles by recombination between the markers and target alleles. To avoid this problem, it is necessary to use the target alleles themselves as the DNA markers. In addition to *Wx* and *Sd1*, several genes controlling agronomically important traits have been identified (Song et al. 1995; Yano et al. 2000; Ashikari et al. 2005). We propose a new breeding method tentatively named "DNA-selection breeding," where most of the genes for the agronomically important traits are selected by SNP analyses without investigating the plants in the field, only the final test for the productivity and adaptability of the lines is being performed in the field so as to reduce the labor, cost, and time required for plant breeding. Dot-blot-SNP analysis may contribute to the development of this new breeding method.

The high ability of the dot-blot-SNP technique to analyze many individuals may facilitate various genetic studies, e.g., gene mapping, QTL analysis, and population genetics. The genes controlling quantitative traits, many of which are important breeding objectives, can be mapped with QTL analysis (Yano et al. 2000). For the identification of genes in QTLs, fine mapping using closely linked DNA markers is indispensable. Many agronomical traits important for plant breeding cannot be assessed using segregating populations derived from the distantly related parents, such as *indica* rice and *japonica* rice. SNPs can be useful as markers for gene mapping using a population derived from a cross between closely related lines. In our preliminary study of QTL analysis of high-temperature tolerance at grain-filling period of rice, SNP analysis using dot-blotting was ten-times more efficient than electrophoretic analyses of the SSLP and CAPS markers. Further identification of SNPs and production of dot-blot-SNP markers are required for genetic study of rice.

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