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Mutation detection in rice waxy mutants by PCR-RF-SSCP

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Abstract PCR-RF-SSCP (PRS), which combines cleaved amplified polymorphic sequence (CAPS) and single-strand conformation polymorphism (SSCP), is expected to be a useful technique for DNA polymorphism analysis. We evaluated the ability of PRS to detect single nucleotide polymorphism (SNP) using the *Waxy* gene, *Wx*, of rice, and subsequently were able to identify point mutations in *wx* mutant lines. The approximately 6-kb *Wx* gene was divided into five regions for PCR amplification. Two regions, in which most of the point mutations of the *wx* mutants have been identified, were amplified by PCR and cloned into a vector, and those clones containing SNPs produced as a result of the inherent inaccuracy of PCR were used for the evaluation of PRS. The efficiency of PRS in the detection of SNPs of these clones was over 70%. PRS analysis of the *wx* genes in 18 waxy mutants was carried out in the five regions using two different restriction endonucleases and two gel conditions, with and without glycerol. Of the 18 lines tested, 17 showed band patterns different from that of the wild type. Most of the mutations identified in this study were nucleotide changes in exons, which result in amino acid changes. One mutation generated an in-frame stop codon, and another was a frame shift mutation by one-base deletion. Two mutations found at a splice site were considered to inhibit normal splicing of mRNA. These results show that PRS is a useful technique for detecting point mutations in large plant genes.

Keywords DNA polymorphism · Point mutation · SSCP · SNP · *Wx*

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Introduction

Point mutations, which are responsible for most genetic variation, can be detected by single nucleotide polymorphism (SNP) analysis. Although several techniques have been developed for SNP analysis, most require information on the position of the mutation (Neff et al. 1998; Drenkard et al. 2000). In order to determine the position of the SNP, nucleotide sequence analysis is performed, which is difficult for a large gene.

SSCP (single-strand conformation polymorphism), in which single-strand DNA fragments are electrophoresed in a native gel, is one of the easiest methods for detecting an SNP (Orita et al. 1989a; Dean et al. 1990). Since this method can detect an SNP in only small DNA fragments – between 100 bp and 400 bp – many primer pairs are therefore necessary for the screening of a point mutation in large genes of more than 2 kb by the polymerase chain reaction (PCR)-SSCP. Cleaved amplified polymorphic sequence (CAPS) (Konieczny and Ausubel 1993) can detect sequence polymorphism only at restriction sites. The combined method of PCR-SSCP and CAPS (designated here as PRS, from PCR-RF-SSCP) has been used for DNA polymorphism analysis of plant pests (Burban et al. 1999) and animal parasites (Wu et al. 2000) and as DNA markers for genetic mapping in plants (Slabaugh et al. 1997, Schneider et al. 1999, 2001), but not so frequently as simple sequence length polymorphism, amplified fragment length polymorphism, and CAPS (Phillips and Vasil 2001).

The *Waxy* gene, *Wx*, encodes granule-bound starch synthase and controls amylose content in endosperm, an important quality trait of rice, corn, barley, and other crops (Hirano and Sano 1991). Many *wx* mutants having glutinous endosperm have been obtained in these crops by spontaneous and induced mutation. *Wx* genes in rice and corn are more than 6 kb, including a promoter and 14 exons (Hirano and Sano 1991; Isshiki et al. 1998). It is difficult to determine the position of a point mutation in *wx* mutant alleles because of the large size of the *Wx* gene. For elucidating the positions of point mutations in

Wx, fine structure genetic maps have been constructed using intragenic recombination values at the *Wx* locus by observing pollen grains stained with I_2/KI in an F_1 hybrid between different *wx* mutants (Nelson 1968). However, this method is applicable only to a limited number of genes because of the difficulty of estimating the intragenic recombination values.

Intraspecific variation of the *SLR1* gene in *Brassica* has been detected by PRS in a previous study (Sato and Nishio 2002). In the investigation reported here, we evaluated the ability of PRS to detect SNPs and subsequently used it for the detection of point mutation in mutant alleles of the *Wx* gene of rice. We found that the detection level of PRS with respect to SNPs was high and that PRS was useful in identifying the point mutation in large genes or in large genome regions.

Materials and methods

Plant material and DNA isolation

In order to evaluate the ability of PRS to detect SNPs, we used mutated DNA fragments produced by the innate inaccuracy of DNA replication of PCR analysis. The *Wx* gene of *japonica* rice cv. Nipponbare, which has the Wx^b gene (Sano 1984), was used as a template DNA.

PRS analysis was applied to the detection of point mutations in 19 waxy mutant lines. Ten lines, namely, N08 (73wx1N1A), N09 (74wx1N1A), N15 (74wx7N1A), N16 (74wx8N1A), N18 (75wx1N1A), N21 (75wx5), N23 (76wx2), N26 (KURwx4N1), N27 (KURwx5N1), and N32 (82Gwx1), were derived from *japonica* rice cv. Norin 8. Five lines, namely, R43 (82RGwx2wx), R45 (84REwx3+), R52 (84REwx11), R65 (85REwx3), and R69 (85REwx7), were derived from *japonica* rice cv. Reimei. Four lines, namely, Ni41 (83GMM1-31wx), Ni73 (WX1), Ni76 (WX15), and Ni78 (WX23) were derived from *japonica* rice cv. Nihonmasari. The mutations of these lines were induced by ethylmethane sulfonate, ethylene imine, gamma-rays, and thermal neutrons at the National Institute of Genetics and Institute of Radiation Breeding in Japan (Amano 1985). Most of the mutant lines have 0% amylose in the endosperm, but N18, N27, and Ni41 have 8–9% amylose, and N16 and R45 have about 4% amylose. The original cultivars, Norin 8, Reimei, and Nihonmasari were used as controls.

Total DNA was isolated from leaves by DNeasy Plant mini kit (QIAGEN, Valencia. Calif.).

DNA amplification of the Wx gene by PCR

The *Wx* gene containing a 5′ non-coding region, approximately 6 kb, was divided into five regions, i.e., waxy 1, waxy 2, waxy 3, waxy 4, and waxy 5 (Fig. 1), because of the difficulties involved

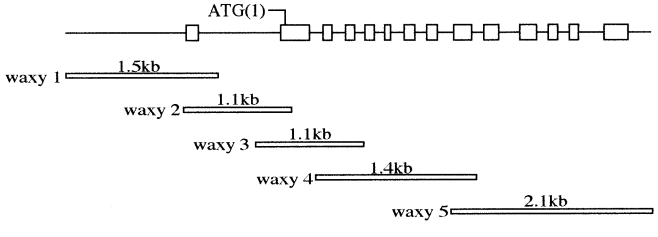

Fig. 1 Physical map of rice *Wx* gene. *Boxes* indicate exons, *bars* under the physical map show the DNA fragments amplified by PCR

Table 1 Nucleotide sequences of primers and restriction endonucleases used for the PRS analysis of the *Wx* gene

Waxy regions	Primer sequences		Restriction endonucleases	
	Forward primers (5′-3′)	Reverse primers (5′-3′)	1	2
waxy 1	CTCTCTAGCTTATTACAGCC	GTATGAGACTACTTGTAAGG	*Taq*I	*Afa*I
waxy 2	TCATCAGGAAGAACATCTGC	TGAATTGTTTAAGGTTTGGTGAGCC	*Alu*I	–
waxy 3	ATCTGATCTGCTCAAAGCTCTGTGCATCTC	TCCACGCTTGTAGCAATGGAAAAACCTCAC	*Taq*I	*Hpy*CH4IV
waxy 4	GTTCTTGATCATCGCATTGG	ACTTGTCCTTGCTAGGATCC	*Alu*I	*Taq*I
waxy 5	GAAGATCAACTGGATGAAGG	GGCATGGTATAATATGGAAC	*Hha*I	*Taq*I

with PCR amplification of the whole *Wx* gene from rice genomic DNA using a single pair of primers. PCR was conducted in each region using the primers shown in Table 1.

The conditions for the amplification of the waxy-2, waxy-4 and waxy-5 domains were as follows: 1 μl (40 ng) of sample DNA was mixed with 2 μl of 10× Ex Taq buffer (TaKaRa Biomedicals, Japan), 1.8 μl 2.5 m*M* of each dNTP, 1 μl 20 μ*M* primers, 0.1 μl 5 U/μl *Taq* DNA polymerase (Ex Taq, TaKaRa Biomedicals), and H_2O to give a final volume of 20 μl. DNA was amplified under the following thermal cycling conditions: one cycle at 94 °C for 30 s; 40 cycles of a 30-s denaturation at 94 °C, 30-s annealing at 60 °C and a 1-min extension at 72 °C; a final extension at 72 °C for 1 min. As Waxy 1 could not be amplified under the same conditions as those for amplification of waxy 2, waxy 4, and waxy 5, 2 μl of 10× PCR enhancer solution (GIBCO, Gaithersburg, Md.) was added to the mixture for PCR of the former. DNA was amplified under the same thermal cycling conditions as that of waxy 2, waxy 4 and waxy 5. The conditions for amplification of waxy 3, which contains a GC-rich region, was as follows: 1 μl (40 ng) of sample DNA was mixed with 10 μl of 2× LA Taq buffer (TaKaRa Biomedicals), 3.2 μl 2.5 m*M* of each dNTP, 1 μl of 20 μ*M* primers, 0.1 μl 5 U/μl *Taq* DNA polymerase (LA Taq, TaKaRa Biomedicals), and H_2O to give a final volume of 20 μl. DNA was amplified under the following thermal cycling conditions: one cycle at 94 °C for 1 min; 40 cycles of a 30-s denaturation at 94 °C, 30-s annealing at 60 °C, and 2-min extension at 72 °C; a final extension at 72 °C for 1 min.

PRS analysis of the Wx gene

PCR products were precipitated with ethanol/sodium acetate and dissolved in 10 μl water. Five microliters of the sample was digested with restriction endonucleases. The restriction endonucleases used for PRS analysis are shown in Table 1. One volume of the sample digested with the endonuclease was mixed with four volumes of denaturing solution [96% (v/v) formamide, 0.1% (w/v) xylene cyanol, 0.1% bromophenol blue, and 20 m*M* EDTA] and heated at 90 °C for 10 min. The denatured samples were immediately placed on ice. The sample was loaded on a 6% native polyacrylamide gel (18 × 20 cm) in 0.5× TBE buffer (45 m*M* Tris-borate, 1 m*M* EDTA) with or without 10% glycerol and run at 300 V or 400 V, 5 °C. After electrophoresis, the gel was stained with a Silver Stain kit (ATTO, Japan).

Sequence analysis

PCR products amplified in each region showing different band patterns were electrophoresed on a 1% agarose gel and then extracted from the gel using the MinElute gel extraction kit (QIAGEN). The eluted DNA was directly sequenced with the ABI PRISM 310 genetic analyzer. Rive cvs. Reimei, Nihonmasari, and Norin 8 were used as controls.

Results

Evaluation of the ability of PRS to detect SNPs

Regions of waxy 3 and waxy 4, where most of the point mutations of the *wx* mutants have been identified (Inukai et al. 2000), were amplified by PCR, and the PCR products were cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, Calif.). These clones were considered to have mutations due to an innate inaccuracy in the PCR analysis. Sixteen randomly chosen clones were subsequently subjected to PRS analysis. in which waxy-3 and waxy-4 clones were digested with restriction endonucleases *Taq*I and *Alu*I, respectively.

Nucleotide sequences of the 16 waxy-3 clones were determined. The nucleotide sequence of waxy 3 of Nipponbare was deduced from the consensus sequence of the 16 clones. The sequences of waxy 3-8 and waxy 3-14 were the same as that of Nipponbare and, therefore, the band pattern of waxy 3-8 was used as a wild type in this PRS analysis of waxy-3 clones. Thirty-eight SNPs were detected (Table 2); 36 were transition mutations and the other two were transversion mutations. There was no mutation at the *Taq*I sites of the wild type of waxy 3. However, a point mutation in waxy 3-13 produced a *Taq*I site in the 211-bp restriction fragment.

Of the 14 waxy-3 PCR clones having SNPs, 12 showed band patterns different from that of the wild type in the

Table 2 SNPs detected by PRS in the waxy-3 PCR clones

Clones	223-bp fragment		538-bp fragment		211-bp fragment	
	No. of SNPs	Detection of a polymorphism[a]	No. of SNPs	Detection of polymorphism	No. of SNPs	Detection of polymorphism
waxy 3-01			2	+		
waxy 3-02	1	+	5	+	1	+
waxy 3-03	1	+	1	+	1	+
waxy 3-04					1	+
waxy 3-05	1	–	1	+		
waxy 3-06			1	–	1	+
waxy 3-07	2	+	1	+		
waxy 3-08						
waxy 3-09			2	+	1	+
waxy 3-10			1	–		
waxy 3-11	1	+	1	+	1	+
waxy 3-12			1	+		
waxy 3-13	1	+	2	–	4 bp/207 bp[b]	
waxy 3-14						
waxy 3-15	1	–	1	+		
waxy 3-16			2	–	1	–

[a] +, Different band patterns between a PCR clone and the control; –, the same band pattern between a PCR clone and the control
[b] Nucleotide substitution producing a recognition site of *Taq*I

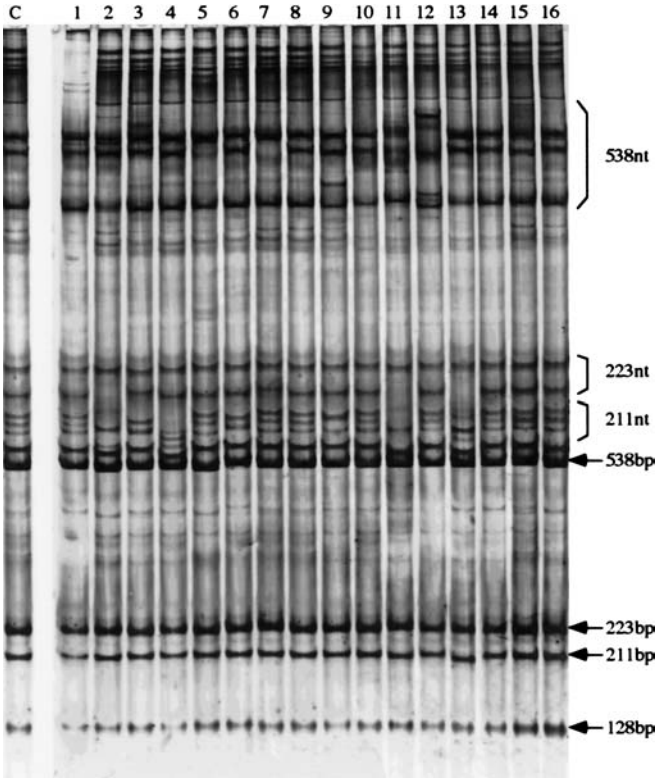

Fig. 2 PRS analysis of waxy-3 clones (*lanes 1–16*) obtained by PCR from the Nipponbare *Wx* gene. *Arrows* and *braces* indicate CAPS bands and SSCP-specific bands, respectively. *Lane C* Band pattern of waxy 3-8 having the consensus sequence was used as a control

PRS analysis. Although waxy 3-10 had one SNP and waxy 3-16 had three SNPs, they showed the same band pattern as that of the wild type. Since most waxy-3 clones had more than one SNP, the ability of PRS to detect one SNP is considered to be lower.

When waxy 3 was digested with *Taq*I, four restriction fragments – 538 bp, 223 bp, 211 bp, and 128 bp – were obtained. In the PRS analysis, the bands found in CAPS were detected together with SSCP-specific bands because of incomplete denaturation of the restriction fragments. Based on the electrophoretic mobility and the polymorphism of SSCP-specific bands, we assigned the restriction fragments to the SSCP bands (Fig. 2). For example, waxy 3-12 had only one SNP in the 538-bp fragment, and the band showing a different pattern in this clone was the SSCP-specific band derived from the 538-bp fragment. The SSCP-specific band derived from the 128-bp fragment was too thin to be analyzed. A total of 28 restriction fragments had more than one SNP (except for the 128-bp fragment), and 21 DNA fragments showed different band patterns (Table 2), indicating that the efficiency of PRS in the detection of DNA polymorphism was 75% per restriction fragment. In the case of DNA fragments containing only one SNP, the efficiency was 77%.

The result of PRS analysis of 16 waxy-4 clones and the number of mutations in each clone are shown in Table 3.

Table 3 SNPs detected by PRS in the waxy-4 PCR clones

Clones	Number of SNPs	Detection of a polymorphism[a]
waxy 4-01	1	–
waxy 4-02	2	+
waxy 4-03	4	+
waxy 4-04	0	–
waxy 4-05	2	+
waxy 4-06	2	+
waxy 4-07	3	+
waxy 4-08	3	–
waxy 4-09	3	+
waxy 4-10	1	+
waxy 4-11	2 SNPs and a 3-bp deletion	+
waxy 4-12	1	–
waxy 4-13	1	–
waxy 4-14	1	+
waxy 4-15	0	–
waxy 4-16	3	+

[a] +, Different band patterns between a PCR clone and the control; –, the same band pattern between a PCR clone and the control

The nucleotide sequences of waxy 4-4 and waxy 4-15 were the same as that of Nipponbare, which was determined from the consensus sequence of 16 waxy-4 clones. The band pattern of waxy 4-4 was therefore used as a wild type. Of the 30 mutations observed, 26 were transition mutations, three were transversion mutations, and one was a 3-bp deletion. Waxy 4-7 lost one of the recognition sites of *Alu*I, and new *Alu*I sites were generated in waxy 4-9, waxy 4-10, and waxy 4-11. Of the 14 clones having SNPs, ten showed different band patterns and, therefore, the efficiency of PRS in the detection of SNPs was 71.4% per clone. In waxy 4, assignment of restriction fragments to the SSCP-specific bands was not possible because of the many restriction fragments obtained (180 bp, 236 bp, 416 bp, 202 bp, 119 bp, 149 bp, 30 bp, and 85 bp), and it was difficult to distinguish SSCP-specific bands of some restriction fragments from the others.

PRS analysis of rice waxy mutants

DNA of one waxy mutant line (N26) originating from Norin 8 was not amplified by PCR with any of the primer pairs, suggesting that the *Wx* gene is deleted in N26 (Yatou 1993). Nine waxy mutant lines originating from Norin 8 (N08, N09, N15, N16, N18, N21, N23, N27, and N32), waxy 5 of N08, waxy 3 and waxy 4 of N9, waxy 4 of N21, and waxy 3 of N23 showed band patterns different from that of the wild type in the PRS analysis using the first set of restriction endonucleases in listen Table 1 (Fig. 3). N15, N16, N18, N27, and N32 were subjected to PRS analysis using the second set of restriction endonucleases in listed Table 1 (Fig. 4, Table 4). Waxy 3 of N15, waxy 4 of N16, waxy 5 of N18, waxy 4 of N27, and waxy 5 of N32 showed band patterns different from that of the wild type. Mutations of N08, N09, N15, N16, N21, and N23 have been identified

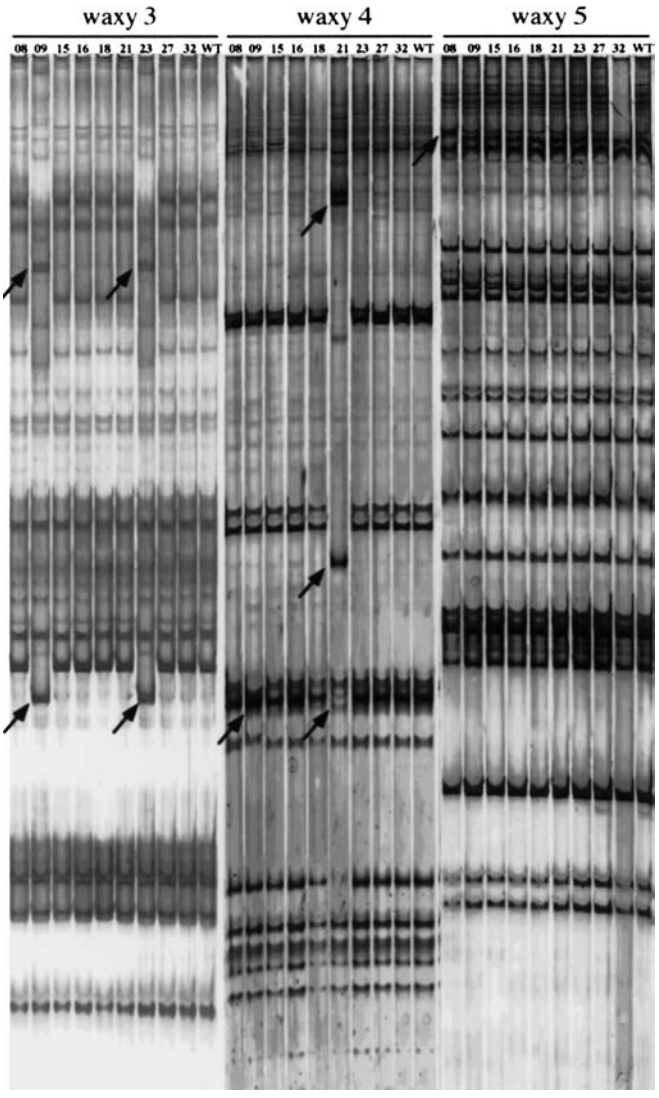

Fig. 3 PRS analysis of waxy mutants originating from Norin 8. *WT*: Norin 8 was used as a control. *Arrows* indicate bands showing different patterns. *waxy 3 Taq*I digestion, *waxy 4 Alu*I digestion, *waxy 5 Hha*I digestion

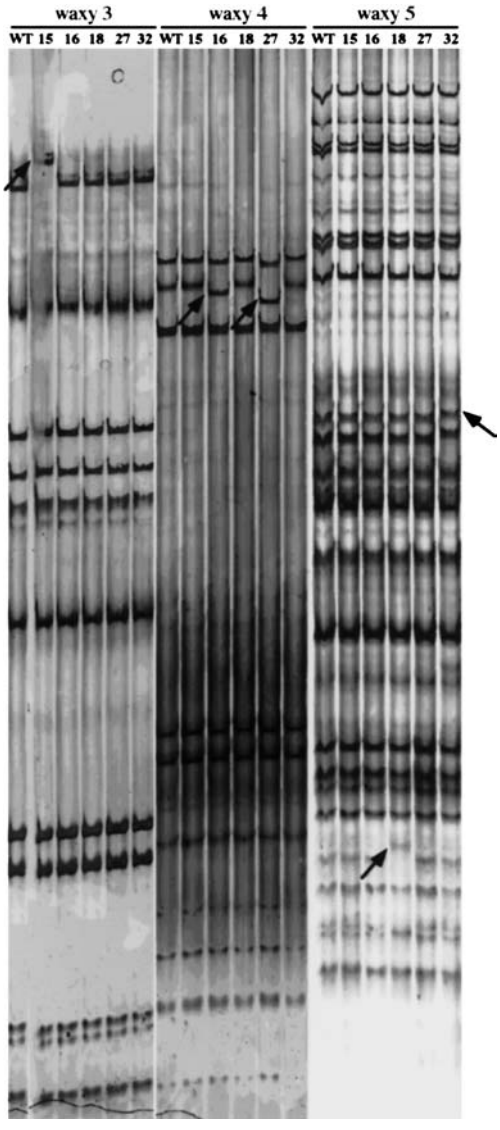

Fig. 4 PRS analysis of waxy mutants originating from Norin 8 using the second set of restriction endonucleases. *WT* Norin 8 was used as a control. *Arrows* indicate bands showing different patterns. *waxy 3 Hpy*CH4IV digestion, *waxy 4 Taq*I digestion, *waxy 5 Taq*I digestion

by Inukai et al. (2000). The regions that showed different band patterns in these mutants corresponded to the regions having the point mutations reported by them.

Nucleotide sequences of the regions showing different band patterns were determined. Mutations of N18, N27, and N32, which have not been analyzed, were identified (Table 5). In waxy 5 of N18, we found a nucleotide substitution from G to A in exon 12, resulting in an amino acid change from Gly to Asp. In N27, a nucleotide substitution from A to G in exon 5 was identified, resulting in an amino acid change from Lys to Glu. In N32, one base had been deleted in exon 9, causing a frame shift.

In the PRS analysis of waxy mutants originating from Reimei (R43, R45, R52, R65, and R69), waxy 3 and waxy 5 of R43, waxy 4 and waxy 5 of R52, and waxy 5 of R65 showed different band patterns, but R45 and R69 did not. Differences in the band patterns between waxy 3 and waxy 5 of R43 were not clear and, therefore, R43, R45, and R69 were subjected to PRS analysis using the second set of restriction endonucleases. R43 clearly showed a different band pattern in both waxy 3 and waxy 5, but R45 and R69 did not. It has been reported that band patterns can be changed by the addition of glycerol to the polyacrylamide gel during SSCP (Orita et al. 1989a, b). Therefore, the waxy mutant lines (R45 and R69) that did not show different band patterns were subjected to PRS analysis using a polyacrylamide gel containing glycerol, R69 subsequently showed a band pattern different from that of the wild type, but R45 did not (Table 4).

In waxy 3 of R43, a nucleotide substitution from C to T in exon 2 generated a stop codon, and this mutation is

Table 4 Detection of mutations in waxy mutants by PRS

Waxy mutant lines	Different band patterns detected in waxy fragments					Total
	waxy 1	waxy 2	waxy 3	waxy 4	waxy 5	
Norin8						
N08	–[a]	–	–	–	+	+
N09	–	–	+	+	–	+
N15	–	–	+	–	–	+
N16	–	–	–	+	–	+
N18	–	–	–	–	+	+
N21	–	–	–	+	–	+
N23	–	–	+	–	–	+
N27	–	–	–	+	–	+
N32	–	–	–	–	+	+
Reimei						
R43	–	–	+	–	+	+
R45	–	–	–	–	–	–
R52	–	–	–	+	+	+
R65	–	–	–	–	+	+
R69	–	–	–	+	–	+
Nihonmasari						
Ni41	+	–	–	–	–	+
Ni73	–	–	–	–	+	+
Ni76	–	–	–	+	–	+
Ni78	–	–	–	+	–	+

[a] +, Different band Patterns were detected; –, the same band pattern was detected

Table 5 Characterization of waxy mutants showing different band patterns in PRS

Waxy mutant lines	Location[a]	Codon change	Amino acid change	Note
N18	2,704 (exon 12)	GGT → GAT	Gly → Asp	
N27	857 (exon 5)	AAG → GAG	Lys → Glu	
N32	1,835 (exon 9)			1-bp deletion
R43	2,122 (exon 2)	TCC → TCT	synonymous	
		CAG → TAG	Gly → stop	
R52	1,678 (exon 9)	ACC → ATC	Thr → Ile	
R65	2,499 (exon 11)	GGA → GAA	Gly → Glu	
R69	1,089 (intron 6)			Splice site (GT–AG → AT–AG)
Ni73	2,501–2,505 (exon 11)	GCC → CGA	Ala → Arg	
		GAC → CCC	Asp → Pro	
Ni76	510 (exon 3)	TGG → AGG	Trp → Arg	
Ni78	1,089 (intron 6)			Splice site (GT–AG → AT–AG)

[a] The numbers indicate the positions of nucleotides from the translation initiation codon in exon 2

thought to be the cause of the waxy phenotype of R43. In waxy 4 and waxy 5 of R52, a nucleotide substitution from C to T in exon 9, resulting in an amino acid change from Thr to Ile, was found. In waxy 5 of A65, we detected a nucleotide substitution from G to A in exon 11, resulting in an amino acid change from Gly to Glu. Nucleotide substitution from G to A in the splice site of intron 6 was identified in R69 (Table 5).

In the PRS analysis of waxy mutants originating from Nihonmasari (Ni41, Ni73, Ni76, and Ni78), waxy 1 of Ni41, waxy 5 of Ni73, and waxy 4 of Ni76 showed band patterns different from that of the wild type, but Ni78 did not. Ni78 did show a band pattern different from that of the wild type in the PRS analysis using the polyacrylamide gel containing glycerol (Table 4).

In waxy 4 of Ni76, we found a nucleotide substitution from T to A in exon 3, resulting in an amino acid change from Trp to Arg. Ni73 had a five-bp substitution (GCCGA → CGACC) causing amino acid changes (Ala → Arg, Asp → Pro) in exon 11. We identified a nucleotide substitution (G → A) at the splice site of intron 6 in Ni78.

Discussion

Using the *Wx* gene, we have demonstrated here that PRS can be used to detect SNPs with a high efficiency; it is also more suitable than PCR-SSCP for larger DNA fragments. Point mutations can be detected by an enzymatic method based on RNase A cleavage of mismatches in RNA/DNA or RNA/RNA heteroduplexes (Myers et al. 1985, Winter et al. 1985) and by electrophoretic analysis of heteroduplexes (Hauser et al. 1998). While both methods are able to effectively detect point mutations, they are usually used in an analysis of DNA

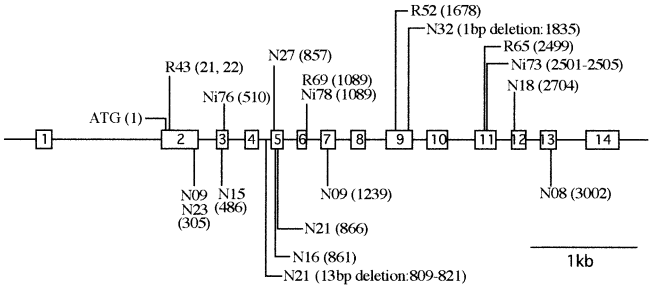

Fig. 5 Physical map of the *Wx* gene. Exons and introns are indicated by *white boxes* and *black bars*, respectively. The locations of the mutation sites in the waxy mutant lines are indicated by *vertical lines*. Point mutations of the mutant lines *under* the physical map have been identified by Inukai et al. (2000); point mutations of the mutant lines *above* the map were identified in this study

fragments less than 1 kb in length. Compared with these methods, PRS is better able to detect a point mutation in fragments as long as 2 kb. In the investigation reported here, the mutations in the 2.1-kb waxy-5 fragment of seven waxy mutant lines were detected by PRS.

Of 18 waxy mutant lines, 17 showed band patterns different from that of the wild type, as determined by PRS, indicating that PRS is useful in determining the position of point mutations. This PRS analysis was done under different conditions, namely, by using two different restriction endonucleases and gels with or without glycerol. The ability of SSCP to detect the SNP depends on the position of the SNP and the size of the DNA fragment and, therefore, different restriction endonucleases can be used effectively in the PRS analysis. In the five lines showing the same band pattern as that of the wild type in the PRS analysis using one restriction endonuclease in each region, band patterns different from that of the wild type were obtained by digestion with the second set of restriction endonucleases. The addition of glycerol to the polyacrylamide gel also enabled the detection of SNPs in the two lines.

The positions of mutations detected in the mutant lines are summarized in Fig. 5. There were seven single-base substitutions; Six of which were transition mutations and one was a transversion mutation. R43 had a two-base substitution, Ni73 had a five-base substitution, and N32 had a one-base deletion. Most of the mutations identified in this study were in exons, resulting in amino acid changes. A stop codon was generated by a mutation in exon 2 in R43, and the deletion of one base in N32 caused a frame shift. Mutations of R69 and Ni78 were at the splice site of intron 6, suggesting that these mutations inhibit normal splicing of mRNA. These mutations are considered to be the cause of the glutinous phenotype of these lines. The amino acid changes in R65, Ni73 and N18 are present in the conserved amino acids in rice granule-bound starch synthase (*Wx*), rice-soluble starch synthase, and *E. coli* glycogen synthase (Baba et al. 1993), which are considered to be important for the enzyme activity. The mutations in Ni76, N27, and R52 are also present in the amino acids conserved in granule-bound starch synthase in four plant species, namely, rice (Hirano and Sano 1991), pea (accession number: AJ345045), potato (accession number: X83220), and barley (Rhode et al. 1988). Therefore, it is inferred that these mutations affect the enzymatic activity of the *Wx* gene products. However, without any analysis of the function of the mutated genes, there remains the possibility that other mutations undetected by the present PRS analysis are responsible for the waxy phenotype of these lines.

The complete sequence of the *Arabidopsis thaliana* genome has been determined (The *Arabidopsis* Genome Initiative 2000). Ninety-three percent of the rice genome sequences have been determined (Goff et al. 2002), and the complete sequence of the rice genome will be published soon. In the next step of our plant genome study, we will analyze the nucleotide sequence variation of many genes in a species and elucidate the relationship between phenotypic variation and differences in nucleotide sequences. Therefore, SNP analysis will be increasingly important for the study of the function of genes.

An SNP in the gene controlling heading date, *Hd6*, has been identified between Nipponbare and Kasalath (Takahashi et al. 2001). Sasaki et al. (2002) have revealed point mutations of a gibberellin-synthesis gene in semi-dwarf rice cultivars. Information on these agronomically important genes will rapidly increase, and the SNP analysis of these genes will contribute to the development of plant breeding methodology. For use in plant breeding, however, detection of SNPs should be cost-effective. Since PRS analysis is simple and rapid and requires no special equipment, it can be applied not only to mutation analysis but also to practical plant breeding.

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